Isolation of a novel chromium(III) binding protein from bovine liver tissue after chromium(VI) exposure

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Abstract

In the ongoing investigation into the biological importance and toxicity issues surrounding the bioinorganic chemistry of chromium, the accepted literature procedure for the isolation of the biological form of chromium, low molecular weight chromium binding protein (LMWCr) or chromodulin, was investigated for its specificity. When chromium(VI) is added to bovine liver homogenate, results presented here indicate at least four chromium(III) binding peptides and proteins are produced and that the process is non-specific for the isolation of LMWCr. A novel trivalent chromium containing protein (**1**) has been isolated to purity and initial characterization is reported here. Chromium(III) identification was determined by optical spectroscopy and diphenylcarbazide testing. This chromium binding protein has a molecular weight of 15.6 kDa, which was determined from both gel-electrophoresis and mass spectrometry. The protein is comprised primarily of Asx, Glx, His, Gly/Thr, Ala, and Lys in a 1.00:2.51:0.37:2.09:0.39:1.17 ratio and is anionic at pH 7.4. In addition, the protein binds approximately 2.5 chromium(III) ions per molecule.

1. Introduction

Chromium can exist in a variety of oxidation states but the most common are chromium(III) and chromium(VI). The former is considered a biological trace mineral [1] important in glucose metabolism while the latter is a known carcinogen [2]. The biological importance of chromium(III) in glucose metabolism is controversial, and recent reports suggest that the biological form of chromium is an artifact of the isolation procedure [3]. Others claim there is a specific biological target molecule for chromium(III) [4]. The proposed target molecule is a small metal binding peptide known as low molecular weight chromium binding protein (LMWCr), or chromodulin, which is essential for proper glucose metabolism [5,6]. However, we show that multiple chromium(III) binding proteins are produced through the accepted protocol, and this work is consistent with other recent reports [3]. Even though the existence of LMWCr was reported over 20 years ago, detailed structural information remains missing in the literature. Early work resulted in the identification of two chromium peptides, LMWCr (low molecular weight chromium binding protein 1.5 kDa) and HMWCr (high molecular weight chromium binding protein 2.6 kDa), and the former was initially studied because it was found in higher concentrations [7]. Subsequently, LMWCr was confirmed to be 1.5 kDa and to bind four chromium(III) ions and it was renamed chromodulin [4]. The isolation procedure for chromodulin is initiated by the reaction of bovine liver with an aqueous solution of chromium(VI). Chromodulin, from this reaction, consists of glutamic acid or glutamine, glycine, cysteine and aspartic acid or asparagine amino acids and four chromium(III) ions [7]. These chromium(III) ions result from the reduction of chromium(VI). Evidence indicates that three of the four chromium(III) ions are part of a trinuclear assembly and are

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antiferromagnetically coupled [8]. In addition, there is evidence that this cluster might electronically interact with a fourth chromium center [8]. However, in each of these accounts no evidence was presented that these studies were performed on the pure peptide. In addition the molecular weight of this peptide is based on gel electrophoresis data that have never been published in the literature or the original dissertation.

The biological activity of chromodulin has been postulated to be initiated by the binding of insulin to the α-subunit of the insulin receptor, which triggers a message inside the cell, causing an influx of chromium(III) ions [5]. The chromium(III) ions are postulated to bind to LMWCr, and this binding event induces a conformation change, which allows the metalloprotein to bind to the β-subunit of the insulin receptor [5]. This theory describes both insulin and holo-LMWCr bound to the insulin receptor to initiate the cascade of phosphorylation events which lead to the opening of glucose channels [9].

A recent genomic informatics search suggests that LMWCr could be an acidic region in the α subunit of the insulin receptor and synthetic peptides derived from this sequence bind multiple chromium(III) ions [10]. However this finding contradicts Vincent's assertion that this peptide is found inside all insulin active cells. Regardless of the exact form, LMWCr is currently proposed to exist in all mammalian insulin active cells in an apo form which is considered biologically inactive [4].

