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Iron(II)-Substituted Metallothionein: Evidence for the Existence of Iron–Thiolate Clusters†

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ABSTRACT: Metallothioneins (MT's) are unique low molecular weight (M_r 6000–7000) metal- and cysteine-rich proteins characterized by two tetrahedral tetrathiolate clusters containing three and four metal ions. Naturally occurring proteins usually contain the diamagnetic metal ions Zn(II) and/or Cd(II). We have now succeeded in substituting these ions by paramagnetic Fe(II). Rabbit liver MT-1 in which all seven metal binding sites were occupied by Fe(II) ions displays absorption features typical of tetrahedral tetrathiolate Fe(II) coordination. This is documented by the presence of a ligand field $^5E \rightarrow {}^5T_2$ transition in the near-infrared region centered at about 1850 nm ($\epsilon_{Fe} \approx 100\ M^{-1}\ cm^{-1}$) and a broad charge-transfer absorption in the UV region with a shoulder at 314 nm. A metal–thiolate cluster structure is inferred from the 7 to 20 ratio of metal ions to cysteine residues and from spectral studies in which successive increments of Fe(II) were incorporated into the metal-free protein. Thus, to about 4 equiv, the charge-transfer absorption and magnetic circular dichroism (MCD) features of the complexes formed resemble closely those of reduced rubredoxin from *Desulfovibro gigas* in which tetrahedral tetrathiolate Fe(II) coordination is documented. However, upon further addition of Fe(II) ions, the charge-transfer absorption bands undergo a progressive red-shift until the full metal occupancy of seven Fe(II) ions per molecule is reached. The bathochromic shift which is also manifested in the MCD spectra can be ascribed to the transformation of some of the terminal thiolate ligands to bridging when the full complement of Fe(II) is bound. The concomitant loss in amplitude of the MCD bands above 4 equiv is thought to arise from exchange coupling of vicinal Fe(II) via the thiolate bridges.

Metallothioneins (MT's)[1] constitute a class of low molecular weight (M_r 6000–7000) metal- and cysteine-rich proteins widely distributed in nature (Kägi et al., 1984) for which physiological functions in metal storage and/or heavy-metal detoxification were proposed (Nordberg & Kojima, 1979). The best characterized mammalian forms contain a single polypeptide chain of 61 amino acids, out of which 20 residues are cysteine (Kojima et al., 1976). Typical of all MT's is the occurrence of a number of unique Cys-X-Cys sequences (X = amino acid residues other than Cys) in their primary structures. The naturally occurring MT's usually bind seven

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[1] Abbreviations: MT, metallothionein; apoMT, apometallothionein; MCD, magnetic circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; 2D, two dimensional; 3D, three dimensional; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

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diamagnetic bivalent metals such as Zn(II) and/or Cd(II). Spectroscopic and chemical studies on these unusual proteins have established the existence of two metal–thiolate cluster structures of three and four metal ions, respectively (Otvos & Armitage, 1980; Winge & Miklossy, 1982), in which each metal ion is tetrahedrally coordinated by four thiolate ligands (Vašák, 1980; Vašák & Kägi, 1981). Recently, both the 3D solution structure of rabbit liver Cd_7–MT-2 employing 2D NMR (Frey et al., 1985; Braun et al., 1986) and also the X-ray structure of crystalline rat liver (Zn_2Cd_5)–MT-2 (Furey et al., 1986) have been determined. Although the X-ray structure and 2D NMR solution structure are at variance with each other in certain details of the organization of both metal–thiolate clusters, the overall mode of metal binding within these clusters was found to be identical.

That MT may serve a role in intracellular iron metabolism has been considered ever since it was first identified as a minor constituent in equine renal MT (Kägi & Vallee, 1961). However, because of the failure to observe in vitro binding of Fe(II) ions to metal-free MT (apoMT), this hypothesis has been questioned (Kojima et al., 1982). The present work demonstrates that under appropriate conditions Fe(II) forms the same well-defined complexes as do Zn(II), Cd(II), or Co(II). Electronic absorption and magnetic circular dichroism (MCD) techniques were used to monitor the binding of Fe(II) ions to apoMT.

Experimental Procedures

Chemicals, Instruments, and Physical Measurements. Rabbit liver MT was isolated from rabbits injected with $CdCl_2$ by 20 subcutaneous injections of 1 mg of cadmium sulfate per kilogram body weight at intervals of 2–3 days. MT was purified by a combination of the procedures of Kimura et al. (1979) and Kägi et al. (1974). The purity of each preparation was examined by amino acid analysis (Durrum D-500) and by metal analysis using atomic absorption spectrometry (Instrumentation Laboratory IL 157). All studies reported in this work were performed on the isoform MT-1. All inorganic and organic chemicals were of reagent grade or better, and prepared solutions were stored in polyethylene bottles.

