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ELECTRON PARAMAGNETIC RESONANCE AND LIGHT ABSORPTION STUDIES ON *c*-TYPE CYTOCHROMES OF THE ANAEROBIC SULFATE REDUCER DESULFOVIBRIO

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SUMMARY

1. EPR and optical studies provide for differentiation of the *c*-type cytochromes examined into three groups with different heme contents. The three groups consist of: the mono-heme type, cytochrome *c*-553; cytochromes c_3 and c'_3 containing at least two hemes per molecule; and cytochromes cc_3 and cc'_3 which contain at least four hemes per molecule. This division into three groups agrees with a previous similar grouping based on amino acid composition and/or sequence.

2. EPR studies suggest that heme–heme interaction is manifest in the ferric state of cytochrome c_3. At about a half-reduced state, an EPR-detectable intermediate with a decreased degree of interaction between hemes is observed.

3. Based on EPR and light absorption changes, cytochrome c_3 is extremely stable in 8 M urea in the ferric state. However, repeated reduction and reoxidation in the presence of 8 M urea results in the apparent conversion of the multi-heme system to a mono-heme system.

4. These and other observations suggest a conformation change involving a possible reorientation of the multi-heme moieties in the partially or fully-reduced tate. Depending on the reactant(s) present this process may be reversible.

INTRODUCTION

For many years, following the discovery by POSTGATE[1] of cytochrome c_3, the cytochrome system of the sulfate-reducing bacteria was thought to be quite simple. Cytochrome c_3 is a unique hemoprotein differing from the mammalian type of cytochrome *c* in its redox potential (–210 mV), the presence of at least two hemes per mole (molecular weight, 13000) and its autoxidizability. Although its physiological role is not completely understood, it has been implicated in sulfite, thiosulfate and sulfate reduction[1–3]. It is also a component of the multi-enzyme system which decomposes formate to CO_2 and H_2[4,5].

The complete amino-acid sequence of cytochrome c_3 from *Desulfovibrio vulgaris*, strain Hildenborough, has been established by AMBLER[6]. Cytochrome c_3 is very different from the other *c*-type cytochromes. Two symmetrical parts in the molecule are discernible, with each part featuring the characteristic sequence for the heme

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attachment of all *c*-type cytochromes: Cys–A–B–Cys–His–. But in addition there is a sequence of the type: –Cys–A–B–C–D–Cys–His– which has not been observed in the other cytochromes. It is also noteworthy that no direct relation appears between the sequence of c_3 and the sequences of "mammalian" *c*-type cytochromes.

Cytochrome c'_3 from *Desulfovibrio gigas* has been isolated and purified[7]. It was found to differ from cytochrome c_3 from *D. vulgaris* in its isoelectric point (5.2 instead of 10.5), a fact previously attributed to a difference in amino acid composition. The establishment of this composition[8] has confirmed this hypothesis: about 50 % of the residues are different. However, in both cytochromes eight cysteine residues are conserved in the same positions. This is also true for all but one of the histidine residues[9].

Another type of cytochrome has been found in *D. vulgaris*, strain Hildenborough[10], and its chemical properties have been described[11]. This cytochrome, *c*-553, has a more positive redox potential than c'_3, is autoxidizable and contains only one heme per molecule (molecular weight, 9000). The physiological function of this cytochrome is still unknown. It is also present in *Desulfovibrio desulfuricans*, strain EL Agheila Z, but has not been detected in *D. gigas*[11].

Still another *c*-type cytochrome has been found in the three species and strains of Desulfovibrio just mentioned[12]. This new type of cytochrome has been tentatively named cc_3 by analogy with the cytochrome system of Rhodospirillum and *Chromatium*[13]. Most of the investigations have been made on cytochrome cc'_3 from *D. gigas*. This cytochrome couples the reduction of thiosulfate by molecular hydrogen in cell-free extracts of *D. gigas* and is several-fold more efficient than either cytochrome c_3 or c'_3. It contains 16 cysteine and 16 histidine residues per molecule with a molecular weight of 26000 and is also autoxidizable. It has been impossible to dissociate the molecule into subunits and its amino acid composition gives little evidence that cytochrome cc'_3 could be a dimer of cytochrome c'_3.

Recently, YAGI[14] has described a new cytochrome isolated from a *D. vulgaris* strain different from that of Hildenborough. The cytochrome, also named *c*-553, has a molecular weight of 6500. It is reduced in the presence of formate by a preparation of formate dehydrogenase from the same organism. In contrast with cytochrome c_3 from other *D. vulgaris*[4,5] strains, the cytochrome c_3 extracted from this organism does not react with formate dehydrogenase. Another cytochrome (molecular weight, 70000) is also present.

We have undertaken a detailed examination of the oxidation–reduction properties of these cytochromes by light absorption and EPR methods to clarify the relative relationships of the described cytochromes and to acquire a clearer understanding for the functional role of the multi-heme systems. The results were also intended to shed some light on the nature of these cytochromes in relation to RPH-type cytochromes and other cytochromes containing at least two hemes per molecule.

A second paper[15] on EPR studies of cytochromes from sulfate-reducing bacteria deals with the possible biological significance of the interaction of various exogenous ligands with cytochrome c_3.

MATERIALS AND METHODS

Culture of the microorganisms

D. gigas and *D. vulgaris* are cultivated in media previously described[7].

For the preparation of the cytochromes, the bacteria are cultivated in a 200-l

AMSCO fermenter. Cytochromes c_3, c'_3, *c*-553 and cc'_3 are prepared according to methods already described[7,8,10,12].

