CHROMIUM: BINDING STUDIES WITH TRANSFERRIN

AND PEPTIDE EEEGDD AND ITS EFFECT

ON COLORECTAL CANCER

by

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A DISSERTATION

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ABSTRACT
Chromium as the trivalent ion has been proposed as an essential element for decades. Although that status has recently been discredited, doses of Cr³⁺ have been shown to generate improvements in insulin sensitivity and blood cholesterol levels in animals that have problems with their glucose and lipid metabolism systems, especially in type 2 diabetic rodent models. The mechanism for these effects at a molecular level is unknown.

Transferrins are a class of protein that can reversibly bind 2 equivalents of metal ions. Biologically, transferrins are the main iron transport proteins in plasma. A role for transferrin in the delivery Cr³⁺ from plasma to tissues has been proposed. Studies have shown that Cr³⁺ readily binds to the two metal-binding sites in the two lobes of apotransferrin. The Cr³⁺ binding is accompanied by intense changes in the transferrin’s ultraviolet spectrum. This intense changes arises from chromic ion binding to two tyrosine residues in the two iron-binding sites of transferrin and allow the binding of Cr³⁺ to transferrin to be monitored.

The rate at which Cr³⁺ binds to transferrin and the stability of Cr-transferrin recently has received considerable attention. In vitro spectroscopic studies previously found that the generation of Cr₂-transferrin needs two weeks to guarantee a stoichiometric amount of Cr³⁺ binding. However, this study indicates that in the presence of 25 mM (bi)carbonate, the concentration in human blood, two Cr³⁺ ions bind rapidly and tightly to apotransferrin.

Glycation of transferrin alters how tightly the protein binds iron and may alter the conformation of diferric transferrin, presumably changing its ability to deliver the iron to tissues. Given that Cr³⁺ complexes has been proposed as nutritional supplements to improve
symptoms of type 2 diabetic subjects, understanding the ability of glycated transferrin to bind and transport Cr is significant, especially for determining the appropriate dose of Cr. This study examined the binding ability of Cr$^{3+}$ to glycated serum transferrin and the transport of Cr \textit{in vivo} by glycated transferrin. The results suggest that glycation of transferrin in subjects with elevated blood glucose levels should lower the ability of Cr from pharmacological agents to enter tissues. Additionally, these studies with glycated transferrin also indicate that heat treatment of transferrin makes dramatic change on its conformation and Cr binding ability.
DEDICATION

To my mom and dad, Jinxia Ke and Yimin Deng; who give me life and love.

To my wife, Ying Feng, my love who always stands beside me.

To all my friends past and present.
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<td>AAALAC</td>
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</tr>
<tr>
<td>Cr</td>
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<tr>
<td>Cr(^{3+})</td>
<td>Trivalent chromium ion</td>
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</tr>
<tr>
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<td>g</td>
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<td>G</td>
<td>Glycine</td>
</tr>
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<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>GTF</td>
<td>Glucose tolerance factor</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HCT</td>
<td>High-capacity ion trap</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HSQC</td>
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<td>I</td>
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<td>Iminodiacetic acid</td>
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<tr>
<td>Ir</td>
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<td>kg</td>
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<td>La</td>
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<tr>
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</tr>
<tr>
<td>LMWCr</td>
<td>Low-molecular-weight chromium-binding substance</td>
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<tr>
<td>LSD</td>
<td>Least significant difference</td>
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<tr>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>Pd</td>
<td>Palladium</td>
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<tr>
<td>pic</td>
<td>Picolinate</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<td>Rb</td>
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<td>SE</td>
<td>Standard error</td>
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<td>Sn</td>
<td>Tin</td>
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<td>Sr</td>
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<td>Statistical Package for the Social Sciences</td>
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<tr>
<td>Tl</td>
<td>Thallium</td>
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<tr>
<td>TXI</td>
<td>Triple resonance probe</td>
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<tr>
<td>V</td>
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<td>Zucker diabetic fatty</td>
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CHAPTER 1
INTRODUCTION

1.1 Diabetes and chromium supplements

Diabetes or diabetes mellitus is a group of metabolic diseases in which blood sugar levels are too high over a prolonged period. The typical symptoms of diabetes are polyphagia, polyuria, polydipsia and weight loss. Prolonged diabetes can change the lens shape of the eye, resulting in vision damage. Diabetes is classified into several broad categories, including type 1, type 2, gestational diabetes and “other specific types”. Type 1 diabetes, further classified as immune-mediated, is characterized by loss of the insulin-producing beta cells in the pancreas. The majority of those with type I diabetes are children. Type 1 diabetes must be managed with expensive insulin injections. Type 2 diabetes is characterized by insulin resistance. People with type 2 diabetes create enough insulin in their pancreas, but their body tissues do not respond to the insulin. Initially, over-production of insulin by the pancreas compensates for the insulin resistance. However, with time the function of pancreas will be damaged, resulting ultimately in stopping the production of insulin. Type 2 diabetes makes up about 90% of all diabetes and is related primarily to lifestyle factors, such as obesity, poor diet, and lack of physical activity. Lowering the glucose in the diet and exercising helps alleviate the symptoms on people with type 2 diabetes. However, if the blood glucose level cannot be controlled by changing lifestyle, expensive medicines or painful insulin shots will be necessary. Furthermore, cardiovascular disease is a serious complication associated with diabetes.

Biochemistry research on chromium (Cr) as a potential supplement has been conducted for decades to potentially reduce the diabetic patient’s need of costly drugs. Chromium has been postulated to have an essential function in maintaining normal glucose in rats.
Researchers believed that a new dietary requirement had been identified. They called this new dietary requirement glucose tolerance factor (GTF), which was supposed to be absent from the *Torula* yeast-based diet. In 1955, Mertz’s group found that rats fed a Torula yeast-based diet developed impaired glucose tolerance in response to an intravenous glucose load [1]. Since other inorganic compounds containing Li, Be, B, F, Ti, V, Mn, Co, Ni, Cu, Zn, Ge, As, Se, Br, Rb, Sr, Y, Zr, Mo, Ru, Rh, Pd, Ag, Cd, Sn, Sb, I, Cs, Ba, La, Ce, Ta, W, Os, Ir, Au, Hg, Tl, Bi, Th, and U (200-500 μg/kg body mass) could not restore glucose tolerance, researchers started to believe chromium is an essential element for human. Mertz and Schwarz subsequently proposed that the active ingredient of GTF was Cr$^{3+}$ [2]. Further studies by the Mertz’s group indicated that several inorganic Cr$^{3+}$ compounds could restore glucose tolerance from a $< 2.8\%$ per minute rate of removal of intravenously injected glucose to the approximately 4% rate of control rats [3]. Porcine kidney powder and Brewer’s yeast, identified as natural sources of GTF, could also reverse the glucose intolerance. Mertz and other researchers proposed Cr as an essential element. In 1973, a review by a World Health Organization Expert Committee determined trivalent chromium as an essential nutrient with typical intakes of from 1 to 4 μmol/d [14]. In 1988, the International Programme on Chemical Safety also indicated that intake of trivalent chromium (from 1 to 4 μmol/d) as a nutrient is essential [14]. In 1989, the Food and Nutrition Board of the U.S. National Research Council suggested a range of safe and adequate intakes for chromium of 1 to 4 μmol/d [14].

In 1977, Mertz’s group claimed that GTF had been isolated from Brewer’s yeast. Mertz and coworkers indicated the GTF as a Cr$^{3+}$-glutathione-nicotinate complex. Complexes synthesized from the combination of Cr$^{3+}$, nicotinate, and glutathione were reported to have similar biological activity to Brewer’s yeast, which is rich in GTF [4]. However, several problems were found in the GTF isolation procedures such as an 18-hour reflux in 5 M hydrochloric acid, which would hydrolyze any proteins or nucleic acids, presumably changing the form of Cr. Furthermore, without using Cr-free cages and analyzing the Cr content of the
diets, the early GTF studies with rats were methodologically flawed. The ability of GTF to improve glucose metabolism had been determined by kinetic studies [5]. The results of these studies indicated that GTF did not have any activity in cells. GTF was actually reported to inhibit insulin simulation [6]. This contradicts an earlier proposal that GTF can enhance the binding of insulin to its receptor. Vincent’s group suggested that GTF is possibly an artifact of acid hydrolysis [7]. Cr is separable from agents in yeast that are responsible for the in vitro simulation of glucose metabolism in adipocytes [7]. Previous Cr studies which cited GTF as evidence to prove the essential role should not be accepted.

Focusing on GTF misled early Cr studies. A specific transport mechanism to distribute and deliver the element is unknown. But, Cr is still be proposed to have an effect on insulin-dependent carbohydrate and lipid metabolism. Several Cr compounds have been shown to improve insulin sensitivity and blood cholesterol levels in normal and diabetic model rodents, but only at pharmacological doses. Among many Cr supplements, Cr picolinate is the most popular in 90s. In 1989, Evan’s group reported Cr picolinate leads to body mass losses and lean muscle mass increases [8]. In 1990, Press et al. reported Cr picolinate to improve in symptoms associated with type 2 diabetes [9]. The weight loss and muscle development claims were quickly challenged by Lefavi’s studies [10, 11]. In 1997, the US Federal Trade Commission (FTC) forbid entities to represent the supplement as causing body fat reduction, weight loss, and muscle mass increases [12]. Studies indicating poor solubility at neutral pH and a low absorption rate (only 1% of absorbed Cr from the supplement in bloodstream as Cr picolinate) of Cr picolinate make this compound a poor choice as a nutritional supplement [10, 11]. The effect of Cr picolinate on the symptoms of type 2 diabetes is more problematic to decipher. In 2001, the US Food and Drug Administration found “there was no credible evidence to suggest that chromium picolinate intake may reduce the risk of elevated blood glucose levels” [13].
The Adequate Intake (AI) of Cr has been set at 35 µg and 25 µg for men and women, respectively. Under the AI, <2% of all Americans would theoretically be Cr deficient [14]. In other words, >98% of Americans are not considered to be Cr deficiency while consuming a normal diet.

The human studies examining the effects of Cr on diabetes have generally generated negative results except for a study that used 185 adult-onset diabetic Chinese patients. However, the results of this study are not reproducible on other populations, including Western populations. The American Diabetes Association’s position is that “Benefit from chromium supplementation in people with diabetes or obesity has not been conclusively demonstrated and, therefore, cannot be recommended” [15]. Compared to treatment with insulin or current diabetes medications, Cr\(^{3+}\) complexes have small or no effects to alter glucose and lipid metabolism in human subjects. However, the human studies have used lower doses of Cr (per unit body mass) compared to the doses in the rodents studies. Vincent suggested that Cr supplementation could potentially have a role as an inexpensive treatment to allow the use of lower doses of current medications or treat subjects at the early stages of type 2 diabetes to potentially delay the continued onset of the disease, but observing effects will probably require higher doses than examined to date [2, 16].

1.2 Effects of chromium supplementation on tissue metal concentrations

Chromium, as a metal, has been used in industry area for decades. It can form thin layer on surface by chemical vapor deposition [17-20]. Diabetes results in several metabolic changes, including alterations in the transport, distribution, excretion, and accumulation of metals. The concentration of metal ions in tissues has been examined in some rat diabetes or insulin resistance models. In 2009, Dogukan detected mineral status of serum and tissue in high-fat and streptozotocin-treated type II diabetic rats [21]. Dogukan’s group indicated that the administration to rats of both a high-fat-fed diet and streptozotocin results in increases in Fe concentration in liver and kidney and increases in Cu concentration in kidney. Krol’s group
in 2011 also found Fe concentration increases in the liver and the kidney and Cu concentration increases in the kidney of the same model rats. However, other effects are not consistent with Dogukan’s study [22]. In 2010, Krol’s group determined mineral levels in high-fructose-fed rats with chromium propionate complex supplementation [23, 24]. Their study suggested that a high-fructose diet lowers liver Cu concentration but has little, if any, effect on kidney Cu or kidney or liver Fe, Zn, or Cr concentrations. In 2011, Ozcelik and coworkers evaluated the trace elements and oxidative stress levels in the liver and kidney of streptozotocin-induced experimental diabetic rat model [25]. Ozcelik found increased Fe and Cu concentrations but lower Zn and Mg concentrations in the liver and kidney in Streptozotocin-treated only rat models.

Zucker obese rats and Zucker diabetic fatty (ZDF) rats are common models of insulin resistance and type 2 diabetes, respectively. Zucker obese rats have a mutation in leptin receptor that blocks signaling from the hormone leptin, which makes the rats an insulin resistance and early stage diabetes model [26]. The Zucker diabetic fatty rats developed from Zucker obese rats and possess another unknown mutation giving rise to the development of type 2 diabetes [27]. While changes have been examined in several rat models of insulin resistance and diabetes, the metal ion concentrations in the tissues of Zucker lean, Zucker obese, and ZDF have not previously been examined in detail [28]. In 1987, Donaldson’s laboratory reported decreases of Zn and Cu concentrations in Zucker obese rats compared with Zucker lean rats. The lower concentrations of Zn and Cu per gram dry mass for the obese rats were attributed to the increased fat content of the liver and kidney; after correction, the liver Zn and Cu content was equivalent to those of the lean rats. Donaldson’s laboratory also indicated streptozotocin-treated lean and obese rats possessed an increased liver and renal Cu and Zn concentrations per gram dry mass [28]. In 1988, Serfass’ group reported that the obese rats have higher Cu concentrations than lean rats in the kidney per gram protein at 5 weeks of age and in the kidney and liver at 12 weeks, while no differences per gram protein were
observed for Fe and Zn concentrations in the liver and kidney [29]. However, the use of milligram metal per milligram protein and milligram metal per organ in this work does not allow for direct comparisons with other studies using milligram metal per unit dry tissue mass. Thus, more details on the tissue metal concentrations for Zucker lean, Zucker obese, and ZDF rats are needed.

The concentrations of Cu, Zn, Fe, Mg, and Ca in the liver, kidney, heart and spleen, and Cr concentration in the liver and kidney of the Zucker lean, Zucker obese, and Zucker diabetic fatty rats were examined. The effects of chromium picolinate, [Cr(pic)$_3$], CrCl$_3$, and Cr$_3$, as well as vanadyl on the metal concentrations of tissues in these rats were determined to establish whether supplemental Cr could alter the concentrations of these metals in tissues. The data, results and conclusions are demonstrated in Chapter 2.

1.3 Transferrin and chromium transport

Although the specific transport mechanism of chromium in cells is still unknown, the Fe$^{3+}$ transporter, transferrin has been shown to be the main chromium transport protein to deliver chromium from plasma into the cell as it is for Fe$^{3+}$.

Transferrins are a class of proteins of ~80 kDa that reversibly bind 2 equivalents of metal ions [30]. The protein exhibits amazing selectivity for Fe$^{3+}$ in a biological environment because its metal sites are adapted to bind ions with large charge-to-size ratios. Transferrin, Tf, is a blood serum protein, a β-globulin, although another form of the protein is found in avian egg white, conalbumin. Transferrin is the major iron transport protein in the bloodstream, while conalbumin is believed to have antibacterial roles by depriving bacteria of iron. The transferrin molecule is composed of two lobes with approximately 40% sequence homology; the three-dimensional structures of the lobes are nearly superimposable [30]. Each lobe possesses an iron-binding site, and each Fe$^{3+}$ binds concomitantly with a synergistic anion, usually (bi)carbonate. Fe coordination is essentially identical in each site, comprised of two tyrosine residues, a histidine residue, an aspartate residue, and a chelating (bi)carbonate ion in
a distorted octahedral arrangement. The presence of the anion is essential for Fe binding. The transferrin molecule undergoes a significant conformational change when binding and releasing Fe [30].

The apoprotein possesses a more open conformation. In the Fe-loaded conformation, transferrin binds to transferrin receptor, a transmembrane protein of the cell membrane [30]. The transport mechanism of transferrin is depicted in Fig. 1.1. Diferric transferrin is brought into the cell by endocytosis. Acidification of the resulting endosome releases the Fe$^{3+}$, and subsequent fusion of the endosome with the cell membrane releases and recycles the apotransferrin. In humans, the protein is present at a concentration of approximately 3 mg/mL in serum and is normally about 30% saturated with Fe, allowing it to potentially bind and transport other metal ions [30].

Transferrin was first proposed to have a role in the transport and storage of chromium in the 1960s. Administration of $^{51}$CrCl$_3$ by stomach tube to rats resulted in $\geq 99\%$ of the chromium in blood being associated with non-cellular components [31]. Ninety percent of the Cr in blood serum was associated with the $\beta$-globulin fractions, while 80% immunoprecipitated with transferrin [31]. Cr$_2$-transferrin serves as an inhibitor for the binding of Fe$_2$-transferrin to the surface of reticulocytes [32], presumably at transferrin receptor. The Cr-loaded human transferrin is a better inhibitor than apotransferrin or Cu$^{2+}$-loaded transferrin but not as good as mono- or diferric transferrin [32]. Injection of $^{51}$Cr$_2$-labeled transferrin into the bloodstream results in a rapid and insulin-sensitive movement of Cr into the tissues as Cr-transferrin [31–33]; greater than 50% of the Cr is transported to the tissues within 30 min. Tissue levels of Cr are maximal 30 min after injection; decreases in tissue chromium with time are mirrored by increases in urine chromium. Approximately 50% of the $^{51}$Cr appeared in the urine within 360 min of injection of Cr-transferrin into the tail vein of rats in the absence of added insulin; insulin treatment concurrent with injection of $^{51}$Cr-labeled transferrin results in approximately 80% of the label appearing in the urine within 180 min. Therefore, transferrin,
in an insulin-dependent fashion, can transfer Cr to tissues from which Cr is excreted in the urine [33–35]. Thus, transferrin appears to be the physiological transport agent for Cr$^{3+}$. (Although care must be taken in that when Cr$^{3+}$ is administered intravenously or added *in vitro* to blood or blood serum or plasma, non-physiologically relevant binding of chromium to other species occurs [36–38].)

