The Heme Environment in Ferric and Ferrous Cytochrome *c* Oxidase†

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ABSTRACT: The environment of the heme in ferric and ferrous cytochrome *c* oxidase has been probed by solvent perturbation difference spectroscopy. Using a cytochrome *c* chymotryptic digest as the reference for a fully exposed heme, an accessibility of 20–30% is calculated for the heme *a* in ferric oxidase. The perturbation does not depend upon the size of the perturbant. Ethylene glycol, glycerol, and sucrose are shown not to affect the activity, molar ellipticity, or state of association of cytochrome oxidase. These studies demonstrate that the heme in the oxidized enzyme is buried in a crevice. A portion protrudes from the cavity and is able to interact with the solvent. During reduction a significant structural reorganization occurs which renders the heme inaccessible.

It is now generally accepted that the conformation of cytochrome *c* oxidase (EC 1.9.3.1) is oxidation state dependent. This conclusion has been supported by two main lines of evidence. First, the work of Yamamoto and Okunuki (1970, 1972) showed that the ferric oxidase is much more resistant to proteolysis than is the ferrous form; and retains full enzymatic activity compared with a 60% loss of activity upon proteolysis of the ferrous enzyme. This result is consistent with a more compact structure for the ferric oxidase. Secondly, the extensive spectroscopic investigations of King and his coworkers (Meyer and King, 1969; Yong and King, 1970a,b; King *et al.*, 1971) have conclusively shown that the optical rotatory properties of the protein are oxidation state dependent, and reflect the geometrical relationships between the prosthetic group and the apoprotein. Recently, we have shown (Cabral and Love, 1972) that there is a small change in the sedimentation coefficient which is associated with the oxidation state of cytochrome oxidase. This change in sedimentation coefficient is consistent with a 3% increase in molecular volume accompanying reduction of the enzyme. It is pertinent to ask at this point how these conformational changes affect the heme cavity of the protein. Although studies of chemical reactivity (Morrison and Horie, 1964; Tzagoloff and Wharton, 1964; Takemori and King, 1965; Yamamoto and Orii, 1973a) have shown that the prosthetic group is well sequestered in the molecule, there are no quantitative estimates of the exposure of the heme. One approach to this problem is to use solvent perturbation difference spectroscopy (SPD), which has had wide application in determining the relative exposure of chromophores in both heme and nonheme proteins (Donovan, 1969; Herskovits, 1967). In this paper we will present evidence from SPD that the oxidation state dependent conformation of cytochrome oxidase is associated with an opening and closing of the heme cavity, and a quantitative estimate of the relative exposure of the prosthetic group will be given.

Experimental Section

Materials. Horse heart cytochrome *c* (Sigma, Type III) was used without further purification. Chymotryptic digests were prepared using chymotrypsin from Nutritional Biochemicals Corporation. Ethylene glycol and glycerol were Spectrograde quality reagents from Eastman Organics (Distillation Products Division, Rochester, N. Y.) and were used with no further purification. Analytical grade sucrose was purchased from Mallinckrodt Chemical Co. (St. Louis, Mo.). The water used was distilled from an all-glass Corning still.

Isolation of Cytochrome c Oxidase. Cytochrome oxidase was prepared from beef heart mitochondria by the method of Yonetani (1966) with modifications as previously described (Cabral and Love, 1972). The final dialysis was against 0.05 M phosphate buffer (pH 7.4) containing 0.5% Tween-20. Cytochrome oxidase prepared in this way had the following spectroscopic ratios: A_{280}/A_{418} = 2.64–2.88 and A_{444} (red)/A_{418} (ox) = 1.25–1.38. Heme *a* was determined from the difference in absorbance at 603 nm between the oxidized and reduced enzyme assuming an extinction coefficient, $\Delta\epsilon$, of 10.4 mM^{-1} cm^{-1} (Griffiths and Wharton, 1961). Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Values obtained from these analyses gave 8.9–10.6 nmol of heme *a*/mg of protein for our preparations.