Cytochrome cc_3 is prepared using the following method (all buffers are at pH 7.6). To 1100 g wet weight of cells were added 300 ml of 10 mM Tris–HCl buffer. The resulting suspension was treated in a French pressure cell and the extract obtained diluted with 1 l of the same buffer. 200 ml of Amberlite CG-50, type II, prepared according to HORIO and KAMEN[16], were poured into the diluted extract and the mixture was stirred overnight. The Amberlite, to which the cytochrome c_3 was adsorbed, was removed by low speed centrifugation. The extract was then centrifuged at 30000 × *g* for 1 h in a Sorvall centrifuge, model RC-2. More of the cytochrome c_3 was adsorbed by passing the cleared extract through an Amberlite column (4 cm × 10 cm).

After the removal of almost all the cytochrome c_3, cytochrome *c*-553 could be adsorbed on a silica-gel column (4 cm × 10 cm) equilibrated with 1 mM Tris–HCl buffer, after dialysis of the extract against 20 l of the same buffer. The cytochrome was eluted from the column in a volume of 150 ml, using a 1 M potassium phosphate buffer containing 1 M NaCl.

The proteins not adsorbed on silica gel were passed over a calcinated alumina column (4 cm × 20 cm) equilibrated with 0.1 M Tris–HCl buffer. The column was washed with the same buffer and the protein eluted with 1 M potassium phosphate buffer.

The extract (150 ml) was passed through successive columns of Sephadex G-75 and G-50 (each column 5 cm × 100 cm) and equilibrated with 0.01 M Tris–HCl buffer. Cytochrome cc_3 was separated from a green pigment with maxima at 404 nm and 589 nm. This latter spectrum is very similar to that of sulfite reductase isolated by ASADA *et al.*[17] from spinach leaves. The possible enzymatic activity of this new heme-protein is being tested.

The cc_3 fraction (250 ml) which contains some hydrogenase activity was reduced by hydrogen gas and placed on a DEAE cellulose column (4 cm × 10 cm), equilibrated with 0.01 M Tris–HCl buffer containing 0.5 % 2-mercaptoethanol. The reduced cytochrome was adsorbed on top of the column. The column was washed with 100 ml of the same buffer but without 2-mercaptoethanol. The eluted cytochrome was adsorbed on CM-cellulose (4 cm × 15 cm), equilibrated with 0.01 M Tris–HCl buffer, and eluted with 0.3 M Tris–HCl buffer. After these steps the protein was judged to be more than 98 % pure based on its absorption spectrum and gel electrophoresis. However, cytochrome cc_3 still contained enough hydrogenase activity to be reduced by molecular hydrogen. After lyophilization, 45 mg (dry weight) of cytochrome cc_3 was obtained.

Horse cytochrome *c* was obtained from Sigma Co. A molar absorptivity of 28000 (α peak, ferrous form) was used for calculation of concentration. In the case of cytochromes c_3 and c'_3, a molar absorptivity of 84000 and 76000, respectively, was used, based on the studies of DRUCKER *et al.*[18]. The molar absorptivities for cytochromes *c*-553, cc_3 and cc'_3 were established by comparison with cytochromes *c*, c_3 and c'_3, respectively. Such a comparison is permissible as a first approximation since the amino-acid composition of cytochrome *c*-553 indicates only one heme per molecule (2 cysteine residues) where as cc_3 and cc'_3 each with 16 residues of cysteine may be considered to contain twice the concentration of heme of that present in c_3 and c'_3.

The calculated molar absorptivities were: *c*-553, 36000; cc_3, 168000; cc'_3, 152000.

Absorption spectra were obtained with a Cary Model 15 recording spectrophotometer or a Unicam SP-800 spectrophotometer. Anaerobic measurements were made in Hellma spectrophotometric Thunberg cells of 1-ml volume and 1-cm light path. EPR measurements were made as previously described[19]. Further experimental details regarding absorption and EPR measurements are indicated in the figure legends.

RESULTS

Ferric forms: The optical spectra of cytochromes *c*-553, c'_3 and cc'_3 are shown in Fig. 1. Although the ferric states of each type of cytochrome appear to be qualitatively similar, there are small differences discernible, *e.g.* in the ratio of absorbance of the Soret maximum at 409 nm as compared to the β peak at 524 nm. This ratio is 10.9, 11.9 and 12.5 for *c*-553, c'_3 (or c_3) and cc'_3, respectively. A maximum at 280 nm is observed with *c*-553 but is absent in c_3 (or c'_3) and cc'_3. *c*-553 shows a maximum at 363 nm while c'_3 (c_3) shows maxima at 350 nm. A pronounced maximum (absent in c_3, c'_3 and cc'_3) is observed at 695 nm with *c*-553. Fig. 2 represents the spectra of c_3, *c*-553 and mammalian cytochrome *c* in the 500–700 nm region and clearly indicates a maximum at 695 nm for cytochrome *c* and *c*-553 which is, however, absent in c_3. The presence of this absorption maximum has been atrributed to a possible heme charge transfer interaction with methionine liganded at position 6[20,21]. This absorption maximum appears to be sensitive to changes in conformational states[22,23].