Absorption spectra were recorded on a Perkin-Elmer Model 340 spectrophotometer using 0.2-cm (for measurements with $\lambda < 500$ nm) and 1.0-cm (for measurements with $\lambda > 1000$ nm) quartz cells. Molar absorptivities (ϵ) are given in units of M^{-1} cm^{-1}. A Jasco spectropolarimeter (Model J-500), connected on-line with an Epson-QX personal computer, was used for MCD measurements, employing a constant magnetic field of 15 kG at room temperature. All MCD spectra were recorded with the magnetic field parallel to and in the direction of the light beam. Molar ellipticity ($[\theta]_M$), in units of degrees centimeter squared per decimole per gauss, was calculated according to the equation $[\theta]_M = \theta/10cdH$. θ is the ellipticity measured directly in millidegrees [$\theta = 3300(A_L - A_R)$], c is the molar concentration, d is the path length in centimeters, and H is the magnetic field in units of gauss.

Preparation of ApoMT. ApoMT, the metal-free protein, was prepared by gel filtration of the native form at low pH. A neutral solution of (Zn,Cd)–MT was adjusted to pH 1 and subsequently passed over a Sephadex G-25 column, equilibrated with 0.01 N HCl. The pooled fractions were lyophilized and stored at −20 °C. Protein concentration was determined spectrophotometrically by measuring the absorbance of apoMT in 0.01 N HCl at 220 nm using an extinction coefficient of 48 200 M^{-1} cm^{-1} (Bühler & Kägi, 1979) or by quantitative amino acid analysis. Prior to metal reconstitution, all cysteine residues were determined by using Ellman's reagent (DTNB) in 10 mM potassium phosphate buffer, pH 7.5 (ϵ_{412} = 13 600 M^{-1} cm^{-1}) (McGilvray & Morris, 1971).

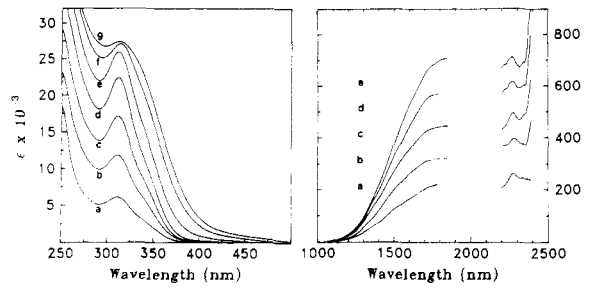

FIGURE 1: Electronic absorption spectra of Fe^{II}–MT in the charge-transfer (left) and near-infrared (right) region as a function of metal to protein ratio. The moles of Fe(II) per mole of apoMT is the following: (left) (a) 1, (b) 2, (c) 3, (d) 4, (e) 5, (f) 6, (g) 7; (right) (a) 2.3, (b) 3.4, (c) 4.6, (d) 5.7, (e) 6.9. Conditions: (left) 0.17 mM Fe^{II}–MT in 160 mM Tris-HCl, pH 8.3, at room temperature; (right) 0.68 mM Fe^{II}–MT in 180 mM Tris-DCl, pH 8.4, at room temperature. ϵ is based on the protein concentration.

Preparation of Fe^{II}–MT. All solutions used in the preparation of Fe^{II}–MT were degassed on a vacuum line prior to use, and the procedures were performed in an argon-purged glovebox. A freshly prepared solution of 0.2 or 1.0 mM apoMT in 0.1 N HCl was mixed with 50 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ in bidistilled water, yielding the desired metal to protein ratio. The solution mixture was then adjusted to pH 8.3 by the addition of 2 equiv of 1 M Tris base (Trizma from Sigma). The spectra in the near-infrared region were obtained in solutions of D_2O (99.8%). The pH value given represents the pH meter reading without correction for deuterons. The metal to protein ratios were determined independently by using a small aliquot of the sample. At neutral pH, the extremely air-sensitive Fe^{II}_7–MT has a yellow color. On exposure of the aqueous solution to air, the color changes immediately to wine-red, and slow protein precipitation sets in.

