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Metal substitutions at the diiron sites of hemerythrin and myohemerythrin: Contributions of divalent metals to stability of a four-helix bundle protein

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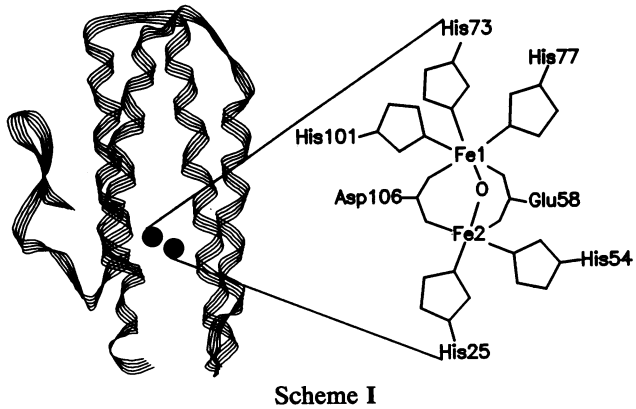
ABSTRACT **A general method is described for substitution of Mn(II) and Co(II) into the diiron sites of hemerythrin and myohemerythrin. Characterizations of these metal-substituted proteins show that their structures closely resemble those of the native proteins. In particular, the four-helix bundle structure appears to be maintained. The apomyohemerythrin retains most of the native helix content but is considerably less stable to denaturation than are the metal-containing proteins. The relative affinities of M(II) for apohemerythrin—namely, Co > Fe > Mn—parallel the stabilities of the M_2myohemerythrins to denaturation by guanidinium chloride. These results indicate that for myohemerythrin (*i*) the majority of the helical structure found in the native protein does not require incorporation of M(II) and (*ii*) stabilization of the native structure relative to the fully unfolded structure appears to be due predominantly to M(II)–protein interactions, at least for M = Fe and Co. Incorporation of M(II) also generates unfolding cooperativity in myohemerythrin. This cooperativity can be attributed to interhelical interactions, which are prevented in the apoprotein by solvation of the seven metal ligand residues. The results are consistent with a minimal model for folding/unfolding of myohemerythrin and hemerythrin subunits consisting of the sequential equilibria, $N \rightleftharpoons I \rightleftharpoons D$, between native, intermediate, and fully unfolded states, respectively. The properties of apomyohemerythrin make it a candidate for the intermediate state, *I*.**

Although the pathways by which proteins fold into their native structures *in vivo* are difficult to examine directly, the large number of *in vitro* refolding studies provides a useful framework for understanding *in vivo* folding (1–5). Prosthetic groups in general are known to stabilize the native folded structure, but only a few examinations of the role of metal ions in nonheme protein folding have been undertaken (e.g., see ref. 3), despite the large and rapidly growing number of nonheme metalloproteins that can be reconstituted by addition of metal ions (6).



Scheme I

For several reasons, the nonheme iron O_2-carrying proteins hemerythrin (Hr) and myohemerythrin (myoHr) are excellent candidates for examining the process of nonheme metal ion insertion and its connection to protein folding and stability. Hr and myoHr are structurally well-characterized and their biological activity—i.e., O_2 binding—can be readily observed and quantitated. The secondary, tertiary, and diiron site structures of the subunits in Hr and myoHr (shown schematically in Scheme I) are quite similar to each other (7–9). Though myoHr is monomeric, Hr is usually octameric; thus, comparisons of the refolding of Hr vs. myoHr would allow assessments of the effects of intersubunit interactions on refolding of two nearly identical protein structures. The subunit in Hr and myoHr has a "four-helix bundle" structure with ≈70% α-helix and contains a diiron active site that binds one molecule of O_2 at Fe2. The four-helix bundle is a structural motif found in several proteins (ref. 10 and references therein), but only in the case of cytochrome b_{562} has an investigation of the role of the prosthetic group in protein folding and stability recently been initiated (11). The two iron atoms comprising the active site in Hr and myoHr are ligated by a total of seven amino acid side chains with either one or two ligand residues from each of the four helical regions. In effect the diiron site cross-links the four helices (see Scheme I). For this reason and because of the relatively small sizes of Hr and myoHr (M_r ≈13,500 and ≈13,900, respectively, per diiron site), one might expect an intimate connection between iron incorporation and protein stability. The "spontaneous self-assembly" of synthetic structural analogues of the diiron site shown in Scheme I from simple reagents (12) suggests that assembly of the diiron site could drive folding of the surrounding polypeptide into the four-helix bundle. Our efforts to address this question led to successful reconstitutions of functional diiron sites in Hr and myoHr from the apoproteins and iron(II) salts (13, 14) and, more recently, to preparation of a cobalt-substituted Hr containing a dicobalt(II) site in each subunit (15). Here we report high-yield preparations of Hr and myoHr containing cobalt and manganese in place of iron and compare stabilities of the native, metal-substituted, and metal-free proteins.