The isolation process and the molecular role of LMWCr binding protein generates several questions that remain unanswered involving the molecular details of chromium(III) speciation, biological importance, toxicity, and biological transport. These concerns include the specific nature of chromium binding by this peptide, the amino acid sequence of the peptide, the details of chromium(III) transport to cells [11] and through cell membranes, and the storage and transfer of chromium between biological molecules. In addition, many questions exist regarding the complex biological redox interchange between chromium(III) and chromium(VI) and other peptides and proteins that are products of this reaction. It is well established that chromium(VI) is reduced to chromium(III) by many biological molecules [12] and there have been recent reports that chromium(III) can be biologically oxidized back to chromium(VI) [13]. To further complicate matters the currently accepted biological form of chromium(III) was isolated through a reaction of tissue with chromium(VI) [7,14,15]. This has brought forward many concerns regarding the validity of LMWCr as the biological form of chromium and the justification of a million dollar chromium(III) supplement business [16–18]. Regardless if LMWCr is specifically produced for glucose metabolism or not, reports show that many chromium(III) compounds, including LMWCr, do aid in glucose metabolism and weight reduction [18–20].

The current literature has largely ignored the possible chromium detoxification role of proteins, and most research groups are currently focusing on the investigation of the biological importance in terms of glucose metabolism [21]. The possible detoxification role brings up the question, whether or not this protein is specific for chromium. If there is a detoxification role what oxidation state of chromium will the protein bind?

To understand the details of both the carcinogenic and trace mineral roles of chromium, it is important to understand the principles governing the conversion between the two important oxidation states of chromium in biological systems. Chromium(VI) is extremely water soluble and found in the environment as chromate or dichromate. These forms are anionic and have a structure similar to sulfate and phosphate, allowing chromium(VI) free passage into biological cells through anionic channels embedded into the cell membrane. Once chromium(VI) enters the cell a number of biological reductants can transform it into chromium(III) [12,19]. Previous studies have focused on proposed intermediates of this reaction which include chromium(V) and chromium(IV) [18,19], and others have identified products of this reaction, including amino acid – chromium – DNA complexes and protein chromium complexes [4,12,14].

Chromium(III) DNA complexes form as a result of chromium(VI) passing into the cell nucleus followed by reduction [22]. In vitro studies show that chromium(VI) can be reduced to chromium(III), which in turns reacts to form chromium(III) amino acid complexes followed by reaction with DNA to form ternary complexes such as amino acid CrDNA [12,23]. These results beg the question of whether chromium proteins cause similar types of DNA damage?

Our laboratory is interested in the isolation and characterization of chromium(III) protein complexes that form as a result of the reaction of chromium(VI) with biological tissue and the systematic study of the structure and chemical reactivity of these resulting molecules. Reported here are initial results of our investigation into the selectivity of the reaction of chromium(VI) with bovine liver to isolate chromium(III) binding peptides and proteins. This work describes the isolation, purification, and characterization of a novel chromium(III) binding protein that formed as an additional and reproducible product of chromium(VI) reacting with bovine liver. However, after multiple isolation attempts no evidence of the existence of LMWCr was observed. The elusive nature of this peptide could be a result of our purification methods or our detection limitations.

2. Materials and methods

Chemicals

Chromatography resins were prepared according to vendor specifications. Other chemicals were analytical or higher purity grade and used as received including: DEAE (Sigma), Sephadex G-25 (Sigma), NaN_3 (Sigma), $KMnO_4$ (Sigma), OPA (Sigma), Chromate and dichromate (Spectrum), ammonium acetate (Fisher), Silver quest silver stain

kit, Nupage 12% bis–tris gel (1.0 mm × 10 well), Mark-12 molecular weight standard and MES running buffer (Invitrogen), HPLC grade acetonitrile (Fisher), TFA (Fisher), OPA (Sigma), sulfuric acid (VWR), sodium bisulfate (Fisher), sodium phosphate (Fisher), phthalic anhydride (Acros), diphenylcarbazide (Sigma), amino acids (MP Biomedical, LLC), ethanol (USP grade from Pharmco).

Protein purification

Proteins were purified by a modification of literature procedure [24]. Protease inhibitors were used in early purification schemes but do not alter the purification and isolation of the protein of interest. Unless otherwise stated all protein manipulation was performed at 4 °C. Bovine liver was cut into small chunks and blended with 8 mM $Cr_2O_7^{2-}$ to create a liquid consistency. This solution was centrifuged at 11,000 g for 10 min to remove cellular debris and the resulting solution was made 50% (v/v) ethanol by volume. This solution was allowed to stir overnight. Proteins that had precipitated overnight were removed by centrifugation at 11,000 g for 10 min. Pellets from the second centrifugation were discarded and the supernatant was made up to 90% (v/v) ethanol by volume and allowed to stir for 2–3 days. The resulting solution was allowed to settle for several hours and the majority of the liquid was siphoned off. The remaining solution was centrifuged at 8250 g for 10 min. The pellets were collected; pooled and residual ethanol was removed by reduced pressure. The proteins were re-suspended in a minimal amount of water and filtered through a 0.2 μm filter. The sample was subjected to several passes through a 30 kDa molecular weight cut-off filter (MWCO) to remove larger proteins. Proteins collected from these steps represent the crude material.

