Effects of Protein-Protein and Protein-Lipid Interactions on Heme Site Conformation in the Mitochondrial b Cytochromes*

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Removal of lipid from detergent-solubilized succinate cytochrome c reductase by a mild method leads to a series of changes in the optical and EPR spectra of the b cytochromes. This culminates in a state that resembles purified b cytochromes from the same source and bisimidazole ferriheme model complexes. Reconstitution of the lipid-depleted complex with phospholipid restores the native spectra in a significant fraction of the complexes in the early stages of lipid depletion. Once the final state has been reached, however, reconstitution has so far been incapable of restoring the complex to its native conformation. The results described in this communication can be related to a model for integral membrane cytochromes.

The b cytochromes of the inner mitochondrial membrane represent heme sites attached to an integral membrane protein. These electron carriers have properties, including redox potential, pH dependence, and spectral characteristics, which are atypical of b type heme sites in soluble proteins (1-4). Two such cytochromes are present in ubiquinol-cytochrome c reductase (cytochrome bc complex). Cytochrome b562 (b1) has an \( E_m \) of about 0 mV in isolated QH2-cytochrome c reductase, whereas b560 (b2) has an \( E_m \) of \(-100\) mV (4).

Both these cytochromes remain associated with the succinate-cytochrome c reductase or QH2-cytochrome c reductase when they are separated from the mitochondrial membrane with deoxycholate or Triton. Purified b cytochromes can be prepared by using higher detergent concentrations. However, the purified cytochromes have very different properties than the cytochromes in more intact systems, notably carbon monoxide sensitivity, redox potentials, spectroscopic features, and the ease of loss of the heme group.1 Presumably, protein-protein and protein-lipid interactions within the complex maintain the native conformation; in the absence of such interactions, the midpoint potential, optical and EPR spectra, stability, and other characteristics change. Cytochromes c and c1, on the other hand, can be purified in a form apparently identical to the native state by these criteria.

The behavior of the mitochondrial b cytochromes on solubilization is not unique, however. It is also difficult to prepare b cytochromes in the native state from bacterial bc complexes and chloroplast b562 complexes. Dissociation of subunits often results in the loss of prosthetic groups or results in profound changes in thermodynamic properties. Succinate dehydrogenase, NADH dehydrogenase, and cytochrome oxidase are examples of systems in which native conformation is not retained after dissociation by the criteria listed here.

Recent communications (5, 6) reported the effects of serial removal of lipid on the spectral characteristics of cytochrome oxidase. Subtle changes in the EPR characteristics of the CuA and the a, a2 heme sites were found; these undoubtedly reflect changes in the conformation of the polypeptide chain, which depends on phospholipid-protein interactions. Lipid depletion has also been shown to affect the activity of succinate cytochrome c reductase (7). In this communication, we report the effects of lipid depletion on the b cytochromes of mitochondrial succinate cytochrome c reductase and describe further changes which are observed during purification. These changes are much more pronounced than those observed in cytochrome oxidase. For the interpretation of results, we have applied the classification scheme developed by Pfeifer and co-workers (8, 9) based on the model of Griffiths (10) for low spin ferriheme complexes. We have also used lineshape analysis techniques described more fully in recent communications (11, 12). The relationship between the results presented here and recent findings based on physical measurements (12, 13, 16), model complex studies (13-17), and sequencing data (18-20) will be explored.

MATERIALS AND METHODS

Reagents were purchased from Sigma and were of the highest available purity. All EPR samples were frozen in isopentane/cyclohexane (5:1) cooled with liquid nitrogen until somewhat viscous. EPR experiments were performed on a Varian E-109 spectrometer. Temperature control was obtained using an Air Products flowing helium cryostat.

EPR lineshapes of the cytochromes were simulated using a recently described model (11, 12) in which the low temperature \( g \)-strain type line width is assumed to be due to variability in the heme ligand field parameters. Distributions in the rhombic parameter appear to be more important than distributions in the tetragonal parameter. The nonlinear relationships between ligand field and magnetic field position in some cases produce unusual lineshapes that carry information about the iron coordination field strength and geometry.