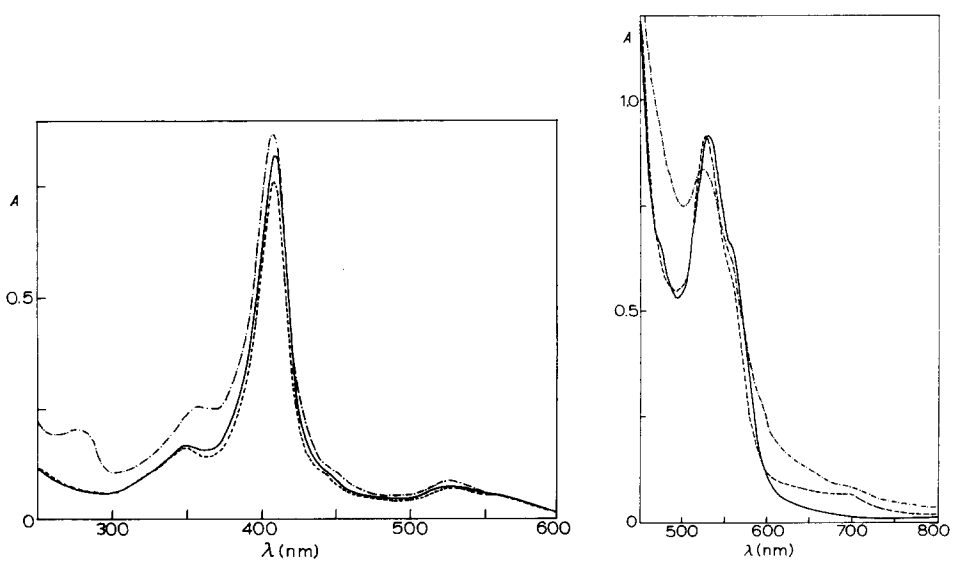


Fig. 1. Absorption spectra of the cytochromes in the ferric form; 10 mM potassium phosphate buffer (pH 7.0). ———, cytochrome cc'_3 (1.3 μM); — — —, cytochrome c'_3 (2.4 μM); –·—·–, cytochrome *c*-553 (5 μM).

Fig. 2. Absorption spectra of ferric forms of the cytochromes in the 500–800 nm region; 10 mM potassium phosphate buffer (pH 7.0) ———, cytochrome c_3 (30 μM); — — —, cytochrome *c*-553 (53 μM); –·—·–, horse heart cytochrome *c* (60 μM).

The EPR spectra of the ferric forms are shown in Figs. 3 and 4. There are three distinct types of EPR spectra obtained. *c*-553 with one heme per molecule exhibits a typical broad low-spin spectrum upper trace (Fig. 3), as has been reported for mammalian cytochrome *c* (ref. 24). The EPR signal of cytochrome *c* sharpens greatly at very low temperatures[25]. c_3 and c'_3 with at least two hemes per mole comprise a

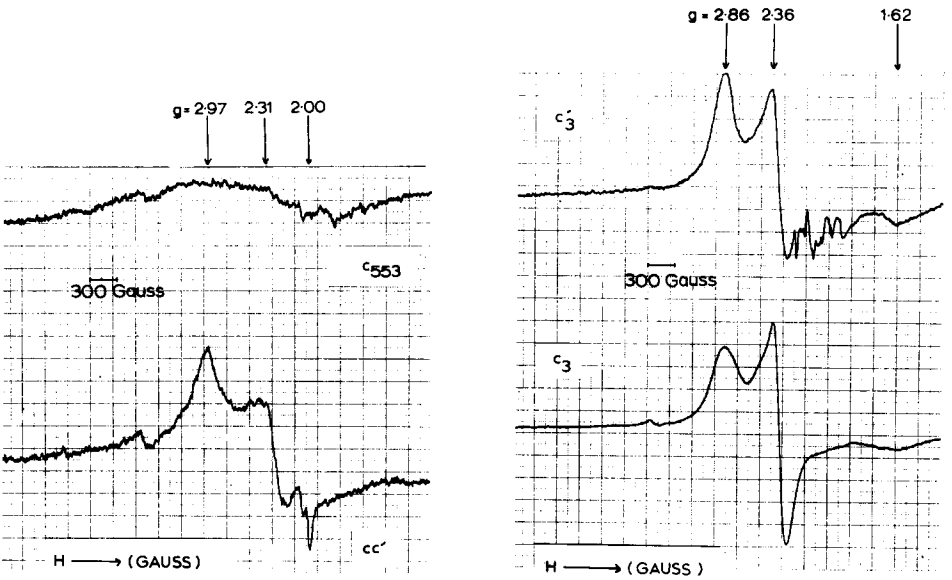


Fig. 3. EPR absorption of ferric cytochrome *c*-553 (upper trace), 5 mg in 0.2 ml 0.10 M potassium phosphate buffer (pH 7.2), and cytochrome cc'_3, 5 mg in 0.2 ml 2 M Tris buffer (pH 8.0). EPR conditions: samples in quartz tubes of 5.0 mm outer diameter; temperature, −180°C; modulation amplitude, 6 G; scanning rate, 1000 G/min; time constant, 0.3 sec; microwave power, 35 mW.

Fig. 4. EPR absorption of ferric cytochrome c_3 from *D. vulgaris* (lower trace) and cytochrome c'_3 from *D. gigas*. Concentrations and EPR conditions as in Fig. 3.

second distinct group of EPR spectra (Fig. 4) exhibiting a low-spin type absorption with three resolved *g*-values. The *g*-values observed are identical for both c_3 and c'_3 and are $g=2.86$, 2.29 and 1.62. The EPR spectra are strictly low spin in the temperature range −20° to −180°. The EPR absorption of c'_3 reveals a small Mn^{2+} EPR signal which is estimated to be less than 1 % contaminant based on protein concentration. The third type of EPR spectrum (lower trace, Fig. 3) is obtained with cytochrome cc'_3. A similar spectrum is observed for cytochrome cc_3. The main *g*-values are at 2.97 and 2.00 and the spectrum is also low-spin in the temperature range, −20 to −180°. Fig. 5 shows the temperature dependence of the EPR signals of cytochrome cc_3 indicating the low-spin character at the temperatures examined. The cc'_3-type cytochrome is sparingly soluble in phosphate buffer and therefore has been examined in 2 M Tris buffer, pH 8, or as a mixture of crystals in suspension. In both instances identical EPR spectra were obtained.