Many experiments were repeated using a King preparation isolated exactly as described by the authors (Kuboyama *et al.*, 1972). This preparation had the following spectroscopic ratios: A_{280}/A_{418} = 2.74 and A_{444} (red)/A_{418} (ox) = 1.40. Using the analytical methods described above, we found 11.4 nmol of heme *a*/mg of protein.

Measurement of Enzymatic Activity. Activities were measured spectrophotometrically at 23° by following the oxidation of reduced cytochrome *c* at 550 nm and at pH 6.0. Molecular activities (MA) were calculated at several cytochrome *c* concentrations and the maximal molecular activity was obtained from the intercept at infinite substrate concentrations of a Lineweaver–Burk plot of 1/MA *vs.* 1/[cytochrome *c*] as described by Yonetani (1966). Using this method, maximal molecular activities of 3800–8100 electrons min^{-1} $heme^{-1}$ were obtained for the Yonetani preparations; and a maximal molecular activity of 5500 electrons min^{-1} $heme^{-1}$ was obtained for the enzyme prepared according to King.

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Preparation of Solutions. All solvent perturbation difference spectra on cytochrome *c* oxidase were taken in 0.05 M phosphate buffer (pH 7.4) containing 0.5% Tween-20 unless otherwise noted. Protein concentrations were 10–20 μM determined spectrophotometrically as described under Methods. The buffer used for the chymotryptic digests of cytochrome *c* was the same except that it contained 0.05 M imidazole and no detergent. The cytochrome *c* concentrations used were the same as for the cytochrome oxidase samples. Chymotryptic digests were prepared exactly as described by Stellwagen (1967) except for the buffer employed in the final solution. Perturbants were prepared as 40% stocks in the appropriate buffer and stored in glass-stoppered volumetric flasks. Sucrose solutions were replaced every few days.

Reduced samples of cytochrome oxidase were prepared by adding a minimal amount of solid sodium dithionite and waiting 30 min before mixing with the perturbant or solvent which also contained a small amount of dithionite. The cytochrome *c* chymotryptic digest was reduced by the addition of a small amount of dithionite directly to the optical cell.

Solvent perturbation difference spectra were recorded at temperatures of 23–25° on a Cary Model 118 spectrophotometer with an expanded scale. Protein solutions, perturbant solutions, and solvent were distributed among the compartments of a pair of matched cylindrical tandem cells (Pyrocell, Inc., Westwood, N. J.) as described by Herskovits and Laskowski (1962). Each cell compartment had a capacity of 3 ml and a path length of 10 mm. Solutions were kept at 0–4° in an ice bath prior to and during mixing in 5-ml test tubes. Immediately after mixing, the tubes were placed in a beaker of water at room temperature for 5 min and the contents of each tube were then transferred to the appropriate cell compartment. The difference spectrum was scanned within 10–15 min after mixing, and scans were repeated every 15 min for a period of at least 1 hr to determine the reproducibility of the spectrum with time.

Urea solutions were prepared as described by Herskovits and Laskowski (1962) at 35°, except that reduction of the protein with thioglycolic acid was omitted.

Other Measurements. Circular dichroism (CD) spectra of cytochrome oxidase solutions in 20% perturbants were measured at 25° on a Cary 60 spectropolarimeter equipped with the 6002 CD accessory. The solutions were prepared as described for the solvent perturbation difference spectra. Molar ellipticity values are based on the molar concentration of heme *a*, measured from the difference in absorbance between 603 and 630 nm for the fully reduced enzyme. An extinction coefficient, $\Delta\epsilon$, of 16.5 mM^{-1} cm^{-1} was assumed for this calculation (Sekuzu *et al.*, 1967).

Sedimentation velocity experiments were performed at 20° on a Beckman Model E analytical ultracentrifuge equipped with electronic speed control and schlieren optics. Sedimentation coefficients were corrected to water at 20° using viscosities and densities obtained from standard tables (Weast, 1972).

Activity measurements in the presence of perturbants were carried out as described above except that the assay mixture contained 20% perturbant, and cytochrome oxidase was incubated in 20% perturbant for 15 min prior to the determination.