Ligand field parameters \( V \) and \( \Delta \), respectively, refer to the rhombic and tetragonal splittings within the \( t_{2g} \) set of ferric d orbitals in units of the spin orbit coupling parameter. For iron, this is about 410 cm\(^{-1}\) in the pure ionic limit and can be expected to be decreased somewhat by covalency. The choice of coordinate axes and sign convention is as in the previous communication (12). It is more important to maintain a consistent system of reference than to choose a proper axis system for each individual species, since comparison of the ligand field parameters would otherwise be difficult.

Pseudo-absorption lineshapes were generated by adjusting passage conditions using the modulation features of the spectrometer under saturating conditions. Optimum conditions can vary considerably across the spectrum of a single species, mainly due to transition probability considerations.

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We usually use the second harmonic mode to acquire such spectra. The essential features of the procedure are simple. Select a temperature at which good quality unsaturated signals can be obtained. Adjust the modulation phase control so that no signal is observed under nonsaturating conditions (90° out of phase). Increase the power and/or lower the temperature until saturation causes a signal to appear, then adjust the modulation amplitude, temperature, and microwave power to obtain the optimum spectrum.

The pseudo-absorption spectrum obtained is dependent on the differences in saturation between modulation phases when the field sweep is moving toward the line center and phases when the field sweep is moving away from the line center. It is usually distorted relative to the absorption spectrum because of differences in saturation across the line, but allows broad features to be observed and their approximate $g$ values determined.

Succinate-cytochrome $c$ reductase (SCR)$^2$ was prepared as previously described (21, 22) with minor modifications. Cytochromes $b$ were isolated from SCR according to Ref. 24. Phospholipid was prepared as previously described from SCR (22). It was determined in various SCR preparations as their phosphorus content using 775 as the average molecular weight.

Lipid-depleted SCR was prepared by mixing 10-11 mg/ml SCR with 0.5% sodium cholate and 20% glycerol. Ammonium sulfate was added to 25% saturation, and the mixture was centrifuged at 30,000 rpm (average acceleration 250,000 x g) for 30 min. The small amount of precipitate formed, if any, was discarded, and the supernatant was brought up to 50% saturation with additional ammonium sulfate; the pH of the mixture was adjusted to 7.8 with NH$_4$OH. This fraction was centrifuged as before, and the resulting precipitate was dissolved in 0.5% cholate, 1 mM EDTA, 0.1 M Tris-HCl at pH 7.8. The ammonium sulfate fractionation was repeated at 40-48% saturation; five or six cycles were needed to produce a "complete" lipid-depleted preparation (containing less than 0.25% phospholipid) in which no "native" cytochrome $b$ remained by spectroscopic criteria, although some changes could be seen even after only one or two cycles of cholate/ammonium sulfate fractionation. The final lipid-depleted sample was dissolved in 0.5% cholate or 1% Triton X-100 with 50 mM phosphate buffer at pH 7.4. All procedures were performed at 0-5°C.

**RESULTS**

Fig. 1A shows the EPR spectrum at 8 K of lipid-sufficient (about 20%) SCR in the $g = 3$ to $g = 4$ region. As described in a recent communication (12), the spectrum can be simulated with only three low spin cytochrome components. Cytochrome $b_{562}$ contributes the sharp asymmetric peak at $g = 3.78$. Overlapping peaks are contributed by cytochrome $b_{566}$ at $g = 3.44$ and cytochrome $c$, at $g = 3.33$. EPR and optical spectra of this preparation closely resemble those of equivalent carriers in mitochondria, suggesting that the conformation of the electron carriers in SCR is close to native.

Removal of lipids led to major changes in the EPR and optical spectra of the $b$ cytochromes. Fig. 1B shows the EPR spectrum, recorded at the same temperature, of lipid-depleted SCR dispersed in cholate (SC-SCR) in the presence of glycerol. The peaks due to the $b$ cytochromes have broadened, and the center of the overlapped peaks has shifted somewhat to higher field. This in general corresponds to an increase in the width of the $V$ distribution and in the average value of $V$ for both $b$ cytochromes (12). However, in the early stages of lipid depletion, the increased rhombicity caused by both these factors applies only to cytochrome $b_{566}$. This can be demonstrated by computer simulation, which will be discussed later. The spectrum of the same sample at 14 K is shown in Fig. 1C. The $g = 3.4$ peak is greatly enhanced relative to the $g = 3.3$ peak. The abbreviations used are: SCR, antimycin-sensitive succinate-cytochrome $c$ reductase; SC-SCR, lipid-deficient SCR solubilized in 0.5% sodium cholate in the presence of 20% glycerol unless otherwise described; T-SCR, lipid-deficient SCR solubilized in the presence of 1% Triton in the presence of 20% glycerol unless otherwise described; mW, milliwatt.