As shown in Fig. 1.2, the movement of Cr$^{3+}$ from plasma to cells is quite similar to that of Fe$^{3+}$. Cr$^{3+}$ in plasma binds to apotransferrin readily, along with binding bicarbonate. After Cr$_2$-transferrin binds to transferrin receptor, the receptor mediated endocytosis brings Cr$^{3+}$ into the cell. After acidification of the endosome, Cr is released from transferrin. The free Cr$^{3+}$ is transported from endosome into cytosol by an unknown transporter. The removal of Cr$^{3+}$ from the endosome is not performed in the same manner as Fe$^{3+}$ because Cr$^{3+}$ cannot be reduced. Fe$^{3+}$ is transported out of the endosome as reduced Fe$^{2+}$ by divalent metal transporters. Finally, chromium binds to apo-chromodulin to form holo-chromodulin, which has four Cr$^{3+}$ ions bound. Then, the holo-chromodulin is rapidly excreted from the cell by some unknown transporter [39, 40].

The rate at which Cr$^{3+}$ binds to transferrin and the stability of Cr-transferrin has received considerable attention [41–45]. Studies indicated the formation of Cr$_2$-transferrin required up to two weeks to guarantee a stoichiometric amount of Cr$^{3+}$ binding. This is too long when considering the half-life of transferrin in serum and that of diferric transferrin is on in the order of hours. Chapter 3 demonstrates the binding of Cr$^{3+}$ to human serum transferrin and conalbumin can be much faster in the presence of appropriate concentrations of bicarbonate.
Fig. 1.1 Cellular uptake and intracellular utilization of transferrin iron.

Fig. 1.2 Proposed chromium transport mechanism.
1.4 Glycation of transferrin

Glycation, sometimes called non-enzymatic glycation, is a site-specific reaction involving covalent bonding of a lipid molecule or protein with a sugar molecule without the enzymatic action. Compared with glycosylation, an enzyme controlled reaction occurring at defined sites on the target molecule and required in order for the molecule to function, the non-enzymatic glycation is a haphazard process that impairs the functioning of biomolecules [46].

Glycation in the bloodstream with a small proportion of the absorbed simple sugars, such as glucose, is the first step in the solution of the sugar molecules through a complex series of very slow reactions: Schiff base reactions, Amadori reactions, and Maillard reactions, which lead to advanced glycation endproducts (AGEs). Glycation interferes with proteins and cellular functioning throughout the body and can result in the release of highly oxidizing side-products such as hydrogen peroxide. Long lived cells and proteins may accumulate substantial damage by glycation over time. Studies have shown AGEs are implicated in many age-related chronic diseases such as cardiovascular diseases, Alzheimer’s disease, cancer, peripheral neuropathy, and other sensory losses such as deafness [47, 48]. Many of the long term effects of diabetes may also be related to the process of protein glycation, but the importance of glycation was underestimated by much of the early laboratory research work.

Under conditions of high blood glucose concentration, as in subjects with diabetes, glucose can bind chemically and irreversibly (glycation) to several proteins in the bloodstream, including transferrin [49]. As shown in Fig. 1.3, the process of transferrin glycation begins with the reaction of a free amine group on transferrin with a reducing D-glucose sugar to form a reversible intermediate product, Schiff base. Then, this intermediate product undergoes a slow rearrangement to generate a more stable Amadori product, fructosamine. Furthermore, the generation of fructosamine can be followed by additional reactions during advanced stage glycation, where the generation of AGEs occurs [49]. The high blood glucose levels in diabetic subjects provides an environment for the glycation of proteins in blood plasma. Recently, the
study of proteins with either early stage glycation products or AGEs has attracted great attention due to the suspected effects of glycation on protein function and tissue damage during diabetes. Several studies indicated that this chemical modification can alter the properties and functions of these biomolecules [49].

The effects of glycation of transferrin on iron transport have recently been a matter of concern. Van Campenhout’s studies reported that glycation of transferrin alters how tightly the protein binds iron [50, 51] and may alter the conformation of diferric transferrin, presumably changing its ability to deliver the iron to tissues [52]. In 2014, Silva’s group detected the glycation site specificity of human serum transferrin. Based on Silva’s study, lysine residues in transferrin modified by glycation result in the disruption of hydrogen bonds described as crucial to stabilize iron binding. They reported the steric hindrance caused by the bound glycation motifs bound to obstruct the conformational change occurring between the apo and holo forms of transferrin, increasing the activation energy required for iron binding [53].

Cr$^{3+}$ binds to the two Fe$^{3+}$-binding sites of apotransferrin and in a similar fashion concomitantly binds two equivalents of (bi)carbonate as it does when binding ferric ions. Glycation of transferrin may change its ability of binding and delivery chromium into tissue. Given that Cr$^{3+}$ complexes have been proposed as drugs to increase insulin sensitivity, particularly in type 2 diabetic subjects, understanding the ability of glycated transferrin to bind and transport Cr is important to determining the appropriate amount of Cr necessary for potentially being used as a drug to treat insulin insensitivity and its symptoms.
1.5 Chromium and colorectal cancer

Colorectal cancer, also called as rectal cancer, colon cancer or bowel cancer, is the third most common cancer in men and the second in women [54]. It is the development of cancer from the colon or rectum.

Most colorectal cancers are associated with old age and lifestyle factors. Given a strong relationship between colon cancer and Westernization, an increased risk of developing colorectal cancer for individuals with type 2 diabetes has been paid great amount of attention. Shared risk factors for type 2 diabetes and colorectal cancer include obesity, sedentary lifestyle, and high caloric diet [55]. Recent studies found that different levels of insulin and insulin-like growth factors (IGF) may account for many of the nutritional and other risk factors of colon cancer [56]. In 1998, Yamada and Bayerdorffer suggested that cholesterol levels affects the risk factor of colorectal cancer [57, 58]. Yamada’s study also indicated that different serum triglycerides levels change the risk of colorectal cancer, which was also supported by a German study [59]. Consequently, therapeutic agents that increase insulin sensitivity and improve
cholesterol and triglyceride levels would be expected to potentially reduce the incidence of colorectal cancer.

Cr can generate improvements in insulin sensitivity and blood cholesterol levels in animals with stresses on the glucose and lipid metabolism systems, most notably in rodent models of insulin insensitivity and type 2 diabetes. Chromium therapeutic agents that increase insulin sensitivity and improve cholesterol levels have been proposed to potentially reduce the incidence of colorectal cancer. In 2004, the chromium(III)-based therapeutic agent \(\text{[Cr}_3\text{O(O}_2\text{CCH}_2\text{CH}_3)\text{6(H}_2\text{O})_3]^+\), Cr3, has been examined previously for its ability to reduce the incidence of 1,2-dimethylhydrazine-induced colorectal tumors [60]. Studies of Cr3 indicated the solubility of Cr3 and its stability allow a unique amount of the chromium to enter the circulatory system and tissues [61]. Chapter 5 describes the effects of chromium on chemically induced colorectal cancer in a mouse model.

1.6 Chromium-binding peptide EEEEGDD

The effects of Cr\(^{3+}\) in enhancing insulin sensitivity in rodents have been shown to be supra-nutritional [61]. No symptoms of chromium deficiency have been unambiguously established, and no biomarker of chromium status has been identified [62]. However, this shift raises questions about the role and mode of action of Cr\(^{3+}\) in increasing insulin sensitivity at a molecular level. The lack of the demonstration of unambiguous benefits of chromium supplementation in clinical trials may also be explained by this shift [62, 63]. Vincent’s group and Hua’s group presented several proposals for the mode of action of Cr\(^{3+}\): however, none have been definitely established in vivo [62, 63].

Chromium is passively absorbed from the gastrointestinal tract, transported by serum transferrin from the bloodstream to the tissue cells. Then, chromium is transported from the tissues to the urine by peptide low-molecular-weight chromium-binding substance (LMWCr) [62] (Fig. 1.2). Transferrin and LMWCr have been proposed to be the only two biomolecules known to specifically bind Cr\(^{3+}\) in vivo when Cr\(^{3+}\) is taken orally at nutritionally relevant doses
Cr-containing LMWCr is rapidly cleared from tissues in vivo and has a very low tubular reabsorption rate so that the peptide in the blood is readily removed from the body via the kidneys and urine, although the mechanism is still unknown [62]. In 1987, Yamanoto isolated biologically active LMWCr from rabbit liver. In 1997, Vincent also isolated and characterized biologically active LMWCr from bovine liver. Their studies indicated that LMWCr is a peptide 10 or 11 amino acid in length; it is comprised of only the amino acids glycine, aspartate, glutamate, and cysteine and tightly binds four chromic ions [64, 65]. This oligopeptide has also been isolated from the livers of chicken, and alligator [66] and from human urine [67], where all appear to contain the contiguous peptide of sequence Glu-Glu-Glu-Glu-Gly-Asp-Asp (EEEEGDD) [67]. In 2003, Jacquamet and coworkers characterized LMWCr by X-ray absorption and electron paramagnetic spectroscopies. This spectroscopic studies of LMWCr indicate that the chromic ions are bound to the peptide primarily via the carboxylate side chains of the aspartate and glutamate residues [68]. The Cr(III)-containing form of LMWCr has been proposed to be the biologically active form of chromium. In vitro it has been found to activate the kinase activity of insulin receptor; this activation was proportional to the Cr content of LMWCr [68, 69].

The heptapeptide EEEEGDD can readily be synthesized. However, whether this peptide might bind Cr similarly to LMWCr or have biological activity as a pharmaceutical agent to treat symptoms of diabetes and other conditions related to insulin insensitivity was unknown. In this study (Chapter 6), the binding of Cr$^{3+}$ to the synthetic peptide EEEEGDD and the biological activity of the peptide and its complex with bound Cr were examined.
REFERENCES


CHAPTER 2
COMPARISON OF TISSUE METAL CONCENTRATIONS IN ZUCKER LEAN, ZUCKER OBESE, AND ZUCKER DIABETIC FATTY RATS AND THE EFFECTS OF CHROMIUM SUPPLEMENTATION ON TISSUE METAL CONCENTRATIONS

2.1 Introduction

The concentration of metal ions in tissues has been examined in some rat diabetes or insulin resistance models including rats on a high-fructose diet [1, 2], streptozotocin-induced diabetic rats [3], and high-fat-fed streptozotocin-induced diabetic rats [4, 5]. The varying models have differing tissue metal concentrations compared to healthy controls due to different coexisting factors affecting bioavailability, metabolism, and excretion, whose mechanisms are not fully understood.

For example, a high-fructose diet lowers liver Cu concentrations but has little, if any, effect on kidney Cu or kidney or liver Fe, Zn, or Cr concentrations [1, 2]. Streptozotocin-treated rats have increased Fe and Cu concentrations but lower Zn and Mg concentrations in the liver and kidney [3]. The administration to rats of both a high-fat diet and streptozotocin results in increases in Fe concentration in the liver and kidney and increases in Cu concentration in the kidney [4, 5], while other effects are not consistent between studies. Zucker obese rats and Zucker diabetic fatty (ZDF) rats are common models of insulin resistance and type 2 diabetes, respectively [6]. Zucker obese rats have a mutation in the leptin receptor that blocks signaling from the hormone leptin. The Zucker diabetic fatty rats developed from Zucker obese rats and possess another unknown mutation giving rise to the development of type 2 diabetes. Researchers also use fluorimeter to determine metal concentrations [7, 8]. However, a limited number of studies have examined tissue metal ion concentrations in Zucker obese rats [9–11], while no systematic studies were identified for
ZDF rats. Zucker obese rats have been reported to have lower tissue Cu concentrations than Zucker lean rats, although these differences disappear when the rats are fed a cafeteria-style diet; however, whole rat homogenates, rather than individual tissues, were examined in this study [9]. In contrast, another lab has reported that the obese rats have higher Cu concentrations than lean rats in the kidney per gram protein at 5 weeks of age and in the kidney and liver at 12 weeks, while no differences per gram protein were observed for Fe and Zn concentrations in the liver and kidney [10]. Unfortunately, the use of milligram metal per milligram protein and milligram metal per organ in this work does not allow for direct comparisons with other studies using milligram metal per unit dry tissue mass. Another laboratory reported that Zucker obese rats have lower liver Zn and Cu concentrations than Zucker lean rats [11]. The lower concentrations of Zn and Cu per gram dry mass for the obese rats were attributed to the increased fat content of the liver and kidney; when corrected for neutral fat content, the liver Zn and Cu content was equivalent to those of the lean rats. Streptozotocin-treated lean and obese rats possessed in increased liver and renal Cu and Zn concentrations per gram dry mass [11]. Thus, more data on tissue metal concentrations for Zucker lean, Zucker obese, and ZDF rats are needed. Hence, in this study, the hypothesis that tissue trace metal concentrations will vary between the pre-diabetic and diabetic animal models (i.e., rat models) and the healthy controls was tested.

While originally proposed to be an essential trace element, Cr as the trivalent chromic ion is a therapeutic agent capable of improving insulin sensitivity in rodent models of insulin resistance and diabetes [12–15]. Additionally, the ion is also able to improve lipid parameters in some of these models, and some Cr(III) complexes, such as Cr3, [Cr3O(propionate)6(H2O)3]7+, have been reported to improve insulin sensitivity in healthy rats at high doses [12, 16].

Supplementary minerals, including Cr, can potentially affect mineral status due to possible interactions with other macro- and microelements at absorption, transport,
metabolism, excretion, and other levels. For example, Cr(III) and Fe(III) can compete for
binding sites on the Fe transport protein transferrin [17]. For this and related reasons, the
question of Cr–Fe interactions, for example, should be addressed whenever Cr(III) is to be
administered orally. This is particularly potentially significant when Cr requires
supranutritional doses to be delivered for beneficial effects to be observed [15]. As insulin
resistance and type 2 diabetes result in changes in tissue metal concentrations, then Cr
supplementation might also be able to improve tissue metal concentrations.

Consequently, in this study, the effects of chromium picolinate, [Cr(pic)₃] (the most
popular commercial chromium nutritional supplement), CrCl₃, and Cr₃, as well as vanadyl
(as a model of chromate) on the metal concentrations of tissues in Zucker lean, Zucker obese,
and ZDF rats were examined to test the hypothesis that supplemental Cr could alter
concentrations in tissues.

2.2 Experimental

2.2.1 Rats

One hundred forty-four male rats, 48 Zucker lean, 48 Zucker obese, and 48 ZDF, approximately 6-weeks old were obtained from Charles River Laboratories. Zucker obese rats
are an insulin resistant model of obesity and early stage type 2 diabetes, and ZDF rats are a
type 2 diabetes model. Rats were maintained in an AAALAC-approved animal care facility in
rooms with 22 ± 2 °C, 40–60 % humidity, and a 12-hour photoperiod. Animals were housed
two rats/cage containing hardwood bedding and were provided Harlan Teklad rodent chow
and water. Following a 1-week acclimation period, rats were assigned to the following
treatment groups with treatments administered by gavage daily at circa 9 am for 12 weeks:
(groups 1–3) eight Zucker lean, eight Zucker obese, and eight ZDF as control vehicles; (groups
4–6) eight Zucker lean, eight Zucker obese, and eight ZDF receiving 1 mg Cr per kilogram
body mass per day as CrCl₃; (groups 7–9) eight Zucker lean, eight Zucker obese, and eight
ZDF receiving 33 μg Cr per kilogram body mass per day as Cr₃; (groups 10–12) eight Zucker
lean, eight Zucker obese, and eight ZDF receiving 1 mg Cr per kilogram body mass per day as Cr\(_3\); (groups 13–15) eight Zucker lean, eight Zucker obese, and eight ZDF receiving 1 mg Cr per kilogram body mass per day as [Cr(pic) \(_3\)]; and (groups 16–18) eight Zucker lean, eight Zucker obese, and eight ZDF receiving 2 mg/kg vanadyl sulfate (a source of vanadate \textit{in vivo}) per day. Animals were weighed twice weekly.

After the 12-week treatment period, rats were anesthetized using isofluorane. A bundle of vastus lateralis muscle fibers and the end of one segment of epididymal fat were dissected from the right side of the body for studies beyond the scope of this report. The rats were then treated intravenously with 5 units of insulin (bovine Zn) per kilogram body mass; after 30 min, left muscle and fat samples were collected for studies beyond the scope of this report. The rats were then sacrificed by carbon dioxide asphyxiation, and the liver, heart, spleen, and kidneys were harvested and weighed. Tissues were transferred directly to plastic weigh boats for weighing and then to disposable plastic centrifuge tubes (capable of withstanding at temperature of 105 °C). The heart, spleen, kidneys, and a weighed aliquot of liver from each rat were then dried to a constant mass in a vacuum oven at 105 °C. All procedures with the rats were approved by The University of Alabama Institutional Animal Use and Care Committee.

Nicholas R. Rhodes, Kristin R. Di Bona, Leigh Ann Pledger, Sharifa T. Love undergraduate students from Dr. Rasco group and Dr. Vincent group, and I took care of those rats for 12 weeks, then harvested the tissues and dried them.

2.2.2 Atomic Absorption Spectrometry

Metal analysis were performed by the Krejpcio group at the University of Life Sciences, Poznan, Poland.

For metal analyses, samples were digested with concentrated 65 % spectra pure HNO\(_3\) (Merck) in a Microwave Digestion System (MARS-5, CEM). The concentration of Cu, Zn, Fe, Mg, and Ca was determined by flame atomic absorption spectrometry method F-AAS
The concentration of Cr was measured using a graphite furnace atomic absorption spectrometer (AA EA 5 with background correction, Jenoptik). The accuracy of the determination of Cu and Zn was assured by simultaneous analysis of the certified reference material bovine liver BCR®-185R (IRMM), while analysis of Fe, Mg, and Ca was controlled using the certified reference materials Virginia tobacco leaves CTA-VTL-2 (Poland). Analysis of Cr was assured using the certified reference material mussel tissue ERM®-CE278 (ERM). The recovery for Cu, Zn, Fe, Mg, Ca, and Cr (expressed of the percentage of the mean certified values were 103%, 101%, 97%, 104%, 103%, and 102%, respectively.

2.2.3 Chromium Compounds

Chromium picolinate and Cr3 were prepared as described previously [18, 19]. CrCl3·6H2O (actually trans-[Cr(H2O)4Cl2]Cl·2H2O) and vanadyl sulfate were used as received.

2.2.4 Statistics

Kristin R. Di Bona and Sharifa T. Love analyzed the data. Each data point in the figures represents the average value for eight rats. Error bars in the figures denote standard deviation. Data were tested for homogeneity of variance by means of the Levine statistic and were analyzed by repeated measures ANOVA using SPSS (SPSS, Inc.). Specific differences ($P \leq 0.05$) were determined by LSD and a Bonferroni post hoc test. For eight animals per group, an expected difference between two means would be significant at the 0.05 level if the difference between the means is twice the standard deviation.

