Reconstitution of the Diiron Sites in Hemerythrin and Myohemerythrin†

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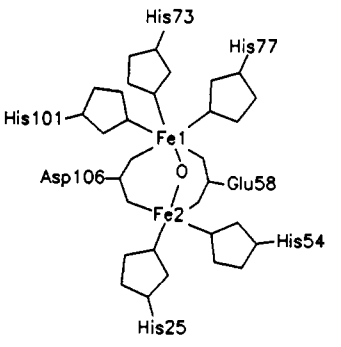
ABSTRACT: The first reconstitutions of functional diiron sites in the nonheme O_2-carrying proteins hemerythrin (Hr) and myohemerythrin (myoHr) have been achieved. Both proteins are reconstituted under anaerobic conditions, and the procedure consists of (i) denaturation of the native met form with 6 M guanidinium chloride in the presence of sodium dithionite and 2,2′-dipyridyl, (ii) separation of the apoprotein from the other reagents and products, (iii) addition of an iron(II) stock solution to the apoprotein in the presence of 2-mercaptoethanol, and (iv) several cycles of slow dilution and reconcentration by ultrafiltration to remove excess reagents. Iron analyses indicate that the apoproteins have been essentially completely freed of iron and that reconstituted Hr contains its full complement of iron, i.e., ~2 Fe/subunit. Ferrous rather than ferric iron appears to be necessary for recovery of the native structures for both myoHr and Hr. In the case of Hr, reconstitution was successful only when iron(II) was added to apoHr *prior* to removal of denaturant. ApoHr is essentially insoluble at pH 7 in the absence of denaturants but remains soluble when denaturant is removed in the presence of ferrous iron, which leads to recovery of the octameric structure containing all of its diiron sites. Iron(II) apparently stabilizes the native or a nearly native structure during reconstitution. OxymyoHr and oxyHr are the major initial products of reconstitution. The yield of oxymyoHr from apomyoHr was ~87%. In contrast to reconstituted oxymyoHr, where essentially all of the iron appears to be functional, approximately 30% of the diiron sites in the reconstituted oxyHr are unable to bind O_2 at ambient $p(O_2)$. Mössbauer spectroscopy of Hr that was reconstituted with iron enriched in ^{57}Fe indicates that the nonfunctional iron is in an environment very similar to but not identical with that in native deoxyHr. The yield of reconstituted oxyHr from native metHr was ~72%, and this value includes both functional and nonfunctional iron. Oxidation of the reconstituted oxyHr with ferricyanide converts the protein to a form that is spectroscopically indistinguishable from native metHr, and the yield of this reconstituted metHr starting from native metHr was ~50%. The reconstitution procedures described here lay the groundwork for detailed examinations of the refolding process and for preparation of metal-substituted Hrs.

Hemerythrin (Hr)[1] is an O_2-carrying protein found in a few phyla of marine invertebrates, most notably sipunculid worms. Despite its name, Hr contains no heme group but rather a nonheme diiron site that reversibly binds one molecule of O_2. Hr thus poses interesting contrasts in evolution, physiology, and molecular structure to the more widespread heme oxygen carriers. The diiron site in Hr has also become the prototype for an emerging group of nonheme, nonsulfur diiron sites in proteins (Que & Scarrow, 1988; Sanders-Loehr, 1989). Several recent reviews have summarized the large body of data on spectroscopy, reactivity, and molecular structure/function relationships of Hr (Wilkins & Harrington, 1983; Kurtz & Klotz, 1984; Wilkins & Wilkins, 1987a; Kurtz, 1990a). Hrs from hemerythrocytes of sipunculids are usually found to be octameric (M_r ~108 000) with essentially identical subunits. Each subunit contains a diiron site that binds one molecule of O_2 according to eq 1. Evidence summarized in the reviews

$$\underset{\text{deoxyHr}}{[Fe^{II}(\mu\text{-}OH)Fe^{II}]} + O_2 \rightleftharpoons \underset{\text{oxyHr}}{[Fe^{III}(\mu\text{-}O)Fe^{III}OOH^-]} \quad (1)$$

cited above indicates that the diiron site structures in both oxy- and deoxyHrs closely resemble that shown for *Themiste dyscritum* $[Fe^{III}Fe^{III}]$metHr (structure **1**), which was determined by X-ray crystallography (Sieker et al., 1982; Stenkamp et al., 1984). Muscle tissues of sipunculids contain a monomeric protein referred to as myoHr. The molecular weight of myoHr from *Themiste zostericola* (~13 900) is very close to that of a subunit in the octameric Hrs (~13 500), as are the secondary, tertiary, and diiron site structures (Sheriff et al., 1987; Sieker et al., 1982). The prototypical tertiary structure consists of four nearly parallel α-helical regions in a left-twisted bundle. The Fe1–Fe2 axis is approximately



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[1] Abbreviations: Hr, hemerythrin; myoHr, myohemerythrin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; GdmCl, guanidinium chloride; Tris, tris(hydroxymethyl)aminomethane; FWHM, full width at half-maximum.

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perpendicular to the four major helical regions in each subunit.

