The Conformational Transition of Horse Heart Porphyrin c*

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The heme iron of horse heart cytochrome c was selectively removed using anhydrous HF. The product, porphyrin c, exhibits the viscosity, far ultraviolet circular dichroic, and fluorescence properties characteristic for native cytochrome c. However, porphyrin c is more susceptible to denaturation by guanidine hydrochloride and by heat than is the parent cytochrome. All of the conformational parameters of porphyrin c exhibit a common reversible transition centered at 0.95 m guanidine hydrochloride at 23 °C and pH 7.0. Guanidine denatured porphyrin c refolds in two kinetic phases having time constants of 20 and 200 ms as detected by stopped flow absorbance or fluorescence measurement, with about 80% of the observed change in the faster phase. The kinetics of porphyrin c refolding are not significantly altered by increasing the viscosity of the refolding solvent 15-fold by addition of sucrose. We suggest that the folding of guanidine denatured cytochrome c is not a diffusion-limited process and that the requirement for protein axial ligation elicits the slow (α) kinetic phase observed in the refolding of cytochrome c.

The crystallographic model for tuna cytochrome c (1) indicates that the native conformation of this protein contains five helical segments each consisting of between six and 17 residues, no β-strands, and six reverse turns. Of these secondary structural elements, the largest two are the terminal helices consisting of residues 2-15 and 87-103. The axis of these two helices form a 90 °C intersection in the native conformation with some of the side chain atoms of one helix packed against those of the other helix.

One of the models for the dynamics of protein folding, the diffusion-collision model of Karplus and Weaver (2), suggests that the rate of folding is proportional to the time required for transiently stable microdomains of the linear sequence, such as helical segments, to collide in an orientation productive to generation of the native conformation. If this model is appropriate to the mechanism of folding of cytochrome c, it is likely that the rate-limiting step involves coalescence of the two most distant helical elements in the sequence, the terminal helices, which should also have the longest transient lifetimes because of their size. Accordingly, substantial increases in the viscosity of the folding solvent for cytochrome c should measurably increase the relaxation time for the folding of this protein. In order to focus on chain folding as opposed to protein ligation of the heme iron, we have examined the effect of sucrose on the folding of porphyrin c, i.e. cytochrome c whose heme iron has been removed, which is reported to fold in a single kinetic phase (3).

EXPERIMENTAL PROCEDURES

Materials—Horse heart cytochrome c, type VI, purchased from Sigma, served as the source material for preparation of horse heart porphyrin c using the procedure of Platman and Robinson (4). About 300 mg of the cytochrome was treated with 6 ml of anhydrous HF for 20 min at 4 °C using a Peninsula Laboratories HF reaction apparatus. The HF-treated protein was fractionated twice using Sephadex G-50 chromatography as described by Fisher et al. (5), lyophilized, and stored at -20 °C before use. Exposure to light was minimized during these procedures. Guanidine hydrochloride was purchased from Heico, Delaware Water Gap, PA.

Chemical Analyses—Protein samples for amino acid analysis were hydrolyzed in 6 n HCl in vacuo for 24 h at 110 °C prior to application to a Beckman model 121 MB analyzer. Protein sulphydryl reaction with 5,5′-dithiobis(2-nitrobenzoic acid) was done in the presence of sodium dodecyl sulfate as described by Habeeb (6). Iron analysis was done using the procedure of Cameron (7).

Spectral Measurements—All spectral measurements were made at 25 °C in 100 mM phosphate buffer, pH 7.0. Fluorescence measurements were made using a Hitachi model MPF-2A fluorometer using a 280-nm excitation beam having a 6-nm band width, and a 340-nm observation beam having a 12-nm band width. Absorbance and circular dichroic spectra were obtained using Cary model 17 and 60 instruments, respectively. Stopped flow measurements were made using a Durrum-Gibson apparatus having a fluorescence attachment.

Other Measurements—Viscosity measurements were made using a Cannon-Ubbelohde viscometer having an outflow time for solvent of 60 s. Thermal transitions were obtained using a Microcal MC-1 differential scanning calorimeter. The enthalpy of denaturation was determined by the area of transition resulting from extrapolation of the changes in the heat capacities of the native and thermally denatured protein.