Ferrous forms: The absorption spectra of *c*-553, c'_3 and cc'_3 after reduction with dithionite are shown in Fig. 6. Typical reduced *c*-type spectra are observed. As with the absorption spectra of the ferric forms, there are differences observed

between the three types of cytochromes. For example the Soret maximum for *c*-553 is shifted 2 nm to the near ultraviolet as compared to c'_3 and cc'_3 which have similar Soret maxima. There are additional differing details in the ultraviolet and near ultraviolet regions. In this instance borohydride is the chemical reductant of choice since it has no absorption in these spectrial regions.

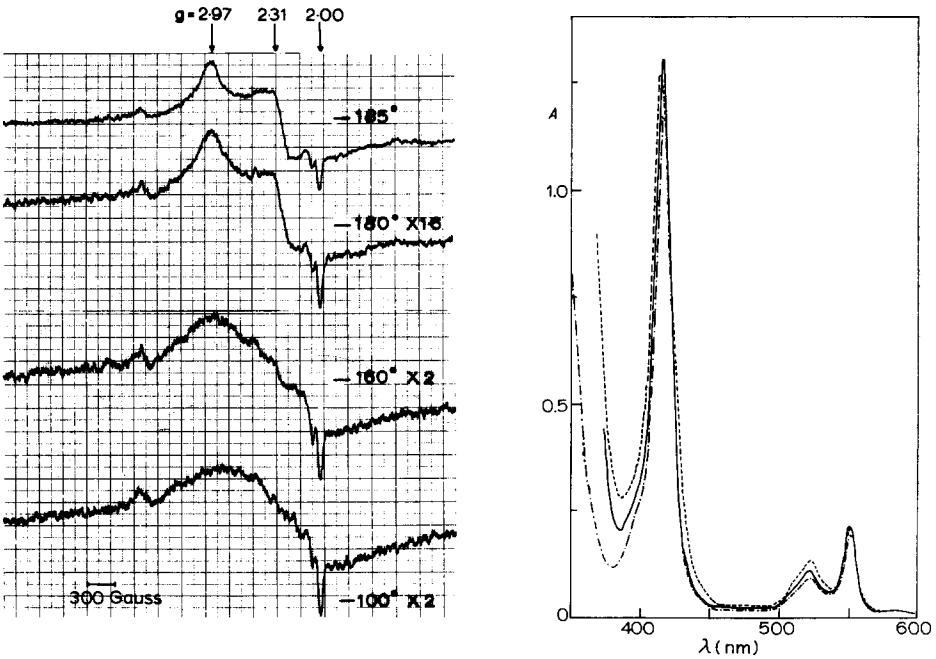


Fig. 5. EPR absorption of ferric cytochrome cc_3 as a function of temperature. Concentration and EPR conditions as in Fig. 3.

Fig. 6. Absorption spectra of the cytochromes reduced with dithionite; 10 mM potassium phosphate buffer (pH 7.0). ———, cytochrome cc'_3 (1.3 μM); -------, cytochrome *c*-553 (5 μM); –·—·–, cytochrome c'_3 (2.4 μM).

Fig. 7 shows the differences observed when cytochrome c_3 is compared to cytochrome *c* which is used here as a protein analogous to *c*-553. No absorption is observed in the 270–280 nm region in the oxidized state of cytochrome c_3. On reduction a distinct maximum at 277 nm emerges (ε band). Also on reduction the maximum at 350 nm (δ band) is shifted to 325 nm as in the case of the aggregate heme undecapeptide (rom cytochrome *c* (ref. 26). On reoxidation the peak at 277 nm disappears and the optical and EPR absorption of the ferric form are obtained. Similarly, a reduced spectrum of cytochrome cc_3 was obtained by reduction with traces of hydrogenase under an atmosphere of hydrogen (Fig. 8). This reduced form also has a maximum at 277 nm and the maximum at 366 nm is shifted to 325 nm. The reversible appearance of absorbance with a maximum at 277 nm obtained on reduction of cytochrome c_3 or cc_3 is attributable to a reduced heme absorption since the aggregate heme undecapeptide, derived from mammalian cytochrome *c* (ref. 26), lacks aromatic amino acids absorbing in the 270–280 nm region but also shows the reversible increase of a absorption on reduction. In contrast, cytochrome *c* which shows a maximum at

280 nm in the ferric state reveals on reduction a shift of this maximum to 275 nm which further increases in absorbance while the maximum at 363 nm is shifted to 315 nm.

It is noteworthy also that the ratio of absorbance of the δ band to the ε band (which varies only between 1.8 and 2.0) is quite similar in reduced cytochromes c_3, cc_3 and the heme undecapeptide from cytochrome *c*. This same ratio is equal to 1 in native mammalian cytochrome *c* (Figs. 7 and 8).