Despite the detailed structural knowledge about Hr, the pathway of protein folding and iron site assembly by which the native structure is attained remains largely obscure. The secondary structure of *T. zostericola* apomyoHr has been reported to contain 25% α-helix compared to 70% α-helix in native myoHr (Fieser et al., 1987). The "spontaneous self-assembly" of synthetic complexes that are structural analogues of the diiron site in Hr from solutions of ferric or ferrous salts, carboxylates, and polydentate nitrogen donor ligands (Kurtz, 1990b) suggests that assembly of the diiron site in Hr could drive the folding of the surrounding polypeptide into the four-helix bundle structure. However, there have been no experimental tests of this hypothesis. In principle, the folding process could be monitored after addition of iron to apoHr or apomyoHr, but all previous attempts at reconstitution of the diiron sites in these proteins have apparently failed (Bradić & Wilkins, 1985; Maret & Zeppezauer, 1988). Therefore, a necessary prelude to any examination of the folding process is the development of reliable reconstitution procedures for Hr and myoHr. Such procedures are reported in this paper along with some properties of the reconstituted proteins. We also report the first enrichment of the diiron site with ^{57}Fe.

Experimental Procedures

Materials

Proteins. MetHr from hemerythrocytes and metmyoHr from body wall tissues of *Phascolopsis gouldii* were the starting proteins for all reconstitution experiments. MetHr was isolated and purified by the procedure described (Bonomi et al., 1989), except that the crystallization step was sometimes omitted. MetmyoHr was isolated, purified, and characterized by procedures recently described (R. S. Long, J.-H. Zhang, and D. M. Kurtz, Jr., unpublished results). The met form of myoHr was normally obtained during purification without exposure to oxidants other than air. The *P. gouldii* metmyoHr consists of two species that can be separated by anion-exchange chromatography and that have slightly different absorption spectra between 300 and 400 nm at pH 7.0–7.5. We label these myoHr species iso I and iso II, and only the former species was used for the reconstitution experiments. The following extinction coeffients [based on total sample iron determined by the method of Rill and Klotz (1970)] were used for metmyoHr iso I in 50 mM HEPES, pH 7.5 [λ_{max} (ϵ_{2Fe}) in nm (M^{-1} cm^{-1})]: 280 (27 700); 324 (6600); 366 (6400). For reconstituted oxymyoHr, ϵ_{2Fe} at 330 nm (6800 M^{-1} cm^{-1}) and 500 nm (2200 M^{-1} cm^{-1}) were assumed to be the same as for native *P. gouldii* oxyHr (Dunn et al., 1977; Garbett et al., 1969); the same assumption was made for ϵ_{2Fe} at 280 nm (35 400 M^{-1} cm^{-1}) for reconstituted oxyHr (Dunn et al., 1977). For metHr at pH 7.0, the literature value of ϵ_{2Fe} at 355 nm (6400 M^{-1} cm^{-1}) was used (Garbett et al., 1969).

Methods

All solutions were prepared in deionized water. Unless otherwise specified, the buffer used was 50 mM HEPES. The pHs of the buffer are specified in the procedures described below. All anaerobic operations were performed 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. Liquid reagents were transferred via gas-tight syringes. UV/vis absorption spectra were obtained on either a Perkin-Elmer Model 554 spectrophotometer or a Perkin-Elmer 3840 λ array interfaced to a PE-7000 computer. Mössbauer spectroscopy was carried out as described in previous studies of Hr (Nocek et al., 1988), except that a sample enriched in ^{57}Fe was used.

Molecular Weight Determination. Analytical gel filtration chromatography was performed at room temperature on a 1.6 × 50 Superose 12 preparative grade column equilibrated with 10 mM phosphate, pH 7.5, containing 150 mM Na_2SO_4 at a flow rate of 0.2 mL/min. A concentration of ~1 mM in Hr subunits in a volume of less than 0.4 mL was applied to the column.

Total Iron and Protein Analyses. Total sample iron was determined either by a standard wet chemical method (Rill & Klotz, 1970) or by inductively coupled plasma emission spectrometry with a Jarrell-Ash Atomcomp 965 spectrometer. For the latter method apomyoHr concentrations of ~0.23 mg/mL were used, and blank samples were solutions of buffer at pH 7.5. Approximately 1 mg/mL (based on the starting metHr concentration) solutions of apoHr in 6 M GdmCl were used, with 6 M GdmCl as the blank. Total Hr concentrations were determined by using the Bio-Rad protein assay with native metHr as the standard protein. When used as the standard, metHr protein concentration was determined with the extinction coefficient cited above.

Reconstitution of MyoHr. (*a*) *Preparation of ApomyoHr.* To a glass vial containing 0.2 mL of ~0.5 mM metmyoHr at pH 7.0 was added 0.1 mL of 30 mM 2,2′-dipyridyl stock solution. Solid GdmCl was then added to a concentration of 6 M, and the resulting light brown solution was made anaerobic. Ten milligrams of solid sodium dithionite (BDH Ltd.) was then added, and the solution immediately developed a red-pink color. This solution was allowed to incubate for ~1 h at room temperature under Ar and then loaded onto a 1 × 25 cm Sephadex G-25 column equilibrated with buffer at pH 7.0. Elution was conducted aerobically with the same buffer. The red-pink band on the column ran well behind the eluting colorless fractions with detectable 280-nm absorbance (in a 1-cm path), which were combined and constituted the apomyoHr solution.

