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Characterization of Chromodulin by X-ray Absorption and Electron Paramagnetic Resonance Spectroscopies and Magnetic Susceptibility Measurements

Lilian Jacquamet,[†,||] Yanjie Sun,[‡] Jason Hatfield,[‡] Weiwei Gu,[†] Stephen P. Cramer,[†,§] Michael W. Crowder,[#] Gary A. Lorigan,[#] John B. Vincent,*[,‡] and Jean-Marc Latour[⊥]

Contribution from the Department of Applied Science, University of California, Davis, California 95616, Department of Chemistry and Coalition for Biomolecular Products, The University of Alabama, Tuscaloosa, Alabama 35487-0336, Lawrence Berkeley Laboratory, Berkeley, California 94720, Laboratoire de Cristallographie et Cristallogénèse des Protèines (LCCP), Institut de Biologie Structurale J.-P. Ebel CEA-CNRS, 41 rue Jules Horowitz, 38027 Grenoble Cedex 1, France, Laboratoire de Physicochimie des Métaux en Biologie FRE 2427 CEA-CNRS-UJF, DRDC/PMB, CEA − Grenoble, 38054 Grenoble Cedex 9, France, and Department of Chemistry and Biochemistry, Miami University, Oxford, Ohio 45056

Received February 19, 2002; E-mail: jvincent@bama.ua.edu

Abstract: The biologically active form of the essential trace element chromium is believed to be the oligopeptide chromodulin. Chromodulin binds four chromic ions before binding at or near the active site of activating insulin receptor and subsequently potentiating the tyrosine kinase activity of the receptor. Charge balance arguments and preliminary spectroscopic studies suggested that the chromic centers might be part of a multinuclear assembly. Using a combination of X-ray absorption and electron paramagnetic resonance spectroscopies and variable-temperature magnetic susceptibility measurements, we found that holochromodulin is shown to possess an antiferromagnetically coupled trinuclear assembly which probably weakly interacts with a fourth chromium center. The chromium centers possess octahedron coordination comprised of oxygen-based ligation, presumably derived primarily from oligopeptide-supplied carboxylate groups. X-ray absorption data cannot be reproduced with the presence of sulfur atom(s), indicating that the cysteine thiolate group does not coordinate to the chromium centers. Thus, chromodulin possesses a unique type of multinuclear assembly, distinct from those known in other bioinorganic systems.

Introduction

When in the +VI oxidation state, chromium is a very toxic metal ion, while it can play a biological role when reduced to the +III oxidation state. Only two naturally occurring chromium-containing biomolecules are known.[1,2] The first is the well-known iron transport protein transferrin that doubles as a chromium(III) transport agent.[2] The second is the oligopeptide chromodulin, the form in which chromium is excreted and which may be biologically active. The oligopeptide chromodulin (also known as the low-molecular-weight chromium-binding substance, LMWCr) may function as part of a novel insulin-signaling autoamplification mechanism in mammals.[1,2] Chromodulin is maintained in its metal-free apo-form inside insulin-dependent cells. In response to insulin, chromium is mobilized from the blood and transported into these cells. Apochromodulin possesses a large chromic ion binding constant of 1.54×10^{21} and a Hill constant of 3.47, suggesting that essentially only holochromodulin and apochromodulin coexist in solution.[3] As a consequence, movement of chromic ions into the cells results in the generation of the holooligopeptide.[3] Holochromodulin, which contains 4 equiv of chromium, binds to the insulin-activated form of insulin receptor with an approximately 100 pM dissociation constant, further stimulating the tyrosine kinase activity of the receptor.[4] To down regulate this further stimulation, chromodulin is removed from the cells and ultimately appears in the urine.

The three-dimensional structure of the anionic oligopeptide has not been elucidated, and only limited information about the environment of the chromic centers is available. The oligopeptide from bovine liver is comprised of the amino acids glycine, cysteine, glutamate, and aspartate in a 2:2:4:2 ratio, respec-

[†] University of California, Davis.
[‡] The University of Alabama.
[§] Lawrence Berkeley Laboratory.
[||] Institut de Biologie Structurale J.-P. Ebel CEA-CNRS.
[⊥] CEA-CNRS-UJF.
[#] Miami University.

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tively.[5] Charge balance considerations suggest that anionic ligands should be present that are not provided by the oligopeptide; combined with the need for a total of 24 coordination sites about the 4 chromium centers, these considerations make the arrangement of the chromium centers into a cluster or clusters probable. The reconstitution of chromodulin from apochromodulin by the addition of chromic ions in the absence of added exogenous ligands suggests the possibility that oxo or hydroxo groups could serve as anionic bridging ligands. Electronic spectroscopic studies indicate that the chromium(III) centers are in pseudo-octahedral ligand fields and that the ligands are predominantly if not solely oxygen based.[5] The electronic spectroscopic studies also are consistent with the arrangement of the chromium centers into a cluster(s), while the results of paramagnetic ^{1}H NMR studies suggest the presence of bridging carboxylate ligands.