Results and Discussion

Electronic Absorption Properties. To determine the binding capacity of this protein toward Fe(II) ions, the stepwise incorporation of these ions into apoMT was investigated. Figure 1 shows the family of electronic absorption spectra of Fe^{II}–MT of increasing intensity formed at various Fe(II) to protein molar ratios. At exactly 7 Fe(II) equiv bound, the increase in absorbance in both near-UV and near-infrared regions levels off, indicating formation of fully saturated Fe^{II}_7–MT (Figure 2, top). Similar conclusions can be drawn also from the corresponding MCD studies (see below). At all stages of filling, the coordination of the protein-bound Fe(II) remains tetrahedral as found for Co(II)-reconstituted MT (Vašák & Kägi, 1981). This is documented in particular by the occurrence of the characteristic ligand field absorption in the near-infrared region (Figure 1, right). Although the Fe(II)-dependent electronic absorption profile is obscured in part by the vibrational transitions of the solvent, its center can be located at about 1850 nm with a molar extinction coefficient of 100 M^{-1} cm^{-1} per Fe(II). Similar absorption profiles in the near-infrared region have been reported for a number of inorganic mononuclear as well as polynuclear tetrahedral tetrathiolate–Fe(II) complexes (Holah & Coucouvanis, 1975; Lane et al., 1977; Coucouvanis et al., 1981; Hagen et al., 1981) and peptide models (Anglin & Davison, 1975; Ueyama et al., 1985). In accordance with these studies, the ligand field absorption in Fe^{II}–MT is attributable to the $^5E \rightarrow {}^5T_2$ transition in the tetrahedral field which corresponds to the elec-

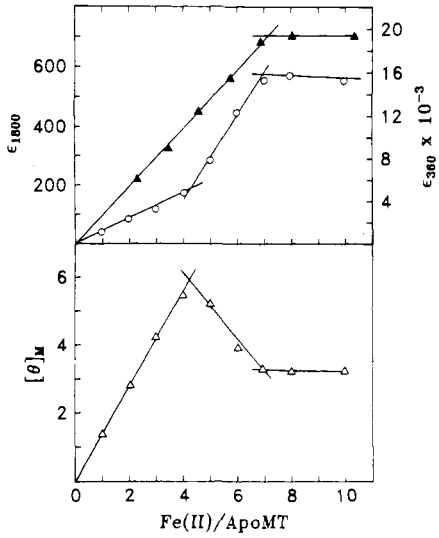

FIGURE 2: Dependency of the absorbance at 360 (○) and 1800 nm (▲) (top) and the magnitude of the low-energy MCD maximum (△) (bottom) on the Fe(II) to apoMT ratio. Conditions are as in Figure 1.

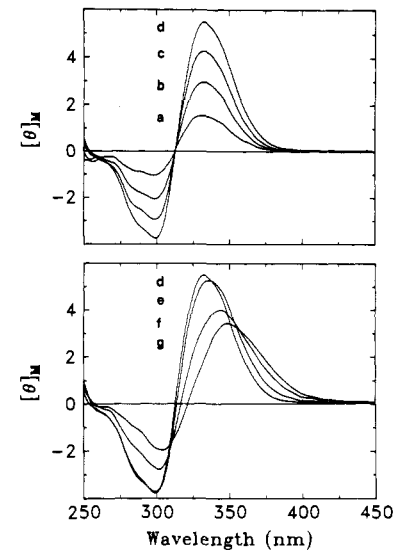

FIGURE 3: MCD spectra of Fe^{II}–MT as a function of metal to protein ratio. The moles of Fe(II) per mole of apoMT is (a) 1, (b) 2, (c) 3, (d) 4, (e) 5, (f) 6, and (g) 7. Conditions as in Figure 1, left. $[\theta]_M$ is based on the protein concentration.

tronic promotion $e^3t_2^3 \rightarrow e^2t_2^4$. The appearance of a rather strong absorption in the D_2O window between 2200 and 2400 nm, when compared with that at 1850 nm, suggests, in agreement with the corresponding Fe(II) model compounds (Lane et al., 1977), the presence of an additional low-energy absorption band arising from the Fe^{II}–thiolate complex. The relatively sharp absorption band at 2380 nm superimposed on the broad electronic band can be attributed to vibrational overtone transitions, either from the protein or from residual HOD. The existence of more than one ligand field band in Fe^{II}–MT suggests a departure from purely tetrahedral symmetry, in agreement with a splitting of the t_2 orbitals. Independent support for this conclusion can be drawn from the ligand field spectrum of Co^{II}–MT, where the occurrence of a large energy separation within the characteristic triplet of the ν_3 transition indicates distorted tetrahedral Co(II) coordination (Vašák & Kägi, 1981). Although the partial masking of the ligand field bands in Fe^{II}–MT does not allow an assessment of Δ_t, this spectral location is in agreement with the weak ligand field nature of the thiolate ligands (Lane et al., 1977).