(*b*) *Iron(II) Stock Solution.* Twenty microliters of 2-mercaptoethanol was injected into 1 mL of anaerobic buffer, pH 7.0. This injection was followed quickly by addition of 2–5 mg of ferrous ammonium sulfate hexahydrate. The resulting solution had a slightly yellow color, presumably due to the $Fe(SR)_4^{2-}$ species (Werth et al., 1989). This anaerobic stock solution was freshly prepared for each reconstitution.

(*c*) *Reconstitution of MyoHr from ApomyoHr.* In a glass vial, 0.5 mL of ~0.2 mM apomyoHr in pH 7.0 buffer was made anaerobic. Five to eight microliters of 2-mercaptoethanol and then 0.1 mL of the iron(II) stock solution were added to the apoprotein solution in a dropwise fashion, with mixing of the solution after addition of each drop. After complete addition, the final Fe(II)/myoHr mole ratio was within the range of 5–12, and the final 2-mercaptoethanol concentration was 160–230 mM. The resulting solution had either no color or a slight yellow tinge. After incubation for ~30 min at room temperature under Ar, the solution was diluted anaerobically by slow addition of ~1 mL of pH 7.0 buffer and incubated for a further 30 min at room temperature. This slow addition was repeated until the total volume was ~5 mL. The solution was transferred anaerobically to a 10-mL Amicon cell equipped with YM-5 membranes and concentrated to ~0.2 mL at 4 °C. More degassed buffer was added to the Amicon cell, and the concentration and redilution cycles were repeated until the eluate had a UV/visible absorption spectrum indistinguishable from that of the buffer. The protein solution gradually became pink during the concentration steps, indicating formation of oxymyoHr. The reconstituted oxymyoHr

(~0.1 mM in ~1 mL of pH 7.0 buffer) was converted to the met form by addition of 0.1 mL of 0.1 M potassium ferricyanide and incubation for either ~2 h at room temperature or overnight at 4 °C. Excess reagents were removed by extensive dialysis against buffer in the 10-mL Amicon cell.

Reconstitution of Hr. (*a*) *Preparation of ApoHr.* The procedure for denaturation and removal of iron from metHr was identical with that described above for myoHr, except that no chromatography was used to separate reagents from apoprotein. Instead, the red-pink solution was incubated for 1 h under Ar at room temperature and then transferred aerobically to a 10-mL Amicon cell equipped with a YM-5 membrane. The solution was then diluted and reconcentrated ~5 times at 4 °C with 6 M GdmCl in pH 7.0 buffer until a clear, colorless solution, which contained the apoHr, was left in the cell.

(*b*) *Reconstitution of Hr from ApoHr.* Iron(II) stock solution was prepared as described above for myoHr. The reconstitution procedure was essentially the same as described above for myoHr, except that the starting apoHr solution contained 6 M GdmCl. After addition of the iron(II) stock solution, buffer was added in a dropwise fashion, typically at a rate of 1 mL per 30 min with rapid mixing after addition of each drop, until the total volume was ~5 mL. The freshly reconstituted oxyHr was oxidized to metHr as described for myoHr. A control reconstitution experiment was also run with buffer in place of the iron(II) stock solution.

(*c*) *Preparation of Hr Enriched in* ^{57}Fe. The procedure for preparing reconstituted oxyHr enriched in ^{57}Fe was the same as for the natural abundance samples, except that the buffer was 50 mM Tris–sulfate, pH 7.5, and the iron(II) stock solution was prepared differently. Fe_2O_3 containing ~75 atom % ^{57}Fe was the starting material. One milligram of the enriched Fe_2O_3 was dissolved in 0.1 mL of concentrated HCl by heating on a water bath at ~80 °C. After being cooled to room temperature, the solution was made anaerobic, and 8–12 μL of 2-mercaptoethanol was added. The resulting solution was then neutralized to pH 7 by dropwise addition of Tris base from a 1 M stock solution and checking the pH with pH paper. During neutralization the iron(III) is reduced to iron(II). The sample of reconstituted oxyHr used for Mössbauer spectroscopy was frozen in a liquid N_2 bath and stored at −80 °C before use. A portion of this sample diluted with buffer prior to freezing had an absorbance ratio A_{280}/A_{330} of ~6.5. The Mössbauer sample was approximately 0.5 mM in oxy subunits based on absorbance at 330 nm.

Results and Discussion

Apoproteins. for apomyoHr prepared as described under Experimental Procedures, the absorbance ratio A_{280}/A_{366} was always found to be greater than 30 and was often between 40 and 60. An $\epsilon_{280} = 23\,800\ M^{-1}\ cm^{-1}$ was determined for apomyoHr on the basis of the assumption of a 100% yield from the starting metmyoHr. This value agrees quite well with that calculated from the tyrosine and tryptophan content of this myoHr (R. S. Long, J.-H. Zhang, and D. M. Kurtz, Jr., unpublished results). This extinction coefficient was used to calculate the starting apomyoHr concentration in the reconstitution procedure. Inductively coupled plasma emission spectrometry showed that the apomyoHr contained 0.016 ± 0.002 mol of Fe/mol of myoHr, which is 0.85 ± 0.10% of that in the native protein, assuming 2 mol of Fe/mol of native myoHr. The absorption spectrum of apomyoHr is shown in Figure 1. In contrast to apomyoHr, apoHr is insoluble in pH 7.0 buffer but is soluble in the buffer plus 6 M GdmCl. Inductively coupled plasma emission spectrometry could detect