MATERIALS AND METHODS

Materials and Instrumentation. Chemicals were purchased from Fisher, Sigma, Bio-Rad, and BDH at the highest available purities. Live specimens of *Phascolopsis gouldii* were obtained from the Marine Biological Laboratory (Woods Hole, MA). MetHr and metmyoHr iso I were isolated and purified from these organisms by previously described procedures (16, 17), and these proteins were used for all of the experiments described below. ApoHr and apomyoHr were prepared from the respective met proteins as previously

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Abbreviations: Hr, hemerythrin; myoHr, myohemerythrin; GdmCl, guanidinium chloride.

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described (13). UV/visible absorption spectra were obtained on a Perkin–Elmer model 554 spectrophotometer or a Perkin–Elmer model 3840 λ array spectrophotometer using quartz cuvettes of 1-cm pathlength. Far-UV CD spectra were obtained on a Jasco J-500c spectropolarimeter interfaced to an IBM PC-XT computer. The instrument was calibrated using a standard androsterone solution. Protein samples of ≈0.5 mg/ml (≈40 μM in subunits) were measured in a cylindrical quartz cuvette of 0.01-cm pathlength. Unless otherwise specified, room temperature and 50 mM Hepes (pH 7.0) were used for all reactions and measurements.

Analytical Methods. The concentrations of met- and oxyHrs were determined by absorbance at 355 nm (ε_{2Fe} = 6400 $M^{-1}\cdot cm^{-1}$) and 330 nm (ε_{2Fe} = 6800 $M^{-1}\cdot cm^{-1}$), respectively (18). DeoxymyoHr concentration was determined after exposure of the sample to air by absorbance at 330 nm using the extinction coefficient for oxyHr. For quantitation of metal/protein stoichiometry in the metal-substituted Hrs and myoHrs, the total protein concentrations were determined using the Bio-Rad protein assay with native metHr as the protein standard, and metal contents were determined by inductively coupled plasma emission spectrometry, as described (13). For other than metal/protein stoichiometry, protein concentrations of the manganese- and cobalt-substituted Hrs and myoHrs in buffer were calculated from A_{280} using $\varepsilon_{subunit}$ = 29,000 $M^{-1}\cdot cm^{-1}$ and 23,800 $M^{-1}\cdot cm^{-1}$, respectively. The former extinction was determined as described for Co(II)-substituted Hr (15). The latter extinction is the published value for apomyoHr iso I (13), the assumption being that Mn(II) and Co(II) make insignificant contributions to the absorbance at 280 nm. Molar ellipticity, [θ], was calculated from the measured ellipticity in the far-UV CD spectra using a standard equation (19). Helix contents of the proteins were calculated from the molar ellipticities at 222 nm using $[\theta]_{222}$ = −35,000 deg·cm²/dmol and +3000 deg·cm²/dmol for 100% and 0% helix, respectively (20, 21). Nondenaturing discontinuous polyacrylamide gel electrophoresis was performed on the native and metal-substituted Hrs following a standard procedure (22) using a 7.5% acrylamide gel and high pH buffers (Tris·HCl, Tris glycine) in a Bio-Rad cell and model 3000xi power supply. Gels were stained for proteins with Coomassie blue R-250.