RESULTS

Preparation of Porphyrin c—Cytochrome c treated with HF was fractionated into two components by chromatography on Sephadex G-50 as shown in Fig. 1A. The major component which elutes at the position found for cytochrome c was pooled, concentrated, and rechromatographed. Two components were seen again in the elution profile, Fig. 1A, but the relative proportion of the minor component was diminished. The major component was pooled as indicated and used for the measurements described below. About 95% of an aliquot of this component pool eluted from a Sephadex G-100 column equilibrated and developed with 6 M guanidine hydrochloride at a position corresponding to the molecular weight expected for monomeric porphyrin c as shown in Fig. 1B. The remaining 5% of the porphyrin c preparation eluted at a position expected for a dimeric form of the denatured protein, i.e. 2.5 × 104. The porphyrin c preparation migrated as a single component during gel electrophoresis in detergent whose mobility was equivalent to that of cytochrome c as shown in Fig. 2.

Iron analysis of monomeric porphyrin c preparations gave no more than 0.05 iron atoms/chain, indicating nearly quantitative removal of iron from the heme. No evidence of a
dominant Soret maximum was seen in the visible spectrum of porphyrin c preparations; rather a five band visible absorbance spectrum was seen having maxima at 404, 505, 540, 565, and 620 characteristic for porphyrin c (4). The visible spectrum was not changed by addition of excess sodium dithionite. However, the visible absorbance of solutions of porphyrin c at pH 7.0 and 23 °C is slowly (h) diminished giving a first order relaxation time of about 600 min independent of protein concentration. In order to minimize this slow reaction, all porphyrin c solutions were prepared just prior to the measurement of interest.

Treatment with HF appeared to selectively remove the heme iron. The amino acid analysis of porphyrin c is comparable to that of the parent cytochrome c as shown in Table I, the high value for histidine content being the most notable feature. This must have resulted from an analytical error since HF treatment should not generate histidine. Reaction of porphyrin c with 5,5'-dithiobis(2-nitrobenzoic acid) resulted in the modification of no more than 0.07 cysteine/chain, indicating that HF treatment had not cleaved a significant number of the thioether bridges linking the porphyrin to the polypeptide chain.

Conformational Transitions—The reduced viscosity of monomeric porphyrin c at neutral pH is 2.5 ml/g, indicating a globular conformation whose gross features are comparable to that of the parent cytochrome c (8). In the presence of 3 M guanidine hydrochloride at neutral pH, the reduced viscosity is 14.5 ml/g, the value calculated for a random polypeptide containing 104 residues (9). The far ultraviolet circular dichroic spectrum of porphyrin c has the profile of a helical protein having a minimum mean residue ellipticity at 222 nm of \(-11.3 \times 10^2\) deg cm\(^2\) dmole\(^{-1}\). The spectrum in 3 M guanidine hydrochloride is that characteristic of a random coil having no minimum at 222 nm and a mean residue ellipticity at this wavelength of \(-2.6 \times 10^2\) degree cm\(^2\) dmole\(^{-1}\). Comparable values for the mean residue ellipticity of native and guanidine hydrochloride denatured horse cytochrome c are \(-9.8 \times 10^2\) and \(-3.0 \times 10^2\) degree cm\(^2\) dmole\(^{-1}\), respectively.

FIG. 1. Exclusion chromatography of porphyrin c. A, elution profiles of porphyrin c chromatographed on a column (3.5 x 125-cm) of Sephadex G-50 equilibrated and developed using 50 mM ammonium acetate buffer, pH 5.0, at 25 °C. The upper curve indicates the elution profile of a fresh preparation of porphyrin c. The slower component was pooled as indicated by the bar, lyophilized, and resubjected to the same chromatographic protocol generating the lower elution profile. The position of the maximum ordinate for the parent cytochrome c subjected to the same chromatographic protocol is indicated by the closed circle. B, estimation of porphyrin c molecular weight. Samples were applied to a Sephadex G-100 column (1.5 x 90-cm) equilibrated and developed with 6 M guanidine hydrochloride solution in 100 mM phosphate buffer, pH 7.0, at 25 °C. Calibration materials, open circles, in descending order of molecular weight are catalase, an intermolecular disulfide dimer of yeast cytochrome c (iso-1), horse heart cytochrome c, horse heart heme peptide 1-80, and horse heart heme peptide 1-65. The volume containing the highest concentration of porphyrin c is indicated by the closed circle.