The synthetic complex, $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$, **1**, has been shown to mimic the ability of chromodulin to stimulate insulin receptor,[6] and when given intravenously to rats for 12 weeks, the compound resulted in dramatic lowering of fasting serum cholesterol and triglycerides levels.[7] This biomimetic activity has been used to suggest that chromodulin may possess an arrangement of chromium centers and ligands similar to that of the synthetic complex. The possibility that chromodulin might contain a tetranuclear chromium(III) assembly has prompted the synthesis of a few tetranuclear chromium(III)-oxo-carboxylate complexes.[8] All such tetranuclear complexes to date contain the butterfly-type $[Cr_4O_2]^{8+}$ core, but have limited solubility in water, which has prevented studies to test these complexes for any ability to stimulate insulin receptor kinase activity.

To get a deeper insight into the structural arrangement of the 4 chromium atoms and about the nature of their ligands, we have performed a thorough examination of holochromodulin using X-ray absorption and EPR spectroscopies and magnetic susceptibility measurements. These studies confirm the existence of a multinuclear Cr(III) assembly in the oligopeptide and indicate that the 4 Cr atoms are arranged in a "3 + 1" mode.

Experimental Section

Bovine liver chromodulin was obtained, and the concentrations of oligopeptide were determined as previously described.[5] Samples of chromodulin possessed 4.6(±0.4) Cr per amino terminus; the isolated chromodulin ran as a single band upon Shodex OH-pak HPLC. $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3](NO_3)$, **1**, was prepared using the method of Earnshaw and co-workers,[9] although it was originally incorrectly formulated as a hydrate of $[Cr_3(OH)_2(O_2CCH_2CH_3)_6](NO_3)$; $[Cr_4O_2(O_2CCH_3)_7(bpy)_2](PF_6)$, **2**, was prepared by the method of Bino and co-workers.[8c] $Cr(acac)_3$ was prepared as described by Fernelius and Blanch.[10]

EPR Spectroscopy. X-band EPR spectra were taken on a Bruker EMX spectrometer equipped with an Oxford liquid He cryostat and an Oxford Model ITC 4 temperature controller. Operating temperatures were read directly from the controller, which was calibrated with a carbon glass sensor. EPR samples were made by pipetting 300 μL of chromodulin (13.4 mM in chromium) into a 4 mm o.d. quartz EPR tube, purging the EPR tube with nitrogen gas for 2 min, and freezing the samples by slow immersion in a liquid nitrogen bath. EPR samples of $Cr(acac)_3$ and $[Cr_3O(CH_3COO)_6(H_2O)_3]^+$ were prepared by dissolving the solids in distilled H_2O until saturated solutions were obtained, and the solutions were transferred to EPR tubes and frozen as described above. Simulations of the EPR spectra were performed using WinSimfonia version 1.25. W-band EPR spectra were taken on a Bruker ELXSYS E-600 spectrometer with a three-channel temperature control system.

Magnetic Susceptibility Measurements. Inside a glovebox under argon was transferred 115 μL of chromodulin solution into a quartz sample bucket. Chromium analysis performed at the "Service Central d'Analyse du CNRS" (Vernaison, France) indicated that the concentration of the solution was 6.46×10^{-3} mol L^{-1} and therefore that the samples contained 7.43×10^{-6} mol of Cr. The samples were quickly frozen in liquid nitrogen outside of the glovebox and introduced in the magnetometer. Magnetization experiments were performed with a SQUID magnetometer SHE 500 at six different fields in the range 0.5−5 T in the temperature domain from 5 to 200 K. The data were analyzed according to the procedure outlined by Day.[11] In this treatment, the diamagnetic contribution of the protein is eliminated along with the ferromagnetic contamination of the quartz sample holder. In the present analyses, this contamination was introduced as a parameter (Y) to be adjusted in the fitting procedure. The best fit of the data obtained at 0.5 T was obtained with $Y = 0.0024(5)$ cm^3 mol^{-1}. The calculations were performed using a full matrix diagonalization procedure with a software written by P. H. Fries and E. Belorizky.[12] The R index for the "3 + 1" models was comprised between 2.1×10^{-7} and 2.6×10^{-7} with $R = \sum(\chi T_{exp} - \chi T_{cal})^2/\tilde{N}\cdot\sum(\chi T_{exp})$ and the number of measurements $N = 32$.

