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Refolding a Disulfide Dimer of Cytochrome *c*†

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ABSTRACT: A covalent dimer of *Saccharomyces cerevisiae* iso-1 cytochrome *c* is stabilized by an interchain disulfide bond involving the cysteine residue penultimate to the C-terminus. The individual chains in the dimer appear to retain the tertiary structural features characteristic for monomeric cytochrome *c* albeit with some perturbation. The dimer is reversibly denatured by heat, urea, or guanidine hydrochloride in a single cooperative transition whose midpoint is less than that of the monomeric protein. The kinetic profile observed for the refolding of the denatured dimer is characteristic for monomeric cytochromes except for a markedly enhanced slow-phase amplitude.

Crystallographic studies of a variety of globular proteins indicate that polypeptide chains having 200 or more amino acid residues are commonly folded into contiguous globular units each containing about 100 residues called domains. It is likely that the individual domains within contemporary proteins once represented independent stable proteins whose structural genes were linked by either gene duplication or gene fusion. In the case of gene duplication, the primary sequences of the linked domains can be quite distinct even though the polypeptide chain fold in each domain remains quite similar. This situation probably reflects the independent genetic drift of the fused structural gene elements, the tolerance of a tertiary fold to sequence variation, and, in some cases, an advantageous asymmetric interaction of adjacent domains.

Cytochrome *c* may be considered to be a protein having a stable single-domain structure (Takano et al., 1977) containing about 100 residues. The domain of all the cytochromes *c* examined can be reversibly unfolded by using denaturants such as urea or guanidine hydrochloride. While most cytochromes *c* do not contain a free sulfhydryl group, the iso-1 isomer purified from *Saccharomyces cerevisiae* contains a single free sulfhydryl group located on a cysteine residue penultimate to the C-terminus (Yaoi, 1967; Narita & Titani, 1969). Formation of a disulfide dimer of the iso-1 monomer would then emulate a two-domain protein resulting from recent duplication of a structural gene. In this report, we inquire whether covalent linkage of two identical polypeptide chains significantly perturbs either the folding or the stability of the individual domains. We use a selectively alkylated monomer to distinguish whether any observed differences between the monomeric and dimeric iso-1 cytochromes result from simple modification of the penultimate cysteine as opposed to linkage of two chains.[1]

Experimental Procedures

Materials. Saccharomyces cerevisiae cytochrome *c* type VIII was purchased from Sigma. Preparations of the protein were subjected to exclusion chromatography at 25 °C using a calibrated 2.2 × 65 cm column of Sephadex G-50 equilibrated with either 50 mM phosphate buffer, pH 7.0, or 50 mM

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[1] The sequence numbering system employed assigns the two cysteine residues linked by thioether bonds with the porphyrin to sequence positions 14 and 17.

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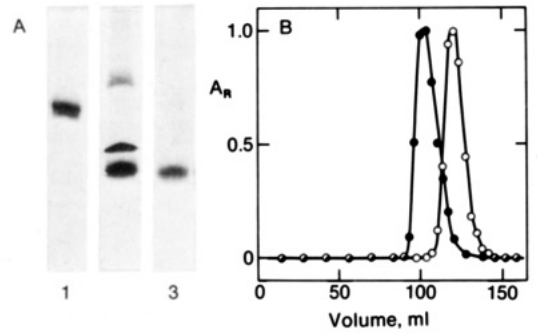

FIGURE 1: Resolution of molecular forms. (A) Polyacrylamide gel electrophoresis measurements. Protein samples were applied to a slab gel polymerized from a solution containing 15% acrylamide, 0.1% sodium dodecyl sulfate, and 0.4 M Tris-HCl buffer, pH 8.8, using ultraviolet radiation for free radical initiation. Protein eluted from the exclusion chromatograph as a single component centered at 100 mL and at 128 mL was applied to lanes 1 and 3, respectively. A mixture of horse heart cytochrome *c*, sperm whale myoglobin, and rabbit lactate dehydrogenase was applied to lane 2. Samples were applied to the top of each lane as oriented in the figure, and electrophoresis was done for 150 min at 30 mA. The gels were then removed from the electrophoresis cabinet, treated with the silver stain procedure, dried, and mounted. (B) Exclusion chromatography. Protein samples were applied to a 2.2 × 65 cm column of Sephadex G-50 equilibrated and developed with 50 mM phosphate buffer, pH 7.0. The absorbance of each fraction measured at 525 nm is expressed as a fraction, A_R, of the maximum absorbance measured in the profile. The closed circles describe the elution profile of a sample of the monomeric protein preincubated for 5 h in 3 M guanidine hydrochloride. The open circles indicate the elution profile of a sample of the alkylated monomer treated similarly.