From the 7 to 20 ratio of Fe(II) to cysteine residues, a metal–thiolate cluster structure is inferred. More direct support for such aggregates is afforded by the spectroscopic changes attending the corresponding titration studies in the UV region (Figure 1, left). Since aromatic amino acids are lacking in MT, the electronic absorption profile in the near-UV region arises exclusively from metal binding (Vašák & Kägi, 1983). This is clearly seen from the rise in absorption envelopes upon incremental addition of Fe(II) ions. These spectral features which are characterized at low metal occupancy by an absorption maximum at 311 nm and a shoulder at 333 nm (Figure 1, left) match those found for reduced rubredoxin from *Clostridium pasteuranium*, a protein for which a tetrahedral Fe(II) coordination by four cysteine thiolates is well documented (Eaton & Lovenberg, 1973; Watenpaugh et al., 1979). Bands and/or shoulders at similar positions have been observed also for a number of model complexes possessing the overall tetrahedral tetrathiolate Fe(II) coordination (Anglin & Davison, 1975; Lane et al., 1977; Ueyama et al., 1985). According to Bair and Goddard (1978), these transitions were attributed to metal ligand charge-transfer excitations. Above about 4 Fe(II) equiv, the absorption increments accompanying Fe(II) binding become progressively smaller, and a red-shift of the charge-transfer envelope sets in, leading to a loss of resolution. An illustration of this effect is given by the plot of the absorbance at 360 nm as a function of the Fe(II) to apoMT ratio which is characterized by the change of the slope at about 4 Fe(II) equiv (Figure 2, top). As documented by the electronic absorption spectra of well-defined mononuclear and oligonuclear inorganic tetrahedral tetrathiolate Fe(II) model complexes, this red-shift reflects most likely the formation of bridging thiolate ligands. Thus, both the binuclear $[Fe_2(S\text{-}o\text{-}xyl)_3]^{2-}$ and the tetranuclear adamantane-type $[(FeSPh)_4(\mu_2\text{-}SPh)_6]^{2-}$ complexes feature in the charge-transfer region a characteristic red-shift with respect to the spectral location of the corresponding mononuclear complexes (Lane et al., 1977; Coucouvanis et al., 1981; Hagen et al., 1981). The occurrence of the red-shift in the course of the filling-up process of the metal binding sites of apoMT with Fe(II) would then indicate the progressive transition of some nonbridging thiolate ligands into bridging ones, resulting in the formation of a cluster structure.

MCD Properties. Since MCD was informative in the studies of iron–sulfur proteins (Johnson et al., 1982), this technique has been used in order to monitor spectral changes on going from the nonclustered to the clustered structure in Fe^{II}–MT. At low metal occupancy, the MCD profiles display a maximum at 332 nm and a trough and shoulder at 299 and 286 nm, respectively (Figure 3). The observed MCD profiles resemble those found in the mononuclear tetrahedral tetrathiolate–Fe(II) complexes in the reduced rubredoxins from *C. pasteurianum* and *D. gigas* as well as that of desulforedoxin of the latter species (Eaton & Lovenberg, 1973; Johnson et al., 1982). The MCD features in the former case have been suggested to mainly arise from *B* or *C* terms although a contribution of *A* terms to the high-energy MCD band has not been ruled out (Eaton & Lovenberg, 1973). The presence of *C* terms has been confirmed by Johnson et al. (1982), who recently measured the MCD spectra of reduced rubredoxin and desulforedoxin, both from *D. gigas*, as a function of temperature down to 17.5 K. By analogy to the electronic absorption behavior of Fe^{II}–MT (see above), substantial spectral changes above 4 Fe(II) equiv bound are also seen in the corresponding MCD spectra. They are characterized both by a similar red-shift, mainly of the low-energy MCD band, and

by a substantial amplitude decrease. An illustration of the latter effect is given by the plot of the molar magnetic ellipticity of the first low-energy MCD maximum as a function of the Fe(II) to apoMT ratio (Figure 2, bottom). This effect is thought to reflect changes in electronic states upon cluster formation that are brought about by the exchange coupling of vicinal Fe(II) via bridging thiolate ligands. Attempts to document such exchange coupling by EPR measurements in Fe^{II}–MT at 4 K remained unsuccessful. Presumably because of the persistence of the large zero-field splitting in both oligonuclear and mononuclear complexes of Fe(II), no signals were observed. These results taken together suggest that the metal ions in $Fe^{II}{}_7$–MT are organized in clusters similar to those found in the native protein. To our knowledge, they constitute the only Fe^{II}–thiolate cluster structure thus far reported in a biological molecule. These results together with similar studies on Zn(II)-, Cd(II)-, and Co(II)-substituted MT emphasize the determining influence of the protein on cluster geometry and organization in this protein (Vašák & Kägi, 1983). The biological relevance of $Fe^{II}{}_7$–MT remains to be established.

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