X-ray Absorption Spectroscopy. Cr K-edge XAS data for chromodulin were collected on beamline 7-3 at the Stanford Synchrotron Radiation Laboratory (SSRL) with the SPEAR ring running at 3.0 GeV and 50−100 mA average storage current. The samples were studied in frozen solution with a typical Cr concentration of 20 mM for a 100 μL volume. Data were collected in the range from 5980 to 6880 eV at the temperature of approximately 15 K in a liquid helium flow cryostat, with scans of ca. 30 min each (8−10 scans per sample). The energy was calibrated by using Cr foil as an internal standard, and the energy scale was assigned to the first inflection point (5989 eV) of the Cr foil spectrum. Harmonic rejection was accomplished by detuning the monochromator crystals to 50% of the maximum flux. Incident and transmitted current were measured using N_2 filled ion chambers. Fluorescence data were obtained using a 13-element Ge detector (Canberra). The average count rate at any individual detector element was ca. 15 000 cps. Data for the two model compounds were collected on finely ground samples mixed with boron nitride. Data were analyzed as previously described[13,14] with the SEDEM software package.[15] All E_0 values were arbitrarily chosen at 6003.5 eV, the value of the maximum of the spectrum derivative of chromodulin. FT have been calculated between 2 and 12.5 Å^{-1}. Curve fittings were performed on 0.9−3.3 Å Fourier-filtered data.

The structural parameters were obtained as described earlier.[13,14] Ab initio calculations have been performed with the FEFF 7.02 code[16] for a single absorber-scatterer pair Cr−O at 1.9 Å distance, a single Cr−C

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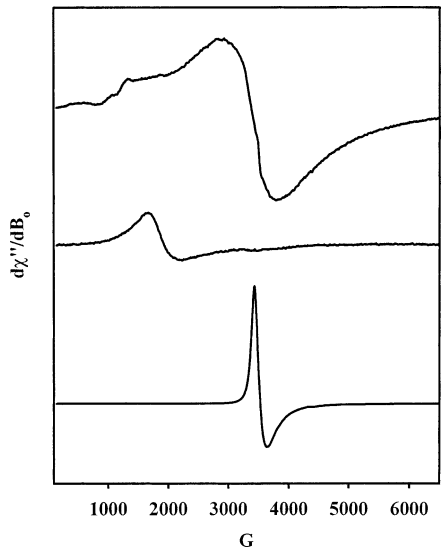

Figure 1. EPR spectra at 4.7 K of (top) chromodulin (13.8 mM in chromium), (middle) 2.7 mM $Cr(acac)_3$ in water, and (bottom) 17.2 mM $[Cr_3O(CH_3COO)_6(H_2O)_3]^+$ in water. EPR parameters were gain, 2.0×10^4; modulation frequency, 100 kHz; modulation amplitude, 1 G; conversion time, 40.96 ms; time constant, 81.92 ms; sweep time, 41.943 s; field center, 3350 G; sweep width, 6400 G; frequency, 9.48138 GHz; and power, 2.0 mW.

interaction at 3.0 Å, and a single Cr−Cr interaction at 3.3 Å. The reduction factor S_0^2 and the energy shift ΔE_0 (shift in E_0 from 6003.5 eV) were calibrated by fitting data from the two model compounds whose X-ray structures have been solved.[8c,17] The optimal values obtained were $S_0 = 0.85$ and $\Delta E_0 = -3.0$ eV for the absorber-scatterer pairs Cr−O/C and $\Delta E_0 = -10.0$ eV for Cr−Cr. N_i was held fixed, and R_i and σ_i^2 were optimized. The goodness of the fit is given by the fit index, FI.[13]

Results

EPR Spectroscopy. EPR studies have been undertaken to elucidate the electronic properties of the Cr assembly of chromodulin. The EPR spectrum of chromodulin at 4.2 K is shown in Figure 1(top), and the spectrum shows a broad signal centered near $g = 2.0$ and several smaller features at g values centered about $g = 5$. Spectra collected at 2 mW and between 4.2 and 40 K show that the signal at $g = 2.0$ had different temperature-dependence properties than the signal at $g \approx 5$ (Figure 2). The relative peak heights for the $g = 2.0$ (■) and $g \approx 5$ (●) signals were plotted separately against $1/T$; the resulting plots were linear, indicating that both signals obey the Curie law (signal $= a/T + b$), as described by Benton et al.[18] The slopes (a in equation above) of these plots were 29 and 18, respectively. Curie law is expected to strictly hold for isolated $S = {}^1/_2$ systems; however, in $S > {}^1/_2$ systems, low-lying excited states often become populated with increasing temperatures. The facts that both signals follow Curie law and that there are no new signals as the temperature is raised indicate that the $g = 2.0$ and $g \approx 5$ signals are due to ground spin state transitions.[18] This result indicates that the EPR spectrum of chromodulin arises from two spectroscopically distinct metal centers: one that yields the predominant, broad signal at $g = 2.0$ and one that yields a signal at $g \approx 5$. Further support for this hypothesis

