The Reversible Unfolding of Horse Heart Ferricytochrome *c**

Earle Stellwagen

ABSTRACT: Physical and chemical measurements of ferricytochrome *c* in 8 M urea indicate that the native conformation of the protein is unfolded in this solvent. The intrinsic viscosity increases from 2.5 to 14.9 ml/g in the presence of 8 M urea and the sedimentation coefficient decreases from 1.9 to 1.2 S. The Soret absorbance undergoes a blue shift and the conformationally sensitive absorbance band at 695 mμ disappears. The buried or partially buried functional groups of the native conformation are exposed in 8 M urea. The two unreactive histidyl and one unreactive methionyl residue are carboxymethylated by bromoacetate; the absorption spectra of the two buried tyrosyl residues are perturbed by ethylene glycol and the ionization of their phenolic hydroxyl groups normalized; the absorption spectrum of the single tryptophanyl residue is perturbed by ethylene glycol; and the Soret absorbance of the heme moiety is fully perturbed by ethylene glycol. Removal of 8 M urea by dialysis gives a protein with the physical–chemical properties of native ferricytochrome *c*. This recovery indicates that the native conformation of cytochrome *c* is reversibly unfolded in 8 M urea.

The reversibility of the unfolding of a variety of proteins (Reithel, 1963) has provided experimental verification of the proposal (Lumry and Eyring, 1954) that the native conformation of a protein is determined by its amino acid sequence. Since the single polypeptide chain of horse heart ferricytochrome *c* contains no intrachain covalent bonds, it should be possible to refold the polypeptide chain into its native conformation by simply removing the unfolding reagent under appropriate conditions. The demonstration of such a reversibility would permit a study of the kinetics of the refolding process using the changes in the absorption spectrum of the covalently bound heme moiety as a natural reporter group, and also facilitate an examination of the effects of chemical modification of specific side chains on the kinetics and extent of refolding. This report describes the reversibility of the unfolding of ferricytochrome *c* in 8 M urea.

Materials and Methods

Materials. Horse heart cytochrome *c*, type VI, was obtained from the Sigma Chemical Co. All protein solutions contained 0.2 M KCl–0.01 M phosphate buffer (pH 7.0). An Ultra Pure Grade of urea was purchased from Mann Research Laboratories, Inc. Solutions of protein in urea were allowed to stand at least 1 hr at room temperature before use. Urea was removed by dialysis against 0.2 M KCl–0.01 M phosphate (pH 7.0) at 2° for 24 hr.

Protein concentrations were determined spectrophotometrically, using the extinction coefficients of Margoliash and Frohwirt (1959). Protein solutions containing urea were diluted into 0.1 M phosphate buffer (pH 7.0) containing excess sodium dithionite, to give a final concentration of urea of less than 0.5 M. The protein concentration was then calculated from the absorbance at 550 mμ using the extinction coefficient of the native protein. This procedure was found to be in error by no more than ±1%. The concentrations of urea solution were determined by refractive index measurements using a standard curve based on dry weight measurements.

Sedimentation Studies. Sedimentation velocity experiments were performed with a Spinco Model E ultracentrifuge using a capillary-type, filled-Epon, double-sector synthetic boundary centerpiece. All experiments were performed at constant temperature (19–23°) with a rotor speed of 59,780 rpm. Photographs were taken with Kodak spectroscopic I-N photographic plates using a Kodak No. 25 red filter. Schlieren patterns were analyzed with the aid of a Nikon microcomparator. Sedimentation coefficients were calculated as described by Schachman (1957). Values for the density and relative viscosity of urea solutions were obtained from Kawahara and Tanford (1966).

Pycnometry. The apparent specific volume of ferricytochrome *c* was calculated from pycnometric measurements as described by Schachman (1957). All measurements were made at 20.0° using a 5-ml pycnometer and a protein concentration of 40 mg/ml. Four successive measurements gave a value of 0.724 ± 0.001 ml/g for the apparent specific volume, which was assumed to be the same as the partial specific volume.

