The circular-dichroic properties of the ‘Rieske’ iron–sulphur protein in the mitochondrial ubiquinol:cytochrome *c* reductase

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We have studied the c.d. spectra of the ‘Rieske’ iron–sulphur protein isolated from the ubiquinol:cytochrome *c* reductase (*bc*₁ complex) of bovine heart mitochondria. Both the oxidized and the reduced form of the ‘Rieske’ protein display a series of well-resolved c.d. features resembling those reported for the ‘Rieske’-type iron–sulphur protein purified from the bacterium *Thermus thermophilus* [Fee, Findling, Yoshida, Hille, Tarr, Hearshen, Dunham, Day, Kent & Münck (1984) *J. Biol. Chem.* **259**, 124–133]. In particular, the difference spectra, reduced minus oxidized, of both proteins have a distinctive negative band at 497 nm. The c.d. features characteristic of the isolated ‘Rieske’ protein were found in the dichroic spectra of the whole *bc*₁ complex in the region between 450 and 520 nm. The reduction of the enzyme by ascorbate or ubiquinol is accompanied by the formation of a negative band at about 500 nm that corresponds, in all its c.d. properties, to the specific dichroic absorption of the reduced ‘Rieske’ iron–sulphur protein.

INTRODUCTION

The ubiquinol:cytochrome *c* reductase (*bc*₁ complex or respiratory Complex III; Rieske & Ho, 1985) is the central redox enzyme in the mitochondrial respiratory chain, and also in the electron-transfer chain of many aerobic and photosynthetic bacteria (Hauska *et al.*, 1983). This multiprotein complex contains four prosthetic groups: two *b* cytochromes, distinguished by their different potentiometric and spectroscopic properties (Hauska *et al.*, 1983; Rich, 1984; Rieske & Ho, 1985), cytochrome *c*₁, and a high-potential [2Fe-2S] iron–sulphur protein, which is usually known as the ‘Rieske’ protein (Rieske *et al.*, 1964; Trumpower, 1981). The three cytochromes possess well-resolved spectroscopic features of optical absorbance (Van Gelder, 1978; Rieske & Ho, 1985), of circular dichroism (Yu *et al.*, 1971; Reed *et al.*, 1978; Myer, 1985) and of magnetic circular dichroism (Tsai, 1983; Tsai & Palmer, 1983).

In contrast with the cytochromes, the ‘Rieske’ iron–sulphur protein possesses a weak and poorly resolved optical spectrum (Rieske *et al.*, 1964; Shimomura *et al.*, 1984), which is completely obscured by the intense absorption bands of the cytochromes throughout the spectrum of the *bc*₁ complex (Trumpower, 1981; Tsai, 1983). Fee *et al.* (1984) have studied the c.d. and magnetic-c.d. properties of an iron–sulphur protein purified from *Thermus thermophilus*, which resembles the mitochondrial ‘Rieske’ proteins in many respects, but no work has been reported on the c.d. or the magnetic c.d. of the iron–sulphur protein of the mitochondrial *bc*₁ complex.

The lack of spectroscopic properties that are specific to the ‘Rieske’ centre, besides low-temperature e.p.r. (Rieske, 1967b; Orme-Johnson *et al.*, 1974; Trumpower, 1981), has prevented detailed studies on its kinetics of reduction and oxidation, whereas the transient kinetics

of the redox changes of cytochrome *c*₁ and of *b* cytochromes can readily be obtained by spectrophotometric means (Trumpower, 1981; De Vries, 1983). Hence the functional role of the iron–sulphur protein in the electron transfer of the *bc*₁ complex has mostly been investigated after extraction and reconstitution of the protein (Trumpower, 1981; Shimomura *et al.*, 1984).

In the present paper we demonstrate that the c.d. spectra of the bovine heart *bc*₁ complex contain, after partial reduction, a characteristic band at around 500 nm that can be ascribed to the specific c.d. features of the ‘Rieske’ protein.

MATERIALS AND METHODS

The *bc*₁ complex was purified from bovine heart mitochondria essentially by the method of Rieske (1967a) but with some modifications (Degli Esposti *et al.*, 1986). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The enzyme preparations usually contained 3.2–3.8 nmol of cytochrome *c*₁/mg of protein.