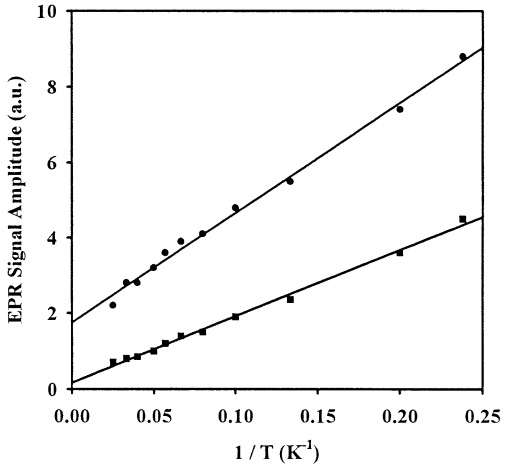

Figure 2. Temperature dependence of chromodulin EPR signals. The (●)'s represent the $g = 2.1$, and the (■)'s represent the $g \approx 5$ signals. [chromodulin] = 13.8 mM. EPR peak amplitudes were measured from baseline to signal peak height.

comes from EPR spectra of chromodulin collected at W-band; the spectrum at 6 K has a large feature at $g = 1.97$ and a smaller feature at $g = 4.2$ (data not shown).

In an effort to determine the nature of the metal centers in chromodulin, EPR spectra were collected on mononuclear ($Cr(acac)_3$) (Figure 1 (middle)) and trinuclear $[Cr_3O(CH_3COO)_6(H_2O)_3]^+$, **1**, (Figure 1 (bottom)) Cr(III) model complexes. Although not very soluble in aqueous buffer, $Cr(acac)_3$ yielded a broad, nearly axial signal with simulated g values of $g_x = 3.8$, $g_y = 3.8$, and $g_z = 1.9$, consistent with a $S = {}^3/_2$ center. The chromiums in the trimeric compound, **1**, were expected to be spin-coupled, resulting in a $S = {}^1/_2$ center.[19] The EPR spectrum of **1** consists of an asymmetric broad $g = 2$ peak. These results suggest that the $g = 2$ signal in the chromodulin sample could be due to a spin-coupled trimer that has an overall spin of $S = {}^1/_2$. One possibility to explain the breadth of the $g = 2.0$ signal in chromodulin is that the spin−lattice relaxation rate is faster than that in the trimer model complex; supporting this possibility are power saturation studies that demonstrated that chromodulin could not be saturated at microwave powers up to 200 mW. Thus, the mononuclear Cr(III) ($S = {}^3/_2$) site may provide an alternative dipolar relaxation pathway for the trimeric Cr(III) cluster in chromodulin. To test this hypothesis, EPR spectra of a mixture of 2.7 mM $Cr(acac)_3$ and 1.1 mM **1** were obtained; there was significant broadening of $g = 3.8$ and 2.0 signals, suggesting a coupling of the isolated centers (data not shown). Because the mononuclear Cr(III) site is tethered by a peptide chain to the trinuclear Cr(III) site in chromodulin, coupling via intramolecular pathways (chromodulin) would be expected to be stronger than that via intermolecular pathways (model complex mixture), resulting in broader signals in the case of chromodulin. Additional support for this hypothesis is the observation that the $g = 2.0$ feature in the spectra of chromodulin at X- and W-bands is sharper at 30 K than it is at 5 K (data not shown). As the temperature is raised, the dipolar coupling between the mononuclear and trinuclear centers in chromodulin would be expected to be overcome, leading to a sharper line.[20] On the other hand, the spectra of $[Cr_3O(CH_3COO)_6(H_2O)_3]^+$ become much broader as the temperature is

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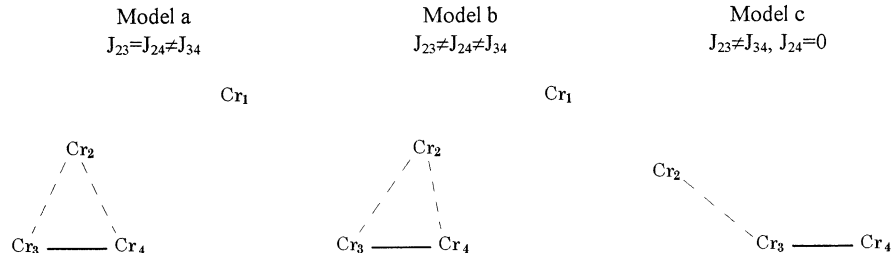
Scheme 1



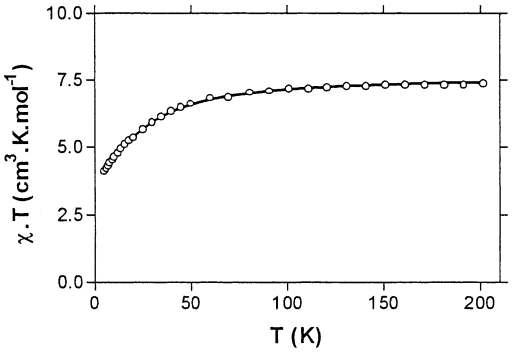


Figure 3. Temperature dependence of the product of the molar magnetic susceptibility by temperature: experimental values (○), calculated values (−). The data were corrected from the ferromagnetic/diamagnetic contributions (see text).

raised, supporting the prediction from the XAS studies that the structure of the trimeric Cr(III) center in chromodulin is different than that in the trimeric model complex.

