The oxidation-reduction potential of horse heart cytochrome c, pH 7.0, at various urea concentrations in the range 0 to 9 mol/liter has been measured. The urea-midpotential profile, unchanged up to about 5 M urea and asymptotically decreasing at higher urea concentrations with a limiting value of about 118 mV at 8.7 M urea, has been analyzed through comparison with the urea-midpotential profiles calculated on the basis of the urea denaturation mechanism, \[ N \rightleftarrows X_1 \rightleftarrows X_2 \rightleftarrows D \] (Myer, Y. P., MacDonald, L. H., Verma, B. C., and Pande, A. (1979) Biochemistry, in press).

It has been found that the midpotential is not governed by either the \( N \rightleftarrows X_1 \) transition, the loosening of the front section of the heme crevice, the exposure of the axial ligands, and the destabilization of the heme coordination configuration, or by the \( X_1 \rightleftarrows X_2 \) transition, the perturbation of the polypeptide conformation. Of the two probes defining the \( X_2 \rightleftarrows D \) transition, the quenching of the 695 nm band and the changes in fluorescence efficiency of the tryptophan residue, the latter generates a potential-urea profile indistinguishable from the observed profile of the protein, whereas the profile from the former, although very similar to observed profiles, lags during the early stages.

It is concluded that neither the hydrophobicity of the heme environment in general, nor the extent of solvent exposure of the heme group, determines the oxidation-reduction potential of the protein, but rather, the integrity of the tryptophan-heme domain of the crevice, i.e. its deepest part, which may also include other protein functional groups in its proximity in cooperation with the Met-80 linkages. The lag in the concurrence between the oxidation-reduction potential-urea profile calculated from the quenching of the 695 nm transition and that from tryptophan fluorescence is taken as a reflection of the role of the tryptophan-heme domain in the maintenance, or the stability of the Met-80—S—iron linkage or both.

The oxidation-reduction potentials of various components of the mammalian electron transport chain constitute one, and possibly the only, physical parameter which provides a sound basis for not only the sequential localization in the chain, but also the thermodynamic feasibility of a specific biological function. The oxidation-reduction potentials of the heme-containing components vary from a value as low as 38 mV for cytochrome b to as high as 365 mV for cytochrome c of cytochrome oxidase (1), with a potential of 250 mV for the only soluble component, cytochrome c (2), which is one of the better understood proteins among all the hemoproteins with regard to protein structure, conformation and function (3-6).

The oxidation-reduction potentials of the cytochromes vary not only among themselves over a wide range, ±200 to 300 mV (7), but they also exhibit differentials of +300 to 550 mV from the potentials of simple heme systems containing identical ligands at the two axial positions of heme iron (7-9). Structure-potential relationships of cytochrome c and of hemoproteins in general are as yet a poorly understood facet of these systems.

Rodkey and Ball in the early 1950s established the pH dependence of the oxidation-reduction potential of cytochrome c, especially toward the higher limits of the pH scale. Present day knowledge of structural and functional details of the molecule (3) indicates that this dependence is due to the effect of the destruction of the Met-80—S—iron linkage of heme through a proton-linked function of the protein. Studies of the oxidation-reduction behavior of simple heme c systems undertaken by Harbury and co-workers (10) further ascertained that the difference of about 450 mV between the cytochrome c potential and those of simple heme or heme c systems (7-9) could in part be the result of differential affinity of the Met-80—S for iron in the two valence states. A rise in the oxidation-reduction potential of simple heme has also been reported by Warme and Hager (11) upon formation of a mixed ligand configuration at the two axial positions of iron, methionine/histidine. The potentials of any of these model systems, although they contain a coordination configuration like that of cytochrome c, methionine/histidine (3), fall short of that of the protein itself, -50 and -110 mV versus 250 mV for the protein, which is far from providing an unambiguous answer for the behavior of the protein. Recently, Kassner has shown, through both experimental observation (19) and theoretical considerations (13), that the abnormally high oxidation-reduction potentials of heme iron in hemoproteins could be because of the localization of heme in a low dielectric, hydrophobic environment, and thus provided a generalization for the oxidation-reduction potentials of these systems. An analysis of the extent of the hydrophobicity, nonpolar and polar environments of the crevice, and the solvent exposure of heme in a number of hemoproteins with known three-dimensional structures in relation to their oxidation-reduction potentials, on the other hand, has led to the conclusion that it is the extent of solvent exposure of the heme group which...
relates inversely to the midpoint potential of the protein, rather than the hydrophobicity (14). In contrast, a preliminary report of our work (15) showed that neither the hydrophobicity nor the extent of solvent exposure of the heme group per se can detect the oxidation-reduction potential of this protein, but a transition localized in the high molar urea limb of the absorption-urea denaturation profile possibly relates to the oxidation-reduction behavior. In this report we present an analysis of the oxidation-reduction potential-urea behavior of the protein on the basis of the urea denaturation mechanism and its structural details (16) in order to discern the nature and extent of the structural relationship to the oxidation-reduction behavior.