Preparation of Metal-Substituted Hrs and MyoHrs. All solutions were prepared in distilled, deionized water. Stock solutions were prepared and reactions were conducted under Ar in septum-capped vials connected to a vacuum manifold by hypodermic needles. Solutions were made anaerobic by several cycles of evacuation and flushing with Ar. Liquids and solutions were transferred via gas-tight syringes. The methods used for metal substitution in Hr and myoHr were essentially the same as those developed previously to reconstitute Hr and myoHr with iron (13) except that freshly prepared M(II) (M = Co or Mn) stock solutions were used in place of the Fe(II) stock solution. These stock solutions were prepared by dissolving 5–8 mg of cobalt(II) nitrate tetrahydrate or manganese(II) chloride tetrahydrate in 1 ml in buffer containing 300 mM 2-mercaptoethanol. Ten to 15 μl of 2-mercaptoethanol followed by 0.2 ml of the M(II) stock solution were added dropwise to 1 ml of ≈0.2 mM apomyoHr in buffer or ≈0.2 mM apoHr subunits in buffer plus 6 M guanidinium chloride (GdmCl). The resulting solutions were then diluted to a total volume of 5 ml with buffer at a rate of ≈1 ml/30 min. Metal-substituted Hrs were also prepared in the same fashion using mixed-metal stock solutions containing M(II)/Fe(II) mol ratios of 0.2, 1, or 5. These mixed-metal stock solutions were prepared as described above for the homo-metal stock solutions at roughly the same total [M(II) + Fe(II)] concentration but including various amounts of ferrous ammonium sulfate to give the appropriate M(II)/Fe(II) mol ratios. For both Hr and myoHr the total [M(II) + Fe(II)]/protein subunit mol ratio in the reaction mixture was always ≈10. As the final step in their preparation, all metal-substituted Hrs and myoHrs were passed through an anaerobic 2.5 × 25 cm Sephadex G-25 gel filtration column to separate excess reagents from protein. After this step no special precautions were taken to exclude air from the protein samples. The metal-substituted Hrs were stored either at 4°C for use within a few days or at −80°C for up to several months. No significant differences in properties were seen for the samples stored in these two ways. The M(II)-substituted myoHrs were stored at 4°C and were used within 1 week of preparation.

Denaturation Titrations of MyoHrs. Freshly prepared solutions of apomyoHr, M(II)-substituted myoHrs, and native deoxymyoHr were used. The deoxymyoHr was prepared by dialyzing 2 ml of ≈0.5 mM metmyoHr against 200 ml of buffer containing 3 mM sodium dithionite at room temperature overnight under Ar followed by dialysis against 200 ml of anaerobic buffer for 8 hr under Ar. For each of the myoHr derivatives except deoxymyoHr, the denaturation titrations were carried out aerobically. Aliquots (50 μl) of the protein stock solution (0.1–0.2 mM) in buffer were added to a set of Eppendorf tubes. Then, a different amount of an 8 M GdmCl (high-purity grade from Sigma) stock solution was added to each tube followed immediately by buffer such that the final sample volume in each tube was 200 μl. Thus, for each denaturation titration, the final protein concentration was fixed at a value between 25 and 50 μM, and the final GdmCl concentrations varied from 0 to 6 M. The samples were mixed thoroughly and allowed to stand at room temperature for 4 hr. Each sample was then transferred to a cylindrical quartz cuvette of 0.01-cm pathlength and the ellipticity at 222 nm was measured. For deoxymyoHr the same general procedure was used, but the samples were prepared in an O_2-free glove box and each solution was transferred to the cuvette anaerobically and kept under an Ar atmosphere during measurements of ellipticity.

RESULTS

Metal Substitution Procedures. The metal-substituted Hrs and myoHrs were all obtained in ≈50% yields based on total protein analyses of the product for multiple preparations. Our previously published observations concerning the procedures for reconstitutions of Hr and myoHr with iron (13) also apply to the metal substitution procedures. The relative insolubility of apoHr in the absence of denaturants required addition of the M(II) stock solution to apoHr in 6 M GdmCl, followed by slow dilution with buffer to minimize precipitation of the apoprotein. The self-purifying nature of this procedure results in essentially only metal-substituted Hr remaining in solution after dilution of denaturant. Freshly prepared apomyoHr is soluble to at least 0.2 mM at pH 7 in Hepes buffer without denaturant, and the initial presence of denaturant was found to be unnecessary for successful incorporation of Co(II) and Mn(II) into apomyoHr.