Results

Choice of Model Compound for Fully Exposed Heme. Several possible models were considered for determining the perturbability of a fully exposed heme. Cytochrome oxidase, denatured in an 8 M urea solution containing buffer salts and Tween-20 was tried but gave values for $\Delta\epsilon/\epsilon$ that were far below those obtained for the native enzyme (see Table I). Addition of disulfide cleaving reagents was not used since these caused a shift in the position of the Soret band of urea denatured oxidase from 423 to 437 nm, implying that the heme was being reduced. The very low values of $\Delta\epsilon/\epsilon$ determined in 8 M urea led us to suspect that the heme, which is not covalently bound to the enzyme, is released into solution and becomes entrapped in detergent micelles, rendering it inaccessible to the perturbants. This possibility is supported by the observation of Simplicio and Schwenzer (1973) that hemin in detergent solutions is intercalated into the detergent micelle.

TABLE I: Results for 8 M Urea Denatured Cytochrome Oxidase and Schiff Base Derivative of Cytochrome Oxidase as Model Compounds for Fully Exposed Heme.

Sample	% Tween-20	20% Perturbant	$\Delta\lambda_{max}$	$\Delta\epsilon/\epsilon$
Oxidase	0.5	Sucrose	423	0.0083
Schiff base	0.1	Sucrose	414	0.011
Oxidase	0.0	Sucrose	423	0.0330
Schiff base	0.0	Sucrose	414	0.0366

To avoid this problem a Schiff base derivative of cytochrome oxidase was prepared according to the method of Takemori and King (1965). It was hoped that the polypeptide chains would exert a protective effect on the covalently bound heme, inhibiting its interaction with detergent micelles. A greater perturbation resulted (see Table I) although the magnitude of $\Delta\epsilon/\epsilon$ was still smaller than observed for the native enzyme. Attempts to remove the detergent by prolonged dialysis gave limited success. The $\Delta\epsilon/\epsilon$ values for both cytochrome oxidase and the Schiff base derivative increased substantially, but increase in scattering made the measurement of $\Delta\epsilon/\epsilon$ very difficult. Furthermore, the values are still lower than the value obtained by Stellwagen (1967) for the cytochrome *c* chymotryptic digest in 20% sucrose.

For these reasons we decided to use a chymotryptic digest of cytochrome *c* as the model compound for a fully exposed heme in our own experiments. This choice has several advantages. There is precedent for its use as a model compound (Stellwagen, 1967) and this allows a more direct comparison of the cytochrome oxidase results with results obtained for other hemoproteins. The structure of heme *c* differs from the structure of heme *a* only in substitutions at positions 2, 4, and 8 of the porphyrin ring. The chymotryptic digest is water soluble, and so detergents are not required. Since the heme is fully exposed it is not necessary to use 8 M urea, thus simplifying the experimental operations. Finally, the solutions do not scatter and the values of $\Delta\epsilon/\epsilon$ are very reproducible.

Exposure of the Heme in Cytochrome Oxidase. The solvent perturbation difference (SPD) spectra in 20% ethylene glycol of ferric and ferrous cytochrome oxidase and of the oxidized and reduced chymotryptic digests of cytochrome *c* are shown in Figure 1. The spectrum beyond 500 nm (not shown) is generally flat due to the low extinction coefficient of the heme in this region and to the small exposure of the heme to perturbants (see Tables II and III). As observed previously for cytochrome *c* (Stellwagen, 1967), the main effect of the perturbant on cytochrome oxidase is to cause an enhancement of the Soret absorbance. Similar spectra are obtained in 20% glycerol