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**Fig. 1. EPR spectra of SCR preparations showing the effects of lipid depletion.** Conditions were: field scan, 0.13-0.4 Tesla; scan time, 4 min; modulation amplitude, 10 Gauss; time constant, 0.25 s; microwave power, 20 mW. A, SCR dispersed in cholate, no additions; temperature, 8 K; cytochrome $b$ concentration, 86 x 10^{-6} M in terms of heme. B, lipid-depleted SCR dispersed in cholate with glycerol, temperature, 8 K; cytochrome $b$ concentration, 75 x 10^{-6} M. C, as in B except temperature was 14 K. D, as in B except without glycerol. E, lipid-depleted SCR dispersed in Triton X-100, with glycerol; temperature, 8 K; cytochrome $b$ concentration 130 x 10^{-6} M. F, as in E except temperature was 14 K. G, as in E except without glycerol. The 3.8 feature, obviously the result of differences in power saturation which will be explored later. In Fig. 1D the EPR spectrum at 8 K of lipid-depleted SC-SCR in the absence of glycerol is shown. The spectrum is similar to that in Fig. 1B, but the changes are more pronounced in the $g = 3.7-3.8$ region. The spectra are broader and considerable definition has been lost.
the 560-563 nm region than lipid-sufficient SCR (not shown).

Fig. 2 shows the detailed lineshape of the native SC-SCR preparations in the g = 3 to g = 4 region. Fig. 2A is the spectrum of native SCR at 10 K and 100 mW. The low temperature and high power conditions emphasize the contribution of the b cytochromes, especially cytochrome b_{560} at g = 3.8, Fig. 2, B and C, shows the EPR spectra of lipid-depleted SCR dispersed in Triton in the presence of glycerol at 100 and 5 mW, respectively. Power saturation characteristics vary continuously across the lines due to variation in relaxation times and transition probability. In this situation, especially with overlap from a saturated resonance at g = 4.2, detailed power saturation plots are probably less meaningful. However, it is clear that the g = 3.7 region is more difficult to saturate than the g = 3.4 region. It is apparently slightly easier to saturate the g = 3.7 region than the sharp g = 3.8 peak of cytochrome b_{560}, whereas the saturation of the g = 3.4 region is comparable to that of cytochrome b_{562}. Fig. 2, D and E, shows the corresponding spectra of lipid-depleted SC-SCR with glycerol. The spectra are similar to 2, B and C.

Fig. 3 shows computer simulations of the EPR spectra of the b cytochromes in lipid-extracted SCR (see "Materials and Methods"). We have not attempted to display simulations of all the lipid-depleted states which were observed, although these can be generated by adjusting the parameters slightly. Instead, the simulations shown are representative of states encountered in the early stages of lipid depletion as illustrated in Fig. 2, C and D. Two b cytochrome components were needed to reproduce the experimental spectra of Fig. 2. The initial changes observed in the EPR spectra are clearly mainly due to cytochrome b_{560}. The observed line broadening and g value shift is initially due to an increase in the center of the V distribution from near zero to 0.4; the increased line width at this stage is not due to an increased width in the V distribution, but merely to a shift in the center of the distribution to a region in which the derivative of g, with respect to V is larger. The parameters used to simulate the cytochrome b_{560} component at this stage fall within the normal range for lipid-sufficient SCR, and may even become slightly more axial initially. At later stages of lipid extraction, both the b cytochrome simulation parameters must be modified. The major differences between these simulations and the previously reported simulations of the EPR spectra of the cytochrome components of native SCR (11, 12) are increases in the widths of the distributions in V and shifts in the centers of the distributions toward higher values of V. The simulations suggest that the b cytochromes remain distinguishable during the first stages of lipid extraction. The ability of the protein conformation to enforce the native ligand geometry at the heme site is reduced, however, leading to a broadening of the distributions and therefore of the EPR lines. The effects of serial removal of lipid on the EPR spectra of the b cytochromes are illustrated in Fig. 4. Fig. 4A shows the spectrum of native SCR; Fig. 4, B–D shows spectra after increasing cycles of lipid extraction. As lipid was removed, the relatively sharp spectra of the b cytochromes in native SCR gradually broadened and coalesced, with the peak g, value moving slightly upfield. A small amount of intensity began to appear at higher field (g < 3.0) as more lipid was extracted (see Fig. 1G).