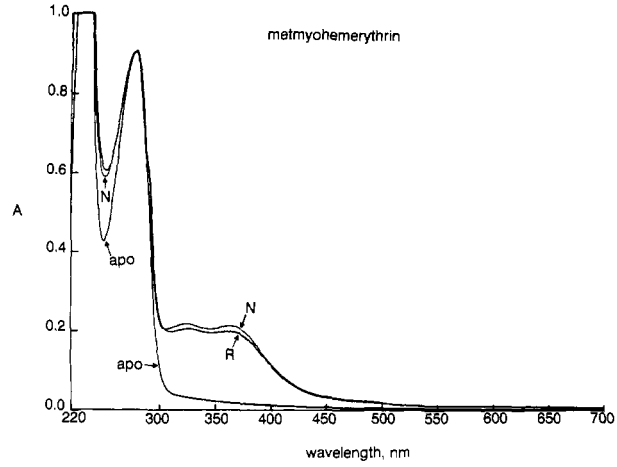

FIGURE 1: UV/vis absorption spectra of apomyoHr, native metmyoHr (N), and reconstituted metmyoHr (R) from *P. gouldii*. Spectra were obtained in 50 mM HEPES, pH 7.0, and have been scaled to equal absorbance at 280 nm.

no iron in apoHr prepared and analyzed as described under Experimental Procedures.

Reconstitution Procedures. When the protocol described under Experimental Procedures was followed, the reconstitutions of both myoHr and Hr were quite reproducible. In the case of Hr, the slow, anaerobic dilution with buffer following addition of iron(II) to the apoprotein in 6 M GdmCl was found to be critical. Rapid mixing was essential to avoid locally high dilutions, which caused protein precipitation. The slower the dilution process, the less protein precipitation was observed. In fact, lack of precipitation is a good indicator of successful reconstitution in the case of Hr. This precipitation also serves as a self-purification step for the reconstituted Hr, since essentially only iron-containing protein remains in solution, which can be readily separated from the precipitate. A control reconstitution in which anaerobic buffer was used in place of the iron(II) stock solution resulted in precipitation of nearly all of the apoHr upon dilution of the GdmCl. Kinetic competition between reconstitution and aggregation into nonnative structures during refolding of proteins is not an uncommon occurrence, and the relative rates of these two competing processes can be functions of both protein and denaturant concentrations (Jaenicke, 1987). Prosthetic groups often increase the yield of reconstitution by stabilizing the native or a nearly native refolded structure. Iron(II) may be playing such a role under our conditions for reconstitution.

Despite the Ar atmosphere in the Amicon cell, the reconstituted proteins reacted with the trace amounts of O_2 present in the cell or buffers during the repeated concentrations and redilutions that were used to remove excess reagents. This reaction is not surprising given the high O_2 affinities of native deoxyHr and deoxymyoHr ($p_{1/2} \leq 3.5$ mmHg; Chadwick & Klippenstein, 1983; Richardson et al., 1987). In fact, the pink color of oxyHr and oxymyoHr that develops during this dialysis serves as a convenient indicator of successful reconstitution. In one experiment, an alternative to dialysis in an Amicon concentrator, namely, anaerobic Sephadex G-25 column chromatography, was used to remove excess reagents following reconstitution of Hr. In this case rapid transfer of the eluted Hr to an anaerobic cuvette followed by repeated injections of small volumes of air resulted in the gradual intensification of the characteristic pink color and absorption spectrum of oxyHr (Garbett et al., 1969). This result qualitatively indicates that O_2 uptake by the reconstituted Hr is similar to that of native Hr.

Table I: Absorbance Ratios for *P. gouldii* ApomyoHr, Native (N) and Reconstituted (R) MetmyoHr, OxymyoHr, MetHr, and OxyHr in 50 mM HEPES, pH 7.0[a]

	A_{280}/A_{366}	A_{280}/A_{355}	A_{280}/A_{330}	A_{330}/A_{500}
apomyoHr	40–60			
metmyoHr (N)	4.3			
metmyoHr (R)	4.4–4.8			
oxymyoHr (R)			5.2–5.3	3.1–3.7
oxyHr (N)			5.1[b]	3.1[b]
oxyHr (R)			7.4–8.5	3.1–3.4
metHr (N)		5.5[b]		
metHr (R)		5.6–6.4		

[a] Ranges for (R) are for four separate reconstitutions conducted as described under Experimental Procedures. [b] Values are from Dunn et al. (1977) and Garbett et al. (1969).

Yields of reconstituted myoHr were calculated from absorbances at 280 nm for apomyoHr, 330 nm for oxymyoHr, and 366 nm for metmyoHr by using the extinction coefficients listed under Experimental Procedures. The average yield of reconstituted oxymyoHr starting from apomyoHr was 87 ± 11% for four reconstitutions. The average yield of reconstituted metmyoHr starting from native metmyoHr was 74 ± 13% for four reconstitutions. Due to the insolubility of apoHr in the absence of GdmCl, we based yields of reconstituted oxyHr on the starting native metHr concentration, which was calculated from absorbance at 355 nm. Quantitation of reconstituted oxyHr was also complicated by the presence of a minor portion of nonfunctional iron (vide infra). We assumed that the published extinction coefficient at 280 nm for native oxyHr (Dunn et al., 1977) also applied to the reconstituted oxyHr, and the calculated yields are, therefore, an estimate of total protein including functional plus nonfunctional iron. The average yield of reconstituted oxyHr calculated in this fashion was 72 ± 4% for four reconstitutions.[2] As described below, oxidation to the met form with ferricyanide converts all of the reconstituted Hr to a form that is spectroscopically indistinguishable from that of native metHr. The yield of reconstituted metHr starting from native metHr was 41%, 47%, and 56% for three experiments.