Other Measurements. The reduced viscosity, solvent perturbation difference spectra, ionization of phenolic hydroxyl groups, iodination of the tyrosyl residues, and alkylation of histidyl residues were measured as previously described (Stellwagen and Van Rooyan, 1967). The number of carboxymethylated methionyl residues was calculated from the methionyl sulfone content of an acid hydrolysate of the performic acid oxidized

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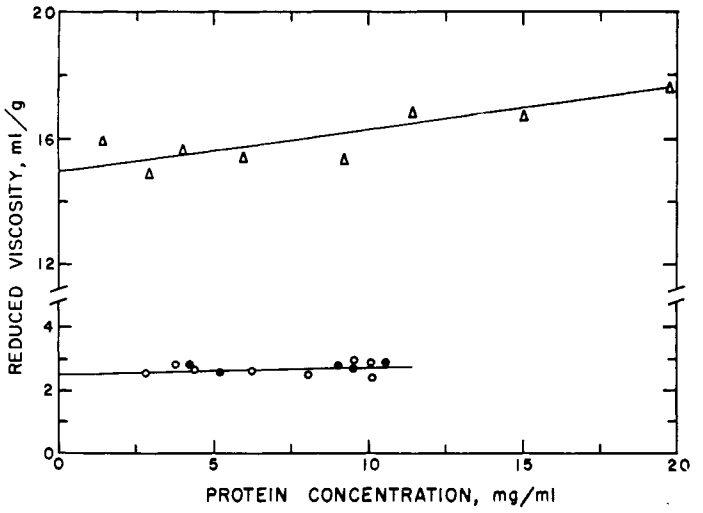

FIGURE 1: Reduced viscosity of ferricytochrome *c*. (○) Native ferricytochrome *c*; (△) ferricytochrome *c* in 8 M urea; (●) ferricytochrome *c* exposed to 8 M urea and then dialyzed to remove the urea. The solvent in all experiments contained 0.2 M KCl and 0.01 M phosphate, and had a final pH of 7.0. All viscosity measurements were performed at 25.0°.