2.3 Results and Discussion

2.3.1 Rat Strains

This research reported herein is one part of a two part study. One part of this study was designed to test the effects of supplementation of a variety of Cr(III) compounds and vanadyl sulfate on the insulin signaling cascade in control rat and rat models of insulin-resistance and
type 2 diabetes. This part of the study required the surgeries and insulin treatment. However, the rat carcasses remaining at the end of the study provided a unique opportunity to also (in the portion of the study reported herein) to compare the mineral distribution in tissues of Zucker lean, Zucker obese, and Zucker diabetic fatty rats and to study the effects of the Cr(III) compounds and vanadyl sulfate on the mineral distribution of tissues of these animals. Blood mineral concentrations were not determined because of the known effect of insulin to result in movement of various ions to or from the bloodstream. For example, a pool of transferrin is mobilized from the pool to the tissues in response to tissue, resulting in the transport of Cr(III) and Fe(III) from the blood to the tissues [17, 20]. However, for many metal ions, multiple storage pools exists. The blood usually represents a small, but rapidly mobilizable pool of the metal ion. In contrast, tissues, such as the liver, represent a large storage pool of the metal, which exchanges only very slowly with the pool of metal in the bloodstream. For example with Cr, most of the metal in the body resides in a large pool in the tissues that very slowly exchange over a period of months with the pool of Cr ion the bloodstream [21–23]. Consequently, the insulin treatment (followed by a 30-min wait and the time required for the surgery and other operations) is anticipated to have little if any effect on the metal concentrations in the selected tissues. This is indeed borne out in the control Zucker lean rats for which metal concentrations were found to be in normal ranges.

No effects on body mass were observed as a function of diet for the Zucker lean, Zucker obese, or ZDF rats (Fig. 2.1). Cr complexes have previously been shown to have no effect on body mass of Zucker lean and ZDF rats [13]. Cr3 at an oral dose of 1 mg Cr/kg body mass has previously been found to result in an increase in body mass of Zucker obese rats [16] in contrast to the results of the current study. An intravenous dose of Cr3 at 20 μg/kg had no effect on body mass [24].
Fig. 2.1 Effects of diets on body mass. (Top) Zucker lean rats (middle) Zucker obese rats (bottom) ZDF rats. No statistically significant effects on body mass were found.

Tissue levels of Cr, Cu, Zn, Fe, Mg, and Ca in the Zucker lean, Zucker obese, and ZDF rats were generally similar, although some statistical significant differences were identified.
(Figs. 2.2, 2.3, 2.4, 2.5, 2.6, and 2.7). ZDF rats possessed an increased concentration of Cu in the kidneys (Fig. 2.3b) compared to the Zucker lean and Zucker obese rats, while no other differences were observed. This is similar to rats with streptozotocin-induced or streptozotocin- and high fat diet induced diabetes [3–5]. Effects on metal concentrations were more common in the Zucker obese rats. Zucker obese rats had a reduction in the concentrations of Cu and Zn compared to that of ZDF rats, a reduction in the concentration of Fe in the liver compared to those of the Zucker lean and ZDF rats, and a reduction in the concentration of Mg in the liver compared to that of the Zucker lean rats. Spleen Fe concentrations were decreased in Zucker obese rats compared to the lean rats. However, the Ca concentration was increased in the kidneys of the obese rats compared to those of the Zucker lean and ZDF rats. These changes presumably arise as a result of the increased fat content of the liver of the obese rats. Lower Cu and Zn concentrations in the liver of Zucker obese rats have been reported previously [11]. Increased kidney Ca concentrations in Zucker obese rats compared to those of lean rats has been observed previously [25], where the increased Ca concentration was attributed to a specific impairment in Ca-ATPase activity in the obese rats.
Fig. 2.2 Chromium concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A Liver and B kidney. (†) statistically significant difference in concentration from that of Zucker lean rat ($P \leq 0.05$).

Fig. 2.3 Copper concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A Liver, B kidney, C spleen, and D heart. (*) significant difference in concentration from those of other two rat strains ($P \leq 0.05$). (**) values for all strains are significantly different from each other ($P \leq 0.05$). ‡ Difference in concentration from that of ZDF rat ($P \leq 0.05$).
Fig. 2.4 Iron concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A Liver, B kidney, C spleen, and D heart. (*) significant difference in concentration from those of other two rat strains ($P \leq 0.05$). (**) significant difference in concentration among all three rat strains ($P \leq 0.05$). ‡ Difference in concentration from that of ZDF rat ($P \leq 0.05$).
Fig. 2.5 Zinc concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A Liver, B kidney, C spleen, and D heart. (*) significant difference in concentration from those of other two rat strains ($P \leq 0.05$). ‡ Difference in concentration from that of ZDF rat ($P \leq 0.05$).
Fig. 2.6 Magnesium concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A Liver, B kidney, C spleen, and D heart. (†) significant difference in concentration from that of Zucker lean rat ($P \leq 0.05$) (*) significant difference in concentration from those of other two rat strains ($P \leq 0.05$). ‡ Difference in concentration from that of ZDF rat ($P \leq 0.05$).
Fig. 2.7 Calcium concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A Liver, B kidney, C spleen, and D heart. (*) significant difference in concentration from those of other two rat strains ($P \leq 0.05$).

2.3.2 Chromium and vanadium supplementation

Cr(III) complexes have been studied as potential nutrition supplements and therapeutic agents for over 50 years; the complexes have been touted as weight loss agents and muscle development agents, although studies have conclusively shown that the complexes have no such effects [15]. Yet, supranutritional doses of Cr(III) complexes have been shown to have pharmacological effects in rodent models of insulin insensitivity and diabetes [15]. The current study is designed to test whether high doses of Cr(III) complexes could lead to alterations in
metal levels of healthy; obese, insulin resistant; and diabetic rats. Three Cr compounds were chosen: CrCl$_3$, [Cr(pic)$_3$], and Cr$_3$. CrCl$_3$ and [Cr(pic)$_3$] are the most studied forms of Cr(III) as nutritional supplements or pharmacological agents [15], while Cr$_3$ has also been extensively studied and possesses some unique characteristics such as a high degree of absorption [15]. Most notably Cr$_3$ has previously been studied in Zucker lean, Zucker obese, and ZDF rats [16, 24].

Lay and coworkers have proposed that the pharmacological effects of Cr(III) are actually toxic effects arising from the generation of chromate from Cr(III) in the body; the chromate, as a structural analogue of phosphate, is then proposed to inhibit phosphatase enzymes [26]. Chromate, CrO$_4^{2-}$, was not used in this study as chromate is readily reduced to Cr$^{3+}$ in the gastrointestinal tract and would primarily (if not solely) serve as another source of Cr$^{3+}$. To model the potential effects from the generation of chromate, a vanadium compound was utilized. Administration of a variety of vanadium compounds has been shown to have beneficial effects in diabetic rodent models [27]. The vanadium source appears to be converted into vanadate, VO$_4^{3-}$, a phosphate analogue that inhibits phosphatase enzymes including PTP1B that deactivates phosphorylated insulin receptor. Unfortunately, the inhibition of a variety of phosphatases appears to result in harmful side effects [27]. Vanadyl sulfate at 2 mg/kg was used as the source of vanadate in vivo as this is the source and dose most commonly used in studies of the effects of vanadyl in rodent models [28].

The doses of the Cr(III) complexes were carefully chosen. The dose of 1 mg Cr/kg body mass used for CrCl$_3$, [Cr(pic)$_3$], and Cr$_3$ was chosen as this dose for Cr$_3$ has been observed to result in increased insulin sensitivity and improved cholesterol levels in Zucker lean, Zucker obese, and ZDF rats [16]. Also, some data exist on the accumulation of Cr in the kidney and liver of rats given this dose of CrCl$_3$, [Cr(pic)$_3$], and Cr$_3$ [16, 29]. The smaller dose of Cr$_3$ was used to account for the difference in absorption (<2 % for CrCl$_3$ and [Cr(pic)$_3$]) [30–32] compared to 40–60 % for Cr$_3$ [33]), while the two different concentrations of Cr$_3$
allowed for concentration dependent effects to be examined. For comparison, the Harland Teklad diet contains ~0.4 mg Cr/kg food [16]. Given that a 100-g rat eats about 15 g of food daily [22], this amount corresponds to the rats receiving approximately 60 μg Cr/kg body mass from the diet. Thus, the 33 mg Cr/kg body mass as Cr3 represents an approximately 50 % increase in oral daily Cr. The 1 mg Cr/kg body mass doses of the Cr compounds represent an approximately 17-fold increase in oral daily Cr, clearly a pharmacologically relevant dose.

The time interval for administration of the Cr and V compounds was chosen so as to guarantee that the model rats fully developed their insulin insensitivity and diabetes and to allow for the potential effects of Cr treatment to be manifested. The rats were approximately 6 weeks old at the initiation of the study, while treatment proceeded for 12 weeks. In previous experiments with Cr3, differences in insulin sensitivity and triglyceride and cholesterol levels were significant after only 4 weeks of treatment [16]. ZDF rats start to show signs of the manifestation of diabetes at week 7 or 8 of age [6]; for males, plasma glucose levels rapidly increase until approximately 16 weeks of age. Thus, ZDF rats at 18 weeks of age (6 weeks old at start plus 12 weeks of treatment) were ideal for these experiments.

2.3.3 Effects on tissue metal concentrations

The administration of the Cr(III) compounds and the vanadyl sulfate had few effects on tissue metal concentrations (Fig. 2.8). Cr levels were elevated in the kidneys of lean rats and kidneys of obese rats receiving either 1 mg/kg Cr as Cr3 or CrCl3 but not [Cr(pic)3] or the smaller amount of Cr3 (Fig. 2.8a, b). No statistically significant effects on the Cr concentration of the liver or kidneys of the other rats were observed. The increased Cu content of kidneys of the ZDF rats was reduced by administration of 1 mg/kg Cr as Cr3 and CrCl3 (Fig. 2.8d). Thus, CrCl3 and Cr3 at doses of 1 mg Cr/kg, but not [Cr(pic)3], had a restorative effect on these symptoms of diabetes in the ZDF rats. Only one other effect was identified. The liver Ca concentration in the lean rats were significantly increased by Cr3 and [Cr(pic)3] when administered at the 1 mg Cr/kg level (Fig. 2.8c). Levels of Ca tended to increase for all
treatments except the lower dose of Cr3, although the effects were not statistically significant for CrCl3 and vanadyl sulfate. The significance of this observation is uncertain at present.

The ability of Cr3 to have a beneficial effect on a diabetic symptom of the ZDF rats is consistent with effects of Cr3 supplementation on the ZDF rats. In fact, Cr3 has been reported to have beneficial effects on several rat models of diabetes and insulin resistance by Vincent and coworkers. Healthy Sprague–Dawley rats treated daily with 20 μg Cr/kg body mass as
Cr3 intravenously for 12 weeks had lower blood plasma insulin, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides, but not glucose, levels [34]. When given intravenously at 20 μg Cr/kg body mass, Cr3 lowered blood plasma insulin, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides, but not glucose, levels in healthy Sprague–Dawley rats after 4, 8, 12, 16, 20, and 24 weeks; also 2-h plasma glucose and insulin levels after a glucose challenge were lowered [34]. In rats with streptozotocin-induced diabetes, this treatment had no consistent statistically significant effects although plasma insulin, total cholesterol, and triglycerides tended to be lower [34]; the streptozotocin treatment appeared to have increased the spread of the values of the measured variables resulting in a loss of sufficient power to resolve any effects. Finally, similar intravenous treatment of Zucker obese rats resulted in lower blood plasma insulin, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides, but not glucose, levels; also 2-h plasma insulin, but not glucose, levels after a glucose challenge were lowered [24]. In contrast, Cr3 only lowered plasma insulin levels in Zucker lean rats, suggesting a difference between effects in healthy strains; however, total cholesterol and triglycerides tended to be lower in these animals [24].

The effects of gavage administration of Cr3 have been examined [16]. At levels of 250,500, or 1,000 μg Cr/kg body mass, the treatment at all concentrations lowered fasting plasma insulin, triglycerides, total cholesterol, and LDL cholesterol levels of healthy Sprague–Dawley rats while having no effect on plasma glucose or HDL cholesterol. These levels were lower after 4 weeks of treatment and remained lower for the next 20 weeks of treatment. The maintenance of glucose levels with less insulin indicates increased insulin sensitivity. Both plasma glucose and insulin levels were lowered in 2-h glucose tolerance tests. In Zucker obese rats receiving 1,000 μg Cr/kg body mass, the results were similar to those from intravenous administration. The effects of Cr3 on ZDF rats were also examined using 1,000 μg Cr/kg body mass. Again, fasting plasma insulin, triglycerides, total cholesterol, and LDL cholesterol levels were all lower while glucose concentrations were consistently but not statistically lower. HDL
levels were lowered from their very high levels; 2-h plasma insulin levels were also lowered. Plasma glycated hemoglobin levels, a measure of longer term blood glucose status, were examined in the healthy, Zucker obese and ZDF rats after 4, 12, and 24 weeks of treatment. No effect was seen for the healthy rats; however, significant effects were noted for the diabetic models. For the ZDF rats, glycated hemoglobin was lower after 12 and 24 weeks of treatment, reaching almost a 22% drop compared with ZDF controls by week 24; for the Zucker obese rats, glycated hemoglobin was 27% lower at week 24 [16].

The effects of the cation on healthy and model diabetic rats have also been examined by Krejpcio and co-workers. Male Wistar rats were provided a control diet or a diet containing 5 mg Cr/kg diet as Cr3 for 10 weeks [35, 36]. Blood plasma insulin levels were lowered 15.6% by the Cr-containing diet, while glucose transport by red blood cells was increased 9.6%. In another study, Krejpcio and coworkers utilized male Wistar rats with streptozotocin-induced diabetes. Using similar diets for 5 weeks, the rats that had the Cr diet had lower blood serum glucose levels (26%) and increased HDL levels (14%) [37]. Cr3 supplementation of the diet (AIN-93M or high-fructose diet) of male Wistar rats for 8 weeks (0, 1, and 5 mg Cr/kg body mass daily) has been shown to result in increased insulin sensitivity without affecting blood plasma glucose or lipid levels; no effects were observed on body mass as a result of supplemental chromium [15]. Thus, the results of the short-term study with male Wistar rats are very similar to those of the current study. In another study, male Wistar rats were fed a control (AIN-93M) diet or high-fat diet with or without chromium supplementation as Cr3 (0, 1, or 5 mg Cr/kg body mass) for 5 weeks; rats were subsequently injected with streptozotocin before being fed the same diets for another week [38]. Cr3 increased insulin sensitivity and lowered serum total and LDL cholesterol and triglycerides levels but had no effect on blood glucose levels. No effects on body mass were observed except the group receiving high-fat diet containing the highest dose of Cr3 had lower body mass weeks 3 through 5 than rats on
the high-fat diet not receiving any supplemental chromium; the effect disappeared after streptozotocin treatment.

The accumulation of Cr in the kidney and liver of rats receiving supplemental chromium has been observed previously, although the results are dependent on the form of Cr and dose. A detailed study of chromium accumulation as a function of dose has been reported for CrCl\textsubscript{3} and [Cr(pic)\textsubscript{3}] [29]. Cr content of kidneys and livers of Sprague–Dawley rats increased in a linear fashion when the rats received daily doses of CrCl\textsubscript{3} or [Cr(pic)\textsubscript{3}] for 24 weeks ranging from 750 μg Cr/kg body mass to 15 mg Cr/kg [29]. In contrast to the current work, the tissue concentration was greater for [Cr(pic)\textsubscript{3}] than CrCl\textsubscript{3}. Unfortunately, one cannot determine from the data presented whether the Cr concentration of the tissues using the source of Cr is significantly greater than that of rats fed an unsupplemented stock diet. The results are consistent with a recent study on absorption of CrCl\textsubscript{3} and [Cr(pic)\textsubscript{3}] that suggested CrCl\textsubscript{3} was better absorbed by rats that [Cr(pic)\textsubscript{3}] when tissue Cr concentration were considered in addition to urinary output of Cr [32].

Previously, no change in kidney or liver Cr concentrations of Zucker obese or ZDF rats receiving 1 mg Cr/kg body mass as Cr\textsubscript{3} orally daily for 6 months has been reported [16]; this was accompanied by no changes in liver Fe concentration but by a small, but statistically significant drop in kidney Fe concentration in the Zucker obese rats but not the ZDF rats [16]. The lack of Cr accumulation from Cr\textsubscript{3} in the Zucker obese and ZDF rats is consistent with the current study. However, healthy Sprague–Dawley rats receiving Cr\textsubscript{3} orally daily for 6 months at doses of 250,500, or 1,000 μg Cr/kg also displayed no accumulation in liver or kidney Cr [16], in contrast to the observed statistically significant increase in liver Cr for the Zucker lean rats in the current study. Whether this difference results from the use of different strains of rats will require more study. For Wistar rats on a high fructose diet supplemented daily with 1 mg Cr/kg body mass or 5 mg Cr/kg as Cr\textsubscript{3}, the liver and kidney levels of Cr of the rats receiving 1 mg Cr/kg were not increased compared to those of rats on a normal diet or on an
unsupplemented high-fat diet, while the Cr contents of the organs for the rats on the 5 mg Cr/kg diet were increased [1]. However, a different study from the same laboratory found a significant increase in kidney but not liver Cr concentration in Wistar rats fed a high fructose diet supplemented daily with 1 mg Cr/kg as Cr3 [2]. Another study of the effects of Cr3 on streptozotocin-treated Wistar rats on a high-fat diet found that supplementing the diet daily with 1 mg Cr/kg or 5 mg Cr/kg as Cr3 had no effect on liver Cr while the kidney Cr concentrations were raised by both levels of supplementation in a dose dependent fashion [4]. Perhaps, the daily 1-mgCr/kg dose of Cr3 is near the borderline for where rats can effectively remove the supplemental Cr compared to accumulating the metal in the kidneys and liver.