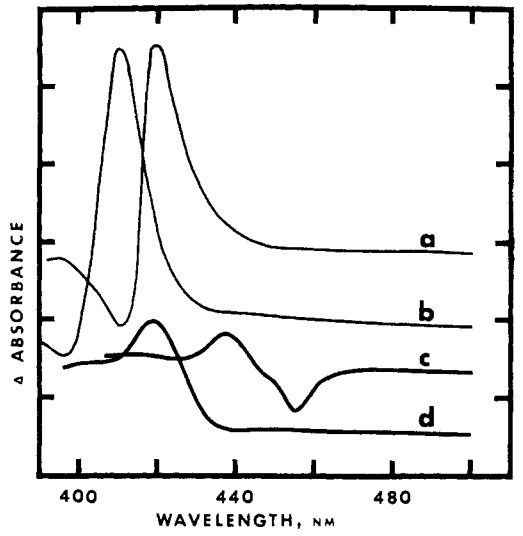

FIGURE 1: SPD spectra of cytochrome oxidase and cytochrome *c* chymotryptic digest in 20% ethylene glycol. The spectra are displaced along the ordinate to minimize overlapping: (a) ferrocytochrome *c* chymotryptic digest, 0.25×10^{-5} M; (b) ferricytochrome *c* chymotryptic digest, 0.55×10^{-5} M; (c) ferrocytochrome oxidase, 1.0×10^{-5} M; (d) ferricytochrome oxidase, 1.0×10^{-5} M. The buffer was 0.05 M phosphate (pH 7.4). In addition, cytochrome *c* chymotryptic digests contained 0.05 M imidazole and cytochrome oxidase solutions contained 0.5% Tween-20. Each unit on the ordinate represents a change in absorbance of 0.01.

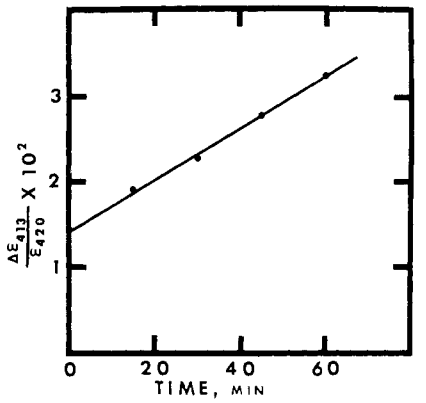

FIGURE 2: Variation of $\Delta\epsilon/\epsilon$ values of ferricytochrome oxidase with time. The perturbant is 20% sucrose and the protein concentration is 1.1×10^{-5} M in 0.05 M phosphate buffer (pH 7.4) containing 0.5% Tween-20. Time after mixing the protein with perturbant is plotted on the abscissa.

and in 20% sucrose except that the position of the difference maximum for the oxidized enzyme occurs at 413 nm for the latter two perturbants rather than at 420 nm as found for ethylene glycol. The cytochrome *c* chymotryptic digest exhibits identical maxima for all three perturbants in either the oxidized or reduced state. Difference maxima in the reduced enzyme were located at 444 and 446 nm for the perturbants sucrose and glycerol, and at 438 nm for ethylene glycol. These results along with the $\Delta\epsilon/\epsilon$ values calculated for the various species examined are summarized in Tables II and III. Each of the values in these tables represents the average of three to seven determinations with an average deviation of ±10%.

The results show an exposure of the heme in ferric cytochrome *c* oxidase of about 30% relative to the chymotryptic digest of cytochrome *c*. It was noted during the course of these experiments that the magnitude of the ferric oxidase SPD spectrum in sucrose and in glycerol increases with time. The increase was found to be linear for approximately the first 60 min, after which it rapidly levels off. A plot of $\Delta\epsilon/\epsilon$ *vs.* time for ferric cytochrome oxidase in 20% sucrose is shown in Figure 2. The values of $\Delta\epsilon/\epsilon$ for glycerol and sucrose in Table II were calculated from the intercepts of similar plots at zero time. No change with time was observed for ferricytochrome oxidase in 20% ethylene glycol or for ferrocytochrome oxidase in any of the three perturbants.

The SPD spectra of ferrocytochrome oxidase was obtained on samples that were fully reduced prior to mixing with the perturbant. This was done to ensure that perturbant molecules able to penetrate certain regions of the macromolecule in the oxidized state would not become "trapped" in those regions during reduction of the enzyme. As noted above, for those cases where the enzyme was fully reduced, the SPD spectrum

TABLE II: Effect of Perturbants on Ferricytochrome Oxidase and a Chymotryptic Digest of Ferricytochrome *c*.