ammonium acetate buffer, pH 6.0. All preparations were resolved into two components whose elution was centered at 99 ± 2 and 127 ± 3 mL. These elution volumes are appropriate to globular proteins having the molecular weights of dimeric and monomeric cytochromes *c*, respectively. This assignment was confirmed by polyacrylamide gel electrophoresis measurements done in the presence of detergent but in the absence of reducing agents as illustrated Figure 1A. Different preparations of the protein exhibited widely different proportions of the monomeric and dimeric forms presumably resulting from variable prior exposure to conditions promoting oxidation. We chose exclusion chromatography in preference to ion-exchange chromatography (Motonaga et al., 1965a) for the resolution of the two forms of the protein in order to obtain the unmodified monomeric form in the desired solvent just prior to measurements. The interval between resolution and measurement is crucial to the integrity of both the native and denatured unmodified monomer as illustrated in Figure 1B.

To increase the amounts of the monomeric or dimeric forms of the protein recovered, preparations were preincubated with a redox reagent prior to exclusion chromatography. The monomeric form was obtained exclusively by preincubation of the protein with a 10-fold molar excess of β-mercaptoethanol in 50 mM phosphate buffer, pH 7.0, overnight at 4 °C. The dimeric form was obtained exclusively by preincubation in a 20-fold molar excess of $CuSO_4$ in 200 mM ammonium acetate buffer, pH 7.0 (Motonaga et al., 1965a), for 2 h at room temperature.

The alkylated protein was obtained by reaction of about 50 mg of the freshly prepared monomeric protein with 0.1 M iodoacetate in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8.6, for 15 min at room temperature as described by Crestfield et al. (1963). The excess iodoacetate was removed by ion-exchange chromatography and the protein subjected to amino acid analysis following hydrolysis in 6 M HCl for 24 h at 110 °C. The hydrolysate contained 1.0 ± 0.1 residue of (carboxymethyl)cysteine per polypeptide chain. No other amino acids in the hydrolysate appeared to have been modified within the precision of measurement, ±4%.

Methods. Absorbance spectra were obtained by using either a Cary Model 17 or a Gilford Model 2600 recording spectrophotometer. The concentrations of ferricytochrome *c* solutions were measured spectrophotometrically in 50 mM phosphate buffer, pH 7.0, using an extinction coefficient at 409 nm of 106 mM^{-1} cm^{-1}. Fluorescence measurements were obtained by using a Perkin-Elmer Model MPF-2A spectrophotofluorometer. The concentration of cytochrome *c* solutions was limited to a maximal value of 2 μM to retain a linear relationship between emission and concentration. Samples were placed in square quartz cells having an optical path of 10 mm. The cells were placed in a hollow brass cell holder through which thermoregulated water was circulated using a Neslab Model RTE-9 refrigerated bath. Routine measurements were made by using an excitation wavelength of 295 mn, an excitation slit of 6, an emission slit of 8, and a sensitivity of 3. Circular dichroic spectra were obtained by using a Cary Model 60 CD spectropolarimeter, cylindrical cells having an optical path of 10 mm, a full scale of 0.1°, and a time constant of 0.3 s.