2.4 Conclusion

The concentration of Cu, Zn, Fe, Mg, and Ca was compared and contrasted for the first time in the liver, kidney, heart and spleen and Cr concentration in the liver and kidney of Zucker lean, Zucker obese, and ZDF rats. Zucker obese rats displayed a reduction in the concentration of Cu, Zn, Fe, Mg in the liver compared to ZDF and/or lean Zucker rats, presumably as a result of the increased fat content of the liver of the obese rats. ZDF rats possessed increased concentrations of kidney Cu compared to the lean rats, while kidney Ca concentrations were increased in the Zucker obese rats. Spleen Fe concentrations were decreased in Zucker obese rats compared to the lean rats. Cr(III) complexes previously have been shown to have beneficial effects on the symptoms of insulin resistance resulted in surprisingly few changes in tissue metal concentrations. Treatment with CrCl3 and Cr3, but not [Cr(pic)3], at 1 mg Cr/kg body mass resulted in accumulation of Cr in the kidney of lean and obese but not ZDF rats and resulted in lowering the elevated levels of kidney Cu in ZDF rats, suggesting a beneficial effect on this symptom of type 2 diabetes.
REFERENCES


CHAPTER 3
BINDING OF TRIVALENT CHROMIUM TO SERUM TRANSFERRIN IS SUFFICIENTLY RAPID TO BE PHYSIOLOGICALLY RELEVANT

3.1 Introduction

*In vitro* studies have shown that Cr\(^{3+}\) readily binds to the two Fe\(^{3+}\)-binding sites of apotransferrin and concomitantly also binds two equivalents of (bi)carbonate as it does when binding ferric ions [1], resulting in intense changes in the protein's ultraviolet spectrum. The amount of bicarbonate bound was determined by measuring the release of CO\(_2\) after the addition of acid; 1.09 equivalents of CO\(_2\) were released per bound Cr\(^{3+}\) for human transferrin [1]. The changes in the ultraviolet spectrum suggest that each chromic ion binds to two tyrosine residues, suggesting that chromium binds specifically in the two iron-binding sites; the ultraviolet absorbance maximum for human Cr\(_2\)-transferrin is at 293 nm [1]. The involvement of tyrosine ligands has been confirmed by Raman spectroscopy [2]. Human Cr\(_2\)-transferrin has been described as pale blue in color with visible maxima at 440 and 635 nm [1], while Cr\(_2\)-lactoferrin (a multifunctional protein of the transferrin family) has been described as gray-green with maxima at 442 and 612 nm (\(\varepsilon = 520\) and 280 M\(^{-1}\) cm\(^{-1}\), respectively) [3]. The visible spectra are typical for Cr(III) centers in a pseudo-octahedral environment. The oxidation state of the bound chromium has been confirmed by variable temperature magnetic susceptibility studies, whose results are consistent with the presence of S = 3/2 centers, and by electron paramagnetic resonance (EPR) studies [1]. The two Cr-binding sites can readily be distinguished by EPR (frozen solutions at 77 K) [2]. At approximately pH 7.7, chromium binds to both sites on the protein. At pH 4.8 to 5.9, chromium binds to only one site. This tighter binding site possesses an EPR signal centered at \(g = 5.43\). At near neutral pH, the Cr\(^{3+}\) in the
tighter binding site that binds chromium at the lower pH cannot be displaced by Fe\(^{3+}\), while Fe\(^{3+}\) readily displaces Cr\(^{3+}\) from the other site [2]. The weaker binding site Cr\(^{3+}\) gives rise to EPR signals at \(g = 5.62, 5.15, \) and 2.42 [2]. Mixed metal complexes (and their EPR spectra) with Cr\(^{3+}\) in its tight binding site and Fe\(^{3+}\) or VO\(_2^+\) in the other binding site have been described [4]. Binding of Fe\(^{3+}\) at physiological concentrations of iron to transferrin has been reported to not be affected by a physiologically relevant concentration of Cr\(^{3+}\); excess Fe\(^{3+}\) decreased the ability of Cr\(^{3+}\) to bind [5]. Cr\(^{3+}\) added to transferrin loaded 50% with Fe\(^{3+}\) (with the C-terminal lobe theoretically filled with Fe\(^{3+}\)) results in displacement of a few percent of the Fe\(^{3+}\) with concomitant binding of Cr\(^{3+}\) [5].

The rate at which Cr\(^{3+}\) binds to transferrin and the stability of Cr-transferrin has received considerable attention recently [5-8]. The generation of Cr\(_2\)-transferrin for the \textit{in vitro} spectroscopic studies generally used samples of transferrin that had been allowed to come to equilibrium with Cr\(^{3+}\) ions over the course of up to two weeks to guarantee a stoichiometric amount of Cr\(^{3+}\) binding. (Additionally, questions over the extinction coefficients for the ultraviolet absorption bands used to measure Cr\(^{3+}\) binding have arisen [6].) However, whether even this leads to stoichiometric binding has been questioned in mass spectrometric studies [5, 7]. This brings about an even more significant question as to how transferrin could be responsible for Cr\(^{3+}\) transport \textit{in vivo} if Cr\(^{3+}\) binding actually requires days or weeks to achieve equilibrium given the half-life of transferrin in serum and that of transferrin-bound iron are on the order of hour [9, 10]. Herein are reported studies on the binding of Cr\(^{3+}\) to human serum transferrin and conalbumin that reconcile these disparate results by demonstrating the importance of using carbonate concentrations that closely model physiological systems.

3.2 Experimental

3.2.1 Materials
Iron-free human serum transferrin and chicken conalbumin (egg white transferrin) were obtained from Aldrich. Doubly deionized water was used throughout. All reagents were used as received unless otherwise noted. Monoferric transferrin with iron in the C-terminal metal-binding site was generated by adding Fe\(^{3+}\) as Fe(nitriloacetate)\(_2\) [11]. Monoferric transferrin with iron in the N-terminal metal-binding site was generated using ferrous ammonium sulfate by the method of Aisen and coworkers [12]. All Cr-binding studies were performed in HEPES buffer, 0.1 M, pH 7.4 in plastic centrifuge tubes. Buffer pH was adjusted by the addition to NaOH to the free acid form of HEPES. For solutions containing KHCO\(_3\), pH was readjusted to 7.4 after addition of KHCO\(_3\) by addition of HCl. Cr\(^{3+}\) solutions were prepared using CrCl\(_3\)\(6\)H\(_2\)O. Co\(^{2+}\) solutions were prepared using CoCl\(_2\). Apotransferrin and apoconalbumin concentrations were determined using the extinction coefficient (\(\varepsilon = 9.12 \times 10^4\) M\(^{-1}\) cm\(^{-1}\)) at 280 nm [13]. Solutions containing transferrin or conalbumin were prepared immediately before use except as otherwise noted. Similarly solutions containing added bicarbonate were prepared immediately before use. All results are the average of at least triplicate experiments.

Kristi Wu assisted me in performing kinetic and titrations experiment with the proteins.

3.2.2 Instrumentation

Ultraviolet-visible spectra were obtained using a Cary 100 or Beckman Coulter DU800 UV-visible spectrophotometer. Binding of Cr\(^{3+}\) to transferrin and conalbumin was monitored at 245 nm. Studies were initiated by the addition of Cr\(^{3+}\). Solutions were continuously stirred using a Starna “Spinette” electronic cell stirrer. Continuous wave (CW) EPR were measured on a Bruker ELEXSYS E540 X-band spectrometer with an ER 4102 ST resonator. CW spectra were measured at 9.45 GHz with a microwave power of 8.39 mW using a magnetic field modulation frequency of 100 kHz with an amplitude of 30 gauss. Spectra were taken at liquid nitrogen temperatures with a quartz insertion Dewar. To prepare EPR samples, 2 \(\mu\)L of the solution of the appropriate metal ion were added to 45 mL of 0.86 mM apoconalbumin in 100
mM HEPES buffer, pH 7.4. After the appropriate time interval, 15 μL of glycerol was added to the samples, which were rapidly frozen in liquid nitrogen. Data analysis was performed using SigmaPlot 11. EPR sample was prepared by me. Cruce run EPR and analyzed EPR data.  

3.3 Results and discussion  

3.3.1 Conalbumin  

Given the apparent discrepancies in the ability of transferrin to serve as the physiological carrier of Cr$^{3+}$ from the blood to the tissues and the times reported required for Cr$^{3+}$ binding to transferrin to reach equilibrium, the binding of Cr$^{3+}$ to conalbumin and to human serum transferrin has been re-investigated. In particular, the binding of Cr to these transferrins has been examined in the presence of varying concentrations of bicarbonate, from ambient to 25 mM, the approximate concentration in human blood plasma.  

The addition of Cr$^{3+}$ to conalbumin at ambient CO$_2$ concentration results in the binding of chromium to the protein and resulting in a concomitant increase in the intensity of bands in protein’s ultraviolet spectrum at ~245 and 295 nm; these changes arise from perturbing two tyrosine residues that become ligands for each bound Cr$^{3+}$ [14]. As shown in Figure 3.1, at pH 7.4 and ambient CO$_2$ concentrations, the binding of chromium by the protein is very slow. The binding was monitored by following absorbance changes at 245 nm. Over 2 days are required for the binding to approach equilibrium. This is consistent with the results of Tan and Woodworth [14] who reported more than 40 hours were required for the ultraviolet spectrum to become constant, and samples of Cr-bound conalbumin were allowed to sit for 2 days before subsequent analysis. They found a change in extinction coefficient for the ultraviolet maximum at 245 nm of 38,743 ± 2,806 cm$^{-1}$M$^{-1}$ [14]. This value is also consistent with that in Figure 3.1. The time dependence behavior displayed in Figure 3.1 is also complex and strongly suggests that multiple processes are involved between binding of the metal cation and structural
rearrangements of the protein. The complexity can be examined in more detail by looking at changes in this behavior as a function of bicarbonate concentration.

![Graph showing binding of Cr³⁺ to apoconalbumin over time](image)

**Figure 3.1** Binding of Cr³⁺ to apoconalbumin as a function of time in 0.1M HEPES, pH 7.4 with ambient HCO₃⁻.

Given that (bi)carbonate is an important synergistic anion in the binding of Cr³⁺ to chromium, the rate of binding of Cr³⁺ to transferrins would be expected to be quite sensitive to the concentration of bicarbonate. The bicarbonate concentration of human blood plasma is approximately 25 mM. In comparison, the pH of the albumen of a fresh egg ranges from about 7.6 to 8.5; the albumin of an egg having a pH of 7.5 has been reported to have a bicarbonate concentration of 0.0528 g/L [15], which corresponds to slightly under 1 mM HCO₃⁻. The effects of changing the HCO₃⁻ concentration on the rate of Cr³⁺ binding to conalbumin are evident in Figure 3.2. As the concentration of HCO₃⁻ is increased from ambient to 26 mM, the rate of chromium binding increases until an apparent equilibrium is reached within 30 minutes for HCO₃⁻ concentrations of 15 and 25 mM. These increase in extinction coefficient with time can
readily be fit for [HCO$_3^-$] = 25, 15, and 5 mM to a single exponential rise to a constant value yielding apparent rate constants of 0.246±0.001, 0.159±0.001, and 0.0358±0.0001 min$^{-1}$, respectively. The curve at ambient HCO$_3^-$ can likewise be fit giving an apparent rate constant of 0.00122±0.00001 min$^{-1}$. However, notice that the extinction coefficients fall far short of the expected ~36,000 cm$^{-1}$M$^{-1}$ as described above. The slope of these curves do not actually approach zero at the end of two hours as suggested by Figure 3.2 but continue to increase with time in a similar but less dramatic fashion than the curve for ambient HCO$_3^-$ concentration in Figure 3.1. Thus, Cr$^{3+}$ binding would appear to be rapid and accelerated at increasing HCO$_3^-$ concentrations and followed by a slower conformational change in the protein resulting ultimately in a change in $\varepsilon_{245}$ of ~35,000 cm$^{-1}$M$^{-1}$, although this conformational change is also accelerated by increasing HCO$_3^-$ concentrations. This extent of this rapid initial rise in the extinction coefficient upon Cr$^{3+}$ binding appears to be very dependent on the conformation of the apoprotein. For the data in Figure 3.2, solutions containing conalbumin were prepared immediately before use. Delays in time between preparation and use result in decreases in the rise in the extinction coefficient and in difficulties in maintaining reproducibility from experiment to experiment; this presumably must be explained by changes with time in the apoprotein conformation before Cr$^{3+}$ addition. If one uses the assumption of Tan and Woodward [14] that deprotonation of a tyrosine residue is accompanied by $\Delta\varepsilon_{245}$ of ~10,000 cm$^{-1}$M$^{-1}$, then the change in the extinction coefficient at 245 nm in the first two hours for 5, 15, and 25 mM HCO$_3^-$ of ~22,000 cm$^{-1}$M$^{-1}$ could suggest that binding of each Cr$^{3+}$ is accompanied by ionization of one tyrosine, followed by a slow ionization of the second tyrosine of each binding site. This is an area that requires further research.
Figure 3.2 Binding of Cr$^{3+}$ to apoconalbumin as a function of time in 0.1M HEPES, pH 7.4 with ambient HCO$_3^-$ (squares), 5 mM HCO$_3^-$ (large circles), 15 mM HCO$_3^-$ (small circles), and 25 mM HCO$_3^-$ (triangles).

That this initial increase is extinction coefficient arises from binding of two Cr$^{3+}$ is readily shown by titrating apoconalbumin with chromic ion at pH 7.4 and 25 mM HCO$_3^-$ (Figure 3.3). Solution of apochromodulin and chromic ions were allowed to sit for 60 minutes before ultraviolet spectra were collected. The figure suggests that approximately two chromic ions are bound. Additionally, the initial slope for the increase in $\varepsilon_{245}$ as a function of added Cr$^{3+}$ gives a $\Delta \varepsilon$ of $1.14 \times 10^4$ cm$^{-1}$M$^{-1}$ per Cr$^{3+}$ or $2.28 \times 10^4$ cm$^{-1}$M$^{-1}$ for 2 Cr$^{3+}$, again well short of
~35,000 cm$^{-1}$M$^{-1}$. The binding appears to be quite tight as the curves can be fit by two straight lines without appreciable curvature near the intersection point.

![Graph showing extinction coefficient vs. Cr:Tf ratio](image)

**Figure 3.3** Titration of apoconalbumin with Cr$^{3+}$ in 25 mM HCO$_3^-$, 0.1 M HEPES, pH 7.4. The time between additions of Cr$^{3+}$ was 3 hours.

The effective thermodynamic binding constants for chromium conalbumin have been determined previously [6, 8]. In both studies, the addition of chromic ions to apoconalbumin at pH 7.4 in 0.1 M HEPES buffer and ambient HCO$_3^-$ was monitored by following the enhancement of the intensity of the ultraviolet absorption bands. The value of $\Delta \varepsilon$ increased rapidly upon the initial additions of chromium but rapidly levels off after the addition of approximately 1.5 chromic ions, indicating the chromium was occupying both metal binding sites. In neither study could the conalbumin be saturated with two equivalents of Cr by adding up to 3.5 equivalents of Cr per transferrin molecules, although saturation was approached at a Cr:transferrin ratio of 6:1 in Ref. 16. $\Delta \varepsilon_{Cr}$ was determined by the slope of the linear portion of a plot of $\Delta \varepsilon_{Cr}$ vs. amount of added Cr at low Cr:transferrin ratios (by assuming the extinction
coefficients for both binding sites were equivalent) was found to be $7.94 \times 10^3 \text{ cm}^{-1}\text{M}^{-1}$ at 245 nm (or $15.8 \times 10^3 \text{ cm}^{-1}\text{M}^{-1}$ for 2 Cr$^{3+}$) [16] or $17.5 \times 10^3 \text{ cm}^{-1}\text{M}^{-1}$ at 257 nm (or $35.0 \times 10^3 \text{ cm}^{-1}\text{M}^{-1}$ for 2 Cr$^{3+}$) [18], a significant discrepancy. In the former study, absorbance was monitored until “equilibrium” was reached (vide infra); in the latter, spectra were measured every least 30 minutes after Cr addition under the absorbance was constant. Neither indicates how much time was required to reach equilibrium. The low extinction coefficient reported in ref. 16 is a result of the researchers confusing the linear portion of the ambient HCO$_3^-$ concentration curve corresponding to the apparent equilibrium described above with a true equilibrium state. The researchers also did not use the apochromodulin solutions immediately upon use, resulting in a lower value of $\Delta \varepsilon$ than reported in the current work.

Fitting the data gave effective binding constants at ambient bicarbonate concentration of $K_1 = 1.42 \times 10^{10} \text{ M}^{-1}$ and $K_2 = 2.06 \times 10^5 \text{ M}^{-1}$ [6] or $K_1 = 1.2 \times 10^{13} \text{ M}^{-1}$ and $K_2 = 4.5 \times 10^5 \text{ M}^{-1}$ [8] when $K_1 = [\text{Cr-Tf}]/[\text{Cr}][\text{Tf}]$ and $K_2 = [\text{Cr}_2\text{-Tf}]/[\text{Cr}][\text{Cr}\text{-Tf}]$ where Tf represents transferrin and [Cr] represents free Cr$^{3+}$. This gives the overall effective binding constant $K = K_1 \times K_2 = [\text{Cr}_2\text{-Tf}]/[\text{Cr}][\text{Tf}]$ to be $2.92 \times 10^{15} \text{ M}^{-2}$ [16] or $5.4 \times 10^{18}$ [18] for the equilibrium

$$2 \text{ Cr}^{3+}(\text{aq}) + \text{Tf}(\text{aq}) \leftrightarrow \text{Cr}_2\text{-Tf}(\text{aq}) \quad \text{(Eqn. 1)}$$

Both studies determined similar values for $K_2$ while values for $K_1$ differ appreciably.