FIG. 2. Slab gel electrophoresis in sodium dodecyl sulfate. The gel was made using a 15% acrylamide solution containing 0.4% bisacrylamide and electrophoresis was done according to the procedure of Laemmli (22). The three protein samples applied to the gel reading from right to left contained 2 μg of porphyrin c, 2 μg of cytochrome c, and 1 μg each of porphyrin c and cytochrome c. The upper arrow indicates the top of the running gel and the lower arrow, the position of the tracking dye, bromphenol blue, at the termination of electrophoresis.

| Residue | Asp | Thr | Ser | Glu | Pro | Gly | Ala | Val | Met | Ile | Leu | Tyr | Phe | Lys | His | Arg |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Residues/Chain | 8.0 | 7.8 | 4.1 | 11.9 | 12 | 4.5 | 11.6 | 6.2 | 3.0 | 1.5 | 5.9 | 4.1 | 4.4 | 18.4 | 3.6 | 2.0 |
| Sequence (21) | 8 | 10 | 0 | 6 | 2 | 12 | 6 | 3 | 2 | 6 | 4 | 4 | 19 | 3 | 2 |
where the observed parameter for denatured porphyrin guanidine hydrochloride assuming a two state transition. The slope hydrochloride concentration as generally observed for native cyto- region in order to calculate the percentage change. dependence was extrapolated through the conformational transition alicty dependent on guanidine hydrochloride concentration, the phan fluorescence; for native porphyrin chromes 222 nm; for native porphyrin c 0, 7.0.

The fluorescence of the single tryptophan in native porphyrin c is quenched 90% relative to that of a comparable concentration of free tryptophan in the same solvent. By comparison, the fluorescence of the same tryptophan in the parent cytochrome c is quenched 98%. This quenching is considered to result from the proximity and orientation of the buried heme and tryptophan 59 in the native cytochrome c structure (5, 10). Addition of guanidine hydrochloride to porphyrin c causes a discontinuous blue shift in the porphyrin absorbance spectrum generating a major difference maximum at 375 nm. Such a blue shift is characteristic of the exposure of a buried chromophore.

Normalisation of the change observed in each of these conformational parameters as a function of guanidine hydrochloride concentration generates a common transition having a midpoint at 0.95 mM denaturant as shown in Fig. 3A. Analysis of this reversible transition in terms of a two state model (11) indicates that the ΔCp for unfolding of porphyrin c is 1.3 kcal/mol (Fig. 3B). Corresponding analysis of the conformational transition for the parent cytochrome c gives a value of 7.3 kcal/mol. The structure of porphyrin c melts in a broad

![Fig. 3. Conformational transition for porphyrin c in guanidine hydrochloride. A, percentage change observed for O, tryptophan fluorescence; , reduced viscosity; mean residue ellipticity at 222 nm; a, absorbance at 375 nm. The total change observed for each measurement has been normalized to 100%. The observed parameter for native porphyrin c was assumed to be independent of guanidine hydrochloride concentration as generally observed for native cytochromes c below their conformational transition zones. In those cases where the observed parameter for denatured porphyrin c was systematically dependent on guanidine hydrochloride concentration, the dependence was extrapolated through the conformational transition region in order to calculate the percentage change. B, dependence of fractional change in tryptophan fluorescence on the concentration of guanidine hydrochloride assuming a two state transition. The slope of the line drawn is 2.28 M⁻¹ and the concentration required to generate equivalent concentrations of the two states is 0.95 M. All measurements were done at 25 °C in 100 mM phosphate buffer, pH 7.0.](http://www.jbc.org/)