The multiple unresolved signals at higher g values ($g \approx 5$) are possibly due to the mononuclear Cr(III) center in chromodulin. The inability to fully resolve these higher g-value features prevented our simulation of these signals; therefore, determination cannot be made as to whether these features are due to (1) a single Cr(III) center with $S = {}^3/_2$ that exhibits hyperfine coupling or splitting from the Cr(III) trimer or (2) several EPR signals arising from uncoupled Cr(III)'s in slightly different electronic environments. Indeed, the model of the tethered mononuclear Cr(III) in chromodulin would predict many possible structural arrangements of the mononuclear site with respect to the trimeric center. ENDOR experiments are planned to further test this hypothesis.

Magnetic Susceptibility Measurements. The magnetic properties of chromodulin are illustrated in Figure 3 as the temperature dependence of the product of the molar magnetic susceptibility with temperature ($\chi \cdot T$). The $\chi \cdot T$ product shows a high-temperature value close to 7.55 cm^3 K mol^{-1} at 200 K, decreasing slightly to 6.9 cm^3 K mol^{-1} at 70 K and then more abruptly to reach 4.0 cm^3 K mol^{-1} at 5 K. The high-temperature value is close to 7.5 cm^3 K mol^{-1} as expected for four uncoupled spins $S = {}^3/_2$ and in agreement with the reported chromium content. These data do not exhibit any significant field dependence (data not shown), and, therefore, no zero-field splitting parameter was introduced in the spin Hamiltonian (see Experimental Section). The overall temperature dependence is consistent with the occurrence of an antiferromagnetic interaction between the Cr atoms. Nevertheless, the $\chi \cdot T$ value at 5 K is not low enough to indicate an $S = 0$ ground state. It is worth noting that overall the magnetic behavior of chromodulin differs from those of **1** and **2**.

The simulation of the magnetic data was attempted using several plausible arrangements of the four Cr atoms that have precedent in the literature of inorganic complexes. High symmetry arrangements such as a cubane or a diamond or even a dimer of dimers could not account for the experimental data. On the other hand, the data could be satisfactorily fitted when assuming that the Cr_4 assembly was comprised of a mononuclear Cr atom and a trinuclear unit. Three different arrangements within the trinuclear unit were considered (Scheme 1): (a) an isosceles triangle with two independent J values, $J_{23} = J_{24}$ and J_{34}, (b) a triangle with three independent J values, J_{23}, J_{34}, and J_{24}, and (c) a "linear" arrangement with J_{23} and J_{34} (assuming that $J_{24} = 0$). The R indexes were, respectively, for a, 2.61×10^{-7}, for b, 2.06×10^{-7}, and for c, 2.53×10^{-7}. The best simulation (Figure 3) was obtained for b with the following parameters: $g = 2.02(2)$, $J_{23} = 0.25(10)$ cm^{-1}, $J_{24} = 1.3(5)$ cm^{-1}, and $J_{34} = -5.1(5)$ cm^{-1}. The values obtained in the simulation using models a and c are consistent with those from model b (model a: $g = 1.99$, $J_{23} = 0.69$ cm^{-1}, and $J_{34} = -4.6$ cm^{-1}, model c: $g = 2.03$, $J_{23} = 1.3$ cm^{-1}, and $J_{34} = -5.0$ cm^{-1}). Because of the small statistical preference (ca. 20%) for model b, it is not possible to discriminate firmly between the three models, but, in this respect, it is worth noting that a Cr_4 arrangement involving an equilateral triangle of Cr atoms does not fit the experimental data. Worthy of note also is that the magnetic interactions within the trinuclear unit clearly indicate the occurrence of a Cr pair loosely associated to a single Cr atom. This should reflect different bridging modes between the related Cr atoms.