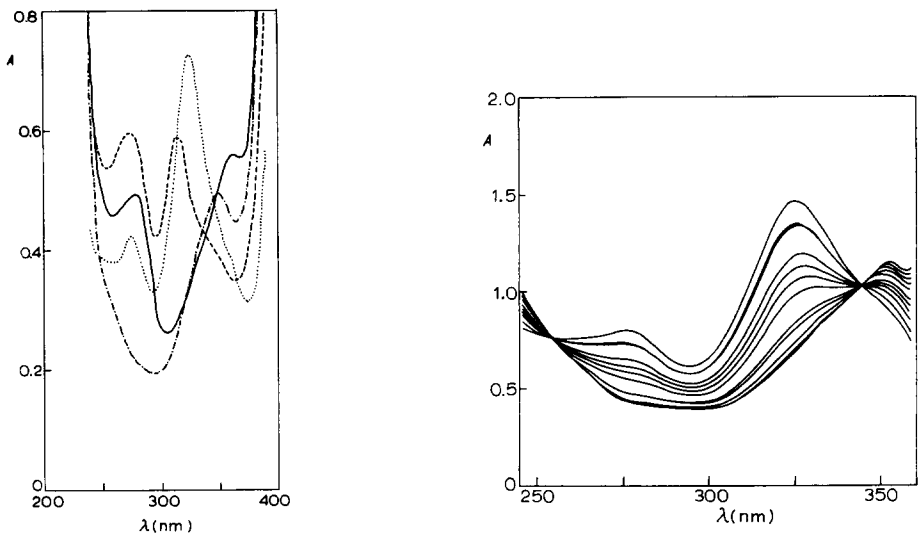


Fig. 7. Absorption spectra of horse-heart cytochrome *c* and of cytochrome c_3 in the ultraviolet region; 10 mM potassium phosphate buffer (pH 7.0); reductant: sodium borohydride. ———, cytochrome *c*, ferric form (17 μM); -------, cytochrome *c*, ferrous form; –·—·–, cytochrome c_3, ferric form (7.3 μM); ·····, cytochrome c_3, ferrous form.

Fig. 8. Optical spectrum of cytochrome cc_3 in the ultraviolet region; the cytochrome (7 μM) was in 10 mM potassium phosphate buffer (pH 7.0). Reduction by hydrogen in a Thunberg cuvette of 1-cm light path.

The ratio of absorbance of the Soret band in the oxidized versus the reduced states for cytochromes *c*-553, c_3, c'_3, cc_3 and cc'_3 are quite similar.

The EPR absorption of all these cytochromes in the ferric state disappears on reduction to the ferrous low-spin (diamagnetic) form, and reappears on reoxidation with sufficient time. In the case of cc'_3 the complete disappearance of EPR resonance on reduction required several minutes.

Since the amounts of *c*-553, cc_3, cc'_3 and c'_3 were rather limited, the remainder of this paper deals primarily with properties of c_3 from *D. vulgaris*.

Alkaline Form of c_3: At a pH of 10.5, a different low-spin form of c_3 was observed by EPR spectroscopy. This low-spin form in which hydroxide is presumably liganded at the sixth coordinate position (identical to that for c'_3) was found to have *g*-values of 2.72, 2.26 and 1.75 (Fig. 9). No significant optical differences were observed for this low-spin compound in both the oxidized and reduced states. Fig. 10 shows the effect of pH on the EPR absorption of cytochrome c'_3 and the gradual disappearance of Mn^{2+} absorption on conversion to the alkaline low-spin form.

Reaction of CO with c_3: No reaction of CO was observed with cytochrome c_3 in the ferric state but on reduction a new spectral species was observed with maxima at 413 nm and 531 nm and a shoulder at 561 nm. No EPR absorption was observed with this CO complex in the reduced state, in agreement with other studies in which EPR absorption was absent for the CO-compounds of various hemoproteins, *e.g.* myoglobin and hemoglobin, (*cf.* ref. 27).

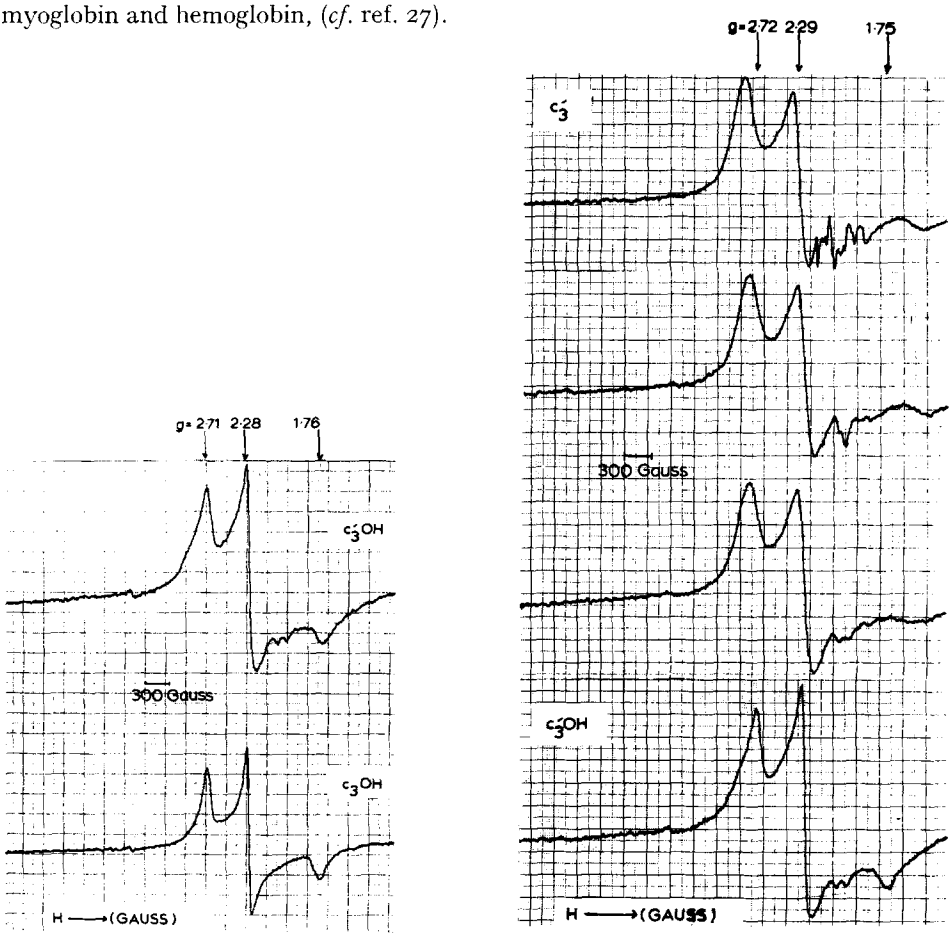


Fig. 9. EPR absorption of ferric cytochrome c'_3 (upper trace) and c_3, in 0.10 M potassium phosphate buffer, carefully adjusted to pH 10.5. Concentrations and EPR conditions as in Fig. 3.