Chromium mass balance was not followed during this procedure or previous work [24]. Current protein purifications are following the total chromium concentration, and account for the fate of chromium throughout this process.

Ion exchange chromatography

The crude material was re-suspended in buffer and eluted on a DEAE column using a step gradient of ammonium acetate buffer at pH 7.2. Buffer concentrations were 0.05 M, 0.10 M, 0.20 M, 0.30 M, 0.40 M, 0.60 M, 0.80 M and 1.0 M. A greenish band adhered to the column and a yellowish material was eluted with the 0.10 M ammonium acetate buffer. The greenish band was eluted using a 0.30 M ammonium acetate buffer. Fractions containing chromium(III) were determined by optical spectroscopy, pooled, de-salted and lyophilized.

Size exclusion chromatography

Pooled DEAE samples were fractionated by size using a Sephadex G-25 column and eluting with 50 mM ammonium acetate buffer at pH 7.4. Fractions containing both protein and chromium(III) were detected by optical spectroscopy, pooled, de-salted and lyophilized.

HPLC

After DEAE and size exclusion chromatography, samples were subjected to prep-HPLC using a C4 Phenomenex Jupiter 250 × 21.2 mm column and eluted with 1% TFA-water with a 5–40% linear gradient of HPLC grade acetonitrile over 20 min. All solvents were degassed and filtered through a 0.22 μm filter before use.

Gel electrophoresis

Samples for gel-electrophoresis were prepared in accordance with Invitrogen's guidelines for NuPAGE Novex Bis–Tris Mini Gels. Pre-cast NuPAGE Novex Bis–Tris Mini Gels were run in an XCell SureLock gel box from Invitrogen (serial number 1151241-10852) for appropriately 30–40 min. The gel box was connected to a FisherBiotech FB 105 power supply (serial number 52444) set at 200 mV. The running buffer was NuPAGE MES SDS running buffer diluted to 1X (NP0002) from Invitrogen. Molecular mass was checked against Mark-12 molecular weight standard after silver-staining.

Mass spectrometry

Samples were sent to the molecular structure facility at the University of California at Davis (UCD). Samples were run on a MALDI-TOF instrument. Samples were analyzed with an AB 4700 TOF/TOF mass spectrometer (Foster City, CA) equipped with a pulsed Yd:Ag laser, a delayed extraction ion source, and a reflectron, but were analyzed in linear mode (AB 4700, Applied Biosystems, Foster City, CA). Samples to be analyzed for nominal molecular weights were typically exchanged into low salt (<20 mM) buffers for best signal. Samples were then spotted onto the MALDI target with an equivolume amount of MALDI matrix (Sinapinic acid in 50% ACN/0.1% TFA, Fluka, St. Louis, MI) and allowed to air dry. Spectra were typically acquired for 1000–2000 shots with an accelerating voltage of 25,000 V. Calibration with external standards resulted in typical mass accuracies of 0.1%.

Chromium(III) ion determination

The number of chromium(III) ions per protein was determined using the diphenylcarbazide method as described in the literature [25].

Amino acid analysis

Pure protein samples, as determined by SDS-PAGE, were subjected to acid digestion by refluxing the protein in 6 M HCl over 20 h. The resulting solution was lyophilized and re-suspended in 0.001 M HCl. Particulates were

removed by centrifugation. The resulting sample was treated with OPA solution according to Sigma and then was run, on HPLC, using a modified method previously described in the literature [26]. Samples were subjected to analytical HPLC and eluted on a Waters Symmetry C18 5 μm 4.6 × 150 mm column, part number WAT045905. Amino acid ratio and type were determined by comparison to an HPLC profile of standard amino acids. In addition a sample was sent for analysis to UCD for cysteine determination.