**EXPERIMENTAL PROCEDURES**

*Materials*- Horse heart cytochrome c, types III and IV, was purchased from Sigma Chemical Co. and used without further purification. Ultrapure urea, a Schwarz/Mann product, was used after evaporation for a period of at least 12 h, and fresh solutions prepared just before the experiment were always used. Potassium ferric cyanide (Fisher Scientific Co.), (+)-ascorbic acid (Eastman Kodak Co.), methyl viologen (Mann Research, Inc.), dimedon (Alrich Chemical Co.), N,N,N',N'-tetramethyl-phenylenediamine dihydrochloride (Eastman Kodak Co.), phenazine ethosulfate (Sigma Chemical Co.), and indigo carmine (Pfaltz & Bauer, Inc.) are some of the special chemicals obtained from suppliers as noted and used without further purification. All other chemicals were of analytical reagent grade.

*Potentiometric Measurements*- Potentiometric measurements were performed using the set-up described in detail earlier (9). Solutions containing the protein, 20 to 50 μM, and the mediator mixture: dimedon, 35 μM; phenazine methosulfate, 35 μM; phenazine ethosulfate, 35 μM; ferric chloride, 300 μM; EDTA, 10 mM; and N,N,N',N'-tetramethyl-phenylenediamine dihydrochloride, 10 μM (1), after extensive deoxygenation and under anaerobic atmosphere, were titrated with 6 mM ascorbate, or 10 mM sodium dithionite solution for reduction, or both, or with a 13 mM solution of potassium ferricyanide solution for oxidative titration. The potentials were recorded after equilibration of the two gold-plated platinum electrodes against a thermostated calomel electrode, or both, or with a 13 mM solution of potassium ferricyanide as the reference point, the analysis by the Reed and Berkson method yielded midpotentials with a maximum deviation of +6 mV. Any deviation larger than this was considered to be an artifact, and the titrations were accordingly rejected, and then repeated. Since the deviation from the classical case for N = 1 was never found to be larger than 0.2, it was accepted that the oxidation-reduction couple involved only a single equivalent of electron.

All measurements were performed in 0.05 M phosphate, 0.2 M KCl, pH 7.0, unless otherwise specified. The concentration of cytochrome c was determined spectroscopically using an extinction of 1.04 × 10^4 at 528 nm, pH 7.0. All the calculations were performed using the Univac 1100 computer.