Reconstitutions of Hr and myoHr were unsuccessful when ferric citrate stock solutions were substituted for the iron(II) stock solutions. Ferrous rather than ferric iron is apparently required for these reconstitutions, not only because of the insolubility of nonchelated ferric iron in water near neutral pH but also because ferric iron, even when bound to the denatured protein, does not result in renaturation to the native state. When native metHr is denatured with GdmCl in the absence of reducing agents and iron chelators, the ferric iron apparently remains bound to the protein (Bradić & Wilkins, 1985). However, removal of the GdmCl does not result in regeneration of the native protein.

Properties of the Reconstituted Proteins. (a) MyoHr. The absorption spectra of native and reconstituted metmyoHr are shown in Figure 1. Compared with that of apomyoHr, the spectrum of metmyoHr has a much larger absorption between 300 and 400 nm. In addition, distinct shoulders at ~290 and 275 nm are present in spectra of metmyoHr that are absent in the spectrum of apomyoHr. The similarity of the native and reconstituted metmyoHr spectra indicates that the diiron site/protein molecule ratio has been nearly totally restored upon reconstitution. Since the absorbance at 280 nm arises mostly from the protein while the absorbance between 300 and 400 nm arises mostly from the diiron(III) site, the absorbance ratio A_{280}/A_{366} for metmyoHr serves as a useful indicator of the diiron(III) site content of myoHr, the standard of comparison for which is the native metmyoHr. The comparison in Table I shows that the absorbance ratios are slightly higher for reconstituted vs native metmyoHr. If it is assumed that the extra absorbance at 280 nm is due entirely to apomyoHr, then the percentage of apomyoHr present in the preparation of reconstituted metmyoHr is calculated to be ≤11% for each of four reconstitutions. We have found neither extinction coefficients nor absorption spectra for any oxymyoHr in the literature. Therefore, our only possible comparison of absorption spectra is with that of native oxyHr. The absorption spectrum of reconstituted oxymyoHr is shown in Figure 2 and closely resembles that of oxyHr (Garbett et al., 1969). As is the case for metmyoHr, distinct shoulders are apparent at ~290 and ~275 nm in the spectrum of *P. gouldii* oxymyoHr. The close agreement between A_{280}/A_{330} for reconstituted oxymyoHr and native oxyHr listed in Table I probably indicates a similar aromatic amino acid content in the two proteins; the calculated yield of oxymyoHr from apomyoHr cited above indicates that most of the apoprotein has been reconstituted to functional protein. A useful comparison can be made of the absorbance ratio A_{330}/A_{500} for oxymyoHr vs oxyHr. For the oxygenated diiron site, absorptions at 330 and 500 nm are due predominantly to the Fe^{III}–O–Fe^{III} and Fe^{III}–O_2H^- units, respectively (cf. eq 1), and the ratio $\epsilon_{330}/\epsilon_{500}$ is nearly independent of the protein (Dunn et al., 1977). The A_{330}/A_{500} ratios listed in Table I for reconstituted oxymyoHr are close to that of native oxyHr. The higher end of the range of oxymyoHr probably indicates some contamination by metmyoHr due to autoxidation of oxymyoHr. In all other Hrs, the met form has a lower absorbance at 500 vs 330 nm than does the oxy form. We have so far been unable to obtain A_{330}/A_{500} ratios for native *P. gouldii* myoHr that are as low as those for oxyHr by anaerobic reduction of metmyoHr followed by exposure to air. On the basis of the A_{330}/A_{500} ratio, our best preparations of oxymyoHr have been obtained via the reconstitution procedure.

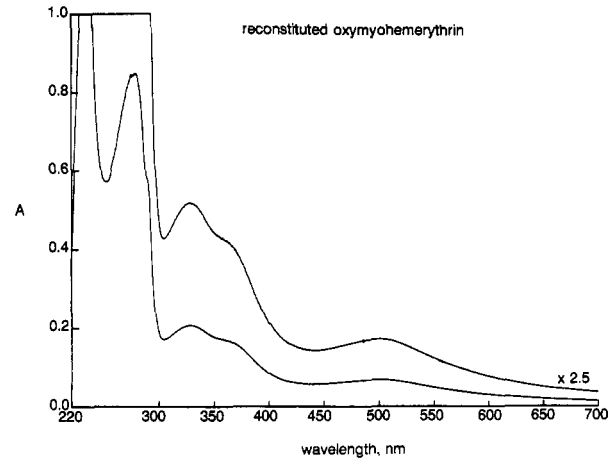

FIGURE 2: UV/vis absorption spectrum of reconstituted *P. gouldii* oxymyoHr in 50 mM HEPES, pH 7.0.

(b) Hr. Analytical gel filtration chromatography indicated that the reconstituted oxyHr is octameric, eluting as a single band at the same volume as for native metHr within experimental error. No protein eluted at the volume for myoHr, indicating the absence of dissociated subunits in the reconstituted oxyHr. Figure 3 shows the absorption spectrum of reconstituted oxyHr. The spectrum between 300 and 700 nm is essentially identical with that of native oxyHr. This identity

[2] This yield may be an underestimate since we expect the presence of nonfunctional iron, which, as described below, is ferrous, to lower ϵ_{280} below the native value.