3. Results and discussion

Chromium(III) binding proteins

The accepted literature procedure for the isolation of the biologically relevant form of chromium(III) (LMWCr) is initiated by a reaction of chromium(VI) with liver [4,7]. During this process biological reductants transform the chromium into the Cr(III) oxidation state to yield both high molecular and low molecular weight chromium binding proteins and peptides. This process reproducibly results in the isolation of a 15.6 kDa protein. Proteins and peptides were selected from the initial reaction mixture by a series of ethanol precipitations, which is a standard literature protocol [24]. A large number of proteins were obtained from the ethanol precipitation as indicated by SDS-PAGE gel electrophoresis (silver staining). Our particular interests are in peptides and low molecular weight proteins that bind chromium(III) since this has been the emphasis in the literature [7,14,24]. To select for smaller molecules the reaction mixture was repeatedly passed through a 30 kDa molecular weight cut off (MWCO) filter and the resulting filtrate was reduced in volume. This process greatly reduced the number of larger molecules as indicated by gel-electrophoresis and HPLC. This mixture is highly reproducible and was used to generate the HPLC trace shown in Fig. 1.

This process resulted in the formation of at least four chromium(III) binding proteins and/or peptides as indicated by the HPLC results shown in the inset of Fig. 1. Protein molecules are indicated by absorbance at 214 nm and chromium(III) detection is shown by absorbance at 570 nm. Since the extinction coefficients for d–d transitions of chromium(III) ions are small, other chromium(III) binding proteins could have been missed in this process. The elution profile shown in Fig. 1 shows a large degree of overlap in retention time between peaks observed for chromium(III) and several protein peaks. Additional peaks observed at 214 nm indicate that this process also yields proteins that do not have an affinity for chromium.

These results indicate that the current process for obtaining the biologically active form of chromium(III) is not selective, but instead leads to the formation of at least four chromium(III) containing protein/peptides. Not surprisingly the process starts with a strong oxidizing agent (chromium(VI)) which is most likely reduced via chromium(IV) and chromium(V) intermediates to form chromium(III) protein–peptide complexes [18,19]. In this work no external reducing agent was added to the reaction, supporting other claims that chromium(VI) is reduced by biological molecules such as thiols, ascorbate, or NADH

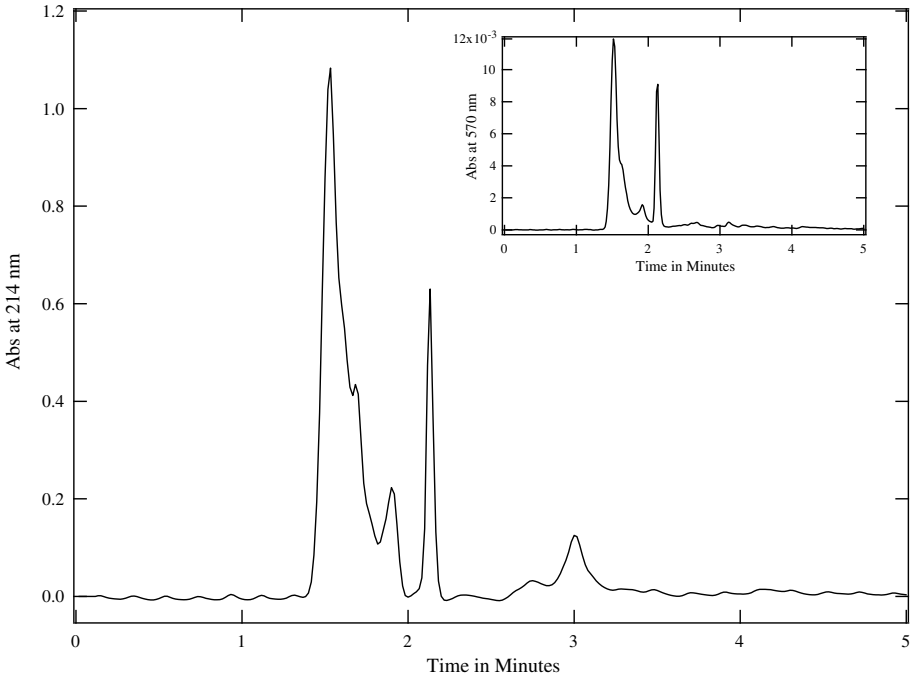

Fig. 1. HPLC chromatogram of crude proteins from bovine liver exposed to chromium(VI). Proteins were detected by monitoring optical density at 214 nm and the inset shows the monitoring of chromium(III) at 570 nm.

[12]. Currently, we are examining the distribution of chromium (both VI and III) throughout the purification process, using mass balance, to determine if the chromium is completely reduced by biological reductants. After filtration of larger proteins, the mixture shows six molecules with molecular masses under 15 kDa. At least four of these proteins contain chromium(III) and it would be interesting to determine if they are related to each other or to LMWCr. This relationship could be through dimerization or protein fragmentation. There have been reports that chromium(III) binding proteins/peptides could be unstable but a systematic protein decomposition study has not yet been undertaken [7,24,27].