Properties of the Metal-Substituted Hrs and MyoHrs. We first sought to establish the similarities in structures of the polypeptides and metal sites in the metal-substituted proteins to those of the native proteins. In the case of Co(II)-substituted Hr, these similarities have already been demonstrated (15). In the present work the following comparisons have been used: metal/protein stoichiometry, which reflects assembly of a dimetal site; far-UV CD spectra, which reflects the protein secondary structure; and nondenaturing polyacrylamide gel electrophoresis, which reflects charge and molecular size. Table 1 shows that the metal/protein subunit stoichiometry is ≈2 for all of the metal-substituted proteins. Little or no iron above background levels was found in any of the homometal-substituted proteins. Based on these stoi-

Table 1. Metal and protein analyses for *P. gouldii* Hr and myoHr iso I substituted with manganese or cobalt

Metal	Protein	Metal, mol/mol of protein subunit*
Co	Hr	1.94 ± 0.03
Co	MyoHr	1.96 ± 0.05
Mn	Hr	1.91 ± 0.15
Mn	MyoHr	1.93 ± 0.10

*Average for two or three preparations. Only trace amounts of iron or other metals were found in all cases.

chiometries, we hereafter refer to the homometal-substituted proteins as M_2Hr or M_2myoHr (M = Mn, Co). Figs. 1 and 2 (and figure 1 of ref. 15) show that the far-UV CD spectra of the M_2Hr and M_2myoHr contain the classic double minimum at 208 and 222 nm, which is characteristic of proteins having a large proportion of α-helix (20, 21) and which closely resemble the far-UV CD spectra of the native proteins. The measured ellipticities at 222 nm were used to calculate the percentages of helix listed in Table 2, assuming only helix and random coil secondary structures [a valid approximation for proteins where helix is the predominant secondary structure (23)]. The calculated helix contents are about the same for native metHr, native metmyoHr, M_2Hr, and M_2myoHr and are also in excellent agreement with the known helix contents of the native proteins, which have been established by x-ray crystallography (7, 8). Nondenaturing polyacrylamide gel electrophoresis showed that native metHr, Mn_2Hr, and Co_2Hr all migrated as single bands and to essentially the same extent, except for the presence of a very small percentage of an unidentified slower migrating species in the M_2Hr lanes (24). Since both native metHr and Co_2Hr have previously been shown to be octameric by gel filtration (15), we interpret the essentially identical behavior of the Mn_2Hr on the nondenaturing gel electrophoresis as evidence for an octameric structure in this protein as well. The absorption spectra of Mn_2Hr and Mn_2myoHr are featureless in the visible region, indicating the exclusive presence of Mn(II) in these proteins. The Co_2Hr absorption spectrum has been discussed in detail elsewhere (15) as reflecting the presence of both five- and six-coordinate Co(II), which is expected if the dicobalt site structure resembles that of the diiron site shown in Scheme I.

Metal Competition During Incorporation into Hr. Metal competition experiments were conducted by reaction of apoHr with mixed-metal stock solutions. The M(II)/Fe(II) mol ratios of these stock solutions are listed in Table 3 along with the resulting metal and protein analyses for the Hrs isolated from these reactions. The mixed-metal substitutions result in ≈2 mol of (M + Fe) per mol of Hr subunit in the isolated proteins, indicating that these reactions proceed similarly to the homo-metal substitutions and that M and Fe are competing for the same sites. UV-visible absorption spectra (not shown) of metal-substituted Hr prepared using stock solutions containing a Co(II)/Fe(II) mol ratio of 5 qualitatively indicated that the product was Co_2Hr. When stock solutions containing equimolar Co(II) and Fe(II) are used, the data in Table 3 show that the vast majority of the product is still Co_2Hr, with ≈5 times more cobalt than iron on a molar basis in the isolated protein. When the Co(II)/Fe(II) mol ratio in the stock solution is 0.2, the cobalt/iron mol ratio found in the isolated protein is ≈0.8. On the other hand, UV-visible absorption spectra (not shown) of metal-substituted Hr prepared using stock solutions containing a Mn(II)/Fe(II) mol ratio of 5 indicated that the majority of the product (after exposure to air) was oxyHr. Similarly, Table 3 shows that for stock solutions containing equimolar Mn(II)

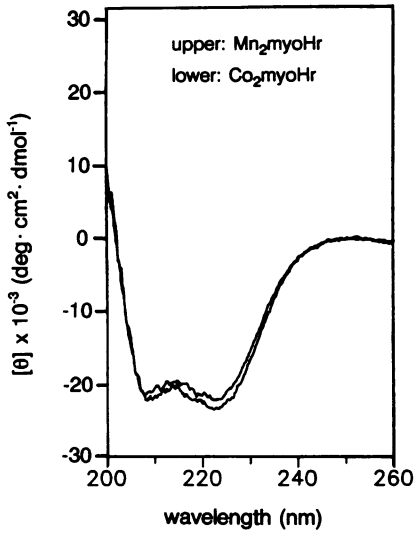

FIG. 1. Far-UV CD spectra of cobalt- and manganese-substituted *P. gouldii* myoHr iso I in 50 mM Hepes (pH 7.0).