Stopped-flow fluorescence measurements were obtained by using a Durrum Model D-110 spectrometer having an instrumental dead time of 6 ms. The mixing syringes and the optical cell block were bathed with circulating water maintained at 25 °C. The quartz observation cell has an excitation path length of 16 mm and an emission path length of 2 mm. An excitation wavelength of 295 nm and a slit of 4 mm were used corresponding to a dispersion of 12 nm. The photometer was placed at a right angle to the excitation beam and the emitted light passed through a Corning 0-54 filter which transmits less than 10% of 305-nm light and more than 80% of 330-nm light. The output from the photometer was digitized by using a Nicolet Model 3091 oscilloscope having 12-bit resolution and a storage capacity of 400 points. The digitized data were delivered to a DEC Model 11/780 VAX computer and analyzed by using a program developed by M. Dunn (University of California, Riverside) based on a series of FORTRAN subroutines for nonlinear least-squares curve fitting (Bevington, 1969). The program facilitates editing, averaging, plotting, and fitting of kinetic data. The kinetic data for cytochrome *c* were analyzed by using the equation:

$$y(t) = A + \sum_{i=1}^{n} B_i e^{-(t/\tau_i)}$$

where $y(t)$ is the fluorescence amplitude at time t, n is the number of kinetic phases, B_i is the change in fluorescence intensity associated with each kinetic phase, and τ_i is the relaxation time of each kinetic phase. The value of n giving the χ^2 value and minimal sum of squared residuals between simulated and observed data was selected. The amplitude of each phase (α_i) was calculated by using the equation:

$$\alpha_i = \frac{B_i}{\sum_{i=1}^{n} B_i}$$

RESULTS

Equilibrium Measurements. All experimental values were obtained in solutions containing 50 mM phosphate buffer maintained at 25 °C unless noted otherwise. The monomer, alkylated monomer, and dimer forms of iso-1 ferricytochrome

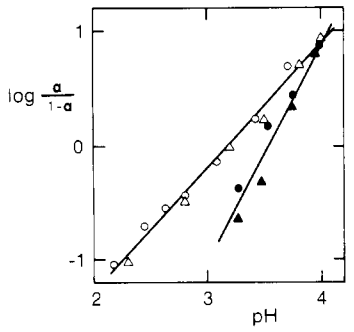

FIGURE 2: Effect of acidification on the spectral properties of a salt-free solution of alkylated iso-1 ferricytochrome *c*. The open circles denote the extinction coefficient at 395 nm which increased from 60 mM^{-1} cm^{-1} at pH 7.0 to a maximum of 173 mM^{-1} cm^{-1} in acid. The open triangles denote the extinction coefficient at 620 nm which increased from 1.7 mM^{-1} cm^{-1} to a maximum of 5.3 mM^{-1} cm^{-1} in acid. The closed circles denote the fluorescence emission intensity which ranged from 10% that of the model tryptophan residue at neutral pH to a maximum 68% of the model at acid pH. The closed triangles denote molar ellipticity measurements at 222.5 nm which ranged as illustrated in Figure 5.

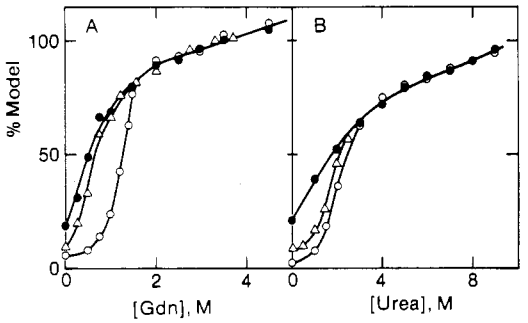

FIGURE 3: Effect of denaturants on the fluorescence of iso-1 cytochromes *c*. The open circles indicate the monomeric protein, the triangles the alkylated monomeric protein, and the closed circles the dimeric protein. All measurements were made with 2 μM protein solutions in 50 mM phosphate buffer, pH 7, and 295-nm excitation. The emission intensity observed at 350 nm is expressed as the fractional percentage of emission intensity observed for an equivalent solution of the model tryptophan chromophore, *N*-acetyltryptophan ethyl ester. Guanidine hydrochloride is abbreviated as Gdn.

c exhibited visible absorbance spectra characteristic for a low-spin coordination complex having methionine and histidine as axial ligands. The positions of the absorbance maxima and their molar extinctions were within ±2% of the values characteristic for horse ferricytochrome *c* (Margoliash & Frohwirt, 1959). Acidification of salt-free solutions of the ferri form of each protein using HCl shifted the Soret maximum from 409 to 395 nm and generated a maximum at 620 nm. These changes were characteristic of formation of a high-spin coordination complex. Analysis of the pH dependence of these absorbance changes as illustrated in Figure 2 indicates that the acid spin-state transition of each form of the protein involves 1.1 ± 0.1 protons having a p*K* value of 3.2 ± 0.1. Polastro et al. (1976) have previously reported a p*K* value of 3.3 for the monomeric protein.