The mass spectrum (Fig. 2) shows the exact mass of several of the proteins, and these peaks correlate with the bands shown in the gel electrophoresis data that have molecular weights less than 30 kDa. The peak at 15.6×10^3 M/Z has been assigned to a chromium(III) binding protein determined by UV–Vis and correlated with gel data. The peak at 7.8×10^3 M/Z has been assigned to the doubly charged ion of the parent 15.6×10^3 M/Z peak. To the best of our knowledge, this is the first mass spectrum of a chromium(III) binding protein to be published in the literature, although the mass spectrum for chromodulin has been discussed [4]. Attempts to obtain the mass spectrum of pure protein have yet to be successful. However, obtaining mass spectra of metal proteins with low binding constants is known to be problematic. In addition, there are limitations to chromium in the laser ablation process, which puts the molecule into the gas phase (David Van Horn, personal communication). We are continuing in our efforts to use MALDI-TOF and electro-spray to obtain a mass spectra/spectrum of the pure protein(s) and the exploration of this technique for protein sequencing.

Purification of 15 kDa chromium(III) binding protein

The crude sample was subjected to various chromatographic methods to separate the chromium(III) binding proteins, which lead to the successful purification of one of the these proteins, as shown by gel electrophoresis (Fig. 3). The first step in the purification process was to elute the mixture through a DEAE column. The mixture adheres to the top of the column, as evident by a green chromium band, supporting the anionic nature of these molecules [7,15]. It is interesting to note that during this process a yellow pigment elutes off the DEAE column with the first wash. The unidentified colored species is shown in the optical spectrum of the mixture in Fig. 4 with an absorbance around 400 nm. The absorbance at 400 nm is not characteristic of chromium(VI) and therefore was not investigated further. Earlier work by Vincent has suggested that this yellow species is bile [28]. Once this yellow pigment had been collected, the concentration of ammonium acetate buffer was increased to elute the proteins of interest. This process is an excellent method to separate the anionic chromium(III) binding proteins from the yellow pigment.

Either a G-25 size exclusion column or direct reverse phase HPLC will yield a pure 15.6 kDa protein. If the G-25 column is used to separate chromium(III) binding proteins, **1** will elute in the void volume. The SDS-PAGE gel shown in Fig. 3, demonstrates that the use of these two methods results in a fairly pure sample of the larger protein and gives an approximate molecular mass of 15 kDa. Using this information in conjunction with our mass

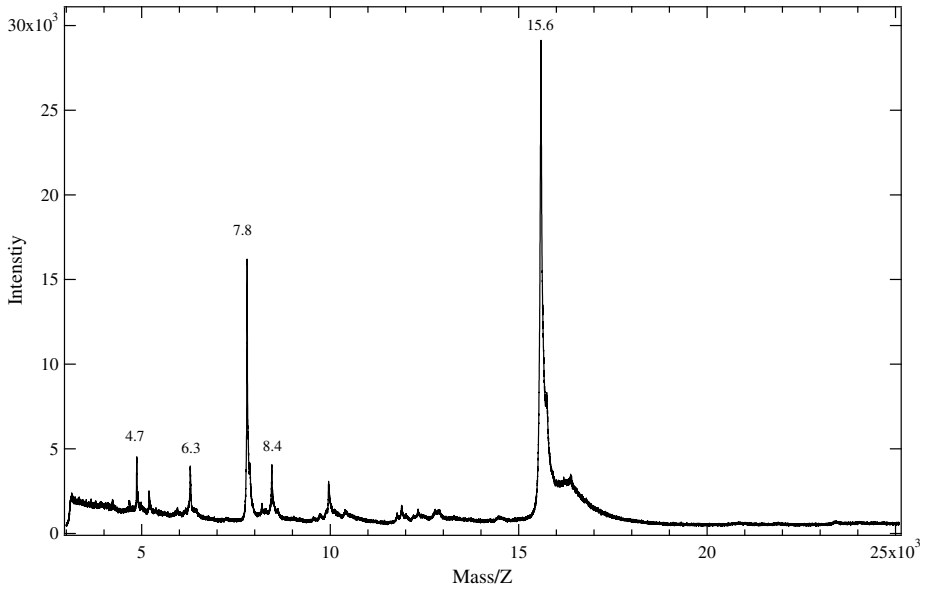

Fig. 2. MALDI-TOF mass spectrum of crude proteins. The most intense peak at 15.6×10^3 M/Z correlates with the band seen in gels around 15 kDa and the next intense peak at 7.8×10^3 M/Z has been assigned as the double charged ion of the 15.6×10^3 M/Z peak. Matrix was sinapinic acid in 50% ACN/0.1% TFA.