X-ray Absorption Spectroscopy. To better characterize the structural properties of the Cr assembly of chromodulin, X-ray absorption studies have been undertaken on the biologically active oligopeptide chromodulin, the synthetic biomimetic **1**, and the tetranuclear chromium(III)-oxo-carboxylate complex **2**. The XANES (X-ray absorption near edge structure) of chromodulin is compared to those of the two model compounds in Figure 4. The edge positions are almost superimposable, which indicates that the mean oxidation state is indeed the same in chromodulin and in the models. The insert of Figure 4 which presents the preedge features, corresponding to 1s → 3d transitions, shows that they are far weaker in chromodulin than they are in the model compounds. Because the preedge intensity is enhanced by distortion from octahedral symmetry,[21] these observations indicate an overall Cr environment less distorted in chromodulin. In addition, the 1s → $4p_z$ transition (at ca. 6002 eV) present in the model compounds is absent in chromodulin. This is in agreement with the very weak preedge intensities between 5992 and 5997 eV associated to the 1s to 3dσ

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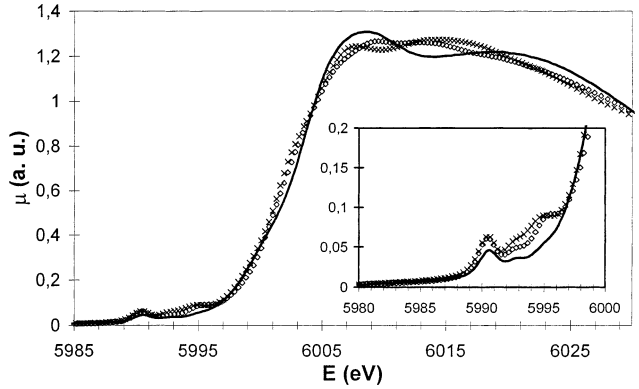

Figure 4. XANES spectra of chromodulin and two model compounds. Chromodulin (−), **1** (◇), **2** (×). The insert is an enlargement of the preedge features.

transitions. Both features are consistent with the absence of axial distortion. These results suggest that oxo bridge(s) usually responsible for an axial distortion are absent in chromodulin. In addition, the intensity of the other 1s → 4p transition (between 6005 and 6015 eV) in chromodulin is similar to those of the two model compounds that possess hexacoordinated chromium. This result suggests mean hexacoordination for the 4 Cr in chromodulin (see below).

EXAFS (extended X-ray absorption fine structure) and corresponding Fourier transform (FT) of chromodulin and of the two model compounds are presented in Figure 5a and b. The FT of model compounds presents two main peaks at ca. 1.6 and 2.8 Å (not corrected for the phase shift) corresponding, respectively, to the first coordination shell of the Cr atoms and to a Cr−Cr shell. The amplitude of the coordination shell is weaker in **2** than it is in **1**, which was expected because crystallographic analyses revealed a wider Cr−O distance distribution.[8c,17] The second peak at approximately 2.8 Å (noncorrected from the phase shift) (Figure 5a and b) corresponds to the Cr−Cr shell at approximately 3.3 Å. This peak is also weaker in **2** because of a wider Cr−Cr distance distribution. EXAFS fits of **1** and **2** match well with the average crystallographic distances[8c,15] for the first coordination shell at 1.97 Å, for the mean Cr−C(carboxylate) distance at approximately 3.0 Å, and for the Cr−Cr distance (wingtip to hinge for the butterfly type Cr_4O_2 core) at approximately 3.3 Å (Table 1). However, in **2**, the two other Cr shells (hinge to hinge and wingtip to wingtip) are not clearly detected probably because those shells do not contribute significantly to EXAFS (only 0.5 Cr is present in those shells) and may give rise to destructive interference with other light scatterers. It is noteworthy that the O shell at approximately 3.3 Å is not detected due to interference with the Cr shell. Trying to replace the Cr shell by an O shell does not fit the data, and adding an O shell to the Cr does not improve the fit.

As shown in Figure 5a and b, the Cr environment in chromodulin is clearly different from those of the model compounds. Indeed, chromodulin FT presents a peak at approximately 2.0 Å corresponding to the first coordination shell. This peak is stronger than those of the model compounds, indicating that the mean Cr environment of the 4 Cr in chromodulin is less distorted and that the distance distribution of the first shell is narrower than those of the model compounds. The chromodulin EXAFS of the first coordination shell can be