Excitation of solutions of each form of the protein with 295-nm radiation results in the observation of emission spectra each having a broad maximum centered at about 350 nm characteristic for emission from a tryptophan residue. In the case of iso-1 cytochrome *c*, this must originate from the single tryptophan in the polypeptide chain located at position 59. However, the tryptophan fluorescence in native iso-1 cytochrome *c* is quenched considerably as reflected in the emission intensity observed at 350 nm which ranges between 5% and 21% of that measured for a comparable molar concentration

Table I: Comparative Properties

parameter	monomer	alkylated monomer	dimer
denaturation midpoint[a]			
guanidine hydrochloride (M)	1.2	0.7	0.5
urea (M)	2.1	1.9	1.3
acid (pH)	3.5	3.7	3.7
heat (°C)	56	52	43
$\Delta G°$, unfolding (kcal/mol)[b]	3.0 ± 0.4	1.9 ± 0.2	1.1 ± 0.4
refolding kinetics[c]			
$\tau_{fast\ phase}$ (s)	1.8 ± 0.5	2.8 ± 1.1	3.4 ± 1.2
$\tau_{slow\ phase}$ (s)	21 ± 3	33 ± 6	25 ± 2
$\alpha_{slow\ phase}$[d]	0.32 ± 0.04	0.21 ± 0.05	0.62 ± 0.03

[a] The indicated values represent the mean of at least three measurements. [b] The values obtained from guanidine hydrochloride and from urea denaturation were averaged. [c] All measurements were obtained by dilution of a solution of denatured protein in 3 M guanidine hydrochloride to 0.5 M guanidine hydrochloride. The values shown represent the mean and standard deviation of at least five measurements. [d] The fraction of the total observed change occurring in the slow phase.

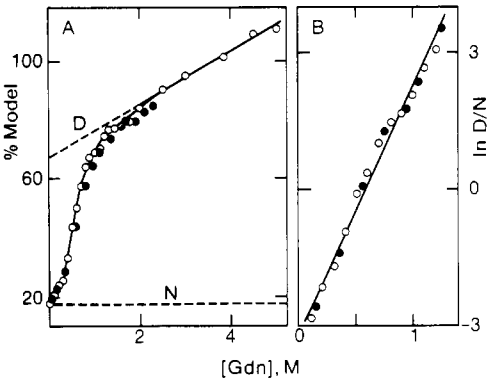

FIGURE 4: Representative calculation of an unfolding free energy. Panel A illustrates the reversibility of the change in fluorescence emission observed for the dimer using the procedures described in the legend to Figure 3. The open circles denote values obtained beginning with the native dimeric protein, and the closed circles denote values obtained beginning with the equilibrium-denatured protein. Panel B illustrates the calculation procedure. The observed experimental values were assumed to represent a mixture of native and denatured protein. Each experimental value was then parsed into the relative amount of native (N) and denatured (D) protein on the basis of the extrapolated values for the pure native and pure denatured material illustrated by the labeled dashed lines in panel A. The unfolding free energy was obtained from the product of the quantities *RT*, the denaturant concentration at ln $(D/N) = 0$, and the slope of the relationship plotted in panel B (Schellman, 1978).

of a model tryptophan residue, *N*-acetyltryptophan ethyl ester, as shown in Figure 3. The relative fluorescence emission intensity measured at 350 nm is increased to 44 ± 4% that of the model upon heating each protein solution at about 70 °C. The increase in fluorescence emission intensity observed upon heating individual solutions of each form of the protein increased in a single cooperative transition whose midpoint ranged from 43 to 56 °C as listed in Table I. Polastro et al. (1976) report a midpoint of 58 °C for the monomer observed at pH 6.5. Cooling solutions of the dimeric protein from 25 to 2 °C did not decrease the fluorescence amplitude below 21 ± 1% that of the model.