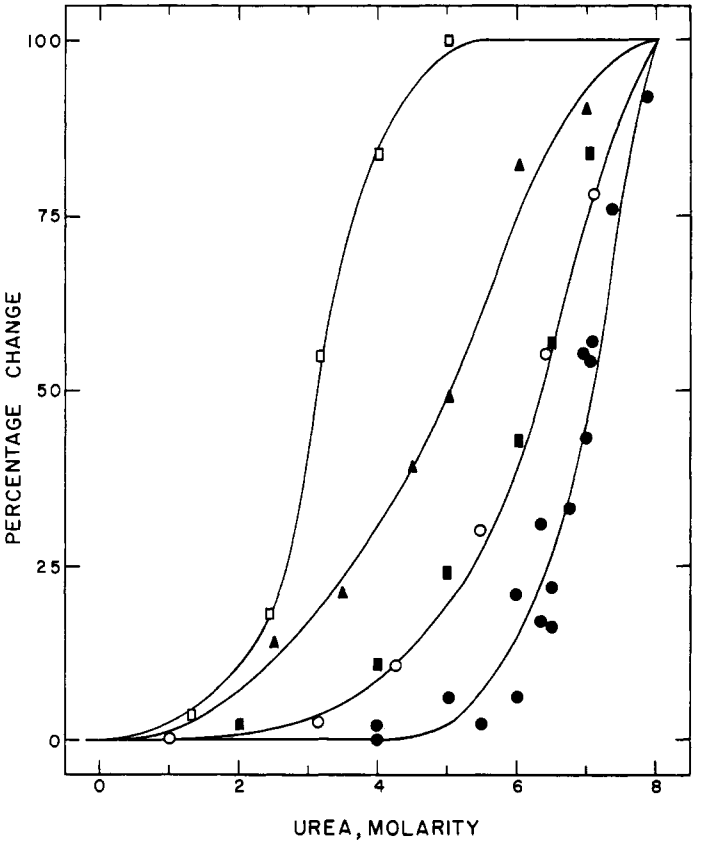

FIGURE 2: Changes in the properties of ferricytochrome *c* as a function of the concentration of urea. (○) ϵ_{695} mμ; (●) reduced viscosity of a 5-mg/ml solution of protein; (▲) reactivity of the histidyl residues with bromoacetate; (□) perturbation of the Soret absorbance by 20% ethylene glycol; (■) $\Delta\epsilon_{403}$.

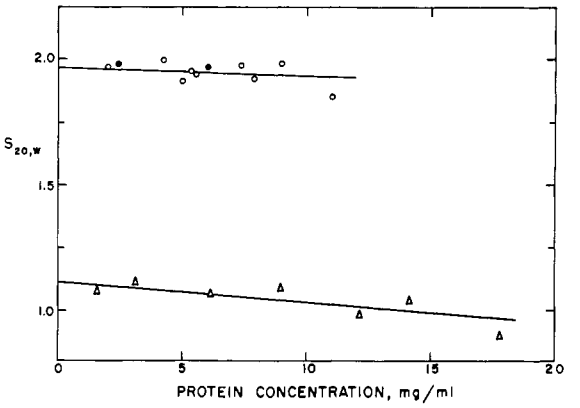

FIGURE 3: Sedimentation coefficient of ferricytochrome *c*. (○) Native ferricytochrome *c*; (△) ferricytochrome *c* in 8 M urea; (●) ferricytochrome *c* exposed to 8 M urea and then dialyzed to remove the urea. The solvent in all experiments contained 0.2 M KCl and 0.01 M phosphate, and had a final pH of 7.0.

carboxymethylated protein as described by Neumann *et al.* (1962). Absorption measurements were made with a Gilford spectrophotometer and a Cary Model 14 spectrophotometer. Refractive index measurements were made with a Bausch & Lomb refractometer.

Results

Hydrodynamic Measurements. Addition of 8 M urea to solutions of ferricytochrome *c* increases the intrinsic viscosity, $[\eta]$, of the protein from 2.5 to 14.9 ml/g, as shown in Figure 1. The value of the constant, k, in the Huggins (1942) equation, $\eta_{sp}/c = [\eta] + k[\eta]^2c$, is decreased from about 3.2 to 0.6 in 8 M urea, the protein concentration being expressed in g/100 ml. The increase in the reduced viscosity of ferricytochrome *c* as a function of urea concentration is shown in Figure 2 (filled circles). Upon removal of 8 M urea by dialysis, the reduced viscosity of the protein returns to values characteristic of the native protein as shown in Figure 1.

The presence of 8 M urea reduces the sedimentation coefficient of ferricytochrome *c* at infinite dilution, $s^0_{20,w}$, from 1.9 to 1.2 S, as shown in Figure 3, and increases the constant, k, in the expression $s_{20,w} = s^0_{20,w}(1 - kc)$ from 0.003 to 0.013 ml/mg. Removal of 8 M urea by dialysis increases the sedimentation coefficient of the protein to values characteristic of native ferricytochrome *c*, as shown in Figure 3.

Both native ferricytochrome *c* and the protein treated with 8 M urea were eluted from a 2.3 × 35 cm column of Sephadex G-75 at the position characteristic of the native monomeric form of the protein (Margoliash and Lustgarten, 1962).

