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THE REDOX POTENTIAL OF HORSE HEART CYTOCHROME c[1]

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Summary: The absolute extinction of the 695-nm band of horse heart ferricytochrome c and its redox potentials as a function of urea concentration at pH 7.0 have been measured. The redox potential is found to be independent up to about the 5 M urea concentration, but drops at higher concentrations, reaching a value of about 175 mV in 8 M urea. Changes in the 695-nm band, on the other hand, are indicative of a weak transition centered at about 2.5 M urea concentration and a major asymmetric transition leading to its quenching at about 6.5 M urea. The insensitivity of redox potentials to the transition at 2.5 M, associated with the loosening of the heme crevice (Myer, Y. P. (1968) Biochemistry 7, 765), clearly demonstrates the unimportance of the integrity of the heme crevice, whereas the drop in potential corresponding to the transition at 6.5 M urea, representing the disruption of the heme coordination and helix coil transition, points to the protein structures as determinants of the high redox potential of the protein. The correlation of the transition profiles further indicates that a process localized toward the high-urea-concentration limb, the disruption of heme coordination, determines the level of the redox potential of this protein.

Introduction: The high redox potentials of hemes in components of the electron transport chain, hemes a_3 and a of cytochrome c oxidase with potentials of 365 and 205 mV, respectively (1), cytochrome c with a potential of 250 mV (2), and cytochrome b with a 38 mV redox potential (1), in comparison to the potentials of either the simple hemes or heme c systems, -50 to -210 mV (3-7), are explained on the basis of the characteristics of a particular heme and its coordination configuration in a given protein (4, 5), and/or the hydrophobicity of the heme environments (8). Theoretical consideration of the effects of the non-polar heme environment on heme redox potentials has added support to the idea that the high redox potentials of these systems are determined largely by the environment of the heme group, i.e. the heme crevice (9). In this report we wish to show that in cytochrome c there is an appreciable degree of freedom between the heme crevice and the protein redox potentials.

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Only when the heme axial bonds are disrupted does alteration of the redox potentials of the protein occur. The dominant role in determining the characteristic redox potential of cytochrome c thus cannot be ascribed to the integrity of the heme crevice, as has been suggested (9, 10), but rather, to the integrity of the heme coordination configuration and/or its structural repercussions in this molecule.

Experimental: Sigma horse heart cytochrome c was used without further purification for these investigations. Absorption spectra were measured with a Cary 15 spectrophotometer, and the absolute extinction of the 695-nm band was determined according to the procedure described by Kaminsky et al. (11). Potentiometric cum spectrophotometric titrations were performed and analyzed according to the procedure already described (6). A mixture of mediators detailed by Dutton et al. (1) was used to facilitate the equilibration of the oxidized-reduced forms. A constant value in the potentials determined over a period of 4-5 min was taken to indicate the attainment of equilibrium. Sodium ascorbate, 6 mM, and/or sodium dithionite, 10 mM, solutions were used as the reducing agents, and a 13 mM solution of potassium ferricyanide was used as the oxidant for oxidative titrations. All potentiometric titrations of cytochrome c, irrespective of the presence or absence of urea, were found to conform to an N, number of electrons, of 1.0 ± 0.2, and yielded midpotentials with an uncertainty of only about 3 mV for a given titration. The composite of uncertainty from multiple titrations was found to be of the order of ±5 mV. The measurements were made using a Calomel electrode as the reference electrode, which was calibrated through determination of the midpotentials of a number of dyes with known midpotentials. All the measurements reported here were made at 25° in the presence of 0.05 M phosphate buffer, 0.2M KCl, unless otherwise stated.