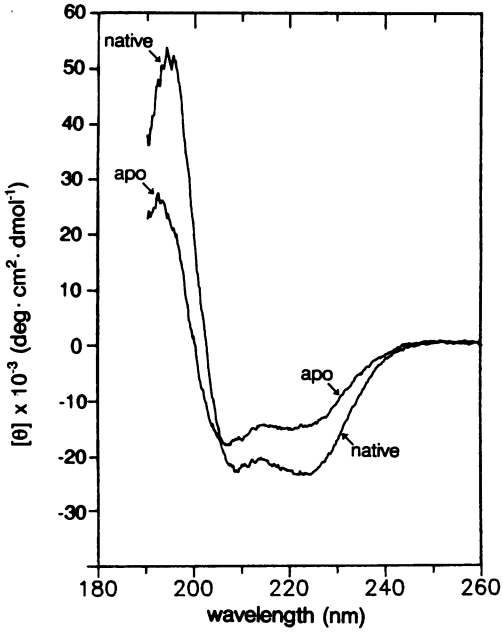

FIG. 2. Far-UV CD spectra of *P. gouldii* apomyoHr iso I and native metmyoHr iso I. Spectra were obtained at 25°C in 10 mM phosphate/30 mM Na_2SO_4, pH 7.2.

Table 2. Helix contents of *P. gouldii* Hrs and myoHrs in 50 mM Hepes (pH 7.0)

Protein	$[\theta]_{222}$,* deg·cm²/dmol	Helix,† %
MetHr(N)‡	23,655	70
MetmyoHr(N)	23,109	69
MetHr(R)‡	22,612	67
Co_2Hr	22,463	67
Co_2myoHr	23,064	69
Mn_2Hr	22,428	67
Mn_2myoHr	22,916	68
ApomyoHr	15,155	48

*Protein concentrations used to calculate these molar ellipticities were determined from absorbances of metHr at 355 nm, metmyoHr at 366 nm, and all others at 280 nm.

†Calculated from the molar ellipticities using the published values (20, 21) for 100% and 0% helix listed in the text.

‡N refers to native protein and R refers to reconstituted protein.

and Fe(II), about 10 times more iron than manganese is found in the isolated Hr. The spectrophotometric results together with the metal and protein analyses in Table 3 indicate that the ordering of metal ion affinities for apoHr under our conditions is Co(II) > Fe(II) > Mn(II). The relative affinities for apoHr are roughly 5 for Co(II) vs. Fe(II) and 10 for Fe(II) vs. Mn(II).

Secondary Structure of ApomyoHr. Fig. 2 compares the CD spectra of native metmyoHr and apomyoHr. The changes in ellipticity at 190 nm, 208 nm, and 222 nm and the isodichroic point at 205 nm are all characteristic of proteins containing a helix–coil mixture (20, 21, 23). The data in Table 2 show that, although the helix content in apo- (48%) is reduced relative to that of the metal-containing myoHrs (67–70%), the apomyoHr still has on average nearly half its residues in helical regions in the absence of denaturant. The corresponding comparison for Hr cannot be easily made, since apoHr is soluble to only ≈1.5 μM in buffer without denaturant (Z.-Q. He and D.M.K., unpublished results).

Denaturation Titrations of MyoHrs. The stabilities of apomyoHr, native deoxymyoHr (i.e., Fe^{II}_2myoHr), Co_2myoHr, and Mn_2myoHr to denaturation were compared by measuring ellipticity at 222 nm after equilibration of the proteins in buffer containing 0–6 M GdmCl. The results are plotted as f, the fraction of native ellipticity vs. [GdmCl] in Fig. 3. The points at 0 M GdmCl reflect the data in Table 2—i.e., metal incorporation into myoHr causes an approximate 30% increase in ellipticity and helix content. The displacements of the curves along the [GdmCl] axis show that the stabilities to denaturation vary in the order apomyoHr < Mn_2myoHr < Fe_2myoHr < Co_2myoHr. Finally, the sigmoidal shapes of the curves for the metal-containing proteins indicate that metal incorporation introduces a considerable degree of cooperativity into the unfolding process. Such sigmoidal curves can be analyzed by assuming a simple equilibrium between native and unfolded forms. In the transition zone, represented by the steepest portion of each curve, the following equation can be used for this analysis (25, 26):