Spectrophotometric Measurements. Addition of 8 M urea to solutions of ferricytochrome *c* causes the absorption band at 695 mμ to disappear and shifts the maximum of the Soret absorbance from 410 to 406 mμ, as shown in Figure 4 (dotted lines). The shift in the Soret absorbance produces a difference spectrum having a maximum at 403 mμ (Stellwagen, 1967). The increase in the extinction of the difference spectrum at 403 mμ

(filled squares) and the decrease in the absorbance at 695 mμ (open circles) as a function of urea concentration are shown in Figure 2. Addition of sodium dithionite to ferricytochrome *c* in 8 M urea produces an absorption spectrum which is quite similar to that of native ferrocytochrome *c*, as shown in Figure 4 (dotted line). Upon removal of 8 M urea from solutions of ferricytochrome *c*, the absorption spectrum of both the oxidized and reduced forms of the protein become virtually indistinguishable from the corresponding spectra of the native protein, as shown in Figure 4 (solid lines).

The solvent perturbation difference spectrum of the Soret absorbance of ferricytochrome *c* in 20% ethylene glycol is markedly increased in the presence of 8 M urea, as shown in Figure 5 (long dashes). The increase in the solvent perturbation difference spectrum as a function of the concentration of urea is shown in Figure 2 (open squares). As shown in Figure 5, upon removal of the urea, the visible solvent perturbation difference spectrum (solid line) returns to that characteristic of native ferricytochrome *c* (short dashes). Similarly, the ultraviolet solvent perturbation difference spectrum of the aromatic amino acid residues in 20% ethylene glycol is increased in the presence of 8 M urea, as shown in Figure 5 (long dashes). The solvent perturbation difference spectrum for native ferricytochrome *c* (short dashes) is equivalent to that expected for two exposed tyrosyl residues, while that observed in 8 M urea is equivalent to four exposed tyrosyl and one tryptophanyl residues (Stellwagen and Van Rooyan, 1967). As shown in Figure 5, upon removal of the urea, the ultraviolet solvent perturbation difference spectrum (solid line) reverts to that characteristic of native ferricytochrome *c* (short dashes).

The spectrophotometric titration of the phenolic hydroxyl groups of the four tyrosyl residues of native ferricytochrome *c*, shown in Figure 6 (open circles), can be resolved into a series of ionizations with apparent pK values of 10.2, 10.5, 12.6, and 12.6 (Stellwagen and Van Rooyan, 1967). In the presence of 8 M urea (open triangles), all four phenolic groups appear to be equivalent with an apparent pK of 11.0. Upon removal of the urea, the spectrophotometric titration curve (closed circles) is identical with that observed for native ferricytochrome *c*.

Alkylation. Only one of the three histidyl residues and one of the two methionyl residues of horse heart ferricytochrome *c* are carboxymethylated by bromoacetate at neutral pH and room temperature, as shown in Table I. In the presence of 8 M urea, all five residues can be carboxymethylated. The increase in the reactivity of the histidyl residues as a function of urea concentration is shown in Figure 2 (filled triangles). The reactivity of the methionyl and histidyl residues of ferricytochrome *c* after treatment of the protein with 8 M urea and removal of the urea by dialysis is the same as that observed for the native protein, as shown in Table I.

Iodination. The $\Delta\epsilon_{315}$ of iodinated ferricytochrome *c* observed between pH 4 and 10 is equivalent to that of two diiodotyrosyl residues (Stellwagen and Van Rooyan, 1967). Exposure of ferricytochrome *c* to 8 M urea followed by removal of the urea by dialysis and iodination with potassium triiodide results in a $\Delta\epsilon_{315}$ of 9.30×10^3, equivalent to the presence of 1.6 diiodotyrosyl residues.