A number of explanations for the differences present themselves. First, the researchers in Ref. 16 determined the binding constants for Cr$^{3+}$ binding to the initial conformation of apochromodulin present in fresh solutions and that potentially leads to only the rapid ionization of one tyrosine residue per binding site. In the latter study, the value of $K_1$ was determined in the presence of a chelating agent (Iminodiacetic acid, IDA - although IDA was never identified, the ligand was probably iminodiacetate) [8]. The use of the competing ligand could potentially provide a better measure of the tight binding constant, which might have been underestimated in the earlier study.
Based on the acidity of Cr\(^{3+}\), its first binding constant, \(K_{1}^{*}\), to human serum transferrin has been estimated to be on order of \(10^{17}\) (and within the range \(10^{15} - 10^{19}\)) [16]. This \(K_{1}^{*}\) is the bicarbonate-independent constant that can be calculated from

\[
\log K_{1}^{*} = \log K_1 + \log a_c \quad (\text{Eqn. 2})
\]

where \(a_c\) is the fractional saturation of the apotransferrin-binding sites with bicarbonate and is given by

\[
a_c = K_c[HCO_3^-]/\{1 + K_c[HCO_3^-]\} \quad (\text{Eqn. 3})
\]

where \(K_c\) is the binding constant of bicarbonate with transferrin [16]. Using the literature value of \(K_c\) [17], the value of \(\log a_c\) at ambient bicarbonate concentration (pH 7.4) is \(-0.01\) (ambient bicarbonate concentration at pH 7.4 is \(\sim 0.2\) mM [11]); hence, effective binding constant is essentially equal to the bicarbonate-independent binding constant under ambient conditions. Both values for \(K_1\) for the conalbumins are below the estimated value for \(K_1\) for human serum transferrin.

To resolve the discrepancies, apoconalbumin was titrated with Cr\(^{3+}\) at pH 7.4 with varying ratios of Cr\(^{3+}\):IDA. The absorbance at 245 nm was measured after 3 hours and after 3 days. The results are shown in Figure 3.4. The ability of IDA to compete for binding Cr\(^{3+}\) is readily observed in the Figure 3.4 as the absorptivity drops as the ratio of IDA:Cr\(^{3+}\) increases.
For the 20:1 IDA:Cr curves, the extinction coefficients plateau at a value of ~12,000 cm\(^{-1}\)M\(^{-1}\) whether the reaction is allowed to go three days or three hours; (in fact, the titration curves for 20:1 IDA:Cr are nearly superimposable for the 3-hour and 3-day experiments). This suggests that only one chromic ion is bound by the protein so that \(K_1\) can be determined. Using the following relationships where \(L\) is the dianion of IDA

\[
[Cr]_{total} = [Cr] + [CrL] + [Cr-Tf] \quad (Eqn. \ 4)
\]

\[
K_L = [CrL]/[Cr] \times [L] \quad (Eqn. \ 5)
\]

then

\[
[Cr] = [Cr-Tf]\left(\frac{1 + K_L[L]}{[Tf]K_1^+} + 1\right) \quad (Eqn. \ 6).
\]
Fitting the data for the 3-day time period and assuming a value of $K_L$ of $5.4 \times 10^9$ [18], yields a value for $K_1$ of $6.5 \pm 4.4 \times 10^{10}$, reasonably similar to the value in Ref. 16 but $10^3$ smaller than the value reported in Ref. 18 using IDA. Ref. 18 did not provide the value of the $K_L$ utilized or its source. (The current fit assumes that $K_L$ does not vary appreciably from its reported value measured at an ionic strength of 0.1 M and a temperature of 20 °C [18].) Thus, given that the value of $K_1$ does not differ appreciable when measured in the presence or absence of a competing chelating ligand, the values of $K_1$ and $K_2$ are, thus, on the order of $10^{10}$ and $10^5$, giving an overall apparent $K$ of $10^{15}$. Counterintuitively, the binding constant also does not appear to change significantly as a result of the conformational change that results in the extinction coefficient of the Cr$_2$-Tf changing from $\sim 2.5 \times 10^4$ cm$^{-1}$M$^{-1}$ to $\sim 3.5 \times 10^4$ cm$^{-1}$M$^{-1}$.

All these studies show that the difference in the binding constant for the two metal-binding sites is consistent with previous EPR studies with Cr$^{3+}$ and human serum transferrin that demonstrated Cr$^{3+}$ binds preferentially to one binding site labeled the B site [14], shown to be the N-terminal site [19]. Iron added to Cr$_2$-transferrin displaces Cr selectively from the A, C-terminal site [20]. Thus, $K_1$ would appear to correspond to the N-terminal metal-binding site, assuming that conalbumin is similar to transferrin in this regard. This would be in contrast to the proposal of Li et al. [8] that $K_1$ is the C-terminal site. The researchers titrated apoconalbumin and N-terminal monoferric conalbumin with Cr$^{3+}$ and monitored the fluorescence intensity of the maximum emission peak (wavelength not given). At low Cr:transferrin ratios, the two titration curves were similar suggesting binding to the C-terminal site; however, while suggestive, these data are not as clear cut as suggested. Recent studies have shown that excess iron can prevent Cr loading of transferrin while Cr will displace a portion of the iron from holotransferrin [7]. The difference of several orders of magnitude in the two binding constants is in contrast to the need to only use one rate constant to fit the time-
dependence of the binding of Cr$^{3+}$ to conalbumin. Attempts to fits the binding curves to two rate constants failed as the resulting rate constants were underdetermined by the data.

Correspondingly, EPR has been used in this study to examine the binding of Cr$^{3+}$ to conalbumin. Unfortunately, EPR spectra could not be collected at 5 mM or greater concentrations of bicarbonate because of the insolubility of Cr$^{3+}$ complexes of bicarbonate. Hence, Cr$^{3+}$ was added to solutions of conalbumin or N-terminal monoferric conalbumin at pH 7.4 in 0.1 M HEPES buffer, and the product was allowed to incubate for 3 days before the solutions were rapidly frozen by submersion into liquid N$_2$. (The preparation of monoferric transferrins is described below). The results are shown in Figure 3.5. The addition of one equivalent of Cr$^{3+}$ to conalbumin or to N-terminal monoferric conalbumin results in the generation of virtually identical spectra of the same intensity, indicating the tight binding of a single Cr$^{3+}$ ion in each case. When two equivalents of Cr$^{3+}$ are added, the intensity of the EPR signal increases, although it does not quite double; additionally an EPR signal originating from [Cr(H$_2$O)$_6$]$^{3+}$ is found at $g\sim2$; hence, at ambient carbonate concentration after 3 days, the binding of Cr$^{3+}$ is not quite stoichiometric. The EPR spectra are similar to those reported for transferrin 30 and 100% loaded with Cr$^{3+}$, where both spectra are qualitatively similar except for the increase in intensity associated with the increased Cr content [1]. If samples are frozen only 3 hours after 3 addition of Cr$^{3+}$, qualitatively similar spectra are obtained, although the intensity is much lower and a more intense signal from free Cr at $g\sim2$ is present. Thus, Cr-binding requires days rather than hours to reach equilibrium consistent with the time-dependent ultraviolet spectra used to generate Figure 3.1.
Figure 3.5 Low field EPR spectra of solutions of conalbumin with 1 (dashed line) or 2 (solid line) equivalents of added Cr$^{3+}$ or N-terminal monoferric conalbumin with 1 equivalent of Cr$^{3+}$ (dotted line).

The EPR spectra contain 3 distinct features in the region around g~5, almost identical to the spectrum of Cr$_2$-human serum transferrin [1]. The features been assigned to particular Cr-binding sites. The N-terminal and tighter binding site gives rise to the signals at g = 5.08 and 5.66, while the C-terminal site gives to the feature at g = 5.42 (and also a feature at g~2 that is not shown) [1, 19]. For human serum transferrin Cr has been shown to selectively bind at low pH (pH 5.9) to the N-terminal site [4], while Co$^{3+}$ [1] or Fe$^{3+}$ [20] will selectively displace Cr$^{3+}$ from the C-terminal site at near neutral pH. When two equivalents of Co$^{3+}$ (Co$^{2+}$ with 1 % H$_2$O$_2$ [1]) is added to the conalbumin stored for 3 d in the presence of 2 equivalents of Cr$^{3+}$, the signal at g=5.42 disappears (Figure 3.6), demonstrating that the signals for
conalbumin can be readily assigned to the same binding sites as done previously for human serum transferrin. This also indicates that the environments of Cr\(^{3+}\) bound to conalbumin and to human serum transferrin are extremely similar. Additionally, the two Cr\(^{3+}\) binding sites can be distinguished in conalbumin as they can in human serum transferrin.

Figure 3.6 Low field EPR spectra of solutions of conalbumin with 2 equivalents of Cr\(^{3+}\) before (dotted line) and after (solid line) the addition of 1 equivalent of Co\(^{3+}\). The arrow indicates the loss of the feature from selective displacement of Cr\(^{3+}\) from one of the metal binding sites.

3.3.2 Human serum transferrin

As shown in Figure 3.7 in the presence of 25 mM bicarbonate, the change in extinction coefficient at 245 nm is linear as the protein is titrated with Cr\(^{3+}\) up until two equivalents of Cr\(^{3+}\) are added. (The solutions of transferrin and chromic ions were allowed to sit one hour
before the absorbance was measured). The slope of this portion of the curve, $\Delta \varepsilon_{\text{Cr}}$, is $1.30 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. Thus, the binding is stoichiometric, and the data cannot be fit to equilibrium expressions to derive effective values of $K_1$ and $K_2$.

![Graph](image)

**Figure 3.7** Titration of human serum apotransferrin with Cr$^{3+}$ in 25 mM HCO$_3^-$, 0.1 M HEPES, pH 7.4. The time between additions of Cr$^{3+}$ was 3 hours.

The binding of Cr$^{3+}$ is also extremely rapid as shown in Figure 3.8. In the presence of 25 mM bicarbonate, equilibrium is achieved within about 15 minutes. As the [HCO$_3^-$] is decreased from 25 mM to 15 mM and then to 5 mM, the rate of binding is decreased. The binding is also clearly biphasic. Fitting the data to the expression,

$$y = a(1-e^{-bx}) + c(1-e^{-dx}) \quad (\text{Eqn. 7})$$

where $a$ and $c$ are the extinction coefficients for the two Cr binding sites and $b$ and $d$ are the respective rate constants for binding, gives rates constants of $3.16 \pm 0.13 \text{ min}^{-1}$ and $0.189 \pm 0.003 \text{ min}^{-1}$ at 25 mM HCO$_3^-$, $2.24 \pm 0.10 \text{ min}^{-1}$ and $0.117 \pm 0.002 \text{ min}^{-1}$ at 15 mM HCO$_3^-$, $1.35 \pm 0.04 \text{ min}^{-1}$ and $0.0479 \pm 0.0010 \text{ min}^{-1}$ at 5 mM HCO$_3^-$, and $0.814 \pm 0.056 \text{ min}^{-1}$ and $0.00901 \pm 0.004 \text{ min}^{-1}$ at ambient HCO$_3^-$. (For 25, 15, and 5 mM HCO$_3^-$, the values of $a$ and $c$ were...
forced to be equal; at ambient $\text{HCO}_3^-$, they were allowed to vary giving values of $5.37 \times 10^3 \pm 1.8 \times 10^2$ and $4.94 \times 10^3 \pm 1.7 \times 10^2$ cm$^{-1}$M$^{-1}$, virtually identical within error).

**Figure 3.8** Binding of $\text{Cr}^{3+}$ to human serum apotransferrin as a function of time in 0.1M HEPES, pH 7.4 with ambient $\text{HCO}_3^-$ (squares), 5 mM $\text{HCO}_3^-$ (large circles), 15 mM $\text{HCO}_3^-$ (small circles), and 25 mM $\text{HCO}_3^-$ (triangles). Lines represent fits of the time dependence of the $\text{Cr}$ binding to apotransferrin to the equation $\Delta \varepsilon = a(1-e^{-bx}) + c(1-e^{-dx})$ where $a$ and $c$ are the extinction coefficients for each metal binding and $b$ and $d$ are the rate constants.

Also notable in Figure 3.8 is that the extinction coefficient at ambient $\text{HCO}_3^-$ concentration appears to level off at a value, $\sim 1.0 \times 10^4$ M$^{-1}$ cm$^{-1}$, well below the values at equilibrium for the other concentrations of $\text{HCO}_3^-$. However, this turns out to be only an intermediate species, probably corresponding to a less stable, but more rapidly forming conformation of the protein than the final conformation. This can better be elucidated by following the behavior of $\text{Cr}$ binding at ambient $\text{HCO}_3^-$ concentrations for longer periods of time (Figure 3.9). Over the course of 48 hours, the protein appears to change conformation
resulting in a form possessing an extinction coefficient ($2\Delta \varepsilon_{Cr}$) similar in value to the human serum transferrin samples treated with 2 equivalents of Cr in the presence of 5 mM, 15 mM, and 25 mM HCO$_3^-$.

Thus, at ambient HCO$_3^-$ concentrations, human serum transferrin appears to behave in a similar fashion to conalbumin. The rapid rise in extinction coefficient to a value of $\sim 2.5 \times 10^4$ M$^{-1}$ cm$^{-1}$ is only observed in the presence of added HCO$_3^-$ if Cr$^{3+}$ is added to the transferrin-containing solutions immediately after their preparation. Otherwise, behavior intermediate between that observed for the added HCO$_3^-$ conditions and the ambient conditions is observed (vide supra, data not shown).

**Figure 3.9** Binding of Cr$^{3+}$ to human serum apotransferrin as a function of time in 0.1M HEPES, pH 7.4 with ambient HCO$_3^-$.

The binding sites can potentially be identified from the rate constants using human serum transferrin labeled in the N-terminal only or C-terminal only (Figure 3.10). Fortunately, both have been well described in the literature [11, 12] and are readily prepared. Similarly, both N-terminal [8] and C-terminal [21] monoferric conalbumin have been prepared previously. The addition of one equivalent of Cr$^{3+}$ to each results in a rapid change in the extinction coefficient until apparent equilibrium is reached. (Note that the extinction coefficients reached at the
apparent equilibrium for both substituted forms of the protein are different. The N-terminal monoferric transferrin was generated *in situ*, while the C-terminal monoferric transferrin was purified by column chromatography. Thus, the time between dissolution of the apotransferrin in buffer and the addition of chromium differed appreciably, leading to these discrepancies as described above. The absorptivities could also be affected by the binding of iron. Rate constants were determined using the single exponential, \( y = a(1-e^{bx}) \) where \( b \) is the rate constant. The rate constants were \( 0.200 \pm 0.001 \text{ min}^{-1} \) for the C-terminal monoferric transferrin and \( 0.587 \pm 0.010 \text{ min}^{-1} \) for the N-terminal monoferric transferrin. The value for the C-terminal monoferric transferrin (and, thus, for binding to the C-terminal metal binding site) is very similar to the slower rate constant for \( \text{Cr}^{3+} \) binding to apotransferrin, \( 0.189 \pm 0.003 \text{ min}^{-1} \). Hence, \( \text{Cr}^{3+} \) appears to bind more rapidly to the C-terminal metal binding site of transferrin; (the limitations because of the conformational effects prevent a more definitive determination).

**Figure 3.10** Binding of \( \text{Cr}^{3+} \) to human serum monoferric transferrins as a function of time in 0.1M HEPES, pH 7.4 with 25 mM \( \text{HCO}_3^- \). Small circles - N-terminal monoferric transferrin; large circles - C-terminal monoferric transferrin.
While the above studies demonstrate that Cr\textsuperscript{3+} binds readily to apotransferrin in the presence of 25 mM HCO\textsubscript{3}\textsuperscript{-}, the blood plasma is a more complicated system that just a buffered bicarbonate solution. Thus, the binding of Cr\textsuperscript{3+} to apotransferrin was followed in the presence of 25 mM HCO\textsubscript{3}\textsuperscript{-} and the additional species most likely to bind chromium at their concentrations in normal blood plasma (1.0 mM phosphate, 1.5 mM lactate, and 0.10 mM citrate [22]). The results are shown in Figure 3.11. The change in absorptivity as a function of time is nearly identical the curve for the binding of Cr\textsuperscript{3+} in when only HCO\textsubscript{3}\textsuperscript{-} was present (in Figure 3.8). The phosphate, lactate, and citrate have one subtle effect, after the curve in Fig. 3.11 reaches a maximum at ~25,000 cm\textsuperscript{-1}M\textsuperscript{-1}, the absorptivity declines slightly before leveling. This suggests that Cr\textsuperscript{3+} under these conditions binds rapidly to human serum apotransferrin before reaching an apparent equilibrium as a small amount of the Cr\textsuperscript{3+} binds more slowly to phosphate, lactate, and citrate. Similar studies using the phosphate, lactate, and citrate one at a time indicate that citrate is primarily responsible for this effect (data not shown).

\textbf{Figure 3.11} Binding of Cr\textsuperscript{3+} to human serum apotransferrin as a function of time in 0.1M HEPES, pH 7.4 with 25 mM HCO\textsubscript{3}\textsuperscript{-}, 1.5 mM lactate, 1.0 mM phosphate, and 0.10 mM citrate.
3.4 Conclusions

*In vivo* studies have previously established that transferrin, the major iron transport protein in the blood, also transports trivalent chromium, despite recent *in vitro* studies suggesting the binding of chromic ions to apotransferrin should be too slow to be biologically relevant. However, in the presence of 25 mM (bi)carbonate, the concentration in human blood, and other potential Cr\textsuperscript{3+}-binding species present in blood plasma, chromic ions bind rapidly and tightly to transferrin. A discrepancy in the literature existed over the value for the apparent binding constant of Cr\textsuperscript{3+} to conalbumin, egg white transferrin; this discrepancy has been resolved.
REFERENCES

CHAPTER 4
THE EFFECTS OF THE GLYCATION OF TRANSFERRIN ON CHROMIUM BINDING
AND THE TRANSPORT AND DISTRIBUTION
OF CHROMIUM IN VIVO

4.1 Introduction

Pharmacologically relevant doses of Cr can generate improvements in insulin sensitivity and blood cholesterol levels in animals with stresses on the glucose and lipid metabolism systems, most notably in rodent models of type 2 diabetes (for a review, see Ref. 1). The mechanism for these effects at a molecular level is not clearly established; but, a role for transferrin in Cr transport associated with insulin action has been proposed. Cr has been proposed to be an artificial second messenger in insulin action [2]. Increases in blood levels of insulin and subsequent activation of the insulin signaling pathway result in a movement of Cr from the bloodstream to tissues where the Cr in turn appears to increase insulin sensitivity by interacting with the insulin signaling machinery in cells [2].