![Fig. 4. Differential scanning calorimetry. Temperature dependence of constant pressure heat capacity at 1.4 mg/ml and in 100 mM potassium phosphate, pH 7.0, with a heating ratio of 1 °C/min observed for porphyrin c, upper curve; and cytochrome c, lower curve. The bar encompasses 0.2 cal degree⁻¹ g⁻¹.](http://www.jbc.org/)

![Fig. 5. Stopped flow kinetic measurements of the refolding of guanidine hydrochloride denatured porphyrin c. A solution of porphyrin c, 0.1-0.15 mg/ml, in 1.4 M guanidine hydrochloride and 100 mM phosphate buffer, pH 7.0, at 23 °C, was diluted with an equal volume of phosphate buffer. A, change in percent transmission at 400 nm where the ordinate is 20 mV/division and the abscissa is 10 ms/division. B, change in fluorescence where the ordinate is 20 mV/division and the abscissa is 20 ms/division. First order plots of transmission (T) and fluorescence intensity (I) changes are shown below each oscilloscope trace.](http://www.jbc.org/)

### Table II

| Sample     | Probe     | [Protein] | [Guanidine] | Time constants | Fractional reaction in fast phase |
|------------|-----------|-----------|-------------|----------------|-----------------------------------|
|            |           |          | mg/ml       |     | ms               |                                |
| Porphyrin c| Absorbance| 0.05-0.5 | 0.7         | 27±15 | 180±73           | 0.76±01                         |
|            | Absorbance | 0.2      | 0.75        | 14     |                   |                                  |
|            | Fluorescence | 0.10-0.15 | 0.7 | 16±4 | 200±70           | 0.78±02                         |
|            | Fluorescence | 0.1      | 0.7         | 12±6 | 130±70           | 0.80±01                         |
| Cytochrome c| Absorbance | 0.13     | 2.5         | 210 | 12×10⁴           | 0.84                            |
|            | Absorbance | 0.04-0.1 | 0.9         | 134±0 (18±2)×10⁴ | 0.74±01                       |
|            | Fluorescence | 0.13    | 2.5         | 250 | 15×10⁴           | 0.73                            |
|            | Fluorescence | 0.2     | 1.5         | 160 | 22×10⁴           | 0.86                            |

* Ref. (3).  
* In 0-50% sucrose.  
* Ref. (13).  
* Ref. (10).
Porphyridin Cytochrome c

**DISCUSSION**

The failure of up to 50% sucrose to measurably effect the kinetics for the folding of porphyrin c suggests that the rate-limiting step(s) in the folding of this protein is not diffusion controlled. Accordingly, it is likely that the rate-limiting step involves interactions between adjacent residue clusters in the linear peptide sequence. A similar conclusion can be drawn from the viscosity-independent folding of ribonuclease A (14).

In pursuing the effect of solvent viscosity on the folding kinetics of porphyrin c, we find that the guanidine denatured protein refolds in two kinetic phases, not one as previously reported (3). We believe that our preparation of porphyrin c is not heterogeneous since it behaves as a single component using chromatographic and electrophoretic criteria. The conformational parameters of the porphyrin c employed are characteristic of the parent cytochrome c. Each of these parameters describes a single common transition upon addition of guanidine hydrochloride. Finally, the kinetic features of the folding of porphyrin c are the same using either porphyrin absorbance or tryptophan fluorescence to monitor the folding process.

The kinetic features of the refolding of guanidine denatured cytochrome c described here, namely the detection of only two kinetic phases with about 80% of the total change in the fast phase, have been previously observed by a variety of investigators using stopped flow measurements (13, 15-17). Since these same kinetic features are observed by stopped flow measurements of the refolding of horse porphyrin c and cytochrome c, it may be concluded that both proteins execute the same folding reactions albeit with different time constants. However, Tsong (10) has noted that the two kinetic phases normally detected by stopped flow fluorescence measurements in the folding of guanidine denatured cytochrome c only account for 80 to 85% of the equilibrium fluorescence change. He has found that the missing fraction constitutes a third kinetic phase, termed here the very fast phase, having a time constant of 3 ms detected by stopped flow measurements using Soret absorbance as the probe.