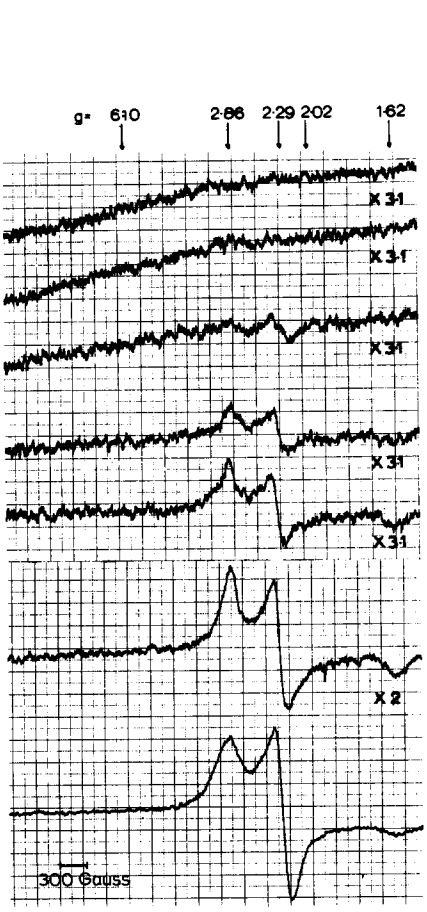
Fig. 10. EPR absorption of ferric cytochrome c'_3 on stepwise addition of 5 M KOH and conversion to the hydroxide low-spin form. Upper trace, pH 7.2; successively lower traces, pH 8.4, pH 9.5 and pH 10.5 (lower trace). Concentration and EPR conditions as in Fig. 3.

EPR spectrum of a half-reduced intermediate of cytochrome c_3. The reoxidation of c_3, reduced with either dithionite or borohydride, leads to the detection of a distinctly different EPR spectrum at approximately a half-reduced state with a signal intensity about half that of the oxidized ferric state. Although similar g-values were obtained (Fig. 11), there was a distinct narrowing of g_x and g_z*. This half-reduced

* g_x, g_y, g_z correspond to the end minimum, midpoint crossing and end maximum, respectively, in the first derivative absorption.

state may be important in the biological reactions catalyzed by this cytochrome (*cf.* ref. 15). The unusual properties of this half-reduced intermediate suggest a heme–heme interaction in the oxidized state which on half-reduction results in a lessening of the interaction*.

Another observation which supports a possible heme-heme interaction in this multi-heme protein involves the effect of ammonia on the reduction and reoxidation of c_3 as monitored by EPR. Cytochrome c_3 in the ferric state shows no changes in the EPR spectrum for several minutes after addition of a 10-fold molar excess of ammonia. On reduction EPR absorption disappears (Fig. 12) and reoxidation leads to the ap-



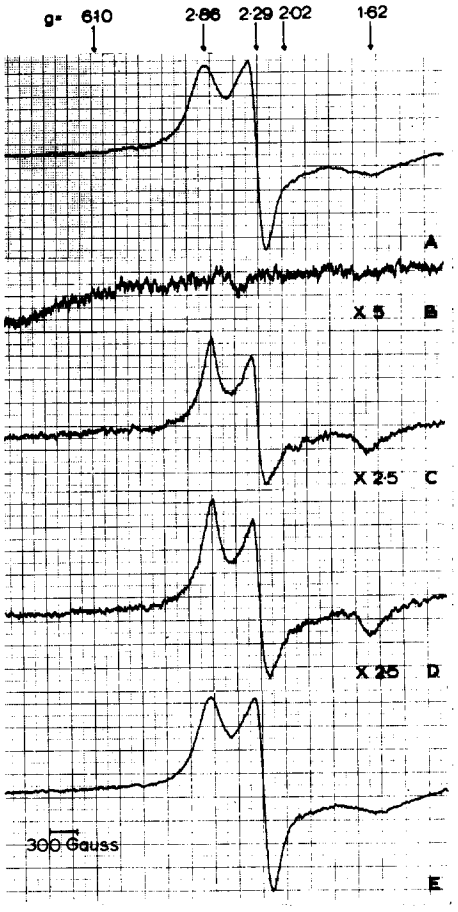


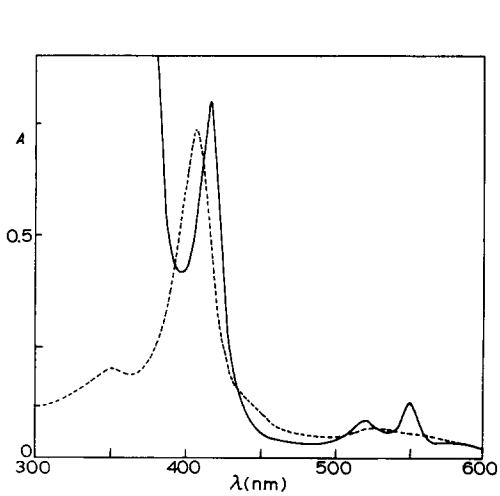
Fig. 11. EPR absorption of cytochrome c_3 in an anaerobic quartz EPR tube, as described in ref. 19, after reduction with sodium dithionite under helium (uppermost trace), followed by stepwise air reoxidation. Lowest trace, fully reoxidized and comparable to initial ferric EPR spectrum. Concentration and EPR conditions as in Fig. 3.