Addition of either urea or guanidine hydrochloride to solutions of each form of the protein also increased the fluorescence intensity observed at 350 nm relative to the model chromophore as shown in Figure 3. The average midpoint value for the increase in fluorescence intensity observed for each protein upon addition of denaturant is listed in Table I. Polastro et al. (1976) report a midpoint value of 1.3 M for the denaturation of the monomer by guanidine hydrochloride.

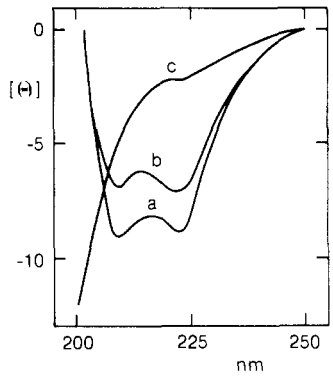

FIGURE 5: Far-ultraviolet dichroic spectra. Spectrum a was obtained by using either monomer or alkylated monomer. Spectrum b was obtained by using the dimer. Spectrum c was obtained by using monomer, alkylated monomer, or dimer either in 3 M guanidine hydrochloride or in salt-free solutions adjusted to pH 2.0 with HCl. The symbol $[\theta]$ represents the molar residue ellipticity.

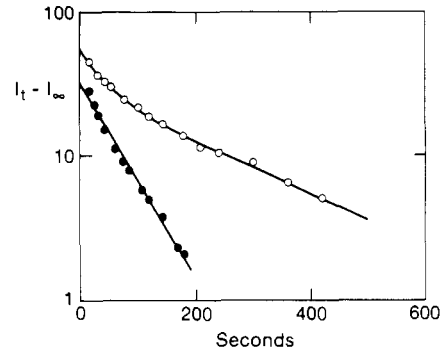

FIGURE 6: Manual multimixing measurements. Native dimer was incubated in 3 M guanidine hydrochloride for either 45 s (closed circles) or 30 min (open circles) prior to refolding in 0.25 M guanidine hydrochloride. I_t represents the fluorescence emission intensity after refolding for time t and I_∞ the intensity at equilibrium. The protein concentration in the refolding solvent was 4 μM. All measurements were obtained at 2 °C.

The increase in fluorescence intensity accompanying addition of denaturant is completely reversible upon dilution of the denaturant as illustrated for the dimeric protein in Figure 4A. The denaturation transitions of the native and modified protein observed in urea and in guanidine hydrochloride were analyzed in terms of a two-state model with no preferential interaction (Schellman, 1978) as illustrated in Figure 4B. The average unfolding free energies so calculated are listed in Table I. The unfolding free energy shown in Table I for the protein alkylated with iodoacetate is within experimental variation of the value reported by Zuniga & Nall (1983) for the protein alkylated with iodoacetamide.

The fluorescence emission intensity of each form of the protein is increased to a maximal value 68 ± 2% that of the model chromophore by acidification of salt-free solutions containing no denaturant. The increase in fluorescence amplitude of each form of the protein upon acidification has a midpoint at pH 3.6 ± 0.1 as illustrated for the alkylated protein in Figure 2.

The far-ultraviolet circular dichroism spectrum of the ferri form of each of the three proteins at neutral pH exhibits minima at 222.5 and 209 nm as shown in Figure 5. A similar dichroic spectrum for the monomer was reported by Looze et al. (1976). The dichroic spectra of the monomer and alkylated monomer are equivalent while that of the disulfide dimer is somewhat diminished. The dichroic spectra of the ferriproteins either in 3 M guanidine hydrochloride at neutral pH or in salt-free solutions at pH 2.0 are distinct in profile and characteristic for denatured ferricytochromes *c* (Myer, 1968a,b) as shown in Figure 5. The pH dependence of the molar ellipticity of salt-free solutions of the ferriproteins is coincident with that of the fluorescence but not with that of the visible absorbance as illustrated in Figure 2. The pH dependence of the fluorescence and dichroic changes observed for each of the three iso-1 ferriproteins indicates the involvement of 2.0 ± 0.1 protons having a pK of 3.7 ± 0.3.