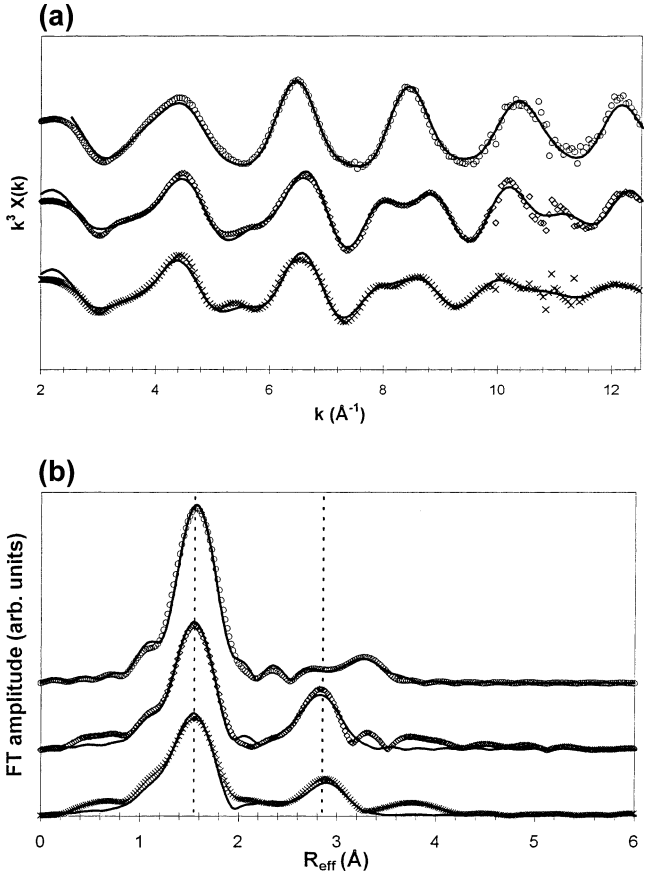

Figure 5. (a) EXAFS spectra and corresponding fits (−) of chromodulin (○) and model compounds **1** (◇) and **2** (×); (b) EXAFS FT spectra and corresponding fits (−) of chromodulin (○) and model compounds **1** (◇) and **2** (×).

fitted with one shell of 6 O/N at 1.98(2) Å. Splitting this coordination shell into two subshells of O/N atoms does not improve the fit. It is worth noting that the first shell cannot be reproduced with the presence of 1 S atom per Cr. On the other hand, 0.5 S per Cr would be very difficult to detect by the fitting procedure. Therefore, while a bis(μ-sulfido) bridging pattern can be ruled out, S coordination to a Cr atom cannot. Furthermore, the peak corresponding to the Cr−Cr distance at approximately 2.8 Å (noncorrected from the phase shift) in the model compounds is not present in chromodulin, but a weaker peak at approximately 3.2 Å (noncorrected from the phase shift) is present. This peak can be assigned to a Cr−Cr shell at 3.8 Å, and the fit can be significantly improved by adding a shell of 5 C at 3.12 Å (Table 1). As in **2**, the fit is further improved by adding a 0.5 Cr shell at 2.79 Å, but the Debye−Waller factor is quite high. Nevertheless, the fit improvement is rather statistically acceptable, and it is identical to that of **2**. In addition, it must be kept in mind that interferences between multiple scatterings of carboxylates and scatterings of other atoms might lead to other equivalent fits.

Discussion

The present spectroscopic and magnetic experiments reinforce some of the conclusions derived earlier. Indeed, magnetic susceptibility and EPR agree with the presence of 4 Cr atoms in holochromodulin. Because of the strong cooperative binding of Cr atoms to apochromodulin, all added Cr atoms are found in holochromodulin. In addition, the edge energy of the XANES

Table 1. Selected Curve-Fitting Results of Filtered EXAFS Spectra of **1** and **2** and of Chromodulin

compound	*N*	*R* (Å)	σ^2 (10^3 Å^2)	FI[10]
1	6 O	1.97(2)	4.3	17.2
	6 O	1.97(2)	4.3	3.9
	2 Cr	3.27(2)	3.6	
	6 O	1.97(2)	4.4	
	4 C	2.98(2)	2.9	1.2
	2 Cr	3.26(2)	5.2	
2	6 O	1.99(2)	7.6	14.0
	6 O	1.99(2)	7.7	5.5
	2 Cr	3.35(2)	8.5	
	6 O	1.99(2)	7.7	3.4
	5.5 C	2.95(2)	17.2	
	2 Cr	3.33(2)	8.6	
	6 O	1.99(2)	7.6	
	0.5 Cr	2.81(2)	3.9	2.4
	5.5 C	2.98(2)	5.7	
	2 Cr	3.35(2)	10.0	
chromodulin	6 O	1.98(2)	2.1	5.2
	6 O	1.98(2)	2.2	3.4
	1 Cr	3.80(2)	3.4	
	6 O	1.98(2)	2.2	
	5 C	3.12(2)	5.9	1.7
	1 Cr	3.79(2)	3.2	
	6 O	1.98(2)	2.2	
	0.5 Cr	2.79(2)	9.1	1.2
	5 C	3.12(2)	5.4	
	1 Cr	3.79(2)	3.3	

spectrum and the value of the magnetic susceptibility confirm the oxidation state +III of all Cr atoms. This is further supported by the analysis of the EXAFS data using the bond valence sum analysis (BVS).[22] The analysis in addition to bond lengths from crystal structures or EXAFS investigations requires only the value of a constant for each bond type (i.e., Cr(III)−O). For chromodulin, the EXAFS data and previous electronic spectroscopic studies[5] indicate that probably only Cr−O bonds are present, and fortunately the necessary constants, R_o, have previously been determined for Cr(II), Cr(III), and Cr(IV).[23] For chromodulin, a chromium oxidation state of 2.9, or essentially Cr(III), is obtained when six bonds with an average bond length of 1.98 Å are considered.