Transferrins are a class of proteins (molar mass ~80 kDa) that reversibly bind two equivalents of metal ions [3]. The rate at which Cr$^{3+}$ binds to transferrin, the rate of the conformation changes associated with Cr binding, and the stability of Cr-transferrin have recently been investigated [4-7]. In vitro studies had suggested that the binding of Cr to transferrin was slow. For example, the generation of Cr$_2$-transferrin for spectroscopic studies generally used samples of transferrin that had been allowed to come to equilibrium with Cr$^{3+}$ ions over the course of up to two weeks to guarantee a stoichiometric amount of Cr$^{3+}$ binding. This brought about question as to how transferrin could be responsible for Cr$^{3+}$ transport in vivo if Cr$^{3+}$ binding actually requires days or weeks to achieve equilibrium given the half-life
of transferrin in serum and that of transferrin-bound iron are on the order of hours [8, 9]. However, in the presence of 25 mM (bi)carbonate, the concentration in human blood, and other potential Cr³⁺-binding species present in blood plasma, two chromic ions bind rapidly and tightly to apotransferrin [7]. Yet, conformation changes associated with metal binding are slow for Cr binding compared to Fe binding [7].

Under conditions of high blood glucose concentration, as in subjects with diabetes, glucose can bind chemically and irreversibly (glycation) to several proteins in the bloodstream, including transferrin. This chemical modification can alter the properties and functions of these biomolecules. The effects of glycation of transferrin on iron transport have recently been a matter of concern [10-13]. Glycation of transferrin alters how tightly the protein binds iron [10, 11] and may alter the conformation of diferric transferrin, presumably changing its ability to deliver the iron to tissues [13]. The degree of glycation has been reported to increase from ~1 to 2 % in healthy subjects to ~5 % in diabetics [14], while this is reported to increase from ~ 4 % in healthy children to ~ 11 % in type 1 diabetic children [15]. Given that Cr(III) complexes have been proposed as drugs to increase insulin sensitivity, particularly in type 2 diabetic subjects [1], understanding the ability of glycated transferrin to bind and transport Cr is important in determining the appropriate amount of Cr necessary for potentially being used as a drug to treat insulin insensitivity and its symptoms. Herein are described studies examining the binding of Cr³⁺ to glycated serum transferrin and the transport of Cr in vivo by glycated transferrin.

4.2 Materials and methods

4.2.1 Materials

Iron-free human serum transferrin and insulin (bovine, zinc) were obtained from Aldrich (St. Louis, MO). Iron-free rat serum transferrin was obtained from Lee Biosolutions (Maryland Heights, MO). ⁵¹CrCl₃ was obtained from Perkin Elmer (Waltham, MA). Doubly deionized water was used throughout. All reagents were used as received unless otherwise noted.
Plasticware was used whenever possible. All Cr-binding studies were performed in HEPES buffer, 0.1 M, pH 7.4 in polypropylene centrifuge tubes. Buffer pH was adjusted by the addition of NaOH to the free acid form of HEPES. For solutions containing KHCO₃, pH was readjusted to 7.4 after addition of KHCO₃ by addition of HCl. Cr³⁺ solutions were prepared by using CrCl₃·6H₂O. Apotransferrin and apoconalbumin concentrations were determined using the extinction coefficient (ε = 9.12 x 10⁴ M⁻¹ cm⁻¹) at 280 nm [16]. Solutions containing added bicarbonate were prepared immediately before use. All kinetics results are presented as the average of at least triplicate experiments. Error bars in figures represent standard deviation.

4.2.2 Instrumentation

Ultraviolet-visible spectra were obtained by using a Cary (Agilent, Santa Clara, CA) 100 or Beckman Coulter (Brea, CA) DU800 UV-visible spectrophotometer. Electrospray ionization (ESI) mass spectral studies were performed on a Bruker (Billerica, MA, USA) HCT Ultra high capacity ion trap mass spectrometer. Gamma counting was performed on a Packard Cobra II auto-gamma counter (Meriden, CT, USA). Continuous wave (CW) EPR were measured on a Bruker (Billerica, MA) ELEXSYS E540 X-band spectrometer with an ER 4102 ST resonator. CW spectra were measured at ~9.43 GHz using 100 kHz magnetic field modulation with an amplitude of 30 gauss with a microwave power of 21.1 mW. After the appropriate binding time, glycerol was added to the samples to 15% v/v, and the solutions were transferred into 4 mm quartz EPR tubes and frozen in liquid nitrogen. EPR measurements were made with samples immersed in liquid nitrogen in a quartz insertion dewar.

4.2.3 Chromium-binding to transferrin

Chromium-binding studies were performed as described previously [7] and were performed at least in triplicate. Binding of Cr³⁺ to transferrin was monitored at 245 nm. Studies were initiated by the addition of Cr³⁺. Solutions were continuously stirred by using a Starna (Atascadero, CA) “Spinette” electronic cell stirrer. Data analysis, calculation of averages and
standard deviations and fitting of curves to the appropriate equations, was performed by using SigmaPlot 11 (SPSS, Inc., Chicago, IL). The iterative curve fitting algorithm of SigmaPlot 11 uses the Marquardt-Levenberg algorithm to find the parameters of the independent variables that provide the best fit between the data and the equation.

4.2.4 Glycation of transferrin

Glycation of transferrin was performed following the procedure of van Campenhout, et al. [11]. Briefly, human or rat transferrin at a concentration of ~5 g/L was incubated in sodium phosphate buffer (0.1 M, pH 7.4) containing 1.0 M glucose for 14 days at 37 °C. As a control for any effects from incubation for 14 days at 37 °C, a sample of transferrin was incubated in a similar fashion using buffer without added glucose. The glucose was removed from the transferrin by cycles of dilution with buffer lacking glucose, followed by concentration via ultrafiltration using a Millipore (Billerica, MA) Biomax 30 kDa membrane.

For ESI mass spectral studies, the solutions of human serum transferrin that had been kept at 37 °C for two weeks in the presence and absence of glucose were desalted by cycles of dilution with doubly deionized water followed by concentration by ultrafiltration using a Biomax 30 kDa membrane membrane.

The extent of glycation of human serum transferrin was measured colorimetrically by the nitroblue tetrazolium method (which assays fructosamine content) using a kit from Pointe Scientific (Canton, MI).

Samantha L. Dyroff assisted me in the preparation of glycated transferrin.

4.2.5 Rats

Twelve male Sprague-Dawley (SD) rats were approximately 6 weeks old and from Charles River Laboratories (Wilmington, MA). The rats were allowed to acclimate for over two weeks before the onset of the study. The rats were allowed to feed *ad libitum* on a commercial rat food and tap water. The rats, thus, received a Cr-sufficient diet.
randomly divided into four groups of 3 rats each: 1) transferrin, 2) transferrin and insulin, 3) glycated transferrin, and 4) glycated transferrin and insulin. Rats were weighed and injected with an aqueous solution (150 μL) containing 0.90 mg of rat $^{51}$Cr-transferrin or 0.28 mg of rat glycated $^{51}$Cr$_2$-transferrin. (The solutions of Cr-containing transferrins were concentrated and unbound Cr removed by ultrafiltration using a Biomax 30 kDa membrane.) The different quantities of $^{51}$Cr-transferrin injected result from different yields of the isolated labelled transferrins; to provide the best chances of observing $^{51}$Cr in all the tissue and fluids, the maximum quantity available was utilized. For rats requiring insulin (bovine, zinc), the solution also contained 25 IU insulin. After injection, rats were transferred into metabolic cages for 2 h for collection of urine and feces. After 2 h, the rats were sacrificed by carbon dioxide asphyxiation. The spleen, pancreas, testes, epididymal fat, kidneys, liver, blood, muscle (musculus triceps surae from right hind leg), and heart were harvested, placed into pre-weighed polypropylene centrifuge tubes, and weighed. All body fluids and tissues were then frozen in liquid N$_2$ and stored at $\sim$-20°C. Because of a technical problem (an imperfect injection), data for the glycated transferrin and insulin group reflect the average of only two rats. The total $^{51}$Cr content of the blood and muscle were calculated assuming that the blood constituted 6% of the rats’ body mass and muscle constituted 30% of the body mass. All work with rats was approved by The University of Alabama Institutional Animal Care and Use Committee. The use of radioisotopes and animal tissue was approved by the institution’s Radiation Safety Committee and Biological Safety Committee, respectively.

Samantha L. Dyroff assisted me to harvest rat tissues.

4.2.6 Statistics

For the rodent studies, data for each tissue or body fluid were analyzed by one-way analysis of variance (ANOVA) followed by a Bonferroni all pairwise multiple comparison $t$-test procedure to determine specific differences ($P \leq 0.05$) using SigmaPlot 11. Data are presented in Figure 4.7 as means ± standard deviations (SD).
4.3 Results and discussion

4.3.1 Glycation

To test whether glycation was successful, the ability of the glycated transferrin to bind Fe$^{3+}$ and the extent of glycation were examined. Glycation of transferrin has been reported to reduce the extent of binding of ferric ion [10, 11]. Figure 4.1 displays the UV/visible spectra of human serum transferrin after storage for 2 weeks at 37 °C in the presence and absence of glucose collected 1 h and 24 h after the addition of two equivalents of Fe$^{3+}$ (in 0.1 M sodium phosphate buffer, pH 7.4 containing 25 mM bicarbonate); Fe was added as ferrous ammonium sulfate. The extent of Fe$^{3+}$ binding to transferrin can readily be determined by the intensity of an intense tyrosine-to-Fe$^{3+}$ charge transfer band in the visible region ($\lambda_{\text{max}}$ 465 nm, $\varepsilon$ 3,750 M$^{-1}$ cm$^{-1}$) [17,18], which gives the Fe-containing protein a distinct salmon color. At both time points, the absorbance at ~465 nm is greater for the non-glycated transferrin. After 24 hours, the non-glycated transferrin has an absorbance maximum at 467 nm with an extinction coefficient of 3,780 M$^{-1}$ cm$^{-1}$, virtually identical to the literature value for Fe(III)$_2$-transferrin. Thus, the storage at 37 °C for two weeks appears to have no effect on the ability of the protein to binds its full complement of iron. Glycation dramatically affects the Fe$^{3+}$ binding ability of the protein; after 24 h, the glycated protein bound only 44% as much Fe$^{3+}$ as the non-glycated protein (assuming glycation has no effect on the extinction coefficient).
Figure 4.1 UV/visible spectra of human serum transferrin (stored in the presence (glycated Tf) or absence (Tf) of glucose for two weeks at 37 °C) after 1 h or 24 h following addition of ferric ammonium sulfate.

ESI mass spectra of glycated human serum transferrin and similarly treated transferrin but in the absence of glucose revealed several multiply-charged species (Figure 4.2). For the non-glycated human transferrin, the molar mass reported for the protein based on its amino acid sequence [19] and assuming the presence of two identical asparagine-linked glycans [20] is 79,550 Da. Estela del Castillo Busto, et al. [21] found a molar mass of human serum transferrin of 79,561 Da using by ESI mass spectrometry. Also using this technique, Thevis, et al. [22] found a value of 79,562 Da. In the current study, the molar mass was found to be 79,568 Da. Thus, the experimental value for the non-glycated is in reasonable agreement with the expected value and similar to other values obtained by ESI MS.
Figure 4.2 ESI mass spectra of human serum transferrin (stored in the presence (bottom) or absence (top) of glucose for two weeks at 37 °C).

The features in the mass spectrum of the glycated transferrin are broader and possess more noise, suggesting that the glycated transferrin is a mixture of species at reasonably comparable concentrations. The average mass is estimated to be between 82,300 and 82,400 Da. Glycation, thus, results in an increase of the molar mass of 2,732 and 2,832 Da. Assuming
that all of the glycation reactions formed an Amadori product generated with the loss of one molecule of water (resulting in a mass increase per reaction of 162.1 Da) as observed by Silva, et al. [13], then approximately 17 sites have been glycated. Silva, et al. [13] using glycated transferrin generated under similar experimental conditions also observed the glycation of 12 lysine residues by analyzing tryptic digests of the glycated protein by ESI mass spectrometry. Given the discrepancy, the extent of glycation was measured chemically. The heat-stored transferrin was found to have 0.051±0.069 glycations sites while the glycated transferrin was found to have an average of 16.8±1.0 glycation sites. Thus, chemical analysis provides a result consistent with the mass spectral results.

4.3.2 Cr binding

The addition of Cr$^{3+}$ to serum transferrins spectrum results in the binding of Cr to the protein with a concomitant increase in the intensity of bands in the transferrin’s ultraviolet at ~245 and 295 nm; these changes arise from perturbing two tyrosine residues that become ligands for each of two bound Cr$^{3+}$ [7, 23]. This allows the binding of Cr to the protein to be readily monitored; (transferrin with bound Cr$^{3+}$ lacks intense charge transfer bands in the visible region as found for transferrin binding ferric ions). After being allowed to reach equilibrium for 40 h in buffer with ambient bicarbonate concentrations after the addition of Cr$^{3+}$, the extinction coefficient change for the binding of two Cr$^{3+}$ at the ultraviolet maxima of 245 nm is 38,743 ± 2,806 M$^{-1}$cm$^{-1}$ [23]. The time required to reach equilibrium does not reflect the amount of time required for Cr$^{3+}$ binding to reach equilibrium but the time required for the conformations of the Cr$_2$-transferrin to reach equilibrium [7]. The time to reach equilibrium can be reduced appreciably by the addition of bicarbonate, at least up to 25 mM HCO$_3^-$ (the concentration of HCO$_3^-$ in human blood) [7].

Titration of glycated human serum transferrin with Cr$^{3+}$ at pH 7.4 and 25 mM HCO$_3^-$ monitored at 245 nm indicates that glycated transferrin binds two Cr$^{3+}$ ions (Figure 4.3). The
binding of Cr is quite tight as the curve can be fitted to two straight lines without appreciable curvature near the intersection point. (The curve cannot appropriately be fit to an exponential rise to a constant). The initial slope for the increase in $\varepsilon_{245}$ as a function of added Cr$^{3+}$ gives a $\Delta\varepsilon$ of $1.73 \times 10^3$ M$^{-1}$cm$^{-1}$ per Cr$^{3+}$ or $3.46 \times 10^3$ M$^{-1}$cm$^{-1}$ per 2 Cr$^{3+}$, well short of ~35,000 M$^{-1}$cm$^{-1}$ per 2 Cr$^{3+}$. This indicates that the conformation of the Cr$_2$-transferrin differs appreciably from that of non-glycated human transferrin [7].

![Figure 4.3](image-url)

**Figure 4.3** Titration of glycated human serum transferrin with Cr$^{3+}$ in 25 mM HCO$_3^-$, 0.1 M HEPES, pH 7.4. Spectra were collected 24 h after Cr addition.

The difference in conformation between the glycated transferrin and freshly dissolved transferrin (as used in Ref. 15) does not simply arise from only glycation. The storage of transferrin for two weeks at 37 °C appears to be non-innocent. As shown in Figure 4.4, titration of human serum transferrin (stored for two weeks at 37 °C) with Cr$^{3+}$ at pH 7.4 and 25 mM HCO$_3^-$ monitored at 245 nm suggests that this protein also binds two equivalents of Cr$^{3+}$ (Figure 4.4). However, if the curve is fit with two straight lines (Figure 4.4 (top)), the lines intercept at a point corresponding to 1 Cr:protein, but the line required to fit the higher
Cr:protein ratios possesses a distinctly non-zero slope. This indicates that Cr binds tightly to one metal-binding site but not the other. To test this, the data were fit to the following function:

\[ y = a(1-e^{bx}) + c(1-e^{dx}) \]

where \( a \) and \( c \) are the extinction coefficient for the two binding sites and \( b \) and \( d \) are the respective binding constants. The best fit is obtained when \( a = 3.17 \times 10^3 \pm 1.40 \times 10^3 \text{ M}^{-1}\text{cm}^{-1} \), \( b = 1.7 \pm 0.8 \text{ Cr/ transferrin} \), \( c = 1.71 \times 10^4 \pm 3.97 \times 10^3 \text{ M}^{-1}\text{cm}^{-1} \), and \( d = 0.06 \pm 0.19 \text{ Cr/ transferrin} \) (\( R^2 = 0.989 \)) (Figure 4.4 (bottom)); however, the parameters for the weaker Cr binding site are poorly refined with large standard deviations, particularly for \( d \) (the binding constant for the second site).
Figure 4.4 Titration of human serum transferrin (stored for two weeks at 37 °C) with Cr$^{3+}$ in 25 mM HCO$_3^-$, 0.1 M HEPES, pH 7.4. Spectra were collected 24 h after Cr addition. (Top) data fit to two straight lines. (Bottom) data fit to two exponential growths to constant values.
The titration differs significantly from the previously reported titration of freshly dissolved human serum transferrin [7]. The binding of Cr to freshly dissolved transferrin is quite tight as the curve can be fit to two straight lines without appreciable curvature near the intersection point at 2 Cr:transferrin [7], similar to the curve for glycated transferrin, although with Δε per Cr³⁺ of 1.30 x 10⁴ M⁻¹cm⁻¹. Thus, two distinct differences exist in the titration curve for the heated transferrin. First, freshly dissolved transferrin binds two equivalents of Cr tightly, whereas the heated transferrin only binds one Cr tightly (the straight fit in Figure 4.4 (top) between 0 and 1 Cr:transferrin). Second, Δε per Cr³⁺ is 1.30 x 10⁴ M⁻¹cm⁻¹ for the fresh transferrin [7] compared to the value of 3.17 x 10³ M⁻¹cm⁻¹ for tighter Cr-binding site of the transferrin stored at elevated temperature and the value of 1.71 x 10⁴ M⁻¹cm⁻¹ for the weaker binding site. Storage at the elevated temperature seems to have a dramatic effect on one binding site by lowering the Cr-binding constant, while dramatically affecting the other site by lowering the extinction coefficient an order of magnitude. Hence, the transferrin stored at elevated temperature does not take the same conformation as the fresh transferrin, and consequently, the storage appears to affect the conformation of the apotransferrin. Curiously, the extinction coefficient for the tight binding site is only about double that of the binding sites in the glycated transferrin (3.17 x 10³ vs. 1.73 x 10³ M⁻¹cm⁻¹ per Cr³⁺), while the extinction coefficient for the weaker binding site is similar to those of freshly dissolved transferrin (1.71 x 10⁴ vs. 1.30 x 10⁴ M⁻¹cm⁻¹ per Cr³⁺, although any significant is difficult to ascertain given the magnitude of the standard deviation associated with this value). (Using the initial points in the titration of the heated transferrin gives an extinction coefficient for the tight binding site of 3.79 x 10³ M⁻¹cm⁻¹, within error of the value from the fit). Glycation actually appears to protect against prolonged heat treatment in terms of the ability of transferrin to tightly bind two equivalents of Cr³⁺.