Reconstitution of lipid-extracted SCR partially restored the native sharp spectra. If reconstitution was attempted at an early stage (corresponding to the broad EPR spectra near g = 3.4), 20–40% of the intensity of the sharp spectra can be restored (see Fig. 4E). If the g < 3.0 form was reached, reconstitution with lipid was so far unsuccessful in restoring the intensity of the native EPR spectra. The signal to noise ratio of the trace decreases because the concentration of native b cytochromes is lower after the extraction/reconstitution. The pain has been increased to compensate for this. In contrast, the native EPR spectra cannot be restored in
cytochrome oxidase after lipid depletion, although partial activity is restored by reconstitution; an explanation has been put forward (5).

The effect of ammonium sulfate/cholate fractionation on the enzymatic activities and the removal of phospholipids is summarized in Fig. 1 of Ref. 7. It was clear that the activities were parallel to the lipid content. Likewise, the absorption spectra of cytochrome b in the reductase were dependent upon the phospholipid content of the preparation as shown in Fig. 5. The difference spectra of succinate-reduced minus ascorbate-reduced samples were presented in order to show the change more explicitly since ascorbate under these conditions only reduced cytochrome c, which was not affected by ammonium sulfate/cholate fractionation (cf. Fig. 1 of Ref. 7). The system contained 50 mM 0.5% cholate/phosphate buffer, pH 7.4, in protein concentration of 3.75 mg/ml for SCR; 4.75 mg/ml for the first cycle; 5.25 mg/ml for the second cycle; 4.75 mg/ml for the third cycle; and 3.5 mg/ml for the fourth cycle. (The figure was kindly supplied by Yan Xu.)

to gross changes in the EPR and optical spectra. Fig. 6, bottom, shows the spectrum of such a cytochrome b preparation containing two "b cytochromes" by the criteria of molecular weight, spectral properties, and oxidation/reduction characteristics. Recent work strongly suggests that both b hemes reside on a single polypeptide (18-20). The origin of the two heme proteins seen here is unclear (25, 31): that it reflects degradation or the migration of one heme to another polypeptide remains to be proved. The optical spectra (not shown) are again of lower resolution in the 560-563 nm region than those of SCR or complex III.

As shown in Fig. 6, bottom, the EPR spectrum is dominated by a single low spin component with features near 2.95, 2.24, and 1.5 on the g value scale; a high spin component with features near g = 6.0 and 2.0 corresponds to a small fraction of the heme present. While the low spin species is similar in some respects to cytochrome b, it exhibits a broader line width that enhances the asymmetry of the peaks. Particular attention should be paid to the long low field tail which extends several hundred gauss until it is cut off near g = 3.8. The complex shape of this tail, which decays more steeply between g = 3.7 and g = 3.8 than in the g = 3.4 region, is of special interest.
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Fig. 6. EPR spectra of SCR and purified b cytochrome. Conditions as in Fig. 1A except temperature was 13 K. Top, native SCR; bottom, purified b cytochrome (see "Materials and Methods"); this preparation (bIII) contains two b peptides, bL and bS; heme content is 67 x 10^{-6} M.

![EPR spectra of SCR and purified b cytochrome](image)

Fig. 7. EPR spectra of SCR and purified b cytochrome using second harmonic out of phase mode under saturating conditions to obtain rapid passage effects. Top, native SCR; temperature, 8 K; microwave power, 50 mW. Bottom, purified b cytochrome; temperature, 14 K; microwave power, 20 mW.