**RESULTS**

A representative sample of a spectroscopic-potentiometric titration of HH cytochrome c in the presence of urea is shown in Fig. 1a, and in Fig. 1b, are compared the observed potentiometric titrations and those generated through calculations based on the extended version of the Reed and Berkson method reported earlier (9) and by least square fitting with a best least square fit to the observed potential titration. Almost all independent titrations under a given set of conditions, the results are reported as the average of two to four titrations. O, observed potentials with respect to calomel; —, freehand drawing through the data points. O, observed electromotive force (EMF); —, calculated curve using the average value of E,, from the extended version of the Reed and Berkson method of analysis, N = 1.0 (see Ref. 9); —, least square fitted titration. For titration in the presence of 4.5 M urea, the best least square fit is obtained with N = 1.17 and an E, of 0.2525. For 8 M urea the corresponding values were N = 1.01 and an E, of 0.1751. The bottom ordinate is for the 8 M urea titration, and the top ordinate for the 4.5 M urea titration. Abscisca at left, observed electromotive force with respect to calomel electrode; abcissa at right, observed electromotive force with respect to hydrogen electrode. 0.2444 V was used as the standard potential of the saturated calomel electrode at 25°C.
midpotential of 248 ± 6 mV and 118 ± 4 mV at 0 and 8.7 M urea concentrations, respectively. Clearly the midpotential of the protein is insensitive to increasing urea concentrations up to about 5 M. At higher urea concentrations the midpotential drops asymptotically, reaching a value of about 118 mV at the highest urea concentration investigated.

**DISCUSSION**

The alteration of the oxidation-reduction potential of a protein is sensitive to the presence of denaturant, and in general can be described in terms of the depletion of the oxidized or reduced forms, or both, of the electrochemically active components. The association of a proton-linked function to one or both forms, depletion through alteration of the coordination configuration, of the state of aggregation, or of the conformationally linked equilibrium, etc., are a few of the possibilities that could be invoked as causes of change in the oxidation-reduction behavior of the protein (2, 6, 8-11, 17). As the studies reported here are at a constant pH of 7, and since in this region the oxidation-reduction potential of both the native and simple heme c systems with strong ligand-field groups is insensitive to pH variation (2, 8, 9), the possibility of the oxidation-reduction potential alterations in urea being a reflection of a proton-linked function of either the oxidized or the reduced form can be ruled out. Also, since cytochrome c under the conditions used is known to be monomeric (3), alteration of the state of aggregation is not the case. As to whether the oxidation-reduction potential-urea behavior, shown in Fig. 2, is a manifestation of the depletion of the oxidized or the reduced state, the nature of the behavior, i.e. the decrease in potential (Fig. 2), reflects the effect of the depletion of the oxidized form, and not the reduced form, as the latter will tend to raise the midpotential, rather than lowering it, as is the case (see "Appendix" and Ref. 17). The observation that ferrocyanochrome c is insensitive to the presence of 9 M urea with regard to not only the conformation of the protein moiety, but also the coordination configuration of heme iron and the conformation of the heme environment and of the aromatic chromophore (3, 6, 18), supports the view that the potential alterations must be linked to the depletion of the oxidized form of the protein.

The understanding of the oxidation-reduction behavior of proteins with increasing concentration of denaturant lies in correlation of the observations to the details of the course of the denaturation processes, with regard to its mechanism and structural details, such as the conformational state, the coordination configuration, the state of the heme crevice, and the extent of exposure, etc. of the intermediates, as each of these aspects has been used, at one time or another, to explain the abnormal oxidation-reduction behavior of the protein (3, 10-14). Recently, we reported results from equilibrium studies of the urea denaturation of this protein (16) through probes such as the absorptivity of the 695 nm band, a parameter linked to the coordination configuration of heme (3), and the fluorescence efficiency of the tryptophan side chain, reflecting the state of the deepest part of the crevice (19). The intermediate forms were characterized through analysis of absorption, fluorescence, circular dichroism spectra, and reactivity to bromoacetic acid, a group-specific reagent for the methionyl and histidyl side chains. A mechanism, \( N \approx X_1 \approx X_2 \approx D \), with midtransition urea concentrations of 2 to 2.5, 6.2, and 7 M, was found to be consistent with the denaturation profiles and other studies as well (20). In terms of the various aspects of protein structure and conformation, each transition has been described.