Fig. 12. EPR absorption of ferric cytochrome c_3 (5 mg in 0.1 M potassium phosphate buffer, pH 7.2, containing 20 mM $(NH_4)Cl$ after 12 min at 25° (trace A); after reduction with dithionite (trace B); on stepwise reoxidation (traces C–E), where E is fully reoxidized and is virtually identical to trace A. EPR conditions as in Figs. 3 and 11.

* A similar intermediate at a half-reduced state has been reported by McDONALD *et al.*[28] by high-resolution NMR.

pearance of a half-reduced intermediate attributable to a new low-spin species presumably with ammonia as the ligand coordinated at the 6th position (*cf.* ref. 24). This intermediate differs from the previously described half-reduced intermediate by virtue of an approximately 52-G shift in g_z to high field although g_x and g_y are similar. Complete reoxidation to the ferric state of c_3 results in the normal low-spin ferric signal.

Effect of urea on the oxidation–reduction states of c_3. No reaction is observed with 8 M urea in the ferric state of c_3 as monitored by optical or EPR spectroscopy when measured after 7 days at 25°. No EPR absorption is observed in the reduced state. Although the urea-treated sample of cytochrome c_3 shows a typical ferric optical spectrum, reduction results in a definitely altered optical spectrum (Fig. 13). The ratio of absorbance at the Soret maximum (reduced *minus* oxidized) declined from 1.56 to 1.09 and the ratio of absorbance of the α to β peaks declined from 1.94 (untreated) to 1.35 in the urea-treated sample. Reoxidation of the urea-treated protein led to a return of the oxidized optical spectrum and almost complete EPR absorption. When the urea-treated enzyme (either reoxidized in the presence of 8 M urea or freshly diluted in 8 M urea) was diluted with 0.025 M potassium phosphate buffer (pH 7.5), the oxidized absorption spectrum appeared to be unaltered. However, on reduction this sample showed a slightly altered reduced absorption spectrum of c_3 (Fig. 14). Because this phosphate-diluted sample was not sufficiently concentrated for EPR spectroscopy, it was passed through a Sephadex G-25 column and reconcentrated. This sample then revealed the same typical ferric low-spin EPR spectrum as the untreated cytochrome although the intensity was not fully comparable to the starting material. Some denaturation was observable primarily in the appearance of a small $g=4.3$ EPR signal attributable to non-heme high-spin ferric iron. The gel-excluded sample after dilution in phosphate buffer revealed a normal oxidized but also slightly altered reduced optical spectrum.



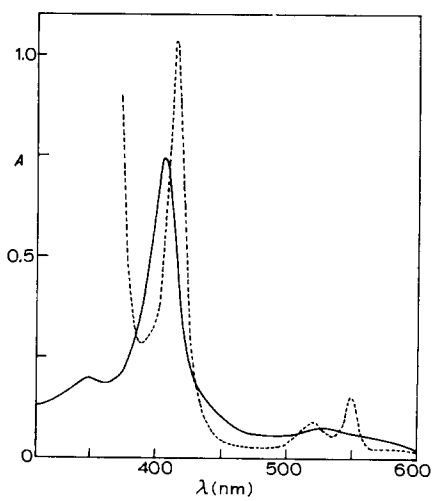


Fig. 13. Absorption spectra of urea-treated cytochrome c_3 in 8 M urea. Reducing agent, dithionite; concentration of cytochrome, 3.5 μM. ———, ferric form; - - - - - - -, ferrous form.

Fig. 14. Absorption spectra of urea-treated cytochrome c_3, (3.5 μM) in 25 mM potassium phosphate buffer (pH 7.5); reducing agent, dithionite. ———, ferric form; - - - - - - -, ferrous form.

Repeated reduction with dithionite and reoxidation in the presence of urea resulted in an irreversible loss of absorption features of the oxidized and reduced states of cytochrome c_3 as well as a gradual conversion of the low-spin ferric EPR absorption to a very broad low-spin ferric spectrum (Fig. 15) suggesting monomeric cytochrome *c* or *c*-553. These results suggest that under reducing conditions in the presence of urea a gradual unfolding of protein occurs during which a disorientation of the hemes relative to each other is evident. A mono-heme type of EPR absorption is then observed. Because of the extremely broad spectrum observed, multiple heme species present under this absorption can not be excluded. In this process an increase in EPR absorption at $g=6$ and $g=4$ is noted. VAN GELDER AND BEINERT[29] have reported that under denaturing conditions cytochrome oxidase revealed an increase in the $g=6$ high-spin ferric heme absorption. Since this signal was also observed in cytochrome oxidase under partially reduced conditions[29], they suggested that in the denaturation process reducing groups became available which led in turn to increased $g=6$ absorption. A similar argument may be applicable in the case of cytochrome c_3