EPR spectra in the second harmonic mode under saturating conditions are displayed in Fig. 7. Rapid passage conditions were obtained using the field modulation; the resulting absorption shaped lines are useful in detecting broad features. In Fig. 7, top the spectrum of SCR is shown; note the sharp rise in the g = 3.4-3.8 region and the long high field tail suggesting |g| values below |g| = 1.0. Fig. 7, bottom, depicts the spectrum of the b cytochrome preparation of Fig. 5, bottom. The spectrum shown in Fig. 6, bottom, was obtained at slightly higher temperatures to enhance the bL type features. All three g values are clearly visible.

Fig. 8 shows power saturation plots of cytochromes b562 and b566 in different preparations at an indicated temperature of 7.5 K. Signal intensity was measured at the g values below |g| = 1.0. Fig. 8, bottom, compares the spectrum of the b cytochrome preparation of Fig. 5, bottom. The spectrum shown in Fig. 6, bottom, was obtained at slightly higher temperatures to enhance the bL type features. All three g values are clearly visible.

**Fig. 8. Power saturation of mitochondrial b cytochromes at an indicated temperature of 8 K.**

- **Top**, g = 3.78 peak of cytochrome b562 in native SCR; +, g = 3.4 peak of cytochrome b562 in lipid-depleted SCR; ▲, g = 3.4 peak of cytochrome b562 in native SCR, identical to g = 3.78 peak of cytochrome b562 in lipid-depleted SCR.

Therefore appeared to sharpen. The saturation of the broad features seen in lipid-depleted SCR followed this general tendency. The low field edge of the signal in these preparations was nearly as difficult to saturate as the sharp peak of cytochrome b562. Experiments showed that the addition of ubiquinone together with phospholipid did not affect the results presented.

**DISCUSSION**

From the data presented, it is evident that the mitochondrial b cytochromes undergo a series of alterations during lipid depletion. Clearly, it is unlikely that phospholipids interact directly with the heme site; rather, phospholipids are in many cases important in stabilizing the native conformational states of membrane proteins. Our results indicate that heme site geometry is sensitive to destabilization of native conformation. The final state reached after repeated extraction resembles the purified b cytochromes by spectroscopic criteria. Several conclusions can be drawn from these observations.

First, the EPR spectra clearly reflect not merely the axial ligation of heme, although this is certainly of importance, but the ligand geometry imposed by the polypeptide. The gradual and partially reversible changes reported here cannot be the result of ligand changes alone; the number of candidates for ligation schemes would be too small for such a series of changes to arise. The participation of axial ligand changes at some point cannot be rigorously excluded, but there is no real evidence for such shifts in this case.

Second, lipid-protein interactions are clearly of great importance in maintaining the native conformation of the b cytochromes. The effects described here are much more profound that those observed in lipid-depleted cytochrome oxidase. However, as should be clear from the discussions to follow, the extent of the effects observed in the two systems is probably as much a measure of the degree of strain imposed in each case as of the relative sensitivity to lipid extraction. Cytochrome c has a ligand field geometry that differs only moderately from that of corresponding model complexes. The geometry of the b cytochrome heme sites is much more strained, so that relaxation of polypeptide-imposed constraints might be expected to produce much larger effects. Lipid extraction eventually leads to an increase in a heme...
component with a $g_s$ in the 2.9–2.95 region in both systems. The purified $b$ cytochrome preparations have interesting features. In some preparations (Fig. 6), there is apparently a single dominant low spin component; a high spin component is present at low concentration. The similarities between this species and cytochrome $b_6$ are obvious; ferric bisimidazole heme model complexes also fall into the same general class. Using the turning point $g$ values, we obtain $V = 1.8$, $\Delta = 3.3$ in units of the spin orbit coupling parameter ($\lambda$). We note that the peak position is not necessarily the center of the $V$ distribution for asymmetric lines, however. The interesting asymmetry of the peaks can be modeled using a distribution in $V$ as the primary source of low temperature line width, as in the previous paper (12). Since $V$ is large, the $V < V$ side of the distribution is the region of lesser slope ($g_s$ approaches an asymptote as $V$ increases without bound). Therefore, the low field side of the line is broader. The changes in slope seen in the $g = 3.4–3.8$ region are due to the $V = 0$ turning point in the plot of $g_s$ versus $V$. While the qualitative lineshape features can be accounted for in this way, the asymmetry of the peak is more pronounced than can be accounted for by a gaussian distribution in $V$. This is a characteristic of rhombic species in general and will be discussed in a later section.