Kinetic Measurements. Stopped-flow measurements of the refolding of iso-1 ferricytochrome *c* were principally done by using solutions of guanidine hydrochloride denatured proteins at neutral pH in order to compare results with those previously reported for the monomeric protein (Zuniga & Nall, 1983). Fluorescence emission rather than absorbance was employed as the observation probe in order to ensure that a conformational transition as opposed to an axial ligation change is observed. Solutions of denatured protein were diluted to span the conformational transition as much as possible given the availability of mixing syringes and the breadth of the equilibrium transitions illustrated in Figure 3. The observed decrease in fluorescence intensity accompanying the dilution of each guanidine hydrochloride denatured protein was resolved into two kinetic phases in accordance with prior measurements (Zuniga & Nall, 1983). The relaxation times and fractional amplitudes for the kinetic phases observed for each form of the protein are listed in Table I. Essentially the same kinetic parameters were observed when refolding was initiated from solutions of the urea-denatured proteins. The only significant difference in the refolding kinetic parameters observed by using the three proteins involves the fractional amplitude of the slow phase which is increased by a factor of 2 in the disulfide dimer relative to either form of the monomer.

The origin of the slow refolding phase was examined by using a multimixing protocol. Since we are not equipped to execute stopped-flow multimixing protocols, the temperature of solutions undergoing conformational transitions was lowered to 2 °C to slow the reactions into a range appropriate to manual multimixing. Native protein was unfolded for a controlled period of time in solutions containing excess denaturant prior to dilution of the denaturant to initiate refolding. A typical result is shown in Figure 6 in which the refolding slow phase was found to be absent following denaturation for only 45 s prior to refolding. Similarly, no slow-phase refolding was observed by using the alkylated monomer denatured for 45 s within the precision of the measurement. An activation energy of 17 kcal/mol was calculated for the slow refolding phase of the disulfide dimer from the relaxation times observed at 2 and 25 °C using the equilibrium-denatured protein.

Discussion

Several qualitative features of the native conformation of monomeric *S. cerevisiae* iso-1 ferricytochrome *c* appear to persist in the disulfide dimer. Each monomeric subunit in the dimer exhibits an absorbance spectrum characteristic for a low-spin coordination complex, a far-ultraviolet circular dichroic spectrum characteristic for a helical heme protein, and a near-ultraviolet fluorescence emission spectrum characteristic for a tryptophan whose fluorescence is considerably quenched by a heme iron. Similarly, Motonaga et al. (1976a) report that the disulfide dimer of *S. oviformis* cytochrome *c*, which has the same sequence as the *S. cerevisiae* iso-1 cytochrome *c*, retains the absorbance spectra and electron transport activity characteristic for its monomeric form. The disulfide dimer of the *S. cerevisiae* cytochrome *c* examined here reversibly unfolds in a single transition as measured by the tryptophan fluorescence emission. The denatured dimeric protein refolds

in two kinetic phases whose relaxation times are comparable to those observed for the denatured monomeric protein. Thus, covalent linkage of two cytochromes *c* with a disulfide bond penultimate to their C-termini produces no major aberrations in either the folding pathway or the conformation of the folded product. Within the precision of the measurements, the monomeric components of the disulfide dimer appear to have equivalent conformations and to refold independently into them.

Nonetheless, quantitative comparisons reveal that aspects of both the folded conformation and the folding pathway of the dimer are perturbed relative to those of either the monomer or the monomer whose cysteine-102 is alkylated with a small reagent. As shown in Figures 3 and 5, the magnitudes of both the tryptophan fluorescence quenching and the far-ultraviolet ellipticity of the dimer in the absence of denaturant are somewhat diminished relative to those of the monomer and the alkylated monomer. Second, as shown in Figure 3 and Table I, the midpoints of the denaturation transitions of the dimer observed by tryptophan fluorescence measurements upon addition of urea or guanidine hydrochloride or application of heat are each diminished relative to the midpoint values observed for the monomer and the alkylated monomer. Third, the relative amplitude of the slow refolding kinetic phase is at least twice that observed with the monomer or the alkylated monomer. Taken together, these comparisons indicate that formation of a cytochrome *c* disulfide dimer diminishes the stability of the monomeric domains and increases the half-time required for their acquisition.