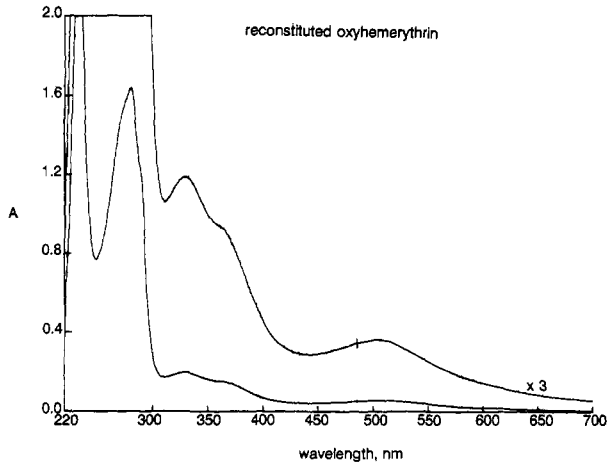

FIGURE 3: UV/vis absorption spectrum of reconstituted *P. gouldii* oxyHr in 50 mM HEPES, pH 7.0.

Table II: Iron and Total Protein Analyses for Reconstituted *P. gouldii* OxyHr

	sample				
	1	2	3	4	5
mol of Fe/mol of Hr subunits[a]	2.01	2.16	2.08	2.13	2.04
mol of functional Fe/mol of total Fe[b]	0.69	0.68	0.81	0.66	0.69

[a] This ratio was calculated from total iron determined by the method of Rill and Klotz (1970) and total protein determined by the method described under Experimental Procedures. [b] Functional iron was determined by using ϵ_{2Fe} = 6800 M^{-1} cm^{-1} at 330 nm for native oxyHr (Garbett et al., 1969). Total iron was determined by the method of Rill and Klotz (1970).

is reflected in the A_{330}/A_{500} ratios of the reconstituted oxyHr from four separate reconstitution experiments (Table I), which are all very close to the published value for native oxyHr (Garbett et al., 1969). Figure 3 and Table I also show that the reconstituted oxyHr has a higher relative absorbance at 280 nm than does the native protein. Two alternative explanations for the higher A_{280}/A_{330} ratio are either that the reconstituted Hr contains less than its full complement of iron or that the reconstituted Hr contains its full complement of iron but a portion of this iron is nonfunctional, i.e., incapable of binding O_2 at ambient pressures. The following results show that the latter explanation applies. The iron and total protein analyses of reconstituted oxyHr listed in Table II show that the reconstituted protein contains 2.08 ± 0.08 mol of Fe/mol of Hr subunits. Thus, the reconstituted oxyHr contains its full complement of iron. However, as shown by the data in Table II, the fraction of total iron that is functional, i.e., capable of binding O_2, is typically 0.7. Upon conversion of the reconstituted oxyHr to metHr, the A_{280}/A_{355} ratio reverts to values much closer to that of native metHr. The absorption spectra of native and reconstituted metHr are shown in Figure 4. Although the published value of $\epsilon_{280}/\epsilon_{355}$ = 5.5 is listed in Table I, our preparations of native metHr show A_{280}/A_{355} ratios in the range of 5.4–6.1, which overlaps the range for reconstituted Hr. The close match of the A_{280}/A_{355} ratio for the reconstituted and native metHrs indicates that, in contrast to reconstituted oxyHr, almost all of the iron in reconstituted metHr exists in diiron sites that are spectroscopically indistinguishable from those of native metHr. Assuming that the diiron sites in reconstituted metHr can be quantitated by using ϵ_{355} = 6400 M^{-1} cm^{-1} (Garbett et al., 1969), two samples of reconstituted metHr gave a ratio of diiron site iron/total iron = 0.92 ± 0.05, where total iron was determined by the wet chemical method of Rill and Klotz (1970). This ratio was found to be 0.97 ± 0.01 for two samples of native metHr

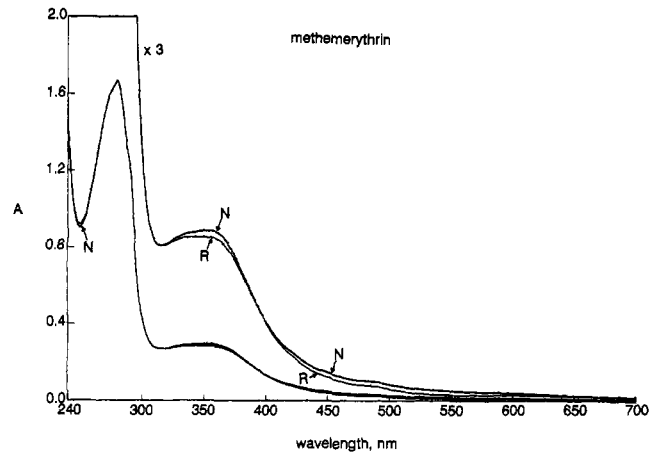

FIGURE 4: UV/vis absorption spectra of native (N) and reconstituted (R) *P. gouldii* metHr in 50 mM HEPES, pH 7.0. Spectra have been scaled to equal absorbance at 280 nm.