	Ferric Oxidase		Ferric Cytochrome *c* Digest		Relative	Effective Radius (Stellwagen,
Perturbant 20%	$\Delta\lambda_{max}$	$\Delta\epsilon/\epsilon$	$\Delta\lambda_{max}$	$\Delta\epsilon/\epsilon$	Exposure[a] (%)	1967) (Å)
Ethylene glycol	420	0.0146	411	0.0630	23	2.3
Glycerol	413	0.0155	411	0.0570	27	2.6
Sucrose	413	0.0163	411	0.0460	35	3.6

[a] The relative exposures are for ferricytochrome oxidase relative to ferricytochrome *c* digest.

TABLE III: Effect of Perturbants on Ferrocytochrome Oxidase and a Chymotryptic Digest of Ferrocytochrome *c*.

	Ferrocytochrome Oxidase		Ferrocytochrome *c* Digest		Relative	Effective Radius (Stellwagen,
Perturbant 20%	$\Delta\lambda_{max}$	$\Delta\epsilon/\epsilon$	$\Delta\lambda_{max}$	$\Delta\epsilon/\epsilon$	Exposure[a] (%)	1967) (Å)
Ethylene glycol	438	0.0049	420	0.0970	5	2.3
Glycerol	446	0.0038	420	0.1030	4	2.6
Sucrose	444	0.0041	420	0.0690	6	3.6

[a] The relative exposures are for ferrocytochrome oxidase relative to ferrocytochrome *c* digest.

remained constant for all three perturbants for periods of at least 1 hr. Reduction of the cytochrome *c* chymotryptic digests presented some problems. Exposed heme *c* is autoxidizable and therefore a small excess of dithionite is necessary to keep it reduced. Too large an excess, however, causes the Soret absorption band to decrease in intensity. Furthermore, the absorbance at 416 nm is not stable and the spectrum must therefore be obtained shortly after adding the reductant. To overcome these problems, a minimal amount of dithionite was added directly to the cell and the SPD spectrum was immediately scanned. Following this, the absolute spectrum was quickly scanned and compared to a spectrum taken before the addition of dithionite. SPD spectra for the ferrous digest were used only in those instances in which the ratio A_{416}(red)/A_{406}(ox) was at least 1.22, indicating that full reduction had occurred (Margoliash and Frohwirt, 1959).

The results for ferrocytochrome oxidase, summarized in Table III, indicate that the heme in the reduced enzyme is almost totally inaccessible to perturbant molecules. Thus a significant structural rearrangement must occur in the region of the heme cavity when cytochrome oxidase is reduced.

Tables II and III contain the radii of the perturbant molecules used. These radii were taken from Stellwagen (1967) and are based on a finding by Lee (1966) that the perturbation of model chromophores by a flexible molecule is related to the radius of the monomeric unit of that molecule. The results presented in these tables indicate that the opening of the heme cavity does not restrict the entry of perturbants according to size if the molecules have an effective radius of at least 2.3 Å. Attempts were made to use smaller perturbants such as methanol and dimethyl sulfoxide to further probe the size of the opening to the heme cavity. These perturbants, however, were found to cause opalescence in the cytochrome oxidase solutions and therefore were not used.

Effect of Detergent. The effect of varying the concentration of Tween-20 on the perturbability of the heme in cytochrome oxidase was studied to determine if detergent molecules limit the accessibility of the heme to the perturbants. It was found that values of $\Delta\epsilon/\epsilon$ increase with detergent concentration up to about 0.1% Tween-20, after which there is a rapid leveling off. Sedimentation velocity experiments indicate that the low values of $\Delta\epsilon/\epsilon$ at low detergent concentrations is due to aggregation of the enzyme. The response is constant and maximal at detergent concentrations of 0.1–0.5%. Similar experiments in 20% glycerol and 20% ethylene glycol could not be quantitated because of sample scattering in these perturbants at the lower Tween concentrations. Thus, all the values of $\Delta\epsilon/\epsilon$ for cytochrome oxidase reported in Tables II and III were obtained in the presence of 0.5% Tween-20 to eliminate scattering and elicit the maximum response.

