Comparative Solvent Perturbation of Horse Heart Cytochrome c and Rhodospirillum rubrum Cytochrome c2

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The extent of exposure of heme to solvent in horse heart cytochrome c and Rhodospirillum rubrum c2 was investigated to determine whether a correlation exists between the properties of these oxidation-reduction proteins and their heme environments. Solvent perturbation absorption difference spectra were measured using ethylene glycol, glycerol, and sucrose at concentrations between 0 and 30%. Cytochrome c appears to exhibit a somewhat greater extent of heme exposure than cytochrome c2 for both the oxidized and reduced states. These results suggest that the lower oxidation-reduction potential of cytochrome c may in part be due to a greater extent of exposure of the heme. The oxidized state of both proteins appears to exhibit a greater exposure than that of the reduced state which is consistent with a more favorable environment for the charge on the ferric heme coordination center.

X-ray crystallographic studies have shown that mitochondrial cytochrome c and the bacterial cytochrome c2 of Rhodospirillum rubrum exhibit extensive structural similarities (1-3). The heme moiety in both cytochromes is situated in a hydrophobic crevice with exposure of heme occurring at only one edge which is surrounded by a ring of positively charged lysine side chains on the surface of the protein. The heme iron in both proteins is coordinated to the imidazole group of a histidyl residue and the sulfur of a methionyl residue in positions 5 and 6, respectively. However, despite such structural similarities, qualitative differences have been observed in the near-ultraviolet circular dichroism spectra of cytochromes c and c2 which have been interpreted in terms of subtle differences in the heme environments of these cytochromes (4). Significant differences in other chemical and physical properties of these proteins have also been reported. Different rates of oxidation and reduction have been observed for cytochromes c and c2 in their reactions with iron hexacyanides (5-7). The reduction potential of cytochrome c2 is 60 mV higher than that of cytochrome c. Furthermore, both the rates of oxidation and reduction (7) and the oxidation-reduction potential of cytochrome c2 (8) have been found to be dependent on pH in the range between 5 and 8, whereas with cytochrome c these properties are independent of pH in this range (9, 10).

It has been suggested by Kassner (11, 12) that the hydrophobic character of the heme environments in these proteins may account for the differences between the oxidation-reduction potentials of these cytochromes and their corresponding model heme-ligand complex. Likewise, differences between the oxidation-reduction potentials of cytochromes may be related to differences in the hydrophobicities of their heme environments which may be manifested by differences in the extent of exposure of the heme to solvent water molecules. In general, the most hydrophobic environment should be characterized by a minimum exposure of the heme to solvent molecules.

The exposure of the heme to solvent has also been considered as a factor in the rates of electron transfer reactions. Marcus theory (13) has been found useful in predicting reaction rates of cytochrome c with a variety of inorganic reactants (14-16) and in accounting for the observed self-exchange rate constant (17) of cytochrome c. The self-exchange rate constant for cytochrome c has been rationalized as being the product of the self-exchange rate constant for the heme moiety and a steric factor which accounts for the fact that the effective area of the heme group occupies only a fraction of the total surface area of the protein (18). Therefore, differences between the self-exchange rate constants for cytochromes c and c2 may be related to differences in exposure of the heme to solvent in these proteins.

The purpose of this study is to explore the differences in the exposure of the heme moiety in cytochrome c and cytochrome c2 and to use them as a basis for understanding the difference in oxidation-reduction properties of these cytochromes.

The relative exposure of the hemes in cytochromes c and c2 is determined through the application of solvent perturbation difference spectroscopy which has had wide application in determining the relative exposure of chromophores in heme proteins (19-21) as well as other proteins (22-24).

EXPERIMENTAL PROCEDURES

Materials

Horse heart cytochrome c (type VI) was purchased from Sigma and used without further purification. Other products used were as follows: sucrose (grade I) from Sigma; ampholyte solutions from LKB-Produkter AB, and glycerol, ethylene glycol, sodium dithionite, and potassium ferricyanide from Fisher.

Methods

Preparation and Purification of Cytochrome c—Cells of R. rubrum were grown anaerobically on the modified Hutner medium (25) under constant illumination. Cytochrome c2 of this organism was extracted and purified essentially according to the procedure of Rattch et al (26). Cytochrome c2 was obtained in pure form after isoelectric focusing in the pH range 5 to 7. The main component with pI (4°C) = 6.2 was used in all subsequent experiments.

Preparation of Solutions—Cytochrome solutions were fully reduced or oxidized with an excess of sodium dithionite or potassium ferricyanide, respectively. These oxidation-reduction agents were subsequently removed by molecular sieve chromatography on Sephadex.
Comparative Solvent Perturbation of Cytochromes c and cz

G-15-80. These solutions were diluted to an appropriate concentration and a minimal amount of sodium dithionite (10⁻² M) or potassium ferricyanide (10⁻⁴ M) added to assure that the resulting cytochrome stock solutions remained in the desired oxidation state. All perturbant stock solutions were volume per cent except sucrose which was weight per cent. For the solvent perturbation measurements, equal volumes of cytochrome and perturbant stock solutions were added directly to cuvettes using 2-ml Gilford micrometer burettes. Final cytochrome solutions contained 1 to 5 × 10⁻⁶ M cytochrome, as determined using the extinction coefficients of Margoliash et al. (27) and Horio and Kamen (28), and 0.1 M potassium phosphate buffer, pH 7.0. Measurements on ferrous cytochrome c in ethylene glycol were made on deaerated solutions under an argon atmosphere.