Other purified preparations have a small amount of residual intensity in the $g = 3.4$ region not associated with the tail of the main peak.

Clearly, the majority species in the purified $b$ cytochrome preparations and the $g = 2.95$ minority species in the lipid-depleted preparations are cases of bishistidine ligation. The ligand field parameters of these species fall into the range of those reported for other bisimidazole systems. The sharp low field ($g_s > 3.2$) $b$ cytochrome spectra in native SCR can be converted to the conventional bisimidazole species through a series of gradual steps. Even in the final state, the broad $2.95$ feature has a tail that extends all the way out to $g_s = 3.8$. The molecules absorbing in the region covered by the tail represent a continuous distribution of crystal field parameters; clearly, a distribution of $V$ values is needed to reproduce the lineshape. The low field edge of the tail is consistent with the upper limit of $g_s$, for $\Delta = 3.3\lambda$, the value calculated from the principal $g$ values. A distribution in $V$ with $V = 1.6\lambda$ and a half-width of $0.6\lambda$ produces reasonable simulations of the $g_s$ lineshape of the $g = 2.95$ species (not shown); the lower value of $V$ obtained is in agreement with the nonlinear relationship between $V$ and field position.

It seems likely that the "low $V$" distributions of cytochromes $b_{600}$ and $b_{690}$ reflect a strained heme geometry imposed by the polypeptide. Conformational changes induced during purification or lipid extraction allow some or all of the heme sites to relax to a state with a more rhombic (higher $V$) character. The lines are much broader than the corresponding features in the spectrum of cytochrome $b_6$, however. This implies that the $V$ distribution is broader; either the denatured conformations of each cytochrome have less ability to specify heme geometry or a broad range of denatured conformations are thermally accessible until "frozen in" as the temperature is lowered. We note that the EPR and optical spectra of SCR during the early stages of lipid extraction resemble the spectra of the $b$ cytochromes in cytochrome $b_6$ complex purified from chloroplasts (27). It is unclear if this reflects a less rigid native state in the chloroplast system or if that system is merely more sensitive to changes during purification. In previous communications, we have suggested that $b_{600}$ and probably also $b_{690}$ are bishistidine cases, since $\Delta$ values near 3.3$\lambda$ (similar to known bisimidazole ferrichrome complexes) produce the best fit to lineshape simulations. Other workers have arrived at the same conclusion through independent evidence. This includes analysis of the spectroscopic properties of model complexes (13–16), spectroscopic studies of the QH$_2$ cytochrome c reductase ("complex III") region (12, 13, 16, 28), and modeling based on sequence data (17–19) primarily deduced from nucleotide sequences.

The lineshape analysis of the tail of the isolated cytochrome provides further support for this suggestion. The fact that the $b$ cytochromes relax to an obvious bishistidine form when purified is suggestive in itself; the extended tail supports the explanation of the origins of lineshape asymmetry on which the estimate is based. As we noted earlier, the obvious existence of a continuous range of crystal field parameter could only be explained in terms of ligand geometry; exchanges of ligands could never account for the number of $\Delta$, $V$ pairs needed to simulate the spectra of lipid-depleted SCR and purified $b$ cytochromes. Taken together, these observations indicate that the $b$ cytochromes maintain bishistidine ligation throughout conformational changes manifested in the spectroscopic effects reported here, but that the ligand geometry at the heme iron is profoundly affected (although still, of course, basically octahedral).

The previous communication (12) presented evidence that no complex II type $b$ cytochrome (29) is present in the $bc_1$ complex or SCR. This conclusion is supported by the spectra of SCR presented here; however, a small amount of complex II type species appears as a shoulder on the $b_{690}$ peak in some preparations of SCR (not shown). It seems possible that the complex II type $b$ cytochrome ("$b_{690}$") is a modified form of $b_{600}$ or $b_{690}$. The $g_s$ peak of this cytochrome is near 3.5; midway between those of $b_{600}$ and $b_{690}$; significant amounts of $b_{690}$ should be easily detectable, but were never observed in SCR. The basis of these arguments is that by definition SCR should be a complex of complexes II and III.