Other Measurements. To correctly interpret the results of SPD it is necessary to show that the perturbants do not cause an alteration of the native conformation of the protein. Figure 3 shows that $\Delta\epsilon/\epsilon$ values for all four species studied are proportional to the concentration of ethylene glycol. Only at perturbant concentrations of 40% or higher can significant deviations from linearity be demonstrated for cytochrome oxidase. Concentrations of 50% produce opalescence in the solution.

The effects of 20% perturbants on activity, molar ellipticity, and sedimentation of cytochrome oxidase were also studied. Results of these experiments are summarized in Table IV. The enzymatic activity is not grossly affected by ethylene glycol, glycerol, or sucrose even after exposure to the perturbant for over 1 hr in the case of glycerol. The molar ellipticity

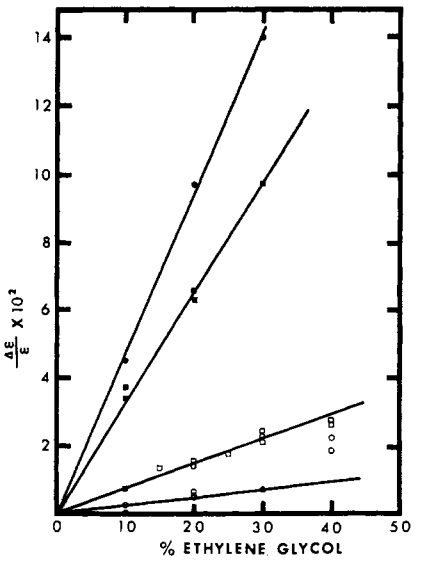

FIGURE 3: Effect of ethylene glycol concentration on $\Delta\epsilon/\epsilon$ values for cytochrome oxidase and cytochrome *c* chymotryptic digest: (●) ferrocytochrome *c* chymotryptic digest, 420 nm; (■) ferricytochrome *c* chymotryptic digest, 411 nm; (□) ferricytochrome oxidase, 420 nm; (○) ferrocytochrome oxidase, 438 nm. The buffer for cytochrome *c* chymotryptic digest was 0.05 M phosphate (pH 7.4) containing 0.05 M imidazole. The cytochrome oxidase buffer was 0.05 M phosphate (pH 7.4) containing 0.5% Tween-20. Protein concentrations ranged from 1.0 to 1.17 × 10^{-5} M.

of the oxidase in the Soret is similarly unaffected. Only the sedimentation coefficients show great variability. Curvature in the base line of the schlieren patterns obtained in the presence of perturbants (especially sucrose) indicates that the perturbant molecules are redistributing themselves under the influence of the centrifugal field, a phenomenon which has been described by Schachman (1959). Thus the sedimentation coefficients in the presence of perturbants are probably anomalous. It is perhaps significant that the base line curvature is greatest for sucrose and least for ethylene glycol, while the $s_{20,w}$ value in ethylene glycol is closest to the buffer value and the $s_{20,w}$ in sucrose is farthest. In no case was there any evidence of heterogeneity.

Discussion

Although it is well recognized that the heme group in cytochrome oxidase is buried in the molecule (Morrison and Horie, 1964; Tzagoloff and Wharton, 1964; Takemori and

TABLE IV: Effect of Perturbants on Some Physical Properties of Cytochrome *c* Oxidase.

Perturbant 20%	Activity (electrons min^{-1} $heme^{-1}$)	[θ] λ_{max}	[θ] (deg cm^2)/dmol	$s_{20,w}$
No perturbant	4450	426	132,000	13.8
Ethylene glycol	4300	425	130,000	12.3
Sucrose	4500	426	133,000	8.66
Glycerol	4400 (4350)[a]	427	132,000	11.4

[a] Measurement made 75 min after mixing cytochrome oxidase with the perturbant.