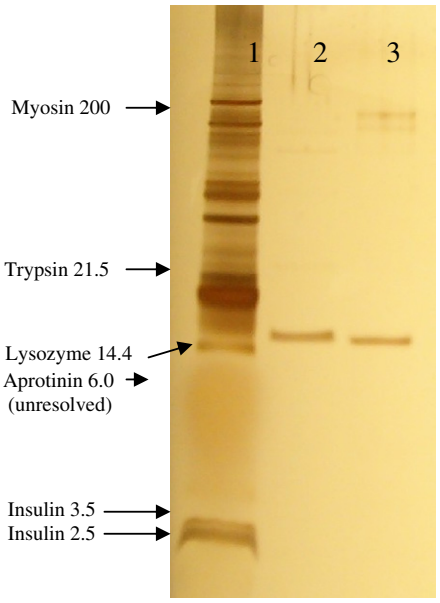

Fig. 3. SDS-PAGE of a purified chromium(III) binding protein. Lane 1 is a molecular weight standards. Lane 2 is the first band from purification by HPLC using a reverse phase column and lane 2 is purification by size exclusion chromatography G-25 (void volume).

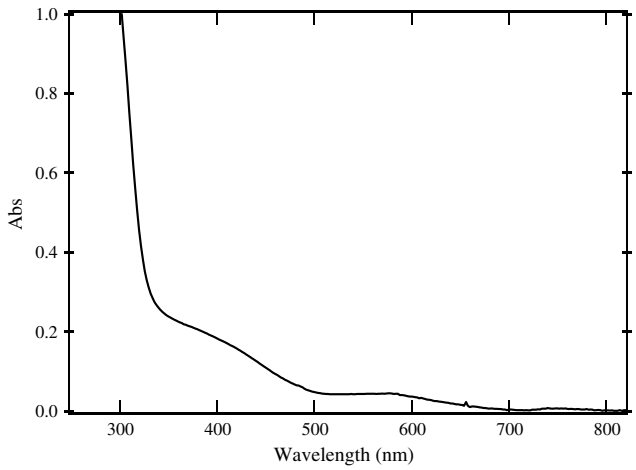

Fig. 4. Optical spectrum of the mixture of proteins showing the absorbance of the yellow pigment (not characteristic of chromium(VI)) that is isolated and the d–d transition of chromium(III) at 570 nm.

spectrum data, we have assigned an exact mass of 15.6 kDa to protein **1**.

The sample was additionally monitored for purity using HPLC, as shown in Fig. 5. This figure shows the elution profile, monitored at 214 nm for protein, and the inset shows the profile monitored at 570 nm for chromium(III). The optical spectrum (Fig. 6) of the purified protein shows that the protein contains chromium, as indicated by d–d transitions at 410 and 570 nm. The inset in Fig. 6 shows an additional peak at 260 nm, characteristic of other chromium(III) binding proteins isolated using this same procedure. This peak is not a common protein peak, and the intensity is too high for a d–d transition. This peak, which is consistently observed in our work and reported in the literature [7,15,29], could be a ligand to metal charge transfer band (LMCT). Significantly, all chromium binding proteins isolated to date display this optical transition at 260 nm.

Samples were shown to be pure by SDS-PAGE gel electrophoresis and silver staining (Fig. 3) and HPLC (Fig. 5). Chromium(III) ions in the protein were confirmed by optical spectroscopy as shown in Fig. 6 and DPC testing as discussed below. Fig. 6 shows peaks at 410 and 580 nm that are characteristic of chromium(III) d–d transitions.

Characterization of the chromium(III) binding protein

To determine the number of chromium(III) ions per protein, the mass from the crude mass spectrum of 15.6 kDa was used as an exact protein mass. Protein mass was converted to moles and compared with the number of moles of chromium(III) ions found from each protein sample. The number of chromium(III) ions were determined using the established DPC method [25], to be 2.5, and results are summarized in Table 1. If this larger protein leads to smaller fragmentation, resulting in LMWCr, we would expect this large protein to have a very high number of chromium(III) ions. This ratio is small compared to the 4 chromium ions per protein reported by Vincent for LMWCr, and 4 chromium ions per protein as reported by Dunham (personal communication) for a chromium binding protein of approximately 4 kDa [4,29]. Our results are derived from an average of three different liver samples, each analyzed in triplicate.