The observed destabilization of iso-1 ferricytochrome *c* following selective alkylation of cysteine-102 is in keeping with the results of Motonaga et al. (1965b), who observed that reaction of this residue with thiosulfate increased the susceptibility of the protein to digestion by three different proteases and to denaturation by urea. They also observed that reaction of cysteine-102 with either iodoacetate, iodoacetamide, or *p*-(chloromercuri)benzoate generated an equivalent increased susceptibility of the protein to digestion by a bacterial protease. In keeping with the latter observation, we find that the susceptibility of the protein alkylated with iodoacetate to denaturation by guanidine hydrochloride is equivalent to the susceptibility of the protein alkylated with iodoacetamide (Zuniga & Nall, 1983). Accordingly, it appears that modification of cysteine-102 and not the polarity or formal charge on the modified residue is responsible for the decreased stability of the protein following reaction with a small reagent.

By contrast, reaction of cysteine-102 with a large reagent, the sulfhydryl of another monomer to form the disulfide dimer, further decreases the stability of the protein to heat and denaturants and causes amplitude changes in the tryptophan emission profile and in the far-ultraviolet circular dichroic profile. We suggest that these changes likely result from noncomplementary interactions between the surfaces of the monomeric units brought into contact by the interchain disulfide bond. Such conformational perturbations in some cases may be advantageous in that they could modulate the biofunction of the individual monomers or create new biofunctions of advantage to the host cell. Thus, gene duplication could lead to new or improved biofunction at the expense of stability. Judicious amino acid additions, deletions, or replacements within the intermonomer contact region could in principle improve the complementarity of the interactions while maintaining the improved or newly acquired biofunction. Such changes may in part account for the difference in sequence but not polypeptide fold of multidomain proteins presumed to be products of gene duplication.

A dominant slow refolding kinetic phase is unique to the disulfide dimer among the guanidine hydrochloride denatured cytochromes *c* whose refolding kinetics have been measured (Nall & Landers, 1981; Brems et al., 1982). Ridge et al. (1981) have shown that the properties of the slow refolding phase of guanidine hydrochloride denatured horse heart ferricytochrome *c* are characteristic of a slow obligatory proline peptide isomerization which precedes more rapid folding. We find that the slow refolding phase of denatured iso-1 monomer and dimer is generated in the denatured state and has a relaxation time and activation energy appropriate to proline peptide isomerization. If the slow refolding phase of denatured iso-1 ferricytochrome also results from an obligatory proline peptide isomerization prior to refolding, then the increase in the amplitude of slow-phase refolding in the dimer compared with the monomer indicates an increase in the population of nonnative proline peptide isomer(s) in the denatured state, an increase in the numbers of proline peptide bonds contributing to the slow refolding phase, or both. Since Lin & Brandts (1983a,b) have observed that the cis/trans ratio of a proline peptide bond is dependent both on the length of the polypeptide chain in which it resides and on the presence of intrachain disulfide bonds, we suggest that the enhanced slow-phase amplitude of the disulfide dimer results from an increased population of nonnative cis isomer(s) in the denatured disulfide dimer compared with the corresponding population in the denatured monomer.

As shown in Figure 2, the acid spin-state transition observed for salt-free solutions of iso-1 ferricytochrome appears to be uncoupled from the acid denaturation of the protein as observed by tryptophan fluorescence and by far-ultraviolet ellipticity. While these two acid transitions are frequently coincident for many cytochromes, studies using a variety of solvents and protein derivatives indicate that the acid spin-state and conformational transitions can be uncoupled. For example, while the acid spin-state and conformational transitions of horse heart ferricytochrome *c* are coincident in salt-free solution (Babul & Stellwagen, 1972), they are uncoupled in the presence of salt (Boeri et al., 1953; Fung & Vinogradov, 1968; Greenwood & Wilson, 1971; Stellwagen & Babul, 1975). Ferrihemopeptides obtained from horse heart cytochrome *c* ranging from 11–21 (Wilson et al., 1977) to 1–65 (Babul et al., 1972) each exhibit an acid spin-state transition free from a conformational change. It should be noted that the uncoupled spin-state transition observed in each of these systems involves a single proton having a pK in the pH range 3.0–4.0 as is the case for iso-1 ferricytochrome *c*.