Results: The redox potential of horse heart cytochrome c with increasing concentrations of urea at pH 7 is found to be unchanged up to a urea concentration of about 5.5 M (Fig. 1), and drops in magnitude at higher urea concentrations. The redox potential-urea profile is typical of a system depleting the electrochemically active ferric form (3) through a transition centered at urea concentrations higher than 6 M. The alteration of absorptivity of the 695-nm band of ferric cytochrome c at pH 7.0 with increasing urea concentrations, however, indicates the presence of at least two transitions: a weak transition centered at about 2.5 M urea concentration and reflected by a slight increase in absorptivity of the band, and the major transition, leading to its complete quenching, centered at about 6.5 M urea concentration (Fig. 1). The major transition, however, is asymmetric, i.e. with a steep drop in absorpti-

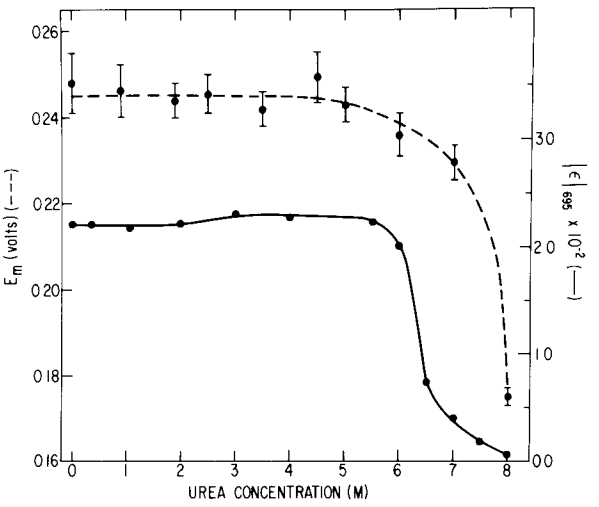

Fig. 1. Effect of increasing concentrations of urea on the absolute extinction of the 695-nm band of horse heart ferricytochrome c and its oxidation-reduction midpotential. ———, 695-nm extinction; - - - , midpotential. Bars show the RMS error from multiple measurements.

vity in the urea concentration range 5.5 - 6.5 M, followed by a relatively slower change with increasing urea concentration.

Discussion: The urea denaturation of horse heart ferric cytochrome c at neutral pH has been shown to proceed through a two-step process, the first centered at about 2.5 M urea concentration, and the second, at about 6.5 M (12). The first denaturation step is the loosening of the heme crevice, without disruption of either the coordination configuration of the heme iron or of the polypeptide conformation, and the second step is a composite of helix coil transition and disruption of the heme coordination configuration (12). The maintenance of the 695-nm band, an indicator of the presence of the Met-80-S bond to heme iron (10, 13), up to 5.5 M urea encompassing the first denaturation step, confirms the integrity of the Met-80-S-iron bond during the first step. The quenching of the 695-nm band with a transition centered at 6.5 M urea (Fig. 1), characteristic of the second denaturation step (12), is consistent with the disruption of the heme-coordinated ligands during this denaturation step.

The state of the molecule, with a loosened crevice, in the mid-range of urea concentration, 4.5 to 5.5 M, is also confirmed through studies of the carboxymethylation reaction in the presence of 4.5 M urea (14, 15). In 4.5 M urea at neutral pH, carboxymethylation with bromoacetic acid results in not only the modification of the Met-65 and His-33 side chains, the two normally reacting groups of the protein (10, 16), but also of both the axial liganding groups, Met-80 and His-18 (14), which are otherwise insensitive to the reaction in the native protein (10, 14, 16). The generation of reactivity to an anionic reagent of both the axially ligated protein groups without the disruption of their bonding to the metal atom could only result from significant disruption of the heme hydrophobic environment, which apparently shields the ligands in the native protein (10). The lowered thermal stability of the protein in the presence of 4.5 M urea, melting temperature about 25^{o} lower than in the native protein, with a denaturation profile that of a single-step transition (17), is also consistent with the above conclusions. Another apparent effect of the loosened heme crevice is the destabilization of the heme coordination configuration, which is also the case in the presence of 4.5 M urea, as the apparent pK_a for the breaking of the Met-S-Fe bond is not 9.2, but rather, is lowered to a value of 8.5 in 4.5 M urea (17).