The \( N \rightarrow X_1 \) transition reflects a loosening of the frontal section of the heme crevice without alteration of the heme coordination configuration, the deepest part of the crevice or the polypeptide conformation of the protein. The extent and nature of crevice loosening is further established through the observation that under solution conditions for form \( X_1 \), i.e. 4.5 M urea, both the axially coordinated amino acid side chains, Met-80—S and imidazole of His-18 (3), are attacked by bromoacetic acid, an anionic reagent, which is otherwise prohibited because of the hydrophobicity of the crevice in the native protein (3, 6, 21, 22).

The \( X_1 \approx X_2 \) transition is simply the solvent exposure of the polypeptide backbone, again without any further perturbation of either the heme crevice, the tryptophan-heme domain or the coordination configuration of heme iron. There was no counterpart to this step of the mechanism in the denaturation profile resulting from alteration of the tryptophan fluorescence efficiency of the molecule.
The $X_i ightleftharpoons D$ transition is characterized as a composite of the disruption of the heme coordination and the further loosening of the heme crevice, the latter involving alteration in the tryptophan-heme domain of the crevice. The reorganization of the protein structures also seems to be likely during this step of the denaturation process. In Fig. 3 we have compared the urea denaturation profiles from alterations of the 695 nm absorbivity and fluorescence efficiency of the tryptophan side chain, leading to the proposed denaturation mechanism (16) with the urea-oxidation-reduction potential behavior of the protein. Also included in Fig. 3 are the urea concentration ranges in which each step of the denaturation mechanism occurs, showing the localization of the states on the urea scale and the urea regions relevant to each step.

The insensitivity of the oxidation-reduction potential to increasing urea concentrations up to 5 M, which encompasses the first urea denaturation step, $N ightleftharpoons X_1$ (Fig. 3), shows unambiguously that the oxidation-reduction potential of heme iron in cytochrome c is independent of the structural perturbation of the molecule constituting this step of the denaturation mechanism. The primary perturbation of the molecule during the $N ightleftharpoons X_1$ transition is shown to be the loosening of the heme crevice, particularly in the front, and extending as far as the two axial ligands are located (16). The heme moiety is localized in a hydrophobic environment (3), and furthermore, it has been suggested that the axial ligands are protected from anionic reagent, bromoacetic acid, because the hydrophobicity prevents the penetration of ions to the core of the crevice (6, 21, 22); therefore, the loosening of the crevice and the generation of reactivity of both axial ligands to bromoacetic acid during the $N ightleftharpoons X_1$ transition (16) imply a definite disruption of the hydrophobicity of the heme environment as well as increased solvent exposure of the heme group. In view of the proposed generalizations, that the oxidation-reduction potential is directly related to the hydrophobicity of the heme environment (12, 13) or inversely to the extent of exposure of heme to solvent (14), and on the basis of the above characterization of the $N ightleftharpoons X_1$ transition, one can expect a decrease in the oxidation-reduction potential of heme. However, this is not observed in the data shown in Fig. 3. Thus this generalization does not appear to be valid.

The localization of the potential alterations in the region above 5 M urea, the region associated with two steps of the proposed denaturation mechanism, $X_1 ightleftharpoons X_2 ightleftharpoons D$ (16) (Fig. 3), confirms the idea that a denaturation process in this range of urea concentration relates to the oxidation-reduction potential behavior of the molecule (15), and it also permits their resolution. On the basis of the proposed mechanism, two possibilities emerge which can be related to the oxidation-reduction potential function of the protein: either the transition $X_1 ightleftharpoons X_2$ is the electrochemically related step, or it is the $X_2 ightleftharpoons D$ step. Through appropriate manipulation of the Nernst equation (see "Appendix"), and using the spectroscopic, or fluorescence data, or both, characterizing each of the two steps of the denaturation mechanism (Fig. 3; see also Ref. 16), the expected midpotential-urea profiles corresponding to the above two possibilities can be easily generated (see "Appendix" for details); these are shown in Fig. 4. In addition to Curves 1 and 2, generated on the basis of the propositions stated above, and using the alterations in the absolute extinction of the 695 nm band as a parameter reflecting both steps of the mechanism (Fig. 3), Curve 3 is the result of transformation of the fluorescence-urea denaturation transition to the corresponding oxidation-reduction potential-urea profile ("Appendix, Case C").