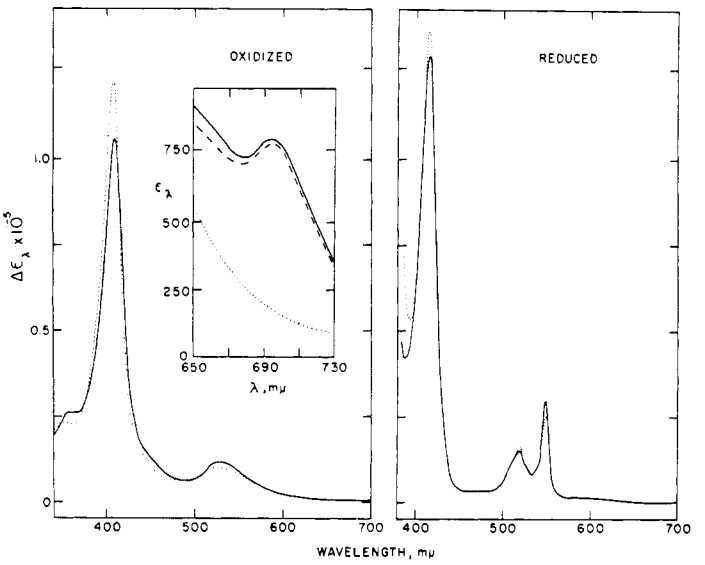

FIGURE 4: Absorption spectra of cytochrome *c*. (———) Native cytochrome *c* or cytochrome *c* treated with 8 M urea and then dialyzed to remove the urea, except for the insert which represents the spectrum of the urea-treated dialyzed protein as a dashed line; (· · · · · ·) cytochrome *c* in 8 M urea. The spectrum of the reduced protein, ferrocytochrome *c*, was obtained by addition of an excess of sodium dithionite to the solution of the corresponding oxidized protein, ferricytochrome *c*. Protein concentrations ranged from 0.6 to 14×10^{-5} M.

Discussion

The physical and chemical properties of ferricytochrome *c* in 8 M urea indicate that the native conformation of the protein is unfolded in this solvent. The intrinsic viscosity, 14.9 ml/g, is nearly identical with a value of 15.1 calculated for a random polypeptide containing 104 amino acid residues using the relationship (Tanford *et al.*, 1966), $[\eta] = 0.684n^{0.67}$, where n is the number of residues per chain. The $s^0_{20,w}$ of 1.2 S measured in 8 M urea is the value predicted by the Scheraga–Mandelkern (1953) equation using a β of 2.50×10^6 for a random coil. The constants calculated in the expressions relating the observed sedimentation coefficients and reduced viscosities in 8 M urea to protein concentration, 0.013 ml/mg and 0.6, respectively, are characteristic of unfolded proteins (Stellwagen and Schachman, 1962; Tanford, 1961).

All amino acid residues examined are exposed to the solvent in the presence of 8 M urea. All three histidyl residues and both methionyl residues are carboxymethylated by bromoacetate at neutral pH. The absorbance of the single tryptophanyl residue and all four tyrosyl residues is perturbed by 20% ethylene glycol. The apparent pK of the phenolic groups of the four tyrosyl residues is 11.0, the same as that observed for the exposed tyrosyl residues of ribonuclease in 8 M urea (Blumenfeld and Levy, 1958).

TABLE I: Comparison of the Properties of Native, Unfolded, and Refolded Ferricytochrome *c*.

Property	Native	Unfolded[a]	Refolded[b]
Hydrodynamic			
$[\eta]$ (ml/g)	2.5	14.9	2.5
$s^0_{20,w}$ (S)	1.95	1.22	1.95
Elution position, G-75	Monomer		Monomer
Spectral			
λ^{Soret}_{max} (mμ)	410	406	410
$\epsilon_{Soret} \times 10^{-3}$	106	122	106
ϵ_{695}	810	180	790
Solvent perturbation			
$\Delta\epsilon_{Soret} \times 10^{-3}$	1.9	4.3	1.9
$\Delta\epsilon_{287} \times 10^{-2}$	2.2	4.2	2.3
Chemical, reactive residues			
Histidyl[c]	1.0	2.9	1.0
Methionyl[c]	0.9	2.0	1.0
Tyrosyl[d]	2.0		1.6

[a] In 8 M urea. [b] Upon removal of 8 M urea by dialysis. [c] Number of residues per molecule carboxymethylated by bromoacetate. [d] Number of residues per molecule iodinated by potassium triiodide.