The present X-ray absorption studies are consistent with electronic absorption studies that indicate the existence of chromic centers in an octahedral ligand field of oxygen-based ligands. EXAFS analysis can totally rule out the occurrence of two sulfur bridges but not the involvement of any of the two cysteines in Cr binding in chromodulin. Indeed, 1 or 2 sulfur atoms among 24 ligands would be very difficult to detect, and, therefore, it cannot be totally ruled out that 1 or 2 S might be coordinated to chromium(s), although this is not likely. Indeed, the FT peak of the O/N shell is narrower and stronger in chromodulin than it is in the model compounds. This indicates a more homogeneous distribution of the bond distances and strongly suggests a purely oxygenated coordination sphere of the 4 Cr atoms, certainly made up of the carboxylate groups of the numerous aspartate and glutamate residues present in chromodulin. In addition, it suggests that neither a contraction (short Cr−O bond) of the coordination shell, such as those characteristic of oxo ligands, nor an elongation (Cr−S bond) is present. These results are also corroborated by the preedge and XANES features. The participation of water molecules to complete the coordination sphere cannot be entirely ruled out, although it is not supported by EPR experiments. Indeed, EPR spectra obtained at 4.2 K on samples of chromodulin in D_2O and in 5 mM NaF showed no appreciable difference from the spectra in H_2O. This seems to suggest that no readily exchangeable coordination sites are available on the chromic centers.

EPR and magnetic susceptibility experiments concur to show that the 4 Cr atoms of holochromodulin are arranged in a "3 + 1" mode. Moreover, magnetic susceptibility allows for the arrangement of the trinuclear unit to be further delineated. Although it is not possible to discriminate firmly between the three arrangements depicted in the scheme, the most symmetrical situation associated to an equilateral triangle is definitely ruled out, and it appears clearly that two Cr atoms are more strongly coupled together ($J \approx -5.0$ cm^{-1}) than with the third one ($J \approx 1.0$ cm^{-1}). This suggests that different bridging ligands should be present within the Cr pair and between it and the third Cr atom. From literature data, it is reasonable to propose that the smaller interaction can be brought about by carboxylate bridges, while the stronger one would require oxygen anions, oxides, or hydroxides. Nevertheless, it is worth noting that oxide bridges usually mediate antiferromagnetic coupling stronger than that observed here. Moreover, the rather symmetric Cr geometry observed by XAS in chromodulin is in agreement with the absence of oxo bridges. EXAFS analysis also clearly rules out in chromodulin the existence of a Cr_3-μ_3-O unit like that in **1**, the biomimetic compound, or any related type of structure giving rise to approximately 3.3 Å Cr−Cr separations. However, EXAFS analysis suggests the presence of 1 Cr at 3.79 Å and 0.5 Cr at 2.79 Å. This result is consistent with model a of the scheme (Cr_3−Cr_2 and Cr_4−Cr_2 distances). The occurrence of hydroxo bridges appears consistent with all experimental data, in particular, the Cr−Cr interaction at 2.79 Å, but this requires further substantiation. Carboxylate bridging between the Cr pair and the third Cr atom is consistent with the small exchange interaction and the Cr−Cr interaction at 3.79 Å. In this respect, it is worth noting that the presence of bridging caroxylates was deduced earlier from ^{1}H NMR spectroscopy.

Conclusion

A combination of EPR and XAS spectroscopic studies and magnetic susceptibility experiments confirms the occurrence of a Cr_4 cluster in holochromodulin and gives insight into its structural arrangement. The Cr environment is mostly if not exclusively composed of O atoms, and the cluster comprises a single Cr atom and a trinuclear unit. The latter is potentially arranged as a hydroxo-bridged pair linked to the third atom through aspartate/glutamate bridges.

Furthermore, the present results allow for the resolution of an inconsistency in the literature. Preparations of chromodulin with approximately 90% of the chromium removed still have appreciable biological activity,[4b] suggesting that the active

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portion of the oligopeptide should have all of the chromium intact. The EPR spectra of these chromodulin preparations at 10 K contain a very weak signal centered at $g \approx 2$. This signal was incorrectly assigned to mononuclear chromium(III) centers[4b] from integration of its intensity with respect to mononuclear Cr^{3+} ($S = {}^3/_2$) standards. Assignment of this signal at $g \approx 2$ to holochromodulin makes the metal removal and activity and reconstitution studies consistent.

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