Spectrophotometric Measurements—Spectra were recorded on a Cary 14R spectrophotometer at ambient temperature (23-25°C). Difference spectra were recorded on the expanded scale (0.0 to 0.1 A). Solvent perturbation difference spectroscopy was performed as described by Herskovits (29), except that standard 1-cm path length, Teflon-stoppered cuvettes were used instead of tandem cells since the perturbants used do not absorb in the Soret region (400 to 420 nm).

Relative exposures were calculated using: percent relative exposure = (Δε/ε)standard/(Δε/ε)model × 100%. The Δε/ε values for the model are those obtained from the ferric (21) and ferrous (30) heme octapeptide of cytochrome c with added imidazole.

The solvent perturbation results for 20% perturbant concentration (Tables I and II) were obtained from the least square lines. Each line was obtained from at least three different perturbant concentrations. Three to five determinations were made at each concentration.

RESULTS

In order to determine the relative exposure of heme to solvent in ferrous and ferric cytochromes c and cz, solvent perturbation absorption difference spectra were measured using ethylene glycol, glycerol, and sucrose at concentrations between 0 and 30%. The principal effect of each of the perturbants on cytochromes c and cz is an enhancement of the Soret absorbance. This effect has previously been observed for cytochrome c (19), cytochrome c550 (21), and cytochrome c oxidase (20).

A linear relationship between Δε/ε and perturbant concentration has been used as an indication that solvent perturbation occurs without changes in the native protein conformation (23) and has been shown to be adequate in detecting solvent denaturation in cytochrome c (19) and other heme proteins (20, 21). In the present study, the relationship between Δε/ε values and perturbant concentration was found to be linear in all cases over the range investigated as shown for

![Graph showing the extent of perturbation](http://www.jbc.org/)

**FIG. 1.** The extent of perturbation, Δε/ε, as a function of glycerol concentration. O, ferricytochrome c, 414 nm; ⊙, ferricytochrome c2, 417 nm. All solutions contained 0.1 M phosphate buffer, pH 7.0. Cytochrome concentration ranged from 1 to 5 × 10⁻⁶ M.

<p>| Table I | Effect of perturbants on ferricytochromes c and cz |
|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Perturbant (20%)</th>
<th>Δλmax (nm)</th>
<th>Δε/ε (±S.D.)</th>
<th>Relative exposure</th>
<th>Δλmax (nm)</th>
<th>Δε/ε (±S.D.)</th>
<th>Relative exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene glycol</td>
<td>408 (± 0.0003)</td>
<td>0.0188</td>
<td>25.9</td>
<td>416 (± 0.0003)</td>
<td>0.0151</td>
<td>20.8</td>
</tr>
<tr>
<td>Glycerol</td>
<td>414 (± 0.0003)</td>
<td>0.0179</td>
<td>27.9</td>
<td>417 (± 0.0003)</td>
<td>0.0162</td>
<td>25.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>414 (± 0.0002)</td>
<td>0.0188</td>
<td>39.2</td>
<td>415 (± 0.0004)</td>
<td>0.0174</td>
<td>36.3</td>
</tr>
</tbody>
</table>

^a Calculated using the Δε/ε values determined for the ferric heme octapeptide of cytochrome c plus added imidazole (21).

^b See Ref. 23.

<p>| Table II | Effect of perturbants on ferrocytochromes c and cz |
|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Perturbant (20%)</th>
<th>Δλmax (nm)</th>
<th>Δε/ε (±S.D.)</th>
<th>Relative exposure</th>
<th>Δλmax (nm)</th>
<th>Δε/ε (±S.D.)</th>
<th>Relative exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene glycol</td>
<td>417 (± 0.0003)</td>
<td>0.0186</td>
<td>20.4</td>
<td>416 (± 0.0002)</td>
<td>0.0151</td>
<td>16.6</td>
</tr>
<tr>
<td>Glycerol</td>
<td>417 (± 0.0002)</td>
<td>0.0176</td>
<td>18.3</td>
<td>417 (± 0.0002)</td>
<td>0.0167</td>
<td>17.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>417 (± 0.0003)</td>
<td>0.0184</td>
<td>32.5</td>
<td>417 (± 0.0002)</td>
<td>0.0179</td>
<td>31.1</td>
</tr>
</tbody>
</table>

^a Calculated using Δε/ε values determined for the ferrous heme octapeptide of cytochrome c plus added imidazole (30).