The ‘Rieske’ protein was isolated from the *bc*₁ complex by following the procedure of Shimomura *et al.* (1984). The isolated protein was eluted from a phenyl-Sepharose column after extensive delipidation of the enzyme, exactly as described by Shimomura *et al.* (1984). The elution peak was concentrated by ultrafiltration on an Amicon YM10 membrane. The final concentration of the protein was 1–2 mg/ml in the elution buffer [25 mM-Tris/HCl, pH 8.0, containing 20% (w/v) glycerol, 1% (w/v) sodium deoxycholate and 1 mM-dithiothreitol]. The dithiothreitol present in the buffer maintained the ‘Rieske’ protein in the completely reduced state (Shimomura *et al.*, 1984), and the preparation was used without further modification to obtain the c.d. spectra in the reduced state. The protein

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was oxidized by dialysing the preparation against 200 vol. of the elution buffer containing $20 \mu\text{M}$ - $\text{K}_3\text{Fe}(\text{CN})_6$ instead of dithiothreitol. Since no significant improvement in the purity of the preparation could be achieved by further gel filtration as described by Shimomura *et al.* (1984), and the 'Rieske' protein was not contaminated to a great extent by other polypeptides, c.d. studies were performed on the crude preparation of the protein obtained as specified above.

SDS/polyacrylamide-gel electrophoresis was carried out in 15% polyacrylamide slab gels as previously described (Degli Esposti *et al.*, 1983, 1986). The electrophoretic pattern of the isolated 'Rieske' protein showed a major band of about 25 kDa, corresponding to the iron-sulphur subunit of the bc_1 complex (Rieske, 1967b; Trumpower, 1981), and two other bands of about 43–45 kDa, which could be ascribed to contaminations

Fig. 1. SDS/polyacrylamide-gel electrophoresis of the bc_1 complex and the 'Rieske' protein

Lane A, molecular-mass standards (Bio-Rad Laboratories), containing phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa); lane B, iron-sulphur-protein-depleted bc_1 complex, obtained after elution of the iron-sulphur protein from the phenyl-Sepharose column (Shimomura *et al.*, 1984); lane C, native bc_1 complex; lane D, crude iron-sulphur protein as eluted from the phenyl-Sepharose column; lane E, iron-sulphur protein further purified by using gel filtration on a Sephacryl-S-200 column as described by Shimomura *et al.* (1984). Preparations similar to that used for lane D were usually employed in the c.d. studies.

of the colourless core proteins of the complex (Rieske & Ho, 1985) (Fig. 1).

The concentration of cytochrome c_1 was measured spectrophotometrically at 553–541 nm after reduction with solid sodium ascorbate by using an absorption coefficient of $17.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Yu *et al.*, 1971). The concentration of the iron-sulphur centre in the isolated 'Rieske' protein was measured both spectrophotometrically from the difference spectrum, ferricyanide-oxidized minus dithiothreitol-reduced, at 570 nm by using an absorption coefficient of $1.55 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Rieske *et al.*, 1964) and by chemical analysis of the non-haem iron carried out as described by Rieske (1967b). The two determinations were in reasonable agreement, and usually gave values of 19–26 nmol of $[\text{2Fe-2S}]$ centre/mg of protein. These values are lower than those expected for the purified protein, namely 35–40 nmol/mg of protein (Rieske, 1967b), owing to the presence of other proteins in the preparations of the crude 'Rieske' protein (Shimomura *et al.*, 1984; see also Fig. 1). All the spectrophotometric measurements were performed in a Jasco UVIDECE-610 spectrophotometer. The enzymic assays of ubiquinol-2:cytochrome *c* reductase were performed as described by Degli Esposti *et al.* (1983). Ubiquinol-2 was prepared from ubiquinone-2 (generously given by Eisai Co., Tokyo, Japan) by the method of Rieske (1967a).

C.d. spectra were obtained in a Jasco J-500 spectrophotometer at room temperature ($20\text{--}22^\circ\text{C}$) in a 1 cm quartz cuvette. The final spectra were the mean of two to eight accumulated scans at a bandwidth of 2 nm, and were usually corrected for the unspecific dichroic absorbance of the medium by computer manipulation. The buffer used for dissolving the iron-sulphur-protein-depleted bc_1 complex was 25 mM-Tris/HCl, pH 8.0, containing 20% (w/v) glycerol and 2% (w/v) Tween-20. The c.d. spectra of the native bc_1 complex were obtained either in the above buffer or in 50 mM-potassium phosphate buffer, pH 7.4, containing 20% (w/v) glycerol and 1 mM-EDTA. No significant spectral changes were found when the enzyme was dissolved in either of the above buffers.