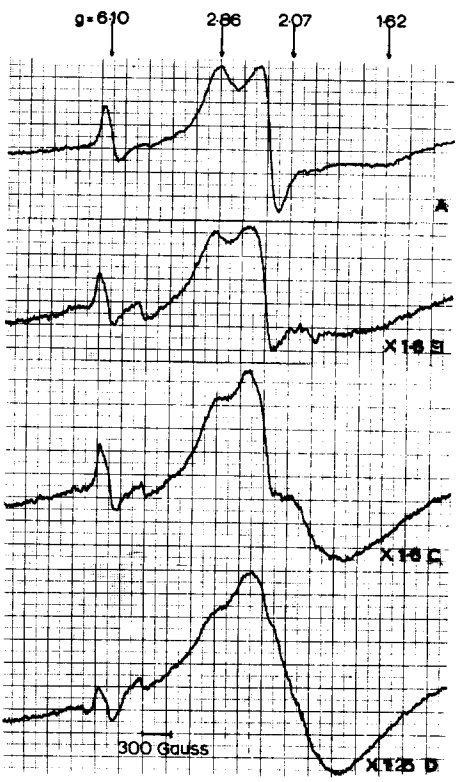


Fig. 15. EPR absorption of ferric cytochrome c_3 (concentration as in Fig. 3 but containing 8 M urea): after a single cycle of reduction with dithionite and complete air reoxidation (trace A); traces B–D correspond to a 2nd, 3rd and 4th cycle of reduction and reoxidation, respectively. The higher amplifications required in traces B and C are due to changes in the autoxidizability of cytochrome c_3. On prolonged exposure to 8 M urea during redox cycling, autoxidizability declines. Trace D is therefore obtained after air exposure for 24 h at 0°. EPR conditions as in Figs. 3 and 11.

since during reduction with borohydride in the presence of 8 M urea, free thiol groups are liberated*. The distorted type of $g=6$ signal observed in cytochrome c_3 has been reported by PEISACH *et al.*[30] in other heme proteins to represent a change from axial to rhombic symmetry.

DISCUSSION

These studies clearly indicate that the *c*-type cytochromes described in this study can be classified into three groups based on their heme contents, as well as absorption and EPR response in both oxidized and reduced states. The number of heme groups per molecule are then clearly reflected in both optical and EPR spectra. The first group consists of the mono-heme type (cytochrome *c*-553); the second group consists of cytochromes c_3 and c'_3 containing at least two hemes per molecule and the third group is composed of cytochromes cc_3 and cc'_3 which contain at least four hemes per molecule. In the latter two groups individual members are virtually identical in optical and EPR properties. These results confirm the recent classification of Desulfovibrio cytochromes mainly based on amino-acid composition and primary structure[31].

The light absorption spectra of cytochrome c_3 in the oxidized and reduced states have been reported elsewhere[1,32]. The circular dichroism (CD) spectrum of cytochrome c_3 in the reduced state indicates a Soret band splitting which has been attributed by DRUCKER *et al.*[32] and earlier by URRY[33] in his studies on the heme undecapeptide of cytochrome *c* to exciton coupling between hemes. In this regard it is interesting to note that as with cytochrome c_3, cytochrome *c*-552 from *Chromatium*[34] and mammalian cytochrome oxidase[35], each of which also contain at least two hemes per molecule, have been reported to exhibit similar Soret splitting in the CD spectra in one or more oxidation–reduction states. A similar Soret band splitting in the CD spectra was observed, as mentioned above, with the autoxidizable aggregate heme undecapeptide. It is interesting to note that the EPR spectrum of the ferri heme undecapeptide aggregate is strikingly similar[19] to that observed for cytochrome c_3 as well as cytochrome *c*-552 and cytochrome oxidase, indicating that heme–heme interaction is reflected in the EPR as well as the CD spectrum.

Certain similarities are noted between cytochrome *c*-553 and cytochrome *c* particularly in terms of the absorption maximum at 695 nm. This band is present in both cytochromes but is more intense in cytochrome *c*-553. Indirect chemical studies (*cf.* ref. 36) as well as definitive high-resolution NMR studies[37] indicate that methionine is liganded at the 6th coordinate position of the heme iron moiety of cytochrome *c*. Evidence has been presented by other workers[20,21] that the 695-nm band is due to an interaction of methionine with heme iron. It is assumed then by analogy that methionine is probably the same ligand in cytochrome *c*-553. In contrast cytochrome c_3 lacks the 695 band and NMR studies[28] indicate that methionine is not the ligand at the 6th coordinative position.

* In the ferric state cytochrome c_3 reveals no free thiol groups. On reduction with borohydride in the presence of 8 M urea, four such groups are liberated[8]. Because a lengthy time is required for their appearance, it is not clear whether this result indicates the presence of disulfide bridges or that the protein is partially destroyed by the treatment. This latter hypothesis is supported by the fact that the Soret band of diluted solutions of cytochrome c_3 disappears slowly in the presence of borohydride and 8 M urea.

The EPR absorption of ferricytochrome c_3 differs considerably from that observed by EHRENBERG AND KAMEN[38] with the RHP-type cytochromes which contain two hemes per molecule. These authors observed a considerably broadened high-spin ferric heme signal at $g=6$ which broadening was attributed to heme-heme interaction or mixed spin-state interaction.

The following observations with cytochrome c_3 suggest a possible disorientation of hemes or conformational change at the half-reduced or fully-reduced states: (1) narrowing of g_x and g_y in the EPR intermediate observed at half-reoxidation (2) reversible formation only at the half-reduced state of an ammonia low-spin EPR detectable intermediate and (3) reaction of CO or NO* only in the half-reduced or fully reduced state (*cf.* refs. 34, 39).

An irreversible unfolding of protein in cytochrome c_3 as a function of repeated oxidation-reduction in the presence of 8 M urea is indicated by an irreversible loss of initial optical and EPR spectra, leading eventually to a quite broad low-spin EPR spectrum suggestive of monomeric cytochrome *c*. As indicated, the ferric state of cytochrome c_3 is extremely stable even to 8 M urea for several days in air at room temperature.

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* Binding of NO with cytochrome c_3 in the ferric state[15] is quite weak based on EPR response. In the reduced state a dramatic reaction is indicated. The inaccessibility of strong ligands like NO to binding with ferric heme iron suggests heme stacking prevents entry of these ligands. In the half-reduced or fully reduced states a decreased heme–heme interaction as a consequence of a conformational change may provide access of strong ligands to heme.

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