The insensitivity of the reduction potentials up to the 5 M urea concentration (Fig. 1), which encompasses the first denaturation step, clearly establishes the absence of any strict interdependence of the redox potentials with the nature of perturbation, the loosening of the heme crevice, destabilization of the Met-80-S-Fe linkage, exposure of the heme axial ligands to a hydrophyllic environment, etc., associated with this denaturation step. The drop in potential at high urea concentrations, seemingly parallel to the transition ascribed to the disruption of the heme axial ligands, on the other hand, points to the importance, direct or indirect, of this structure of the molecule to its high redox potential.

As the second urea denaturation step is constituted of the disruption of both the heme coordination as well as the polypeptide conformation (12), resolution between the two as to their importance in determining potentials of the protein, at least in part, comes from a critical examination of the potential vs. spectral profiles shown in Fig. 1. The former is symmetric, whereas the latter is definitely asymmetric. The major change in the potential is encountered above 7 M urea concentration, whereas 80% of the 695-nm band is quenched below 7 M. The lack of coincidence between the spectral-urea and potential-urea profiles is further emphasized by the disagreement between the observed potentials and the ones calculated[2] on the basis of the spectral transition reflecting depletion of an electrochemically active form. The expected drop in the midpotential at 7 M urea, 80% depletion of the functional form, amounts to about 42 mVolts,[2] whereas the observed drop is only of about 18 mVolts (Fig. 1). Similar deviations occur at all other points of the titration. Although a one-to-one relationship between the spectral titration and the potential-urea profile does not seem to exist (Fig. 1), the fact remains that the two aspects of the protein must be correlated, as both processes are associated with the higher urea concentrations, i.e. above 6 M.

It should be noted that the potentials calculated on the basis of the transition centered at 6.5 M urea yield values[2] which are always lower than the observed potentials (Fig. 1). A process centered at urea concentrations higher than 6.5 M must therefore be linked to the redox potential of the

[2] For a system involving equilibrium between an electrochemically active, N, and an inactive form, D, of the oxidized form with the conditional equilibrium constant, $K_d = [D]/[N]$, $S_o = [N] + [D]$, and S_r the concentration of the reduced form, The Nernst equation for the potential of the cell upon substitution for [N] and simplification becomes:

$E_h = E_1 + RT/NF(\ln(S_o/S_r) + RT/NF(\ln(1/1 + K_d))$. For midpotential conditions, $S_o = S_r$ and the limiting condition $K_d = 0$, the equation becomes

$E_m^{obs} = E_m^o + RT/NF(\ln(1/1 + K_d))$, the equation for the dependence of the midpotential on the equilibrium state of the system. E_m^{obs} is the observed midpotential and E_m^o is the limiting midpotential. From the above equation for a one-electron system, N = 1, temperature 25°, the drop in potential for 80% depletion of the ferric form is 42 mVolts.

protein, as such a process could produce a potential profile coincident with the observed behavior shown in Fig. 1. Since the spectral titration is asymmetric, multiplicity of processes constituting the observed transition is likely. The high-urea-concentration limb of the transition, i.e. above 7 M, could easily be a reflection of a process related to the potentials of the protein. Preliminary analysis of the results of the resolution of the spectral transition and the correlation of calculated vs. observed midpotentials does support the above inference that the high-urea-concentration limb of the spectral transition determines the redox potentials. Since this component of the spectral transition is involved with the quenching of the 695-nm absorptivity (Fig. 1), and since it is acknowledged that the 695-nm band is a manifestation of the Met-80-S-Fe bond (10, 13), the dominant role of the heme coordination in determining the redox potential of the protein (5) seems certain, especially since the interdependence of the two is ascertained without any regard to the integrity of the heme crevice. However, it is still possible that maintenance of the heme coordination configuration determines the integrity of a critical structure of the protein, which in turn determines the potential of the molecule. Investigations related to this aspect of the redox potential-structural relationships of the protein are presently being conducted.

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