In the c.d. spectra of the bc_1 complex the absorption coefficients were calculated on the basis of the spectrophotometric determination of cytochrome c_1 , performed on the same sample as used for the c.d. measurements.

RESULTS

C.d. features of the isolated 'Rieske' protein

The electrophoretic analysis of the preparations of the isolated iron-sulphur protein showed no contamination by proteins carrying chromophores that could noticeably interfere with the absorption properties of the iron-sulphur centre (Fig. 1). However, the spectrophotometric determination revealed the presence of small amounts (below 0.01 mol/mol of iron-sulphur centre) of cytochrome c_1 , which gave significant interference with the optical spectra of the 'Rieske' centre because the absorption coefficients of the purified cytochrome are much higher than those of the iron-sulphur protein (Van Gelder, 1978; Trumpower, 1981). Nevertheless interference by the contaminant cytochrome c_1 with the c.d. spectra of the 'Rieske' protein in the spectral region studied was negligible, since the $\Delta\epsilon$ values of the c.d.

Fig. 2. C.d. spectra of the isolated 'Rieske' protein

(a) -----, C.d. spectrum of the isolated 'Rieske' iron-sulphur protein in the reduced state as prepared in the elution buffer (see the Materials and methods section); —, c.d. spectrum of the same preparation that was then oxidized as described in the Materials and methods section. The final concentration of the 'Rieske' centre was $11 \mu\text{M}$ in both the reduced and the oxidized state. The spectra are the average of two accumulated scans and are corrected for the medium. (b) —, Difference spectrum, dithiothreitol-reduced minus ferricyanide-oxidized, of the isolated 'Rieske' protein calculated from the spectra in (a); ·····, difference spectrum, reduced minus oxidized, of the iron-sulphur protein from *Thermus thermophilus* calculated from the data of Fee *et al.* (1984).

absorption of the purified cytochrome c_1 are comparable with those of the 'Rieske' protein (Yu *et al.*, 1971; Fee *et al.*, 1984).

Fig. 2 shows the absolute c.d. spectra of the oxidized and the reduced 'Rieske' protein isolated from the bovine heart bc_1 complex. Similarly to the c.d. spectra of other iron-sulphur proteins (Stephens *et al.*, 1978), the c.d. spectra of the 'Rieske' protein from bovine heart exhibit much greater resolution and complexity than the broad optical absorption spectra. The oxidized iron-sulphur protein has a weak negative band at 555 nm, a series of positive bands between 490 and 420 nm, an intense negative band at 375 nm and a positive band at 318 nm. The dithiothreitol-reduced iron-sulphur protein has a wide negative band at 507 nm, a set of positive bands between 465 and 420 nm, an intense negative band at 384 nm and a large positive band at around 315–310 nm. C.d. spectra very similar to those shown in Fig. 2(a) were obtained when a preparation previously oxidized by ferricyanide was then reduced by ascorbate or ubiquinol-2 (results not shown).

On the whole, the c.d. features of the bovine heart 'Rieske' protein are quite similar to those found with the iron-sulphur protein purified from *Thermus thermophilus* (Fee *et al.*, 1984). In particular, the dominant negative band around 380 nm, with similar intensity both in the reduced and in the oxidized state (cf. Fig. 2a), appears to be identical in the values of minima and absorption coefficients in the two proteins. The spectral region between 600 and 460 nm is also very similar in the mitochondrial and in the *Thermus thermophilus* iron-sulphur proteins. However, significant differences, particularly in the intensity of the absorption bands, are seen in the near-u.v. region and between 460 and 420 nm.

The difference spectrum, reduced minus oxidized, of the 'Rieske' protein (Fig. 2b, — line) shows a quasi-symmetrical major band at 497 nm with half-bandwidth of approx. 36 nm and $\Delta\epsilon$ of $-13 \text{ M}^{-1}\cdot\text{cm}^{-1}$

on the basis of the concentration of the $[2\text{Fe-2S}]$ cluster. Features very similar to these can be derived from the data presented by Fee *et al.* (1984) for the c.d. spectra of the *Thermus thermophilus* iron-sulphur protein, as is also shown in Fig. 2(b) (····· line).