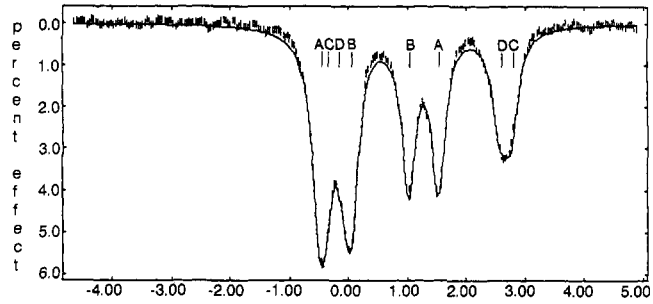

FIGURE 5: ^{57}Fe Mössbauer spectrum at 4.2 K of *P. gouldii* oxyHr reconstituted with iron isotopically enriched with ^{57}Fe. The Hr concentration is ~0.5 mM in oxy subunits in 50 mM Tris–sulfate, pH 7.5. The solid line represents a constrained Lorentzian least-squares fit by four quadrupole doublets, A–D, with line positions indicated by vertical bars. Doublets A and B arise from oxyHr; each component has the same FWHM, Γ = 0.285 mm/s, and fractional area a = 15.6%. Doublets C and D arise from unreactive high-spin ferrous iron. The parameters δ_{Fe}, ΔE_Q, Γ (in mm/s), and fractional area a (in %) are 1.18, 3.11, 0.32, 11.1 and 1.23, 2.63, 0.28, 7.7 for doublets C and D, respectively.

assayed in the same fashion. These results indicate that, upon conversion of reconstituted oxy- to metHr, the subunits containing nonfunctional iron are either lost or converted to subunits that are indistinguishable from those of native metHr. Our present results do not distinguish between these alternative possibilities. However, the fact that both reconstituted oxy- and metHr contain ~2Fe/subunit implies that the nonfunctional iron in reconstituted oxyHr is also present in diiron sites. Since [$Fe^{II}Fe^{II}$]deoxyHr has little or no absorbance at 330 nm, the higher than native A_{280}/A_{330} ratio for reconstituted oxyHr suggests the presence of ferrous iron in the nonfunctional diiron sites.

Mössbauer spectroscopy was used to further investigate the nature of the iron in reconstituted oxyHr. The sample for Mössbauer spectroscopy was reconstituted with iron that had been isotopically enriched in ^{57}Fe, and the 4.2 K spectrum of this reconstituted oxyHr is shown in Figure 5. The spectrum consists of two quadrupole doublets, A and B, due to high-spin ferric sites, which account for approximately two-thirds of the spectral area, and of two broad, unresolved lines due to high-spin ferrous sites. The absence of any magnetically split components implies that all high-spin ferric iron is spin-paired in the ground state. The isomer shifts (quadrupole splittings), δ_{Fe} (ΔE_Q) in mm/s for the two doublets, A and B, 0.51 (2.02) and 0.53 (0.98), respectively, are close to those published for oxyHr at 4.2 K [0.54 (1.92) and 0.51 (1.09); Okamura et al.,

1969)]. The line width of each of the middle two absorption lines, $\Gamma \sim 0.29$ mm/s, is close to the minimum instrumental width (0.24 mm/s), and these two lines have approximately equal intensity. The equality in intensity and line width of the middle two absorption lines indicate little or no contamination by either metHr or other high-spin ferric species. The relatively broad lines due to the high-spin ferrous sites account for approximately one-third of the spectral area. Figure 5 shows an attempt to fit this component by a pair of quadrupole doublets labeled C and D. The weighted averages of δ_{Fe} (ΔE_Q) for these doublets, 1.20 (2.92) mm/s, are very close to the values of 1.21 (2.92) mm/s for native deoxyHr run under the same conditions (Okamura et al., 1969). Within experimental error, the temperature dependence of ΔE_Q is the same as that of deoxyHr, but the line shape is clearly different.

Deoxygenation of this Mössbauer sample by addition of solid sodium dithionite changed the solution from pink-red to colorless, which is characteristic of conversion to deoxyHr. Reexposure to air regenerated the pink-red color characteristic of oxyHr. The Mössbauer spectrum of the sample after this treatment was not significantly different from that in Figure 5. This result indicates that the functional iron in reconstituted oxyHr reversibly binds O_2 but that cycling through the deoxy form does not convert the nonfunctional iron to functional iron.

The proportion of ferrous iron seen in the Mössbauer spectrum of reconstituted oxyHr agrees well with the proportion of nonfunctional iron in Table I. The fits of the Mössbauer data suggest that this iron is similar to but not identical with that in native deoxyHr. The lack of detectable O_2 binding at ambient pressure to this ferrous iron has no precedent in Hr chemistry. Native *P. gouldii* Hr shows no cooperativity in O_2 binding (Richardson et al., 1987). For native deoxyHr, binding of certain exogenous ligands to the sixth coordination site on Fe2 (cf. structure **1**) is known to inhibit O_2 binding (Reem & Solomon, 1987; Wilkins & Wilkins, 1987b), and an allosteric inhibition of O_2 binding by perchlorate has been noted (Richardson et al., 1987). However, these effectors do not prevent quantitative conversion to oxyHr under ambient $p(O_2)$. Furthermore, none of the species known to inhibit O_2 binding by either mechanism in native Hr were present at any stage of reconstitution. The lack of nonfunctional iron in reconstituted oxymyoHr may imply that the nonfunctional iron in reconstituted oxyHr is contained in subunits that became trapped in a nonnative refolded structure by rapid incorporation into the octamer upon removal of denaturant.