King, 1965; Yamamoto and Orii, 1973a), there are at present no quantitative estimates of the degree of heme exposure, and particularly of any change in exposure, between the oxidized and reduced forms of the enzyme. Stellwagen (1967) has previously used the technique of solvent perturbation difference spectroscopy to show that the heme group in cytochrome *c*, although buried, is accessible to a number of perturbant molecules of various sizes and concluded that a portion of the heme extends out of the cavity and is able to interact with the solvent. These conclusions have recently been confirmed for cytochrome *c* by X-ray crystallography (Takano *et al.*, 1971, 1973). Thus SPD has been demonstrated to be a reliable probe for the heme environment in hemoproteins.

We have previously shown that cytochrome oxidase undergoes a 3% expansion in molecular volume when it is reduced (Cabral and Love, 1972). In this communication we wish to demonstrate changes in structure with changes in oxidation state at the more localized heme cavity region by employing the technique of SPD.

As expected, the heme was found to be quite buried in the protein. This is in agreement with the results found for other hemoproteins by Stellwagen (1967) and Herskovits (1969) and for cytochrome oxidase by Yamamoto and Orii (1973a). The last authors studied the reactivity of various alkylisonitriles with heme *a* in solution and with ferrous oxidase, and concluded that the heme *a* was buried in the molecule. Our work supports this conclusion but also quantitates the exposure and compares the ferrous to the ferric enzyme.

Using ethylene glycol as the perturbant we find that the heme is approximately 23% exposed in ferric oxidase relative to the fully exposed heme *c* in cytochrome *c* chymotryptic digest. Herskovits (1969) has found that the multisubunit hemoproteins, hemoglobin and catalase, have hemes which are about 10% exposed to ethylene glycol perturbation. Hemoproteins consisting of a single polypeptide chain such as myoglobin, horseradish peroxidase, and cytochrome *c*, on the other hand, all have heme exposures of about 40%. The value obtained for cytochrome oxidase is lower than 40% as expected for a multisubunit enzyme; but, it is considerably higher than the value found for single polypeptide hemoproteins. This suggests that the polypeptide chain associated with the heme in oxidase is situated in an exposed area on the surface of the molecule. It is pertinent to note here that cytochrome oxidase has two heme *a* prosthetic groups. The studies presented here do not resolve the contributions to the SPD spectrum of the two hemes; and thus only the composite response is given.

The use of several perturbants of varying size provides some information about the opening to the heme cavity. As mentioned in the results (and shown in Tables II and III) the heme exposure is not a function of the size of the perturbant used, indicating that the heme cavity does not restrict the entry of molecules having a radius larger than 2.3 Å according to size. This result combined with the fact that the heme is about 25% exposed to these perturbant molecules indicates that a portion of the heme protrudes out of the hydrophobic cavity in the oxidized state.

Cytochrome oxidase undergoes a significant structural reorganization in the region of the heme cavity upon reduction. This is indicated by the fact that the heme in the ferrous enzyme is almost totally inaccessible to the same perturbants which cause a sizable perturbation in the ferric enzyme. This oxidation state dependent change in the heme exposure is similar to the changes observed in cytochrome *c* by Takano *et al.* (1971, 1973). These authors found that the heme in ferricytochrome *c* sits in a crevice with one edge exposed to the solvent. In the reduced state, however, phenylalanine-82 folds into the opening of the crevice, rendering the heme inaccessible. It was further shown that a contraction of the cytochrome *c* molecule accompanies reduction. In the case of cytochrome oxidase a closing of the heme cavity upon reduction is also observed. Unlike cytochrome *c*, however, the molecule is more expanded in the ferrous than in the ferric oxidation state (Cabral and Love, 1972). Van Buuren *et al.* (1972) have claimed on the basis of cyanide binding that the heme is more buried in the oxidized than in the reduced form. Although we have no explanation for this, we feel that SPD is a more direct and proven means of determining the exposure of groups. The idea of an expanded molecule having a less accessible heme is reasonable in light of studies by Perutz *et al.* (Muirhead and Perutz, 1963; Perutz, 1970; Perutz and Ten-Eyck, 1971) on oxy- and deoxyhemoglobin. Although the molecular volume of deoxyhemoglobin is about 8% greater than it is for the oxy form, the heme group of the β subunit is less accessible to ligands in the expanded molecule due to the movement of valine E11 into a position that blocks the ligand site. Based on the results for hemoglobin and cytochrome *c* it is interesting to speculate that the heme in the reduced form of cytochrome oxidase is less accessible because of the movement of a hydrophobic residue into the heme crevice in such a way as to block the opening to the crevice. In the absence of SPD data with small perturbant molecules, however, we can reach no conclusion regarding the accessibility of the heme to oxygen and carbon monoxide.