$$RT\ln[f/(1-f)] = \Delta G_D^{H_2O} - mC, \quad [1]$$

where C is the denaturant concentration, and $\Delta G_D^{H_2O}$ represents the free energy change of unfolding in the absence of denaturant. Table 4 lists the values of m, which represents a measure of cooperativity for the unfolding transition, $C_{0.5}$, which represents the concentration of GdmCl at which half of the protein is denatured, and $\Delta G_D^{H_2O}$, which is obtained by linear extrapolation of plots of Eq. 1 using the data from Fig. 3. Nearly the same values of $\Delta G_D^{H_2O}$ are obtained from multiplications of m by $C_{0.5}$ (27).† Since the apomyoHr curve does not clearly show a sigmoidal shape, an accurate analysis of this curve using Eq. 1 is difficult. However, for comparison with the $C_{0.5}$ values in Table 4, it can be estimated that apomyoHr loses half of the native ellipticity at ≈0.5 M GdmCl, under which conditions the metal-containing myoHrs retain >90% of the native ellipticity. At ≈1.9 M GdmCl, apomyoHr retains about 10% of the native ellipticity vs. 70–80% for deoxy- and Co_2myoHr.

DISCUSSION

Our use of essentially the same procedures for metal substitution in Hr and myoHr as had been used for our previous reconstitutions of functional proteins with iron (13) was intended to encourage formation of similar native and metal-substituted protein structures. Indeed, we have recently shown that the cobalt-substituted Hr contains a dicobalt(II) site in each subunit (≈3.54 Å Co–Co distance) surrounded by a polypeptide having essentially the same percentage helix content and the same octameric quaternary structure as that of the native protein (15). The results of metal/protein analyses, far-UV CD spectroscopy, and nondenaturing polyacrylamide gel electrophoresis provide further evidence that both Mn(II) and Co(II) have replaced Fe(II) in the metal-substituted Hrs and myoHrs and that their secondary, tertiary, and quaternary structures are quite similar to those of the native, iron-containing proteins. These results indicate that the *P. gouldii* M_2Hrs and M_2myoHrs (M = Mn, Co, and Fe) together with the apoproteins can be used to analyze the relationship between stability of the folded protein structure and metal incorporation.

Table 3. Metal and protein analyses of metal-substituted Hrs prepared using mixed-metal [M(II) + Fe(II)] stock solutions

M	mol of M/ mol of Fe (stock)	mol of M/ mol of Hr* (as isolated)	mol of Fe/ mol of Hr* (as isolated)
Co	1	1.79 ± 0.20	0.37 ± 0.06
Co	0.2	0.89 ± 0.08	1.10 ± 0.20
Mn	1	0.176 ± 0.003	1.81 ± 0.02

Ratios are the average for two or three preparations.
*As Hr subunit.

The trend in M(II) affinities—namely, Co(II) > Fe(II) > Mn(II)—obtained from the metal competition during incorporation into Hr (see Table 3) agrees with the relative values of $\Delta G_D^{H_2O}$ and $C_{0.5}$ for myoHr in Table 4. These parallel trends indicate that the M(II) affinities of Hr and the stabilities of the M_2myoHrs reflect thermodynamics rather than the kinetics of metal ion binding or release. The fact that the aforementioned order is the same as the Irving–Williams order of metal complex stabilities (29, 30) reinforces this statement. Furthermore, although quantitation is difficult, the data in Fig. 3 clearly show that apomyoHr is much less stable to denaturation than are the M_2myoHrs. Taken together, these results strongly suggest that the majority of the stabilization energy for the folded structure of the Hr and myoHr subunits derives