C.d. features of the 'Rieske' protein in the bc_1 complex

Ascorbate can reduce only cytochrome c_1 and the 'Rieske' centre in the mitochondrial bc_1 complex, but not the b cytochromes, since the E_m of this reducing agent is higher than that of the b cytochromes (Rich, 1984; Rieske & Ho, 1985). Therefore the c.d. spectrum of the ascorbate-reduced enzyme is due to the combination of the dichroic absorption of oxidized b cytochromes, reduced cytochrome c_1 and reduced iron-sulphur protein (Reed *et al.*, 1978). The isolated cytochrome c_1 has a positive c.d. band in the reduced state at 417 nm, but does not show any resolved c.d. features in the region between 430 and 520 nm (Yu *et al.*, 1971; Myer, 1985). Although the c.d. properties of the oxidized b cytochromes of the mitochondrial bc_1 complex have not been studied directly (Myer, 1985), it has been suggested that the c.d. spectrum of the b haem groups in the oxidized state should be much weaker than that of reduced cytochrome c_1 (Reed *et al.*, 1978), and should not have any distinctive c.d. features in the spectral region between 450 and 520 nm (Okada & Okunuki, 1970; Myer, 1985). Therefore, assuming that the c.d. properties of the reduced 'Rieske' centre are the same in the whole complex as in the isolated protein, it is to be expected that the c.d. spectrum of the ascorbate-reduced bc_1 complex would reflect the features of the iron-sulphur centre in the spectral region between 450 and 520 nm.

The spectrum of the ascorbate-reduced bc_1 complex (Fig. 3) shows a main positive band at 414 nm resulting from the overlapping of the band of reduced cytochrome c_1 with the absorption due to oxidized b cytochromes (Reed *et al.*, 1978), and a wide negative band at about

Fig. 3. C.d. spectra of the native bc_1 complex

Bovine heart bc_1 complex was dissolved at $3.9 \mu\text{M}$ cytochrome c_1 in 50 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA and 20% glycerol. —, C.d. spectrum of bc_1 complex oxidized by $50 \mu\text{M}$ - $\text{K}_3\text{Fe}(\text{CN})_6$; ----, spectrum of bc_1 complex reduced by solid sodium ascorbate; ·····, difference spectrum ascorbate-reduced minus ferricyanide-oxidized. The spectra, corrected for interference by the medium, are the average of two accumulated scans.

500 nm. The difference spectrum, ascorbate-reduced minus ferricyanide-oxidized, of the whole bc_1 complex has two main resolved features in the 380–520 nm region (Fig. 3, ····· line). The signal with maximum at 420 nm and minimum around 409 nm is due to the shift in the spectrum of cytochrome c_1 upon reduction (Myer, 1985); in fact, isolated cytochrome c_1 has a c.d. maximum at 414 nm when oxidized and at 417 nm when reduced (Yu *et al.*, 1971). The wide negative band around 500 nm possesses peak maximum (497 nm), half-bandwidth (35 nm), absorption coefficient (approx. $14 \text{ M}^{-1}\cdot\text{cm}^{-1}$) and cross-over points that closely correspond to those found in the difference spectrum, reduced minus oxidized, of the isolated 'Rieske' protein (cf. Fig. 2b). Hence the negative band at about 500 nm that is observed in the c.d. spectrum of the ascorbate-reduced bc_1 complex can mostly be attributed to the specific dichroic absorption of the iron-sulphur protein in the enzyme.

To confirm the above, spectra were taken of the bc_1 complex that had been depleted of the iron-sulphur protein, both before and after reconstitution with the isolated 'Rieske' protein (obtained according to the procedure of Shimomura *et al.*, 1984) (Fig. 4). The c.d. spectrum of the ascorbate-reduced iron-sulphur-protein-depleted complex (Fig. 4a, — line) appears to be rather flat in the 450–520 nm region, whereas the c.d. spectrum of the bc_1 complex reconstituted with isolated iron-sulphur protein (Fig. 4a, ---- line) shows a wide negative band in the same spectral region. The difference spectra, ascorbate-reduced minus ferricyanide-oxidized, of the iron-sulphur-protein-depleted complex presented in Fig. 4(b) show that reconstitution with the