The conformational changes responsible for the spectroscopic effects reported here can be related to an emerging model for the integral membrane $b$ cytochromes (12-19, 28). Spectroscopic work on oriented systems has shown that the heme and iron sulfur prosthetic groups associated with mitochondrial complexes I–IV have specific orientations with respect to the plane of the membrane (30–32). The $b$ cytochrome hemes are carried with the plane of the heme perpendicular to the membrane plane (32). The explanation for this, originally suggested to account for the spectroscopic data, is that the prosthetic groups are carried between transmembrane helical segments of the polypeptide (30, 31). The heme orientation would then be constrained to be perpendicular to the membrane to within the accuracy imposed by the disorder of available oriented samples (usually 5–10°). Recently, models constructed from sequence information have pointed out some potentially important common features of $bc$ type complexes (17–19). In particular, regions corresponding to probable transmembrane helical segments have been identified. Two pairs of histidine residues are arranged to provide two potential heme-binding sites; both hemes would be carried between the same two helical segments.

Evidence that the heme axial ligation is indeed bishistidine has, as previously mentioned, been obtained from a number of sources (11–16, 28). An unusual ligand geometry imposed by the polypeptide must then account for the unusual spectroscopic features of the heme sites. As has been previously pointed out (12, 16, 28), a simple mechanism by which the necessary ligand field changes could be produced involves rotation of the imidazole planes about the heme normal. When the imidazole planes are parallel, the rhombic splitting within the $t_{2g}$ set of iron d orbitals is maximized because of the
differences in the abilities of iron d orbitals in the \( xz \) and \( yz \) planes to \( \pi \) bond. The axial case would correspond to perpendicular orientation of the imidazole planes; tilting of the imidazoles with respect to porphyrin could also contribute to the rhombicity of the iron ligand field. Since the rhombic geometry is observed in model complexes, more axial cases are apparently produced by polypeptide-induced strain which is relieved upon denaturation.

Other integral membrane proteins carry their prosthetic groups in a particular orientation with respect to the membrane. For cytochromes \( b_{566}, b_{568}, c_1, a, \) and \( a_6, \) the heme plane is perpendicular to the membrane plane; as previously pointed out (30, 31), this suggests that the heme is suspended between helical segments oriented normal to the membrane plane. The EPR spectra of integral membrane cytochromes suggest a more axial ligand field than corresponding soluble proteins.

It appears likely that the polypeptide-imposed heme site geometry is likely to produce a more axial imidazole ligation, the crystal structures of the EPR spectra require that the distribution of rhombicities allowable in these cases have an upper limit, which may correspond to the parallel orientation of the imidazole planes. Therefore, any effect that the polypeptide has on the heme site geometry is likely to produce a more axial system.

The integral membrane heme proteins generally exhibit more axial ligand fields than corresponding soluble proteins. In our view, this is a result of the general features of the native conformational states of membrane proteins rather than the presence of the membrane per se, although clearly phospholipids can stabilize this native state. The mechanism by which strain is imposed on the heme sites is, however, of variable effectiveness even among hemeproteins that share the same axial ligation and membrane orientation. This mechanism probably involves constraints imposed on the imidazole planes by the helical segments, including steric interactions and the fact that a histidine imidazole in such a structure would have only the degrees of freedom made available by rotations about two single carbon-carbon bonds with which to make the most favorable geometrical compromise with the heme. Several sources of variability are present, including the identity of the neighboring R groups on the helix, and polypeptide-imposed constraints on the distance between adjacent helices. Of particular importance would be the rotational position (about the helix long axis) of each liganding residue relative to the heme.

In conclusion, these results provide a rationale for the difficulty encountered in purifying b cytochromes in a native conformation. Moreover, the heme site geometry and the function of the b cytochromes in mitochondria are dependent on the effects of protein-lipid and protein-protein interactions. The spectroscopic changes observed upon lipid extraction and reconstitution and dissociation of b cytochromes subunits from SCR are consistent with an emerging model for integral membrane cytochromes. Features of this model have been independently deduced in several laboratories.

REFERENCES


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