Registry No. Cytochrome *c*, 9007-43-6.

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Redox Chemistry of Sulfide-Bridged Derivatives of the Binuclear Iron Site in Hemerythrin from *Phascolopsis gouldii*†

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ABSTRACT: Reported are redox potentials and redox chemistry for the binuclear iron site of hemerythrin (Hr), when bridging S^{2-} is substituted for bridging O^{2-} (or OH^-). The sulfide-bridged derivatives can be reversibly cycled between the met and semi-met oxidation levels, μ-S^{2-}met and μ-S^{2-}semi-met, respectively, at pH 8.0. The midpoint reduction potentials for this couple are in the range of 283–312 mV vs. NHE and depend on the presence or absence of perchlorate. μ-S^{2-}met slowly autoreduces to μ-S^{2-}semi-met in anaerobic solutions. The autoreduction, which consistently occurs to the extent of 80–85%, can be explained in terms of the successive reactions μ-S^{2-}met → met + S^{2-} and S^{2-} + μ-S^{2-}met → μ-S^{2-}semi-met + SO_3^{2-}. The reactions of both μ-S^{2-}met and μ-S^{2-}semi-met with O_2 are sulfide based and result in met- and semi-metHr, respectively. The latter reaction appears to proceed through $(\text{semi-met})_O$. O_2 appears to react with bound S^{2-}, since the reactions with O_2 are much faster than anaerobic dissociation of S^{2-}. Unlike O_2, ligand anions accelerate the autoreduction of μ-S^{2-}met. This acceleration causes the reaction of μ-S^{2-}met with O_2 in the presence of N_3^- to proceed through μ-S^{2-}semi-met. Perchlorate slows both oxidative loss of sulfide and autoreduction. μ-S^{2-}semi-met cannot be reduced by $S_2O_4^{2-}$; however, electron paramagnetic resonance evidence indicates that S^{2-} apparently does interact with deoxyHr. From the observation that S^{2-} substitutes at the semi-met but not at the met oxidation level, a μ-hydroxo bridge is proposed to bridge the iron atoms in semi-metHr. Lack of stable anion adducts of μ-S^{2-}met or μ-S^{2-}semi-met suggests severe steric restrictions at both oxidation levels. The ~200-mV positive shift in met/semi-met reduction potential when bridging O^{2-} is replaced by bridging S^{2-} is consistent with hard–soft acid–base theory. The potentials of the μ-S^{2-}met/μ-S^{2-}semi-met couple fall within the range of "Rieske"-type [2Fe-2S] centers. It is proposed that histidine imidazoles provide some of the ligands to the iron atoms in the Rieske center.

The non-heme oxygen-carrying protein hemerythrin (Hr)[1] occurs in certain marine invertebrates, most commonly as an octamer of M_r 108 000. Each of the eight identical subunits contains a binuclear iron site. Upon oxygenation this binuclear site is converted from a form containing two high-spin Fe^{2+} ions (deoxyHr) to a form containing two high-spin Fe^{3+} ions with the bound dioxygen reduced to the peroxide oxidation state (oxyHr) (Kurtz et al., 1977; Sanders-Loehr & Loehr, 1979; Wilkins & Harrington, 1983; Klotz & Kurtz, 1984). DeoxyHr and oxyHr can be oxidized to a form containing two high-spin Fe^{3+} ions per site, which no longer reversibly binds O_2 (metHr). This form does bind a number of small anions, hereafter referred to as ligand anions.

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[1] Abbreviations: Hr, hemerythrin; EPR, electron paramagnetic resonance; Tris, tris(hydroxymethyl)aminomethane; $metN_3^-$, semi-$metN_3^-$, and $deoxyN_3^-$, azide adducts of met-, semi-met-, and deoxyHr, respectively; μ-S^{2-}met and μ-S^{2-}semi-met were previously identified as $metS^{2-}$ and semi-$metS^{2-}$, respectively (Lukat et al., 1984).

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