^b See Ref. 23.
the oxidation-reduction potential of cytochrome c is smaller degree of exposure to solvent. The observation that the heme exposure in ferrous and ferric cytochromes c and c2 was found not to be a function of the size of perturbants used. These results are in agreement with previous results for ferrous cytochrome c (19) and similar to those observed for other heme proteins (20, 21).

As seen in Table I, the extent of enhancement, $\Delta\varepsilon/\varepsilon$, and the resultant exposure of the heme relative to the model chromophore are somewhat greater for ferric cytochrome c than for ferrous cytochrome c2. The same effect is observed for the ferrous cytochromes as shown in Table II. A further comparison of Tables I and II indicates that for both cytochromes c and c2 the relative exposure in the ferric state appears greater than that in the ferrous state.

**DISCUSSION**

The solvent perturbation results summarized in Tables I and II indicate that the heme moiety in both cytochromes c and c2 is partially exposed to solvent and that for cytochrome c2 the folding of the polypeptide chain about the heme restricts its contact with solvent to a greater extent than that in cytochrome c. It has been proposed that a more hydrophilic heme environment stabilizes the ferric state relative to a hydrophobic environment and thus results in a lowering of the oxidation-reduction potential (11). On this basis, one would predict that cytochrome c2 should have a higher oxidation-reduction potential than cytochrome c since a more hydrophilic environment would in general be expected for a smaller degree of exposure to solvent. The observation that the oxidation-reduction potential of cytochrome c2 is about 60 mV higher than that of cytochrome c suggests that a correlation exists between solvent exposure and oxidation-reduction potential. This observation is in contrast to the lack of correlation between the oxidation-reduction potentials of equine heart cytochrome c and Prosthecochloris aestuarii cytochrome c2. The per cent exposure of the heme group in these cytochromes (21). Although cytochrome c2 has also been shown to be characterized by the same axial ligands as cytochrome c and c2, spectroscopic and sequence differences have suggested that it be classified with other cytochromes (33).

The observation that the heme exposure for the ferricyanochromes c is greater than that for the ferrocyanochromes c2 suggests that a conformational change toward a tighter heme crevice occurs upon reduction in both cytochromes c and c2. This result is consistent with the expectation that the oxidized state would be stabilized by a more polar heme environment (11, 12, 34). These results are then also consistent with the concept that differences between the oxidation-reduction potentials of these cytochromes may be due to differences in the change in conformational energy between the oxidized and reduced states.

It has recently been shown that a correlation exists between the oxidation-reduction potential and methionine methyl resonances of various ferrocytochromes which indicates that there is a variation in the iron-sulfur bond length with variation in oxidation-reduction potential (35). This concept suggests that the iron-sulfur bond length in these proteins is determined by the minimal conformational energy of the polypeptide and, therefore, exhibits a value intermediate between the bond lengths of unconstrained ferrous and ferric complexes. However, the change in conformation indicated by the difference in per cent exposure of oxidized and reduced states suggests that the iron-sulfur bond length may not be rigidly constrained. It should be acknowledged that an evaluation of the solvent perturbation of these cytochromes in terms of a per cent exposure of the heme to solvent requires a comparison of the data for the proteins to that of a model chromophore completely exposed to the solvent. In this study, the heme octapeptide in the appropriate oxidation state plus imidazole was used as the model chromophore. Although this complex should suffice as a model chromophore, a more appropriate model would be the methionine-histidine-heme complex. However, added methionine appears to coordinate incompletely to the ferric heme octapeptide at accessible concentrations (30).

The solvent perturbation results show that a decrease in the extent of exposure is not observed with an increase in the size of perturbant. This indicates that the heme crevice in cytochromes c and c2 does not selectively restrict the access of the perturbants to the heme according to size and suggests that a portion of the heme protrudes out of the hydrophobic crevice into the solvent for both the oxidized and reduced states of cytochromes c and c2. These observations and the aforementioned differences between the heme exposures of cytochromes c and c2 are also pertinent to proposed mechanisms of electron transfer. As previously mentioned, Marcus theory has been used in the analysis of the reactions of cytochrome c with various inorganic reactants involving an outer sphere mechanism and in the determination of the self-exchange rate constant of cytochrome c. This self-exchange rate constant has been rationalized as being the product of the self-exchange rate constant for the heme moiety and a steric factor which is essentially an expression of the exposure of the heme moiety. It, therefore, should follow that the difference in the self-exchange rate constants for these two cytochromes should be dependent on differences in the exposure of the heme moiety. Since the heme in cytochrome c2

![Diagram](http://www.jbc.org/)
is less exposed than the heme in cytochrome c, one would predict a smaller steric factor for cytochrome c₂ which in turn would result in a smaller self-exchange rate constant for cytochrome c₂ than for cytochrome c. However, calculated self-exchange rates based on cross-reaction rates with ferri-cyanide or ferrocyanide indicate that cytochrome c₂ has a greater self-exchange rate than cytochrome c (36). Thus, the present results suggest that electron transfer may occur through a mechanism where exposure of the heme is not a limiting factor.

REFERENCES
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