Biochimica et Biophysica Acta, 638 (1981) 86–93
Elsevier/North-Holland Biomedical Press

BBA 48161

STRUCTURAL STUDIES ON THE CYTOCHROME *c* OXIDASE PROTON PUMP USING A SPIN-LABEL PROBE

ROBERT P. CASEY, CLEMENS BROGER and ANGELO AZZI

Medizinisch-Chemisches Institut der Universität, CH-3012 Bern (Switzerland)

(Received June 12th, 1981)

Key words: Cytochrome c oxidase; Proton pump; ESR; Spin label; Enzyme reconstitution; N,N-Dicyclohexylcarbodiimide

We report studies in which we have used *N*-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-*N'*-cyclohexylcarbodiimide, a spinlabel analogue of *N,N'*-dicyclohexylcarbodiimide, to investigate the structural aspects of the cytochrome *c* oxidase proton pump. We establish that the spin label binds to the reconstituted enzyme at the same site as does *N,N'*-dicyclohexylcarbodiimide, i.e., within subunit III. ESR studies of the bound spin label indicate that its binding site is situated in an apolar region of the enzyme, though close to its surface. The binding of the spin label to the free oxidase is different from that with the reconsituted enzyme, leading to spin-spin exchange between the bound probe molecules. From this and the fact that *N,N'*-dicyclohexylcarbodiimide binds to subunits III and IV in the free oxidase, we conclude that these two subunits are at the most 20 Å apart.

Introduction

It now seems clear that cytochrome *c* oxidase of mammalian mitochondria possesses an H^+-translocating activity linked to its redox function, which fulfills at least part of its role in H^+ movement by the respiratory chain (Refs. 1 and 2; but see also Refs. 3 and 4). There is, however, as yet very little information concerning the mechanism and structural basis of the H^+-translocation process at the molecular and submolecular levels. Cytochrome *c* oxidase is structurally complex and it is important in particular at this stage to establish which of the subunits of cytochrome *c* oxidase are directly involved in H^+ translocation.

We have recently reported that DCCD binds covalently and specifically to subunit III of cytochrome *c* oxidase either reconstituted in vesicles or in intact mitochondria, whilst potently inhibiting H^+ translocation by the enzyme [5]. The observation [6] that cytochrome *c* oxidase depleted of subunit III, whilst retaining full oxidative activity, does not translocate H^+ is consistent with this observation as is the fact that cytochrome *c* oxidase from *Paracoccus denitrificans*, which, when extracted lacks a subunit corresponding to subunit III, cannot translocate protons [7].

These findings provide a clear indication that subunit III of cytochrome *c* oxidase has an important role in H^+ pumping and it is of interest to probe further the inhibitory interaction of DCCD with this subunit.

NCCD is a close structural analogue of DCCD, containing a nitroxyl group. This has enabled its use as a spin-label probe of H^+-translocating ATPases where it has been shown to mimic the inhibitory effects of DCCD [8]. Here we introduce the use of this substance as a probe of the cytochrome *c* oxidase H^+ pump. NCCD is shown to inhibit H^+ translocation

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; NCCD, *N*-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-*N'*-cyclohexylcarbodiimide; MSL, 4-maleimido-2,2,6,6-tetramethylpiperidine oxyl; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate; TMPD, *N,N,N',N'*-tetramethylphenylenediamine; SDS, sodium dodecyl sulphate.

whilst binding to the oxidase and studies of the incorporated spin label are used to confirm and extend the earlier investigations of the interaction of DCCD with this enzyme.