The results for Cr³⁺ differ from previously reported results with Fe²⁺. Increasing glycation of transferrin has been shown to result in less Fe binding; however, the reduced
binding results from the formation of a roughly equal mix of monoferric transferrin with either the N-terminal or C-terminal metal-binding site occupied [11]. Neither metal-binding site appears to be selectively influenced. At near neutral pH, the binding constants for both binding sites of unglycated human serum transferrin for Fe\(^{3+}\) are approximately the same [25]. This is not the case for Cr\(^{3+}\), where the binding constants for Cr to non-glycated transferrin differ by 5 orders of magnitude [4, 7]. Thus, for Cr, precedent exists for differential binding of Cr between the two metal-binding sites, and this differential binding can be observed under appropriate conditions.

Chromium binding as a function of time was examined for glycated human serum transferrin and similarly treated transferrin but in the absence of glucose (Figures 4.5 and 4.6). At time zero, two equivalents of Cr\(^{3+}\) were added to the transferrins at pH 7.4 in 25 mM HCO\(_3^−\). For both glycated human serum transferrin and similarly treated transferrin but in the absence of glucose, Cr binds relatively rapidly leading initially to an increase in the protein’s extinction coefficient at 245 nm. For human serum transferrin not exposed to elevated temperature for two weeks, binding of Cr is relatively rapid; at pH 7.4 in 25 mM HCO\(_3^−\), the rate constant for Cr binding is 0.246 \(\pm 0.001\) min\(^{-1}\), although Cr binding is followed by slower conformational changes [7]. Rather than this increase approximately leveling off and slowing increasing with time as observed previously [7], an initial increase in \(\varepsilon_{245}\) rapidly spikes and is followed by complex behavior including reaching a minimum after the initial rise (Figures 4.5 and 4.6). This suggests that binding of Cr is rapid followed by slower conformational changes. The change in extinction coefficient for the heated, non-glycated transferrin is more than double in value that of the glycated transferrin at early times but is less than double at later times. Understanding what is happening as a function of time will require more detailed studies, although EPR studies suggest the transferrin may slowly take the conformation of untreated transferrin over time.
**Figure 4.5** Binding of Cr\(^{3+}\) to glycated human serum transferrin as a function of time in 25 mM HCO\(_3\)\(^-\), 0.1 M HEPES, pH 7.4.

**Figure 4.6** Binding of Cr\(^{3+}\) to human serum transferrin (stored for two weeks at 37 °C) as a function of time in 25 mM HCO\(_3\)\(^-\), 0.1 M HEPES, pH 7.4.

The two Cr-binding sites could potentially be monitored by EPR spectroscopy as the two sites can be distinguished by EPR in samples of transferrin, lactoferrin, and conalbumin [7, 17, 24, 25]. The EPR spectra are comprised of three distinct signals in the region between...
g = 5 and 6. The signals have been assigned to the two tight Cr-binding sites. Signals at g ~ 5.1 and 5.6 arise from Cr$^{3+}$ bound to the N-terminal and tighter binding site; the signal at g ~ 5.4 arises from Cr$^{3+}$ bound to the C-terminal site. Spectra of the heat-treated and glycated transferrins 24 h after the addition of Cr$^{3+}$ (as a solution of CrCl$_3$·6H$_2$O) results in distinctly different spectra than for untreated transferrin. As shown in Figure 4.7 for the heat-treated transferrin with addition of one equivalent of Cr$^{3+}$, the spectrum is dominated by a feature at g = 5.41, suggesting binding mainly to the C-terminal site, although shoulders on each side of this signal suggest some Cr$^{3+}$ is bound to the N-terminal site as well. (Under the conditions of the experiment, Cr$^{3+}$ in the buffer gives rise to signals at g = 5.65 and 5.22).

![Figure 4.7 Low field EPR spectra of solutions of heat-treated transferrin with 1 equivalent of added Cr$^{3+}$ after 1 d (solid line) and 3 d (dashed line).](image)

The spectrum for the addition of two equivalents of Cr is similar (not shown). However, spectra of the heat-treated transferrin 3 d after the addition of 1 or 2 equivalents of Cr$^{3+}$ indicate that distinct changes occur over time (Figure 4.8).
Figure 4.8 Low field EPR spectra of solutions of heat-treated transferrin with 1 (solid line) or 2 (dashed line) equivalents of added Cr$^{3+}$ after 3 d.

The dominate signals now arise from Cr$^{3+}$ in the N-terminal site, although the signal from Cr$^{3+}$ in the C-terminal site can readily be discerned. Spectra for the glycated transferrins are extremely similar (Figure 4.9).
Figure 4.9 Low field EPR spectra of solutions of glycated transferrin with 2 (dashed line) equivalents of added Cr\(^{3+}\) after 1 d (solid) or 3 d (dashed line).

The spectra for the heat-treated and glycated transferrin 3 d after addition of 1 or 2 equivalents of Cr\(^{3+}\) are extremely similar to those of conalbumin with 1 or 2 equivalents of added Cr\(^{3+}\) under nearly identical conditions [7]. However, for conalbumin, the EPR spectrum grows in intensity with time after the addition of Cr; no shift from a C-terminal type signal to a mixture of N- and C-terminal type signals is observed. Thus, the EPR spectra suggest that heat-treatment significantly alters the conformation of the transferrin such that potentially Cr binds first primarily to the C-terminal site; then, over time the conformation appears to change to the normal conformation resulting in Cr being two to both sites in a similar fashion to untreated transferrin. In this case, the N-terminal site of transferrin appears to be altered to a more significant degree than the C-terminal site. However, one cannot rule out other possibilities such as the binding sites being altered by heat-treatment in such a manner that the EPR signals from Cr\(^{3+}\) bound at the sites after 24 h happens to resemble the EPR signals of Cr\(^{3+}\) bound to the C-terminal site of untreated transferrin. Yet, given that the half-life of
transferrin in the serum and of transferrin-bound iron (and thus transferrin-bound Cr as well) are on the order of hours [24, 26], then the slow conformation change of the treated transferrins to a conformation similar to untreated transferrin is not relevant on a physiological time scale.

4.3.3 Cr transport and distribution

Previously [24], the injection of rat $^{51}$Cr$_2$-transferrin into Sprague Dawley has been shown to result after 2 h in most of the $^{51}$Cr being transfer to the urine, while a significant amount remained in the blood stream. Significant amounts of $^{51}$Cr were also found in the liver, spleen, kidney, and muscle. Cr-treatment with insulin resulted in lowering of the amount of radiolabel in the bloodstream while increasing the content of the radiolabel in the urine and in all the tissues examined [24]. Consequently, the effects of heat storage and glycation on the distribution of Cr from rat serum transferrin were examined. To prepare the $^{51}$Cr-containing transferrins, 2 equivalents of $^{51}$CrCl$_3$ were added to solutions of the heat-stored and glycated rat transferrins in 25 mM HCO$_3^{-}$, 0.1 M HEPES, pH 7.4 for 24 h. As shown above, this should produce the protein with 2 bound $^{51}$Cr for the glycated transferrin; but for the transferrin stored at 37 °C for two weeks without added glucose, this should produce transferrin containing only one or slightly more $^{51}$Cr$^{3+}$ ions per transferrin molecule. The proper binding of transferrin to its receptor onto the cell surface requires the conformation of transferrin containing two appropriate metal ions [27]; consequently, the results of the chromium-binding studies above indicating the deviations in the extent of Cr binding or in the conformations of the Cr-bound forms of the heat stored and glycated transferrin suggest the transport and distribution of Cr should be greatly altered.

The $^{51}$Cr content of the tissues and body fluids 2 h after injection of the labeled transferrin or these transferrins together with insulin are shown in Figure 4.10. The data are presented in terms of % of recovered $^{51}$Cr as the $^{51}$Cr content of every body tissue was not examined. For
those not examined, the $^{51}$Cr content after 2 h is expected to be extremely small and not make a meaningful contribution to the total [28, 29].

A significant amount of the $^{51}$Cr is found to still be in the bloodstream, nearly 40 % of the heat-stored transferrin and over 60 % for the glycated transferrin. A greater amount of the $^{51}$Cr appears to clear the bloodstream for the heat-stored transferrin compared to the glycated transferrin. After injection of insulin (which leads to a movement of a pool of transferrin receptor molecules to the cell surface, increasing transferrin movement from the bloodstream to tissue cells), the amount of $^{51}$Cr in the bloodstream decreases for both heat-stored and glycated transferrin. Thus, the movement of the transferrins from the bloodstream is still stimulated by insulin.

The lower amount of $^{51}$Cr in the bloodstream for the heat-stored transferrin is reflected in a greater content of $^{51}$Cr in the urine and in the liver and spleen for the heat-stored transferrin compared to the glycated transferrin. In both cases, insulin results in the amount of $^{51}$Cr in the
urine, the liver, and the spleen increasing, reflecting the movement of $^{51}$Cr from the bloodstream in response to insulin (although the increase for glycated insulin for the spleen is not statistically significant). The relative contents of $^{51}$Cr in the blood and urine for both transferrin are similar to that for Sprague-Dawley rats injected with untreated rat transferrin loaded with $^{51}$Cr [26].

The greatest amounts of $^{51}$Cr in the tissues regardless of which transferrin is administered and whether insulin is co-administered are in the liver, skeletal muscle, and kidney. This is consistent with previous studies using untreated rat transferrin [26]. When given orally, the distribution of Cr from Cr supplements is similar [28, 29], as would be expected if the absorbed Cr is stored and transported in the bloodstream by transferrin.

However for Sprague-Dawley rats injected with $^{51}$Cr-loaded, untreated rat transferrin, the amount of $^{51}$Cr after insulin injection increased significantly in every tissue examined (liver spleen, kidney, pancreas, skeletal muscle, epididymal fat, testes, and heart) [26, 30]. For the heat-stored and glycated transferrin, this is not observed for the tissues other than spleen and liver. This different pattern may suggest that increased metabolism in general from insulin administration could be leading to increased catabolism of the heat-stored and glycated transferrins (with their altered conformations) rather than increased transferrin cycling and chromic ion transport. Although the liver and kidney have been suggested to account for 40-50% of total transferrin catabolism [31], unfortunately, the precise mechanisms of transferrin catabolism are poorly elucidated, limiting further interpretation. Serum transferrin normally has a half-life in vivo of 7-10 days [32], such that storing transferrin at 37 °C for two weeks does not appear unreasonable, although storage in buffer does not model well physiological conditions.

Given that insulin leads to increased glucose metabolism in the fat and skeletal muscle and given that skeletal muscle is believed to be important for Cr-induced increased
insulin resistance [1], the lack of movement of Cr by the glycated transferrin suggests that doses of Cr would be less effective in terms of potentiating insulin action in individuals as the amount of glycated transferrin increases as less Cr would enter the tissues. Larger doses of Cr might be expected to generate similar effects in a subject with elevated blood glucose levels compared to a healthy individual with normal blood glucose levels. However, type 2 diabetes affects many systems in the body. For example, type 2 diabetic model rats absorb a greater percentage of an oral dose of Cr$^{3+}$, presumably as a result of greater water absorption [33]. This increase in Cr$^{3+}$ entering the bloodstream potentially could offset the effects of increased glycation on Cr transport, although such a determination would require more detailed investigations.

4.4 Conclusions

Storing transferrin in the presence or absence of glucose for two weeks at 37 °C for two weeks results conformation changes that affect the binding of Cr$^{3+}$ to the protein. Heat storage alone results in only one of the two metal binding sites binding Cr$^{3+}$ tightly. Glycated transferrin appears to adapt a different conformation than freshly dissolved, non-glycated transferrin after tightly binding two Cr$^{3+}$ ions. The rates of Cr$^{3+}$ binding are also modified. These changes reduce the ability of transferrin to transport Cr in vivo in rats. The results suggest that glycation of transferrin in subjects with high blood glucose concentrations should reduce the ability of Cr from pharmacological agents to enter tissues. However, this is just one piece in understanding the fate and mode of action of pharmacological doses of Cr.
REFERENCES


CHAPTER 5

TRIVALENT CHROMIUM HAS NO EFFECT ON DELAYING AZOXYMETHANE-INDUCED COLORECTAL CANCER IN FVB/NJ MICE

5.1 Introduction

Chromium was utilized in many areas for a long time, such as in electrodeposition to form layer on surface [1-3]. Chromium was first proposed to be an essential trace element for mammals over 50 years ago; as the trivalent ion, Cr has been accepted as an essential element for decades. However, this status has recently been discredited, and the status of Cr is a matter of current debate, although the issue is moving towards settlement with the element no longer to be considered as essential but to have a pharmacological action [4]. For example, in 2014, the European Food Safety Authority removed Cr from its list of nutrients and essential minerals [5]. Part of the confusion with the status of Cr arises from the pharmacological effects of Cr(III) at supranutritional doses. These doses of Cr can generate improvements in insulin sensitivity and blood cholesterol levels in animals with stresses on the glucose and lipid metabolism systems, most notably in rodent models of insulin insensitivity and type 2 diabetes [4, 6]. Cr appears to have no beneficial effects at doses normally found in mammalian diets [4, 5].

Type 2 diabetes and dietary patterns that stimulate resistance to insulin are associated with a higher risk of colon cancer [7–10]. For example, efforts to counter these dietary patterns associated with obesity and insulin resistance are likely to reduce colorectal cancer, as well as cardiovascular disease [7]. The dietary supplements conjugated linoleic acid (suggested to increase insulin sensitivity) and vanadium (an insulin mimic) have been shown to reduce the incidence of 1, 2-dimethylhydrazine-induced colorectal cancers [11, 12]. Consequently, therapeutic agents that increase insulin sensitivity and improve cholesterol and triglyceride
levels would be expected to potentially reduce the incidence of colorectal cancer. Toward this end, the ability of the chromium(III)-based therapeutic agent \([\text{Cr}_3\text{O}(\text{O}_2\text{CCH}_2\text{CH}_3)_6(\text{H}_2\text{O})_3]^+\), Cr3, has been examined previously for its ability to reduce the incidence of 1,2-dimethylhydrazine-induced colorectal tumors [13]. Daily gavage administration of Cr3 (1 mg Cr/kg body mass) to male Sprague Dawley rats for 6 months was found to significantly decrease colon tumor incidence. While no effects on body mass or food intake were observed, the number of rats with observed tumors, the number of tumors, and the mean number of tumors per rat were reduced by the Cr3 treatment [13]. Similarly, visible inspection of the colons of the rats suggested the colons of the rats receiving Cr were healthier.

Cr3 is very water soluble, unlike the popular nutritional supplements chromium picolinate and chromium nicotinate, and can be recrystallized from dilute mineral acids suggesting that it might be reasonably stable in the stomach [7]. The compound is absorbed with between 40 and 60 % efficiency within 24 h of oral dosing; this represents a greater than tenfold increase over those of Cr picolinate (marginally soluble in water, 0.6 mM), CrCl3 (which oligomerizes in water), and Cr nicotinate (\(\text{“Cr(nic)z(OH),”}\) insoluble in water) [4]. The solubility of Cr3 and its stability, thus, apparently allow a unique amount of the material to enter the circulatory system and tissues.

Herein are described follow-up experiments to expand the examination of the effects of Cr on chemically induced colorectal cancer in a mouse model to include concentration and sex dependence.

5.2 Materials and methods

5.2.1 Animals

FVB/NJ mice approximately 12 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were allowed to acclimate for at least 1 week before the initiation of experiments. After acclimation, all animals were singly housed in a shoebox-type caging
with hardwood bedding and with ad libitum access to drinking water (distilled water) and a commercial chow diet (Teklad LM-485 rodent diet (Harlan Teklad, Madison, WI)) and kept on a 12-h light/dark cycle. Mice were uniquely identified via ear punch and cage card. The temperature was maintained at 22 ± 2 °C with 40–60 % relative humidity. Male and female mice were randomly assigned to treatment groups. Water intake and body mass were measured twice a week. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Alabama (Protocol Number 14-01-0058).

5.2.2 Materials

Cr3 was available from previous work in the Vincent Lab and was prepared by the method of Earnshaw et al. [14]. Azoxy methane (AOM) was obtained from Santa Crux Biotechnology (Dallas, TX). AOM was dissolved in distilled water at 10 mg/ml, and aliquots were stored at ~−20 °C. Aliquots were thawed directly before use and diluted 1:10 in saline solution to a final concentration of 1 mg/ml.

5.2.3 Procedures

The procedure for inducing colon carcinogenesis followed the method of Neufert and coworkers [15]. Thirty-two female mice and 32 male mice both were randomly separated into four treatment groups: (1) control, (2) AOM only, (3) AOM and 1.0 mg Cr daily as Cr3/kg body mass, and (4) AOM and 10.0 mg Cr daily as Cr3/kg body mass. On days 1, 8, 15, 22, 29, and 36 of the study, animals received either a sterile saline solution (used in preparation of AOM solution) or AOM solution (10 mg AOM/kg body mass) via intraperitoneal injection. Cr3 was dissolved in distilled water; the solutions were provided as drinking water at concentrations necessary to provide the appropriate daily dose of Cr(III) based on the periodic water intake and body mass measurements. Inclusion of Cr3 in drinking water had no effect on water intake; inclusion of Cr3 in the drinking water was started 2 weeks before the first AOM treatment to be certain that inclusion in the drinking water would not affect water intake.
Animals were examined daily for signs of complications from the development of colorectal cancer (e.g., anal bleeding).

After five and one-half (males) or six (females) months, mice were sacrificed by CO2 asphyxiation. The colon was removed and rinsed with phosphate-buffered saline (PBS). The colon was then opened, and the contents were removed. The colon was transferred to a microscope slide, and the slide was then placed in a petri dish where the tissue was covered with 10% neutral buffered formalin for 24h. Then, slides with the colons were removed from formalin and rinsed with PBS. Tissues were stained using a modification of McGinley and coworkers [16]. The tissues were covered with the stain methylene blue for 5 min. Tissues were destained as needed with distilled water. The crypt morphology was examined using a dissecting microscope under ×40 or ×10 resolution.