Attention should be given to some of the difficulties involved in these studies. Cytochrome oxidase is a complex multisubunit enzyme which is only solubilized in the presence of detergents. The possibility that contaminants or detergent affect the results must always be considered. Contaminants are not believed to be a significant factor in the results presented here, since the values obtained for $\Delta\epsilon/\epsilon$ are highly reproducible not only from run to run with the same preparation but also when several preparations of varying purity are used. Even a highly purified King preparation gave the same results. The effect of detergent is more difficult to assess. Although it was shown that the concentration of Tween-20 used gives the maximal response, the possibility that detergents sequester the heme to some extent cannot be excluded. There are indications that the heme in cytochrome oxidase is bound to one or more of the hydrophilic subunits (Schatz *et al.*, 1972). However, it is more likely that the detergent binds primarily to the hydrophobic regions of the molecule and causes little masking of the heme crevice. Another possible interpretation of the solvent perturbation results is that the preparation contains a large fraction of denatured oxidase with a fully exposed heme in addition to the native enzyme with a completely buried heme. This interpretation is extremely unlikely because different preparations give similar results and because in the reduced enzyme the heme becomes almost totally inaccessible. Thus, if some denatured oxidase exists in the preparation, it cannot account for more than about 5% of the total protein. We also do not find that lipid significantly affects the SPD results. Cytochrome oxidase preparations with widely varying lipid contents yield identical data with all perturbants tested.

The change with time in the SPD spectrum of ferricytochrome oxidase in 20% glycerol and 20% sucrose raises questions about the effect of these perturbants on the oxidase molecule. We do not believe that major structural changes are occurring since properties sensitive to the conformation of the

enzyme remain the same in the presence or absence of these perturbants (see Table IV). More likely, some interaction is occurring between the perturbants and the portion of the heme exposed to the solvent. The fact that values of $\Delta\epsilon/\epsilon$ for ferricytochrome oxidase in 20% sucrose and 20% glycerol obtained by extrapolation of the data to zero time (Figure 2) are very reproducible and agree with the value obtained in 20% ethylene glycol, which does not show these time-dependent changes, leads us to believe that this is a valid procedure for obtaining the exposure of the heme in these perturbants. That these changes exist at all is a further indication that the heme in ferricytochrome oxidase is partially exposed. Their absence in the reduced enzyme reinforces the idea that the heme in this species is completely buried.

We conclude that the heme in ferricytochrome oxidase is buried in the protein but is situated in such a way that a small portion of it can interact with the solvent. Upon reduction a significant structural rearrangement occurs which renders the heme amost totally inaccessible to large solvent molecules. The relationship of these changes to the changes observed by others for cytochrome *c*, and their significance to the mechanism of action of the enzyme remain to be elucidated.

Acknowledgments

We thank Dr. T. T. Herskovits for his suggestions and discussion while this work was in progress. We are indebted to Dr. Alan Poland, Department of Pharmacology, for his generosity in making available the Cary 118 spectrophotometer used in these studies.

Added in Proof

After submission of this manuscript we became aware of a very recent solvent perturbation study of ferric cytochrome oxidase by Yamamoto and Orii (1973b). Although we have also found that the heme is sequestered in the ferric enzyme, quantitative comparison is not possible, since we have adopted different reference states for the fully exposed heme. Furthermore, Yamamoto and Orii have not presented SPD data on the ferrous enzyme.

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