Materials and Methods

NCCD was synthesized in this laboratory according to the method of Azzi et al. [8]. $DyCl_3$ was obtained from Aldrich Chemical Co. and MSL was from Syva, Palo Alto, CA, U.S.A. The sources of other chemicals were as reported previously [5]. Cytochrome *c* oxidase was reconstituted into asolectin vesicles as described previously [9] except that for experiments where H^+ translocation was not studied, the sonication medium was 24.5 mM potassium cholate, 10 mM Hepes, 39.6 mM KCl, 50.4 mM sucrose, pH 7.4, and the mixture of enzyme and sonicated lipid was dialyzed against 10 mM Hepes, 39.6 mM KCl, 50.4 mM sucrose, pH 7.4. For labelling with NCCD, cytochrome *c* oxidase vesicles (15 nmol of enzyme in 10 mM Hepes, 39.6 mM KCl, 50.4 mM sucrose, pH 7.4) or isolated cytochrome *c* oxidase (15 nmol in 0.5% Tween 80, 10 mM Hepes, pH 7.4) were incubated with 1.5 or 6 μmol of NCCD at 4°C for 22 h. Similar results were obtained using both these amounts of spin label. To remove unincorporated NCCD, the suspension was first centrifuged at 2000 × *g* for 10 min to remove any undissolved spin label. Sodium cholate was then added to the oxidase or vesicle suspensions to a concentration of 1.5% and ammonium sulphate was added until the enzymes precipitated. After collection by centrifugation, the enzyme was subjected to further similar washings. Removal of free spin label was facilitated by passage of the enzyme down a column of Sephadex G-25 equilibrated with 1.5% sodium cholate, 50 mM sodium phosphate, pH 7.4, and elution using the same solution.

For labelling with MSL, 160 nmol of spin label in hexane were evaporated to dryness under N_2 and to this were added 90 μl of cytochrome *c* oxidase (32 nmol haem). The suspension was incubated for 60 min on ice with occasional stirring. The labelled enzyme was then reconstituted into vesicles by the cholate dialysis method [9] during which the free spin label was removed.

Labelling of the oxidase with [^{14}C]DCCD was carried out as follows. To 0.5 ml samples of reconstituted cytochrome *c* oxidase vesicles (2.5 nmol enzyme) were added 250 nmol of [^{14}C]DCCD (4 Ci/mol) and the samples incubated for 24 h at 4°C. The suspensions were then layered over 10% sucrose and centrifuged at 300 000 × g_{av} and 4°C for 20 h. The pellets were then dispersed in 40% sucrose , 3% SDS, 0.1 M Tris-phosphate, pH 6.8, and aliquots of the suspensions containing equal amounts of protein were analyzed by polyacrylamide gel electrophoresis as described previously [5]. Following staining and destaining, the resulting slab gel was washed for 2 days using 10% ethanol, 7% acetic acid and then assayed for radioactivity by autoradiographic fluorography according to the method of Bonner and Laskey [10].

ESR spectroscopy was carried out using a Varian E-Line Century Series Spectrometer. Resonance of the nitroxide spin label was centred on 3380 G using a microwave frequency of 9.415 GHz. The frequency of modulation was 100 kHz and the spectrum plotting time was 8 min. Other parameters are given in the figure legends.

Proton translocation and rate of cytochrome *c* oxidation were measured as described previously [9].

Results

When cytochrome *c* oxidase reconstituted in phospholipid vesicles was incubated in the presence of NCCD and subsequently extracted and washed to remove free spin label, a highly broadened ESR spectrum was observed, characteristic of a tightly bound nitroxide spin label (Fig. 1B). The fact that washing the enzyme with $CHCl_3/CH_3OH$ (1 : 4, v/v) did not remove the spin-label signal (not shown) indicates that the binding of NCCD to the enzyme was covalent. Following labelling with NCCD in vesicles, extraction and washing, the oxidase could be reconstituted by the cholate dialysis procedure giving vesicles with a respiratory control ratio of 5. The reconstituted labelled oxidase gave an ESR spectrum with essentially the same properties as the free labelled enzyme (Fig. 1A). Fig. 1C shows the ESR spectrum of cytochrome *c* oxidase labelled with NCCD under exactly the same conditions as for Fig. 1B, except that the vesicles were pre-incubated with DCCD before addition of NCCD. The diminished size of this