The spectral properties of the heme moiety in 8 M urea are characteristic of a fully exposed chromophore. The maximum of the Soret absorbance is shifted from 410 to 406 mμ, the maximum wavelength observed for hemopeptides (Harbury and Loach, 1959). The perturbation of the Soret absorbance is increased to a value characteristic of a hemopeptide in the presence of 8 M urea (Stellwagen, 1967). The conformationally sensitive (Schejter and George, 1964) absorbance maximum at 695 mμ disappears in the presence of 8 M urea.

On the basis of optical rotatory dispersion and circular dichroism measurements, Myer (1968) has proposed

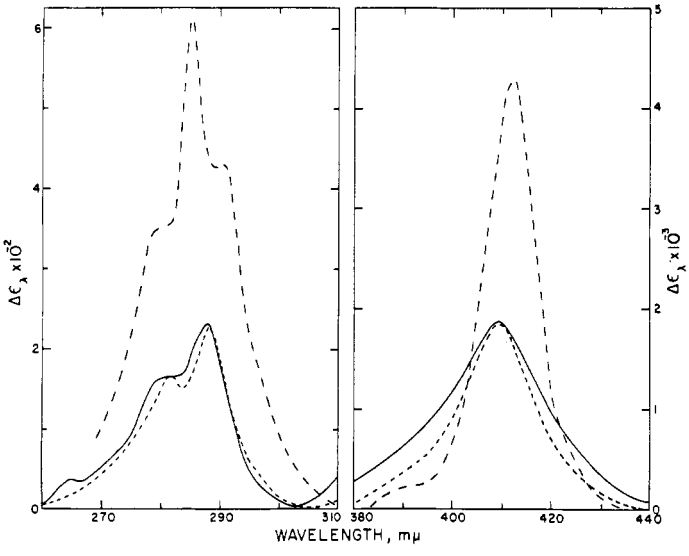

FIGURE 5: Solvent perturbation difference spectra of ferricytochrome *c*. (- - - -) Native ferricytochrome *c*; (——) ferricytochrome *c* in 8 M urea; (——) ferricytochrome *c* exposed to 8 M urea and then dialyzed to remove the urea. Protein concentrations varied from 0.8 to 9 $\times$ 10^{-5} M. The solvent in all experiments contained 0.2 M KCl and 0.01 M phosphate and had a final pH of 7.0.

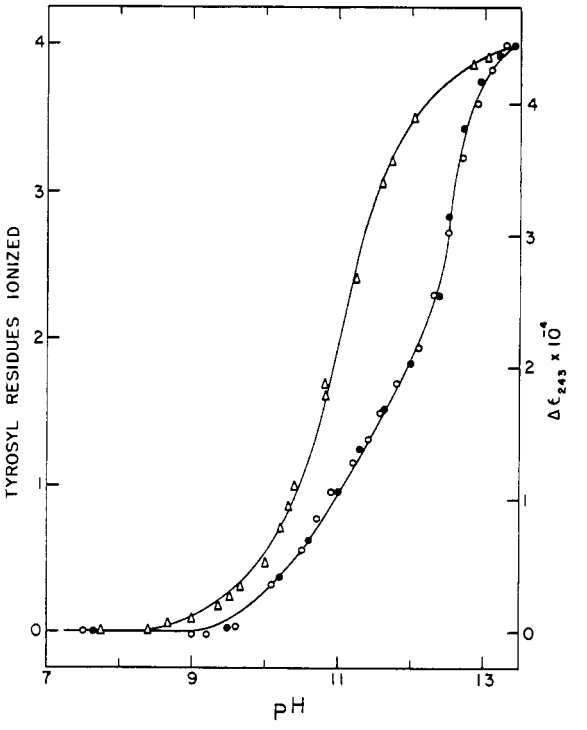

FIGURE 6: Spectrophotometric titrations of the tyrosyl residues of ferricytochrome *c*. (○) Native ferricytochrome *c*; (△) ferricytochrome *c* in 8 M urea; (●) ferricytochrome *c* exposed to 8 M urea and then dialyzed to remove the urea. Protein concentrations ranged from 1 to 2 $\times$ 10^{-5} M. The solvent was 0.2 M KCl.