**APPARATUS** SF SF SF and

**Time constant**

**Very fast** 16 ± 4 
**Fast** 200 ± 70 
**Slow** Not seen

**Fractional reaction**

**Very fast** 0.78 ± 0.02 
**Fast** 0.22 ± 0.02 
**Slow** 0.73 ± 0.65

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* SF, stopped flow; TJ, temperature jump.
* Measurements were made at guanidine hydrochloride concentrations giving equivalent positions in the equilibrium transition zone of porphyrin c and cytochrome c.
* This value was obtained from temperature jump measurements using Soret absorbance as the probe.

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Unfortunately, we cannot choose between these alternative conclusions with the available information. One potential way to resolve this dilemma would be to examine the temperature dependence of the two phases observed during the refolding of porphyrin c, since Ridge et al. (16) have shown that the fast reaction in the refolding of cytochrome c observed by stopped flow measurements has an activation enthalpy of about 12 kcal/mol, while that of the slow phase is about 21 kcal/mol. However, the guanidine-dependent transition for porphyrin c is such that essentially no region exists below the folding transition zone where it is only possible to interpret temperature dependencies in a simple manner as discussed by Hagerman and Baldwin (18). As an alternative route, we have examined the refolding of guanidine denatured cytochrome c at pH 5 and in the presence of 20 mM imidazole at pH 7, conditions found or known (19), respectively, to dissociate at least one protein axial ligand in the denatured state. Preliminary measurements of the refolding of guanidine denatured cytochrome c, both at pH 5 and in imidazole at pH 7, reveal a kinetic phase having a time constant of 13 ± 8 ms involving 87 ± 2% of the total reaction. The remaining 13 ± 2% of the reaction occurs in a single kinetic phase in both measurements having a time constant of 12 ± 2 ms at pH 5 and 227 ms in imidazole at pH 7. Taken together, these results indicate that elimination of one or both protein axial ligands alters the fractional change but not the time constants of the three kinetic phases seen in the folding of cytochrome c by Tsong (10). Accordingly, we suggest that removal of the heme iron from cytochrome c to eliminate axial ligation results in a marked enhancement of the fractional change in the very fast phase, a diminution of the fractional change in the fast phase, and a loss of the slow phase.

Ridge et al. (16) have recently demonstrated that the slow phase in the folding of guanidine denatured horse cytochrome
Porphyrin Cytochrome c

has the activation enthalpy, time constant, and fractional amplitude characteristic for the cis/trans isomerization of likely one, and no more than two, proline peptide bonds. Since horse cytochrome c contains four proline residues located at positions 30, 44, 71, and 76, this suggests that the native conformation of cytochrome c can tolerate a cis proline peptide bond at two, and possibly three, sequence positions. Since horse cytochrome c contains four proline residues located at positions 30, 44, 71, and 76, this suggests that the native conformation of cytochrome c can tolerate a cis proline peptide bond at two, and possibly three, sequence positions. One of these noncritical positions likely involves proline 44 since the features of the slow phase folding of horse and tuna cytochrome c are the same (20) even though proline 44 is replaced by a glutamic acid in the tuna protein. Given that the slow phase in the folding of cytochrome c results from proline peptide isomerization during denaturation, the absence of the slow phase in folding of porphyrin c suggests that the more deformable conformation of porphyrin c, as evidenced by a lower thermal transition and greater susceptibility to guanidine denaturation, can accommodate a cis proline peptide isomer at any of the four sequential positions. Thus, proline peptide isomerization and protein axial ligation could be coupled by the conformational constraints necessary to coordinate at least one protein axial ligand. Since the histidine 18 is adjacent to the covalent attachment of the heme, little, if any, conformational structure is likely required to facilitate its coordination. Indeed, small heme peptides invariably exhibit visible absorbance spectra characteristic of histidine 18 ligation. By contrast, coordination of methionine 80, which is much more remote from the heme attachment sites at positions 14 and 17, requires a distinctive conformational assemblage on the left face of the heme involving two helical segments and a reverse turn (1). Since proline 71 separates the helical segments and proline 76 participates in the reverse turn, we suggest that a cis configuration at one or both of these proline peptide bonds likely generates the slow phase.

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