A significant departure from observed behavior of Curve 1 in Fig. 4, the oxidation-reduction behavior expected if $X_i ightleftharpoons X_i$ is the electrochemically functional step of the mechanism ("Results"), rules out the importance of this step as a determinant of the oxidation-reduction behavior of this protein. As the $X_i ightleftharpoons X_i$ step is shown to be primarily the solvent exposure of the polypeptide backbone (18), a relatively high degree of freedom between the oxidation-reduction potential property and the protein conformation must be the case.

In contrast to the above, the potential-urea profile expected from a consideration of the $X_i ightleftharpoons D$ step as the functional step, Curves 2 and 3, especially Curve 3, is exceedingly close, if not identical, to the observed midpotential behavior of the molecule (Fig. 4). As the $X_i ightleftharpoons D$ step involves the disruption of the Met-80-S-iron linkage as well as alteration in the tryptophan-heme domain of the molecule (16), one of these two structural aspects or a combination thereof must be critical in determining the oxidation-reduction potential of the protein.

The importance of the Met-80-S-iron linkage relative to oxidation-reduction potentials has been well documented. Harbury et al. (19) attributed the abnormal oxidation-reduction potential of the protein to the presence of the Met-80-S linkage because of the differential stability of this structure in the ferric versus ferrous form of heme iron. Rodkey and Ball (2) reported a drop of midpotential by about 130 mV with a pH change from 7 to 10, which is now known to involve the disruption of this structure of the molecule (3). Similarly, the lowering of the midpotential upon chemical modification of Met-80-S resulting in its displacement from heme iron (15) supports the above contention. The importance of the heme environment in the oxidation-reduction potential behavior, on the other hand, is clearly seen in the recent work of Kassner (12, 13). The conclusion consistent with the above is that the observed potential cannot be attributed to either the integrity of the Met-80-S-iron linkage or the hydrophobicity of the tryptophan-heme domain per se, but rather to a composite of these structures of the protein.

Tsung (23) has shown that disruption of the Met-80-S-iron linkage upon addition of cyanide has no effect on the fluorescence efficiency of the tryptophan side chain, thus...
suggesting that the integrity of the structures in the deepest part of the heme crevice is not dependent upon the integrity of the methionine-S-iron linkage. A similar conclusion comes from the observation that at pH 10, where the Met-80-S-iron linkage is disrupted (3), tryptophan fluorescence is little enhanced. We have shown that the modification of Met-80 to methionine sulfoxide results in its replacement from heme coordination by an oxygen atom (25), is also without effect as far as the fluorescence is concerned. In contrast, it is well documented that modification of the tryptophan side chain (24, 25) or of the crevice-located Tyr-67 (26) results in significant destabilization of the Met-80-S-iron linkage or its complete elimination (24, 26). It is thus apparent that the integrity of the tryptophan-heme domain is independent of the integrity of the Met-80-S-iron linkage, but the maintenance or the stability of the Met-80 S iron linkage is dependent upon the integrity of the structures in the deepest part of the crevice. The lower value of the midtransition urea concentration of the transition reflecting the disruption of the tryptophan-heme domain, the fluorescence-urea transition, as compared to that observed for the transition reflecting the quenching of the 695 nm band (16) is consistent with the above. The apparent lag, particularly in the early stages, of the potential-urea profile generated from consideration of the transition reflecting the disruption of the Met-80-S-iron linkage, i.e. from the high molar urea component of the 695 nm absorption-urea transition (Fig. 3, Curve 2 in Fig. 4), when compared to the profile of the interrelationship between the oxidation-reduction potential and the tryptophan-heme domain (Fig. 4, Curve 3), is not only consistent with the idea of the disruption of the Met-80-S-iron linkage following that of the tryptophan-heme domain, but also with the idea that the abnormally high oxidation-reduction potential is determined by the integrity of the tryptophan-heme domain.