Summary and Conclusions

The first reconstitutions of functional diiron sites in Hr and myoHr have been achieved. The reconstitutions using our methods are reliable and reproducible. Met (native)-to-met (reconstituted) yields of ~50% for Hr and ~70% for myoHr are readily achievable; oxyHr and oxymyoHr are the major initial products of reconstitution and are obtained in yields exceeding 70%. Iron analyses indicate that the apoproteins have been essentially completely freed of iron and that reconstituted Hr contains its full complement of iron, i.e., ~2 Fe/subunit. Ferrous rather than ferric iron appears to be necessary for recovery of the native structures of both myoHr and Hr. ApoHr is essentially insoluble at pH 7 in the absence of denaturants but remains soluble when denaturant is removed in the presence of ferrous iron, which leads to recovery of the octameric structure containing its full complement of diiron sites. In contrast to reconstituted oxymyoHr, where essentially all of the iron appears to be functional, approximately 30% of the diiron sites in reconstituted oxyHr are unable to bind O_2 at ambient $p(O_2)$ (see Added in Proof). ^{57}Fe Mössbauer spectroscopy of the reconstituted oxyHr indicates that the nonfunctional iron is ferrous and is in an environment very similar to but not identical with that in native deoxyHr. The recovery of the octameric structure of Hr starting from the denatured apoprotein would appear to be a complicated process requiring refolding into the four-helix bundle structure within each subunit as well as reestablishment of the correct intersubunit contacts. Iron(II) apparently stabilizes the native or a nearly native subunit structure, which can then be incorporated into the octamer. The reconstitution procedures described here lay the groundwork for detailed examinations of the refolding process and for the preparation of metal-substituted Hrs. Such studies are currently in progress in our laboratory.

Added in Proof

We have recently found by both electronic absorption and ^{57}Fe Mössbauer spectroscopy that >90% functional iron can be obtained by reduction of reconstituted metHr back to deoxyHr with $Na_2S_2O_4$ followed by exposure of the sample to air.

Registry No. Fe, 7439-89-6; ^{57}Fe, 14762-69-7.

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Correlation between Mutational Destabilization of Phage T4 Lysozyme and Increased Unfolding Rates†

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ABSTRACT: The thermodynamics and kinetics of unfolding of 28 bacteriophage T4 lysozyme variants were compared by using urea gradient gel electrophoresis. The mutations studied cause a variety of sequence changes at different residues throughout the polypeptide chain and result in a wide range of thermodynamic stabilities. A striking relationship was observed between the thermodynamic and kinetic effects of the amino acid replacements: All the substitutions that destabilized the native protein by 2 kcal/mol or more also increased the rate of unfolding. The observed increases in unfolding rate corresponded to a decrease in the activation energy of unfolding ($\Delta G_u^{\ddagger}$) at least 35% as large as the decrease in thermodynamic stability (ΔG_u). Thus, the destabilizing lesions bring the free energy of the native state closer to that of both the unfolded state and the transition state for folding and unfolding. Since a large fraction of the mutational destabilization is expressed between the transition state and the native conformation, the changes in folding energetics cannot be accounted for by effects on the unfolded state alone. The results also suggest that interactions throughout much of the folded structure are altered in the formation of the transition state during unfolding.

Structural and thermodynamic comparisons of genetically altered proteins have resulted in rapid progress in understanding the factors that contribute to conformational stability (Alber, 1989; Goldenberg, 1988; Matthews, B. W., 1987; Shortle, 1989). Although the effects of amino acid replacements on protein stability are often attributed to alterations of interactions in the native structure, it is generally not possible to determine whether a change in thermodynamic stability (i.e., the free energy change for unfolding) is due primarily to a change in the native state or the unfolded state. This has generated considerable controversy about how the two states are affected by mutations.

One line of evidence indicating that destabilizing amino acid replacements have significant effects on the native state comes from the observation that most destabilizing replacements alter residues that are rigid or buried in the folded structure. This correlation between destabilization and features of the native structure—which has been observed for several proteins including the repressor (Hecht et al., 1984) and cro (Pakula et al., 1986) proteins of bacteriophage λ and bacteriophage T4 lysozyme (Alber et al., 1987a)—would be unlikely if many of the mutations acted primarily through effects on the unfolded state (Alber et al., 1987a).

On the other hand, it has been suggested that the effects of mutations on the stability of staphylococcal nuclease may be attributable to changes in the unfolded protein (Shortle, 1989). Amino acid replacements have been shown to change the steepness of the denaturant-induced unfolding transition (Shortle & Meeker, 1986) and to alter the hydrodynamic volumes of incompletely folded fragments of this protein (Shortle & Meeker, 1989). These observations have been interpreted as indicating that the mutations change the distribution of conformations in the unfolded state and the interactions of the unfolded chain with solvent. However, it is not yet known what effects these changes in the unfolded state have on the free energy change for unfolding.

Additional insights about the effects of substitutions on the different species making up a folding transition can be gained from kinetic studies, since effects on the free energy change for different steps in folding can be compared (Beasty et al.,

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