Fig. 4. C.d. spectra of the iron-sulphur-protein-depleted bc_1 complex

(a) —, C.d. spectrum of the iron-sulphur-protein-depleted bc_1 complex, which was suspended at $3.2 \mu\text{M}$ in 25 mM-Tris/HCl buffer, pH 8.0, containing 20% glycerol and 2% Tween-20, and then reduced by solid sodium ascorbate; ----, c.d. spectrum of iron-sulphur-protein-depleted bc_1 complex reconstituted with 0.7 mol of isolated iron-sulphur protein/mol of cytochrome c_1 for 30 min at 25°C as described by Shimomura *et al.* (1984); the preparation is dissolved in the same buffer as above and reduced with solid sodium ascorbate. The reconstituted enzyme exhibited an ubiquinol-2:cytochrome c reductase activity of 250 s^{-1} , which corresponded to about 60% of the enzymic activity of the native enzyme. The enzymic activity of the depleted complex was about 30 s^{-1} . (b) —, Difference spectrum, ascorbate-reduced minus ferricyanide-oxidized, of the iron-sulphur-protein-depleted bc_1 complex in (a); ----, difference spectrum, ascorbate-reduced minus ferricyanide-oxidized, of the reconstituted complex in (a). The substoichiometric reconstitution with the isolated 'Rieske' protein (0.7 mol/mol of cytochrome c_1) is responsible for the quantitative differences in the spectra of the reconstituted bc_1 complex and of the native complex (cf. Fig. 3).

'Rieske' protein leads to the re-appearance of the negative peak around 500 nm that is specifically due to the dichroic absorption of this component (cf. Figs. 2 and 3).

The spectra in Fig. 5 present the dichroic features that can be attributed to the 'Rieske' protein centre in the region 550–450 nm in more detail. The reduction of the bc_1 complex by ubiquinol, which is the natural reductant of the enzyme (Rieske & Ho, 1985), leads to a slight reduction of the high-potential cytochrome b in addition to the complete reduction of cytochrome c_1 and the iron-sulphur centre (Trumpower, 1981). However, the c.d. features of the reduced 'Rieske' protein around 500 nm are essentially the same in the ascorbate-reduced as in the ubiquinol-reduced bc_1 complex (cf. Fig. 2b). Also confirming that the reduction of the b cytochromes does not significantly affect the c.d. features of the 'Rieske' protein centre in the region of the negative band at about 500 nm, the c.d. spectra of the dithionite-reduced

Fig. 5. Spectroscopic features of the 'Rieske' protein in the c.d. spectra of the bovine heart bc_1 complex

Native bovine heart bc_1 complex was suspended at $6.3 \mu\text{M}$ -cytochrome c_1 in the same phosphate buffer as described in Fig. 3 legend. —, C.d. spectrum of ferricyanide-oxidized bc_1 complex; ----, c.d. spectrum of bc_1 complex reduced by $200 \mu\text{M}$ -ubiquinol-2; ·····, difference spectrum, ubiquinol-reduced minus ferricyanide-oxidized. Note that the small peak at 530 nm, which is not seen in the spectra of the isolated 'Rieske' protein (cf. Fig. 2), is mostly due to the β band of reduced cytochrome c_1 (Myer, 1985). The spectra are the mean of four scans and appear to be slightly different from those shown in Fig. 3, particularly in the oxidized state. The higher concentration of the enzyme employed here produced a slight turbidity of the solution, which may have distorted the signals of the absolute c.d. spectra, thus giving rise to the discrepancy with Fig. 3. However, the distortions are cancelled in the difference spectrum, which is remarkably similar to the difference spectrum in Fig. 3.

enzyme, in which the b cytochromes are completely reduced (Trumpower, 1981; Rieske & Ho, 1985), have the same dichroic absorption as the spectra of the ascorbate-reduced enzyme between 480 and 520 nm (results not shown).

The c.d. features of the 'Rieske' protein centre that are evident in the spectra of the bovine heart bc_1 complex can also be observed in the c.d. spectra of bc_1 complexes extracted from other species. Fig. 6 shows the c.d. spectra obtained with the enzyme extracted from chicken heart mitochondria, which was prepared by the same procedure as that employed for the bovine heart bc_1 complex (Degli Esposti *et al.*, 1986). The negative band around 500 nm is also clearly evident in the ascorbate-reduced chicken heart bc_1 complex, particularly in the difference spectrum, ascorbate-reduced minus ferricyanide-oxidized. Furthermore, a similar negative band at about 500 nm has previously been reported for the ascorbate-reduced bc_1 complex extracted from yeast mitochondria (Tsai, 1983).

Therefore it can be suggested that the c.d. features of the 'Rieske' protein may be very similar in all organisms possessing an ubiquinol:cytochrome c reductase.