that the unfolding of ferricytochrome *c* in urea occurs in two distinct steps. The first step, centered around 3 M urea, is proposed to result exclusively from the displacement of the polypeptide chain from close proximity to the heme moiety. As shown in Figure 2, only one transition, the exposure of the heme moiety to the solvent, as measured by solvent perturbation in 20% ethylene glycol (open squares), occurs in this region. This transition would be expected to reflect the proximity of the polypeptide chain to the heme moiety since in the native conformation the heme resides in a crevice formed by the polypeptide chain (Dickerson *et al.*, 1967). The exposure of the heme moiety at low urea concentrations cannot be the result of ethylene glycol acting as an auxiliary denaturant since the change in the reduced viscosity of ferricytochrome *c* as a function of urea concentration is unaffected by the presence of 20% ethylene glycol (Stellwagen, 1967).

The second denaturation step (Myer, 1968), centered around 6.5 M urea, is proposed to involve disruption of the two protein–heme coordinate covalent bonds and extensive unfolding of the polypeptide chain. As shown in Figure 2, the loss of the absorption band at 695 mμ (open circles) and blue shift in the Soret absorbance (filled squares) exhibit a common transition centered at 6.4 M urea. The maximum of the Soret absorbance is shifted to 406 mμ, a wavelength characteristic of a hemopeptide having both the fifth and sixth coordination positions filled by nitrogenous ligands (Harbury and Loach, 1959). Since the protein is thought to provide one nitrogenous ligand (His 18) and one sulfur ligand (Met 80) (Margoliash and Schejter, 1966; Fanger *et al.*, 1967), such a wavelength shift suggests replacement of one or both protein ligands by urea. Additional evidence for replacement of one or both protein ligands comes from the concomitant loss of the 695-mμ absorption band, since a similar loss is observed upon replacement of a protein ligand, probably Met 80 (Fanger *et al.*, 1967), by cyanide (Horecker and Kornberg, 1946). Eaton and Hochstrasser (1966) have reported that the transition of this band is polarized perpendicular to the plane of the porphyrin ring, suggesting the promotion of an electron from a nonporphyrin ligand into an iron–porphyrin orbital. If this interpretation is correct, substitution of a protein ligand would be expected to alter the 695-mμ absorption band. Replacement of the protein ligand His 18 would be expected to cause this residue to become reactive with bromoacetate. As shown in Figure 2 (filled triangles), the reactivity of both buried histidyl residues (positions 18 and 26) undergoes a transition centered at 5.0 M, not 6.5 M, urea. Since measurement of the histidyl residues requires addition of bromoacetate, it is possible that bromoacetate serves an auxiliary denaturant to urea by displacing an equilibrium between folded and unfolded protein conformations toward the latter at intermediate urea concentrations.

The change in the reduced viscosity (Figure 2, filled circles) suggests that the gross unfolding of the polypeptide chains is centered at 7.1 M urea and not at 6.5 M urea, as proposed by Myer (1968). As shown by Myer (1968), the major changes in the ellipicity at 222 mμ and the mean residue rotation at 231 mμ are also centered about 7.1 M urea, in contrast to similar transitions in the near-ultraviolet and visible spectra which are centered about 6.5 M urea. The rotatory changes observed in the far-ultraviolet spectrum are consistent with the loss of dissymmetry of the peptide chromophores while those in the near ultraviolet and visible spectra reflect changes in the environments of the aromatic side chains and the heme moiety. It is therefore proposed that the replacement of one or both protein ligands precedes the gross unfolding of the polypeptide chain of ferricytochrome *c* in urea.