Further characterization of this novel protein includes amino acid analysis, and these results are summarized in Table 2. HPLC retention times were compared to amino acid standards using OPA. Table 2 additionally summarizes the amino acid analysis found for other chromium(III) binding proteins reported in the literature. One striking difference is the lack of cystine in our protein compared to other reports regarding chromium(III) binding proteins [4,7,29]. However all of the proteins reported thus far contain aspartic acid, or asparagine and glutamic acid or glutamine, which cannot be resolved in amino acid analysis techniques. Due to the hard nature of chromium(III) ions, we have concluded that the oxygen-rich amino acids comprise a chelating environment for chromium(III), which is logical since this is an oxo-philic metal and oxygen is a hard ligand. If the chromium environment is oxygen rich, then the LMCT optical transition at 260 nm (Fig. 6) could further be assigned to an oxo-metal charge transfer band.

There have been ample attempts to elucidate the amino acid sequence of chromium(III) binding proteins/peptides, but to date they have been unsuccessful for reasons that remain unclear [8,29]. Information regarding the secondary, tertiary and quaternary structure of this molecule could not be determined from the experiments presented here but hopefully will become available in future studies.

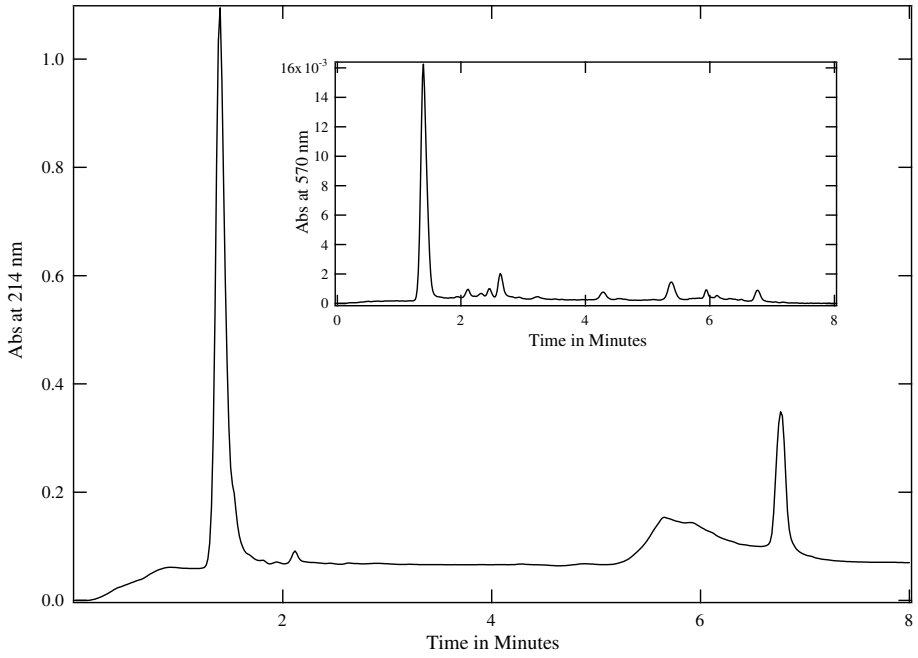

Fig. 5. Analytical HPLC trace of purified chromium(III) protein monitoring for protein content at 214 nm. Inset shows the monitoring at 570 nm wavelength characteristic of chromium(III).