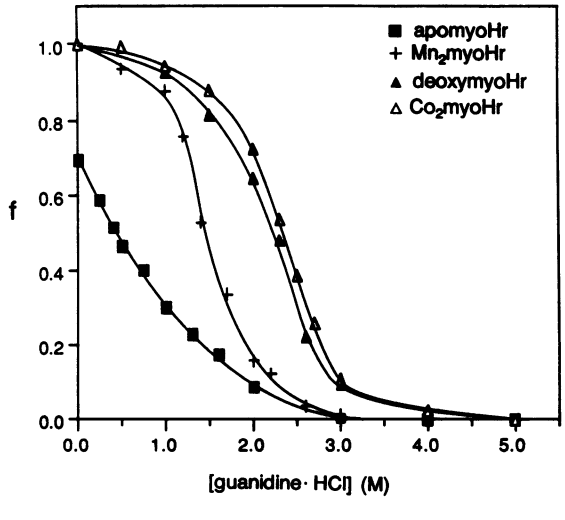

FIG. 3. GdmCl denaturation curves of *P. gouldii* apomyoHr, native deoxymyoHr, Co_2myoHr, and Mn_2myoHr at 25°C and ≈35 μM protein in 50 mM Hepes (pH 7.0). f, Normalized fraction of native ellipticity at 222 nm. Points are the average of two denaturation experiments for all but deoxymyoHr, which are for one experiment.

†Although likely to be accurate in rank order, the absolute values of $\Delta G_D^{H_2O}$ in Table 4 should be regarded as approximations due to possible extrapolation errors and to assumptions made in the derivation of Eq. 1 (25–28). The values of the parameters in Table 4 for M = Fe and Co are within the range or, in the case of $\Delta G_D^{H_2O}$, slightly below the range of those found for denaturation of other small globular proteins by GdmCl (26, 28).

Table 4. Parameters characterizing denaturation of *P. gouldii* M_2myoHrs iso I by GdmCl at 25°C in 50 mM Hepes (pH 7.0)

Protein	$\Delta G_D^{H_2O}$, kcal·mol^{-1}	$C_{0.5}$, M	m, kcal·mol^{-1}·M^{-1}
Mn_2myoHr	3.2	1.5	2.1
Fe_2myoHr*	3.9	2.2	1.8
Co_2myoHr	4.2	2.4	1.8

The parameters are defined in the text. One calorie = 4.18 J.
*Native deoxymyoHr.

from M(II)–protein interactions, at least for M = Fe and Co. On the other hand, our results also clearly show that apomyoHr retains considerable helical structure in the absence of denaturant (see Table 2 and Figs. 2 and 3); in fact, the helix content of apomyoHr is closer to that of the fully folded than fully unfolded protein. This observation indicates that incorporation of M(II) is *not* the predominant driving force for formation of the helical regions.

The sigmoidal shapes of the denaturation curves (see Fig. 3) for the M_2myoHrs indicate a considerable degree of unfolding cooperativity, in contrast to the behavior of apomyoHr. The lack of appreciable unfolding cooperativity in apomyoHr can be attributed to fewer interhelical interactions than are known to occur in the native proteins (9, 31). The reduced number of interhelical interactions would be required to accommodate solvation of the seven iron ligand residues, which are all quite polar (see Scheme I). Binding of M(II) apparently compensates for this solvation, since in the native structures, the iron ligand residues are largely buried in the hydrophobic interior of the four-helix bundle (7, 32).

The presence of M(II) in solutions of apoHr vastly decreases the amount of protein precipitation upon dilution of denaturant and leads to 50–70% yields of an essentially native protein structure (13–15). This observation indicates that binding of M(II) to apoHr removes a folding intermediate that is prone to aggregation. ApomyoHr is much less prone to aggregation, and metal incorporation into apomyoHr can be accomplished in the absence of denaturant. This contrasting behavior may stem from the intersubunit interactions that are required in the octamer of M_2Hr but that do not occur in M_2myoHr. Thus, some of the residues that normally participate in intersubunit interactions in the folded M_2Hr (8, 17, 33) may also initiate the intersubunit interactions that lead to the observed aggregation of apoHr.

The results are consistent with a minimal model for folding/unfolding of the Hr and myoHr subunits consisting of the sequential equilibria, $N \rightleftharpoons I \rightleftharpoons D$, between native, intermediate, and fully unfolded states, respectively. The properties of apomyoHr observed in this work make it a candidate for the intermediate, I. Given the nonsigmoidal denaturation curve for apomyoHr, I could consist of a set of structures whose relative proportions are dependent on denaturant concentration. Nevertheless, I would retain most of the native helical structure in the absence of denaturant but would be much less stable to denaturation and show much less unfolding cooperativity than the M_2myoHrs. Strong binding of M(II) to I would reduce its concentration sufficiently so that essentially only N and D are present, thereby explaining the sigmoidal shapes of the denaturation curves for the M_2myoHrs. Multi-dimensional NMR and kinetics experiments could test the extent to which apomyoHr fulfills the properties of such an intermediate and whether the "molten globule" description (2, 3) applies to apomyoHr.

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