An interpretation consistent with the independence of the Met-80-S-iron linkage and the integrity of the tryptophan-heme domain, on the one hand, and the indication that the abnormal oxidation-reduction potential is a reflection of a composite of the integrity of both of these structures, and also the inference that the observed potential level may be a direct effect of the heme-tryptophan structure, accords to the idea that while the quenching of the 695 nm band is a composite reaction involving the interaction of tryptophan and hemoglobin, it is not a direct measure of the oxidation-reduction potential of the cofactor. The apparent lag in the early stages is consistent with the idea that the effect of depletion of the electrochemically active form in the reduced state, two distinct possibilities

\[ \text{Eh} = \text{E}^0 + \frac{RT}{NF} \ln \left( \frac{[\text{OX}]}{[\text{RE}]} \right) \]

For midpotential conditions, \( S_1 = S_0 \), Equation 1 yields an expression defining the apparent midpotential, \( E_m \), as a function of the fractional coefficients of the oxidation-reduction couple. Introducing the limiting conditions, \( f_o = 0 \) or \( f_o/f_r = 1 \), where \( E_m \) is the limiting midpotential, Equation 1, becomes

\[ E_m = E_o^0 + \frac{RT}{NF} \ln \left( \frac{f_r}{f_o} \right) \]

which defines the effect of depletion of the electrochemically active oxidized, or reduced forms, or both, on the midpotential of the system. It is apparent from Equation 2 that if depletion of the oxidized form occurs, the intrinsic midpotential, \( E_o^0 \) of the oxidation-reductant couple will be lowered, and vice versa. For a system exhibiting an equilibrium of the form \( X_1 \rightleftharpoons X_2 \),

\[ D \]

of the oxidized component, with only one electrochemically active form in the reduced state, two distinct possibilities

\[ 1 \text{ Y. P. Myer, A. Pande, K. Thallam, and B. C. Verma, unpublished work.} \]
emerge: (a) the $X_1 \leftrightarrow X_2$ transition is the depleting step of the active form, or (b) the $X_2 \rightarrow D$ transition is the electrochemically active step. Using Equation 2 and appropriate conditional equilibrium constants, $K_{m1} = [X_1]/[X_1]$ and $K_{m2} = [D]/[X_2]$, the expression for the $E_n$ can be derived as follows.

Case $A$—If $X_1 \rightarrow X_2$ is the electrochemically active form:

$$f_e = \frac{[X_1]/([X_1] + [X_2] + [D]) = (1 + K_{m1} + K_{m2}K_{obs})^{-1}}{[X_1]/([X_1] + [X_2] + [D]) = (1 + K_{m1} + K_{m2}K_{obs})^{-1}}$$

and for the system with $f_e = 1$, Equation 2 yields

$$E_n = E_n^0 - \frac{(RT/N_{F}) \ln (1 + K_{m1} + K_{m2}K_{obs})}{1 + K_{m1} + K_{m2}K_{obs}}$$

which describes the $E_n$ of the system.

Case $B$—If the $X_2 \rightarrow D$ step of the mechanism is the electrochemically active step:

$$f_e = \frac{([X_1] + [X_2])/([X_1] + [X_2] + [D]) = (1 + K_{m2})}{(1 + K_{m1} + K_{m2}K_{obs})}$$

and for $f_e = 1$, Equation 2 yields

$$E_n = E_n^0 - \frac{(RT/N_{F}) \ln (1 + K_{m1} + K_{m2}K_{obs})}{1 + K_{m1} + K_{m2}K_{obs}}$$

The calculated midpotential-urea profile from Equation 5, using the fluorescence-urea transition as the model, is shown as Curve 3 in Fig. 4.

For all the above calculations, a limiting potential, $E_n^0$, of 248 mV, temperature of 25°C, $N = 1.0$, and $2.93RT/N_{F} = 0.099$ V were used.

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