I assisted Pandora E. White to dose mice, harvest tissues and stain tumors.

5.2.4 Statistics

Data were analyzed by Kruskal-Wallis one-way analysis of variance (ANOVA) followed by a Dunn’s method pairwise multiple comparison procedure to determine specific significant differences ($P \leq 0.05$) using SigmaPlot 11 (SPSS, Inc., Chicago, IL). When appropriate, data are presented in figures and tables as means ± standard error (SE). Data were analyzed by Pandora E. White.

5.3 Results and Discussion

FVB/NJ rats were chosen for this study as they tend to not develop spontaneous tumors but are known to be highly susceptible to some chemically induced cancers, such as squamous cell carcinomas [17]. AOM acts as a cancer-initiating agent by resulting in the alkylation of DNA, which ultimately facilitates base mispairing. However, AOM is not the actual alkylating agent; it requires conversion in the body to methyldiammonium through a mechanism that has not yet been entirely elucidated. AOM itself is a metabolite of 1,2dimethylhydrazine [18]. Thus,
either 1,2-dimethylhydrazine or AOM may be used to induce colon carcinogenesis. AOM was chosen for the current work as it has been reported to be more stable in solution and to generate less variability in results [19].

As shown in Figs. 5.1 and 5.2, neither AOM nor Cr3 treatment had any significant effect on body mass. However, at certain time points, some significant differences were observed; these differences were not consistent over time. Administration of chromium(III) complexes has been shown in numerous experiments over a wide range of doses to have no effects on body masses of rodents or humans [4]. Similarly, neither AOM nor Cr3 treatment had any significant effect on average daily water intake (data not shown).

Fig. 5.1 Body mass of male mice in various treatment groups over the course of the study. Data points at a given time labeled with different lower case letters are statistically different. Circles, control; inverted triangles, AOM only; squares, AOM + 1 mg Cr/ kg; diamonds, AOM + 10 mg Cr/ kg.
Fig. 5.2 Body mass of female mice in various treatment groups over the course of the study. Data points at a given time labeled with different lower case letters are statistically different. Circles, control; inverted triangles, AOM only; Squares, AOM + 1 mg Cr/Kg; diamonds, AOM + 10 mg.
Seventeen weeks after the study began, mice in all the AOM treatment groups started to exhibit rectal prolapses (Table 5.1). Three animals were removed prematurely from the study because of the severity of this condition; the colons of one male receiving only AOM and one male and one female receiving AOM and 1 mg Cr/kg were not used for data collection. Anal bleeding in the absence of a rectal prolapse was also observed for animals in all AOM treatment groups except the female AOM only treatment group (no Cr3). Neither rectal prolapses nor anal bleeding was observed in animals in the control treatment group (no AOM). The number of rectal prolapses was lowest among the AOM-treated animals receiving the larger dose of Cr, but the apparent effect is not statistically significant. In addition, female mice were more prone to rectal prolapses as a result of the AOM treatment (10 females compared to 7 males after 5½ months), and this effect was observed regardless of whether Cr3 was also administered. However, the first rectal prolapses in a treatment group were generally observed earlier for a male rather than a female. As noted above, the male mice were sacrificed 2 weeks before the female mice. The rectal prolapses of some of the male mice were not reduced over time; as all three mice that were removed from the study for health concerns had initially presented this
condition, the continued health of the male rats was a concern. Gender differences from AOM and dimethylhydrazine-induced tumor formation have been observed previously [18].

Colons were scored for aberrant crypt foci, ACF (a premalignant colon pathology), adenomas (tumors confined to the mucosa), and carcinomas (tumors that penetrate the mucosa). As shown in Fig. 5.3, none of these features were found in control animals not receiving AOM. In contrast, AOM treatment effectively induced colorectal cancer. The colons of all animals that received AOM possessed adenomas and carcinomas. Thus, a statistically significant difference in the mean total number of ACF and tumors was found between the male and female controls and the AOM male and female treatment groups. Given the differences based on sex noted above, colon data were calculated separately for each sex for statistical analyses. Similarly, for both the mean number of ACF and the mean total of tumors (adenomas and carcinomas), a statistically significant increase was observed for all groups treated with AOM compared to controls (Fig. 5.3). The number of tumors tended to be greater for females compared to males, consistent with the observations on rectal prolapses.

The administration of Cr3 in the drinking water (at either dose) had no statistically significant effects on the mean total number of ACF and tumors, mean number of ACF, or mean number of adenoma and carcinoma (Fig. 5.3). This is in stark contrast to the results of a previous study using Sprague Dawley rats [13] in which Cr3 (1 mg Cr/kg body mass by gavage administration) had a significant effect on lowering the number of tumors induced by dimethylhydrazine.
5.4 Conclusions

While administration of Cr(III) compounds has previously been shown to improve insulin sensitivity and plasma cholesterol and triglycerides levels in rodent models of insulin resistance and to reduce the incidence of chemically induced colorectal cancer in otherwise healthy rats, the current study found no effects from Cr(III) as Cr3 on the incidence of colorectal cancer in male or female FVB/NJ mice. While AOM might induce colorectal to an extent in the mice strain used in this study such that the Cr(III) treatment was unable to overcome its effects, this hypothesis will require additional investigation. Consequently, the value of Cr(III) compounds in preventing the onset of colorectal cancer is currently uncertain at best.
REFERENCES


6.1 Introduction

While chromium as the trivalent ion was proposed to be an essential trace element over half a century ago, a recent paradigm shift in understanding the effects of chromium has occurred [1–5]. The effects of Cr$^{3+}$ in enhancing insulin sensitivity in rodents have been shown to be supra-nutritional [6]. No symptoms of chromium deficiency have been unambiguously established, and no biomarker of chromium status has been identified [1]. However, this shift raises questions about the role and mode of action of Cr$^{3+}$ in increasing insulin sensitivity at a molecular level (at least in rodent models of diabetes and insulin insensitivity). It also may explain the lack of the demonstration of unambiguous benefits of chromium supplementation in clinical trials as the human subjects have failed to receive doses of Cr$^{3+}$ proportionally as high as the doses administered to the rodents [1–3]. To date, several proposals for the mode of action of Cr$^{3+}$ have been presented [1, 7], although none have been definitely established in vivo. Unraveling the mechanism and mode of action at a molecular level of the potentially beneficial action of Cr$^{3+}$ would resolve over five decades of studies in the field of nutritional biochemistry.

Yet, much has been elucidated about the mechanism of transport and movement of chromium in the bodies of mammals. Chromium is passively absorbed from the gastrointestinal tract, transported by transferrin from the bloodstream to the tissues, and transported from the tissues to the urine (via the bloodstream) by the peptide lowmolecular-weight chromium-binding substance (LMWCr); transferrin and LMWCr are currently the only two biomolecules
known to specifically bind Cr\(^{3+}\) *in vivo* when Cr\(^{3+}\) is taken orally at nutritionally relevant doses [1]. Transferrin and LMWCr appear to form a detoxification pathway for passively absorbed Cr\(^{3+}\). Unlike other metal ions, Cr\(^{3+}\) binds first to the C-terminal metal-binding site of transferrin; given that transferrin is only approximately 30% loaded with iron in the bloodstream, the protein appears to be readily available to bind and transport Cr and has been shown *in vivo* to be responsible for Cr transport (reviewed in Ref. [3]). Cr-containing LMWCr is rapidly cleared from tissues *in vivo* (although the mechanism is unknown) and has a very low tubular reabsorption rate so that the peptide in the blood is readily removed from the body via the kidneys and urine [1]. Increases in Cr intake result in increases in urinary Cr loss, presumably as the complex with LMWCr [1]. The movement of Cr(III)–LMWCr is stimulated by increases in blood insulin concentration; thus, increased movement of Cr from the bloodstream to the tissues by transferrin results in a subsequent elimination of Cr from the body bound to LMWCr. The peptide appears to be maintained in the tissue in the apo form; levels of the apo form potentially are under homeostatic control. At a minimum, LMWCr functions to rapidly clear Cr from tissues [1].

LMWCr is a peptide 10 or 11 amino acids in length; it is comprised of only the amino acids glycine, aspartate, glutamate, and cysteine and tightly binds four chromic ions [8, 9]. The oligopeptide has been isolated from the livers of mammals [8, 9], chicken [10], and alligator [10] and human urine [11] where all appear to contain the contiguous peptide of sequence Glu-Glu-Glu-Glu-Gly-Asp-Asp (EEEEGDD) [11]. Cr from oral administration has been found in the bloodstream to be bound to transferrin and a low-molar-mass peptide that co-elutes on size exclusion columns with LMWCr; similarly, injection of Cr-containing transferrin into the bloodstream results in the appearance of a low-molar-mass species that coelutes with LMWCr (reviewed in Ref. [1]). Spectroscopic studies of LMWCr indicate that the chromic ions are
bound to the peptide primarily via the carboxylate side chains of the aspartate and glutamate residues [12]. The chromic ions appear to be arranged in a multinuclear assembly [1].

The Cr(III)-containing form of LMWCr has been proposed to be the biologically active form of chromium. In vitro it has been found to activate the kinase activity of insulin receptor; this activation was proportional to the Cr content of LMWCr [13, 14]. In a proposed mechanistic scheme, at high intakes of Cr$^{3+}$, LMWCr would accumulate to pharmacological levels, bind to insulin receptor (helping to maintain the receptor in its active conformation), and result in increased insulin sensitivity [15]. The validity of this proposal requires testing in vivo.

However, the discovery that LMWCr contains a heptapeptide that could readily be synthesized raises the question of whether this peptide might bind Cr similarly to LMWCr, have biological activity (i.e., increase insulin sensitivity), and thus have potential as a pharmaceutical agent to treat symptoms of diabetes and other conditions related to insulin insensitivity. Herein are reported initial studies to examine the binding of Cr$^{3+}$ to the synthetic peptide EEEEGDD and the biological activity of the peptide and its complex with bound Cr.

6.2 Materials and methods

6.2.1 Materials

The heptapeptide EEEGDD (purity >98 %) was synthesized by GenScript (Piscataway, NJ, USA). Chromic chloride hexahydrate was from Fisher Scientific (Pittsburg, PA, USA). MES (2-(N-morpholino)ethanesulfonic acid) was from Research Organics (Cleveland, OH, USA).

6.2.2 Analytical procedures

The heptapeptide concentration (amino terminus concentration) was determined by the fluorescamine method [16], as the only primary amine group on the peptide is the amino terminus. Fluorescence measurements were obtained on a Jovin Yvon Fluoro Max-3
fluorescence spectrometer. Cr content in peptide was determined by measuring Cr concentration in supernatant; the Cr concentration in supernatant was detected by Cr assay with diphenylcarbazide solin [9].

6.2.3 Miscellaneous

Doubly deionized water [Millipore (Billerica, MA, USA) Milli-Q] was used throughout the mass spectrometric and spectroscopic studies.

6.3 Results and discussion

Stoichiometry of Cr\(^{3+}\) binding

The addition of four equivalents of Cr\(^{3+}\) as CrCl\(_3\) to the EEEEDGG peptide in 20 mM HEPES buffer, pH 7.4, results in the rapid formation of a light purple precipitate. Centrifuging the sample results in a light purple pellet containing Cr and peptide and in a clear, colorless supernatant. The lack of solubility of the Cr-peptide complex limits the ability to perform spectroscopic studies on this complex. This behavior unfortunately is in contrast to that when Cr\(^{3+}\) is added to the peptide pEEEEGDD (where pE is pyroglutamate) [11]. The precipitate removes nearly all the peptide and chromic ions from solution, suggesting the peptide binds four equivalents of Cr\(^{3+}\) forming a neutral species that readily precipitates from aqueous solution. This was confirmed by Cr analysis of the product. The binding of 4 equivalents of Cr would be consistent with 4 equivalents of Cr binding to the peptide pEEEEGDD [9]. After centrifuging the product of the addition of four equivalents of Cr\(^{3+}\) to the peptide, the Cr and peptide concentrations of the supernatant have been measured allowing the determination of the Cr:peptide ratio of the precipitate; the Cr concentration was determined using the diphenylcarbazide method [9], while peptide concentration was determined as described above [16]. The Cr:peptide ratio of the precipitate was 3.2:1. In addition to this representing binding of Cr to form a neutral complex, aggregation of the anionic peptide with bridging chromic ions would also be consistent with the results. However, when 4 equivalents of CrCl\(_3\) are added to
5 mg of the peptide dissolved in 1 L of HEPES buffer, precipitation occurs again, suggesting that the effect is not simply the result of aggregation of the peptide. Increasing the pH above 8 or below 6 results in the precipitate dissolving. Precipitation occurs even if Cr\(^{3+}\) is added to 5 mg of peptide in 1 L of buffer; given this occurs even at this dilute concentration suggests that the Cr-peptide complex is quite insoluble, opposed to Cr\(^{3+}\) and the peptide forming Cr-bridged aggregates that precipitate from solution. These results are consistent with spectroscopic studies on the Cr-peptide complex, which suggest 4 Cr ions are bound to the peptide [12].

6.4 Conclusions

A variety of spectroscopic techniques and mass spectrometry reveal that the heptapeptide EEEEDGG, corresponding to the contiguous peptide component of LMWCr, binds multiple Cr\(^{3+}\) ions in octahedral environments; the carboxylate groups of the peptide are involved in Cr binding. Small anionic ligands, such as hydroxide and oxide that bridge the Cr centers probably also serve as ligands. Preliminary *in vivo* and *in vitro* studies suggest that the heptapeptide-chromium may enhance glucose disposal by augmenting insulin signaling and enhancing cellular glucose uptake. Further in-depth studies with various concentrations of peptide and Cr are necessary to fully characterize the biological role of the peptide.

Currently studies are underway to examine the ability of the heptapeptide when titrated with varying amounts of Cr\(^{3+}\) and other biologically relevant metal ions to activate members of the insulin signaling pathway *in vitro* and *in vivo* and to characterize the resulting metal-peptide complexes and establish whether Cr is unique in its interaction with the peptide. Additionally, efforts to solubilize the Cr-peptide complex at neutral pH so that its structure can be determined are also underway.
REFERENCES


CHAPTER 7

CONCLUSIONS AND FUTURE WORK

The effects of daily gavage administration of chromium picolinate, CrCl$_3$ and Cr3 on metal concentrations (Cu, Zn, Fe, Mg, and Ca) in kidney, liver, spleen and heart were examined. Treatment with CrCl$_3$ and Cr3, but not [Cr(pic)$_3$], at 1 mg Cr/kg resulted in statistically significant accumulation of Cr in the kidney of lean and obese but not ZDF rats but resulted in lowering the elevated levels of kidney Cu in ZDF rats, suggesting a beneficial effect on this symptom of type 2 diabetes.

The in vitro studies that suggested that the binding of chromic ions to apotransferrin is too slow to be biologically relevant have generally failed to adequately take physiological bicarbonate concentrations into account. In aqueous buffer with ambient (bi)carbonate concentrations, the binding of chromium to transferrin is too slow to be physiologically relevant, taking days to reach equilibrium with the protein’s associated conformational changes. However, in the presence of 25 mM (bi)carbonate, the concentration in human blood, chromic ions bind rapidly and tightly to transferrin. At higher bicarbonate concentrations, more of the binding sites contain the bicarbonate anion, which become locked in place, and the bicarbonate provides two metal ligands for metal binding. In the presence of 25 mM bicarbonate, equilibrium is achieved within about 15 min, which is biologically relevant time frame.

When blood concentrations of glucose are high (as in a diabetic subject), transferrin can be glycated, modifying its ability to bind and transport Cr. Storage of transferrin at 37 °C in the presence and absence of glucose has significant effects on the binding of Cr. Transferrin stored in the absence of glucose only binds one equivalent of Cr tightly, compared to the normal
binding of two equivalents of Cr by transferrin. Glycated transferrin binds two equivalents of Cr but the changes in its extinction coefficient at 245 nm that accompany binding suggest that the Cr-bound transferrin possesses a conformation that deviates appreciably from untreated transferrin. These changes have dramatic effects, greatly reducing the ability of transferrin to transport Cr in vivo in rats. The results suggest that glycation of transferrin in subjects with high blood glucose concentrations should reduce the ability of Cr from pharmacological agents to enter tissues.

Both glycated and non-glycated transferrin samples were incubated in 37 °C for two weeks. The conformation of transferrin can be modified by both heat treatment and glycation. Thus, determining the effect from only glycation of transferrin on chromium binding and transport is difficult. Different extinction coefficient values from Cr binding to glycated transferrin and heat-treated only transferrin sample suggest that besides heat treatment, glycation may also change the transferrin conformation after Cr binding. To study glycation of transferrin, the details of conformation of glycated transferrin should be studied in future work. By the way, the mechanism of increased catabolism of the heat-treated and glycated transferrins are unknown. Studying liver and kidney cells for transferrin catabolism is necessary. Glycation of transferrin also affects the iron binding. When iron exists at a similar concentration as Cr, whether glycation of transferrin has effect on the competition of Cr binding to the protein is unknown. If glycation of transferrin provides greater effect on Cr binding than iron binding, presumably, once transferrin is fully glycated, Cr is more competitive than iron in vivo. Currently, the binding of chromium to glycated transferrin is more thoroughly studied than iron binding. Iron binding needs to be probed in greater detail.

A previous study reported the positive effects of Cr3 on 1,2-dimethylhydrazine-induced colorectal cancer in Sprague Dawley rats. In contrast to that study, no effects of Cr3 at daily doses of 1 and 10 mg Cr/kg body mass were observed in the current study. This result left in
question whether administration of Cr(III) compounds can delay or prevent the onset of colorectal cancer. Thus, elucidating the effects of Cr(III) compounds against colorectal cancer requires further studies. The current study only examined the effects of Cr3. The use of the cancer-causing agent used with mice in this study could be examined alone with chromium supplementation in rats to help resolve this discrepancy.

The Cr-EEEGDD complex is quite insoluble. The Cr:peptide ratio of the precipitate is constant with 4 equivalents of Cr binding to the peptide in other studies. The mechanism of Cr-EEEGDD functioning in cells requires further studies. But first, the problem of insolubility of Cr-EEEGDD need to be resolved. The use of alternative solvents or modifying the peptide with polyethylene glycols or other moieties should be examined.