Fig. 6. Spectroscopic features of the 'Rieske' protein in the c.d. spectra of the chicken heart bc_1 complex

Experimental conditions were the same as for Fig. 5, except that the chicken heart enzyme was $4.7 \mu\text{M}$ -cytochrome c_1 . —, C.d. spectrum of ferricyanide-oxidized bc_1 complex; ----, c.d. spectrum of bc_1 complex reduced by solid sodium ascorbate; ·····, difference spectrum, ascorbate-reduced minus ferricyanide-oxidized.

DISCUSSION

There has been some doubt as to whether the protein isolated by Fee *et al.* (1984) from the bacterium *Thermus thermophilus* is actually related to the 'Rieske' protein of mitochondria (Harnish *et al.*, 1985). This doubt has been based on DNA sequencing data (Harnish *et al.*, 1985) and on the fact that the iron-sulphur protein from *Thermus thermophilus* contains two [2Fe-2S] clusters (Fee *et al.*, 1984), whereas the mitochondrial 'Rieske' protein contains only one [2Fe-2S] cluster (Trumpower, 1981). However, the e.p.r. properties, the optical absorbance spectra, the midpoint potential and the molecular mass are very similar for the two proteins (Fee *et al.*, 1984). Here we have reported that the c.d. features of the 'Rieske' protein isolated from bovine heart mitochondria are also very similar to those found in the protein from *Thermus thermophilus* (cf. Fig. 2 and Fee *et al.*, 1984), thereby suggesting that the two proteins are indeed related.

C.d. spectroscopy is able to give an insight into the structure and the conformation of the cluster in iron-sulphur proteins (Stephens *et al.*, 1978), and as such may be used to compare the structural characteristics of the mitochondrial 'Rieske' protein and the 'Rieske'-type protein of *Thermus thermophilus*. The clear similarities between the c.d. spectra of the two proteins (cf. Fig. 2b) indicate that the structure and the conformation of the iron-sulphur cluster are essentially the same in the mitochondrial and in the bacterial protein, confirming the previous suggestion based on other spectroscopic and physical data (Fee *et al.*, 1984). The c.d. spectra of the 'Rieske' protein are quite distinctive and remarkably different from those found for a variety of

[2Fe-2S] proteins (Stephens *et al.*, 1978). Typical [2Fe-2S] proteins, such as ferredoxins, possess four cysteine residues that form the ligands with the iron atoms within the cluster (Palmer, 1973). However, it has been demonstrated (Fee *et al.*, 1984) that each of the two clusters of the *Thermus thermophilus* 'Rieske'-type protein has at least two non-cysteine ligands, which may be histidine residues. The close resemblance of the c.d. features of the bacterial and the mitochondrial 'Rieske' protein suggests that the iron-sulphur cluster of the mitochondrial bc_1 complex may also have one or two non-cysteine ligands. In support of this hypothesis it should be noted that the primary sequence of the 'Rieske' protein from *Neurospora crassa* mitochondria (Harnish *et al.*, 1985) and *Rhodopseudomonas spheroides* (Gabellini & Sebald, 1986) contain, in addition to four conserved cysteine residues, at least two histidine residues that are also conserved.

Another important finding presented here is that it is possible to monitor the redox changes of the 'Rieske' protein centre in the c.d. spectra of the whole bc_1 complex without any significant interference by cytochrome c_1 (Figs. 4 and 6) and/or by b cytochromes (Fig. 5). This arises from the fact that the dichroic absorption of the bc_1 complex in the spectral region from 450 to 520 nm is essentially due to the specific features of the 'Rieske' protein centre alone (Figs. 2–6). Hence c.d. constitutes the first spectroscopic technique that can be exploited to monitor the redox kinetics of the 'Rieske' protein centre in a specific and sensitive fashion at physiological temperatures. The alternative technique available to measure the redox changes of the iron-sulphur protein is low-temperature e.p.r. with the use of rapid-freezing systems (Orme-Johnson *et al.*, 1974; De Vries, 1983). This technique has the disadvantages that it requires high concentrations of the 'Rieske' protein centre (Rieske, 1967b; Orme-Johnson *et al.*, 1974; De Vries, 1983) and cannot be combined directly with spectrophotometric measurements of the redox changes of the cytochromes because they cannot be performed under the same conditions (especially temperature) (De Vries, 1983).

Clearly, c.d. spectroscopy appears to be one of the readiest methods to apply to a complete study of electron transfer in the bc_1 complex, since it is able to monitor the redox changes of all the prosthetic groups of the enzyme under the same conditions.

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