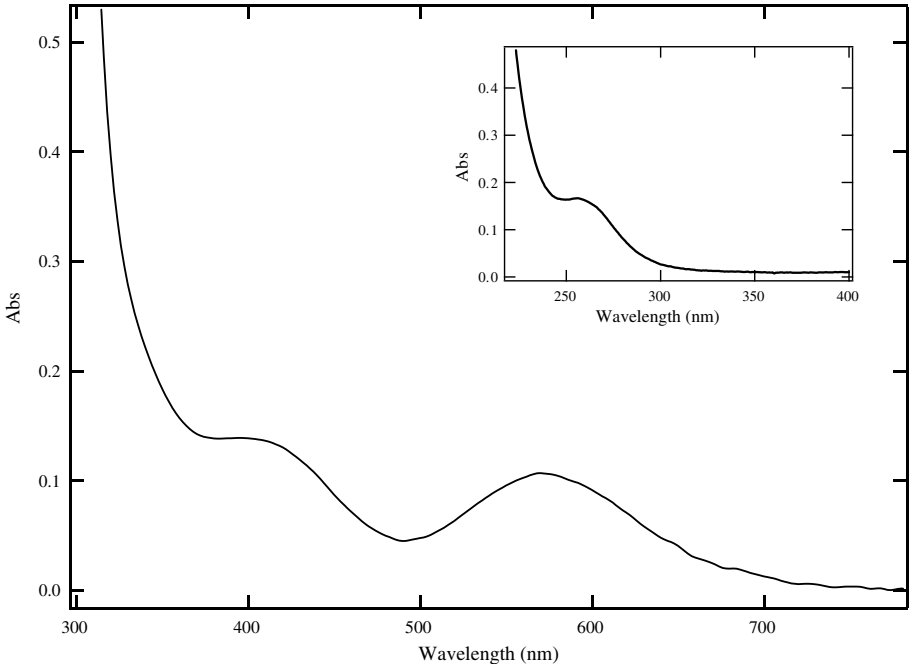

Fig. 6. Optical spectrum of the purified 15.6 kDa protein taken in water and showing the d–d transitions at 410 and 570 nm that are indicative of chromium(III). Inset shows a charge transfer band at 260 nm.

Therefore we cannot comment if this is a signal chromium binding protein or several peptides that are crossed linked by chromium. We are currently working on extracting the chromium, via dialysis, and examining the apo protein by, gel electrophoresis, HPLC and mass spectrometry. In addition we are continuing our efforts to deduce the amino acid sequence, and this report is the first in a series of papers that will fully characterize protein **1** and others from this reaction mixture. Significantly, the previously reported proteins have a very similar amino acid content and the protein reported here deviates from that content. This leads to the conclusion that the molecules are not related but are different products of the complicated redox reaction that takes place in biological tissue exposed to chromium(VI).

Table 1
Chromium(III) ion analysis on three bovine liver samples

Sample	Chromium(III) ions per protein
1	2.3
	2.5
	2.3
Average	2.4 ± 0.1
2	2.9
	2.8
	3.2
Average	3.0 ± 0.2
3	2.3
	2.6
	1.7
Average	2.2 ± 0.5

Each sample was tested in triplicate and three samples were taken. Molar ratios of chromium:protein in the chromium(III) binding protein were calculated on the basis of an approximate molar mass of 15.6 kDa derived from the mass spectrum and gel data.

Table 2
Amino acid composition of the novel chromium(III) protein derived from HPLC (this work) and compared to other previously reported chromium(III) binding proteins [24, 29]

Amino acid	Amount in chromium protein (this work)	UC Davis	Vincent	Dunham
Asx	1.00[a]	1.00[a]	2.00[b]	2.00[b]
Glx	2.51	2.10	4.15	2.00
Gly	2.09	2.20	2.29	0.70
Ala	0.39	0.20		
Cys			2.18	0.60
Lys	1.17	0.80		
His	0.37	1.80		

[a] Asx = 1.0.
[b] Asx = 2.

The protein digest and elution profile of the amino acids show that chromium binding protein **1** is comprised of primarily acidic and small amino acid side chains. The large presence of acidic amino acids is consistent with previous results reported by others, but our protein lacks the presence of cystine and indicates the presence of lysine. In the amino acid analysis there are three unassigned peaks with elution times of 7, 18, and 26 min. These molecules most likely contain one or more primary amines since the OPA derivatizing agent is specific to primary amines that lack a freely reducible sulfur atom. The unassigned peak eluting at 26 min is the only unassigned peak, which could interfere with integration parameters of another amino acid. This overlap is most likely to be responsible for the slightly higher amount of lysine content which is present in the samples diagnosed at Sonoma State University and analyzed at UC Davis.

4. Conclusion

The established literature process for the isolation of LMWCr was examined for protein/peptide selectivity. The process starts by reacting chromium(VI) with liver to produce a mixture of chromium(III) binding proteins/peptides. In this work we have shown that this complicated redox reaction produces at least four chromium(III) binding proteins/peptides and therefore is a non-specific process. In addition this supports previous work that chromium bind proteins are an artifact of the isolation procedure [3]. These four protein/peptide complexes can be reproducibly isolated. Reported here as the initial characterization of a novel 15.6 kDa chromium(III) binding protein (**1**) by optical spectroscopy, analytical HPLC, gel electrophoresis, mass spectrometry and amino acid analysis.

Continuing work in this area will focus on the exact nature of the novel protein presented here and the distribution of chromium in the isolation process. In addition experiments are currently focusing on the biological activity of all chromium(III) binding proteins isolated from the reaction of bovine liver with chromium(VI).

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