The correspondence of the chemical, spectral, and hydrodynamic properties of the protein upon removal of 8 M urea with those of the native protein (Table I) indicates that the native conformation can be unfolded reversibly in 8 M urea. Since the refolded protein was not subjected to any purification procedure prior to measurement of these properties, the refolding appears to be quantitative. The conversion of polymeric forms of cytochrome *c* into the native monomer by treatment with urea, guanidine hydrochloride, or organic solvents (Margoliash and Lustgarten, 1962) indicates that the native monomeric form is the preferred conformation of the protein and that it can be obtained by unfolding the polymeric form and allowing it to refold into the monomer.

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Selenotrisulfides. Formation by the Reaction of Thiols with Selenious Acid*

Howard E. Ganther

ABSTRACT: Selenious acid combines with cysteine, 2-mercaptoethanol, glutathione, or coenzyme A to form moderately stable derivatives having an enhanced absorption in the 260–380-mμ region. The combining ratio for the thiols and selenious acid was found to be 4:1 by spectrophotometric analysis. The over-all stoichiometry thus conforms to the reaction proposed by Painter (Painter, E. P. (1941), *Chem. Rev. 28*, 179), $4RSH + H_2SeO_3 \rightarrow RSSeSR + RSSR + 3H_2O$. The reaction mixtures were resolved by thin-layer chromatography into two spots corresponding to the disulfide and the selenotrisulfide (RSSeSR). A column chromatographic procedure based on chelated copper as a stationary phase was developed which permitted the isolation of selenodicysteine and selenodimercaptoethanol. Selenodicysteine was identified by elemental analysis and by amino acid analysis.

These results establish the above reaction as a plausible means of incorporating inorganic selenite into a stable organic moiety. The chemistry and possible biological significance of selenotrisulfides are under further investigation.

As part of a continuing investigation on the biosynthesis of organoselenium compounds from inorganic forms of selenium, it was shown in a previous study that a rather extensive enzymic conversion of sodium selenite into dimethyl selenide occurs directly in mammalian tissues (Ganther, 1966). Liver microsomal fractions showed an absolute and specific requirement for glutathione in this process. The involvement of glutathione in the metabolism of selenite made it desirable to know more about the possible nonenzymic reactions that might occur between sulfhydryl compounds and selenite.

It has been known for over 30 years that aqueous solutions of selenium dioxide react with sulfhydryl compounds to form relatively unstable derivatives. In a 1941 review, Painter proposed that the reaction between thiols and selenium dioxide takes place as seen in reaction 1. These derivatives of the type RSSeSR will be referred to as selenotrisulfides.[1] Selenotrisulfides are relatively unstable, especially in alkaline solution, decomposing to the disulfide and elemental selenium as shown in reaction 2. Although previous studies

$$4RSH + SeO_2 \longrightarrow RSSeSR + RSSR + 2H_2O \quad (1)$$

$$RSSeSR \longrightarrow RSSR + Se \quad (2)$$

of selenium dioxide–thiol reactions, summarized in the Discussion, are consistent with reaction 1, no selenotrisulfide has ever been isolated from the reaction mixture of thiols and selenious acid in pure form for proper characterization. The potential importance of selenotrisulfides in biological systems, as well as the interesting chemistry involved, made it desirable to establish this class of compounds as genuine chemical entities. The experiments in this paper confirm reaction 1 and describe the isolation and characterization of selenodicysteine.

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[1] No general name has previously been used for these compounds, although specific compounds have been referred to as selenodicysteine and selenodiglutathione (Rosenfeld and Beath, 1964). Although selenodithiol or dithioselenide might be used as trivial names for the general type, selenotrisulfide is preferred because it follows the common practice of using the prefix seleno to indicate substitution of an atom of selenium for an atom of sulfur in the sulfur analog, which in this case is a trisulfide. The names selenodicysteine, selenodiglutathione, etc., will be retained for specific selenotrisulfides.