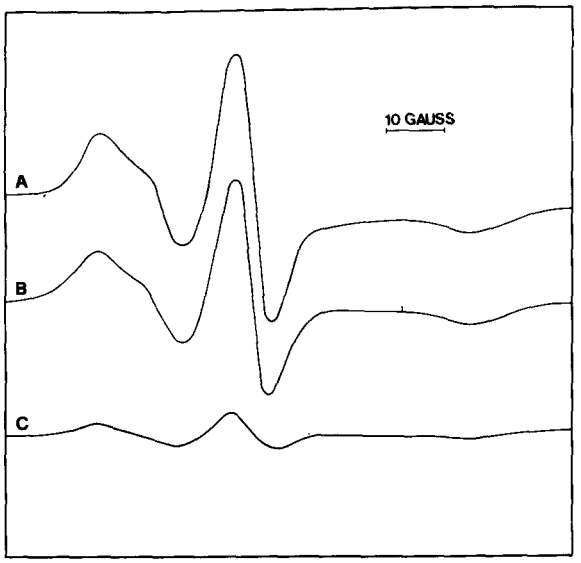

Fig. 1. Binding of NCCD to reconstituted cytochrome *c* oxidase and its inhibition by DCCD. Two identical aliquots of reconstituted cytochrome *c* oxidase vesicles were incubated for 24 h at 4°C, one with 100 mol of DCCD/mol of oxidase and one with an equal amount of ethanol. Both samples were then incubated with NCCD and then processed to extract the enzyme and remove lipid and free spin label, as described in Materials and Methods. The samples were then adjusted so that both had the same protein concentrations. The ESR spectra were measured of the ethanol control (B) and DCCD-treated (C) enzymes, suspended in 1.5% sodium cholate, 50 mM sodium phosphate, pH 7.4, at 23°C. A further sample of reconstituted oxidase was labelled with NCCD, extracted and washed and then re-incorporated into lipid vesicles by the cholate dialysis procedure [9]. The ESR spectrum from the resulting vesicles in 39.6 mM KCl, 50.4 mM sucrose, 10 mM Hepes, pH 7.4, at 23°C is shown (C). The spectra were obtained using a modulation amplitude of 1 G, time constant of 0.5 s and receiver gain of 2000.

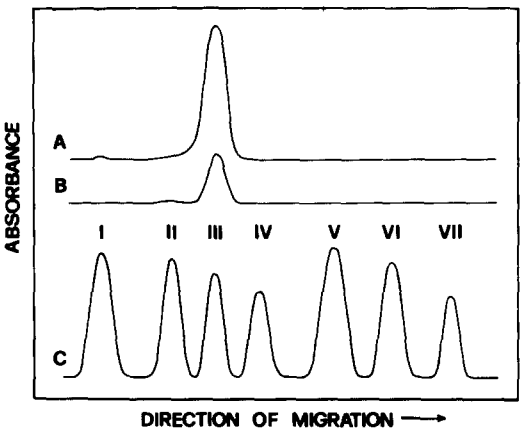

Fig. 2. Binding of [^{14}C]DCCD to subunit III of cytochrome *c* oxidase and its inhibition by NCCD. Samples of reconstituted cytochrome *c* oxidase vesicles were labelled with [^{14}C]DCCD as described in Materials and Methods following incubation for 24 h at 4°C with 250 nmol of NCCD in ethanol or with an equal volume of ethanol. The labelling was then terminated as described. The samples of oxidase were then analyzed by polyacrylamide gel electrophoresis and the resulting gels assayed for radioactivity by autoradiographic fluorography. Taces A and B show the densitometric scans of the autoradiographs from the gels of oxidase labelled without (A) and with (B) pre-incubation with NCCD. Trace C shows the densitometric scan of the Coomassie blue-stained gel of one of the oxidase samples, the staining pattern being the same with both gels.

signal with respect to the former indicates that less NCCD had been bound to the oxidase after this treatment.

The experiment of Fig. 2 shows that, when added in the same stoichiometric amount relative to the oxidase as used in the experiment of Fig. 1, DCCD bound to the oxidase almost exclusively at subunit III with only very minor traces in subunit I and possibly subunit II. These data are consistent with our earlier report [5] and the fact that under these conditions, H^+ translocation by the oxidase was inhibited by 60% (not shown) provides further strong evidence for the involvement of this subunit in H^+ translocation by the enzyme. Fig. 2B shows that pre-incubation of the oxidase vesicles with NCCD before addition of [^{14}C]-DCCD greatly reduced the amount of DCCD incorporated.

When considered in combination, the experiments of Figs. 1 and 2 provide very strong evidence that the NCCD- and DCCD-binding sites are identical.

Having established the binding of NCCD, it was of interest to examine its effects on enzyme function. Interaction of DCCD with cytochrome *c* oxidase leads to inhibition of the H^+-translocating activity of the enzyme [5,11]. The experiment of Fig. 3 shows that NCCD also causes such an inhibition, under conditions where it binds to the enzyme though this was less than that caused by DCCD.

Coin and Hinkle [13] have recently suggested that the inhibition by DCCD of H^+ translocation via cytochrome *c* oxidase is due to a protonophorous effect, though in our experiments this possibility has been

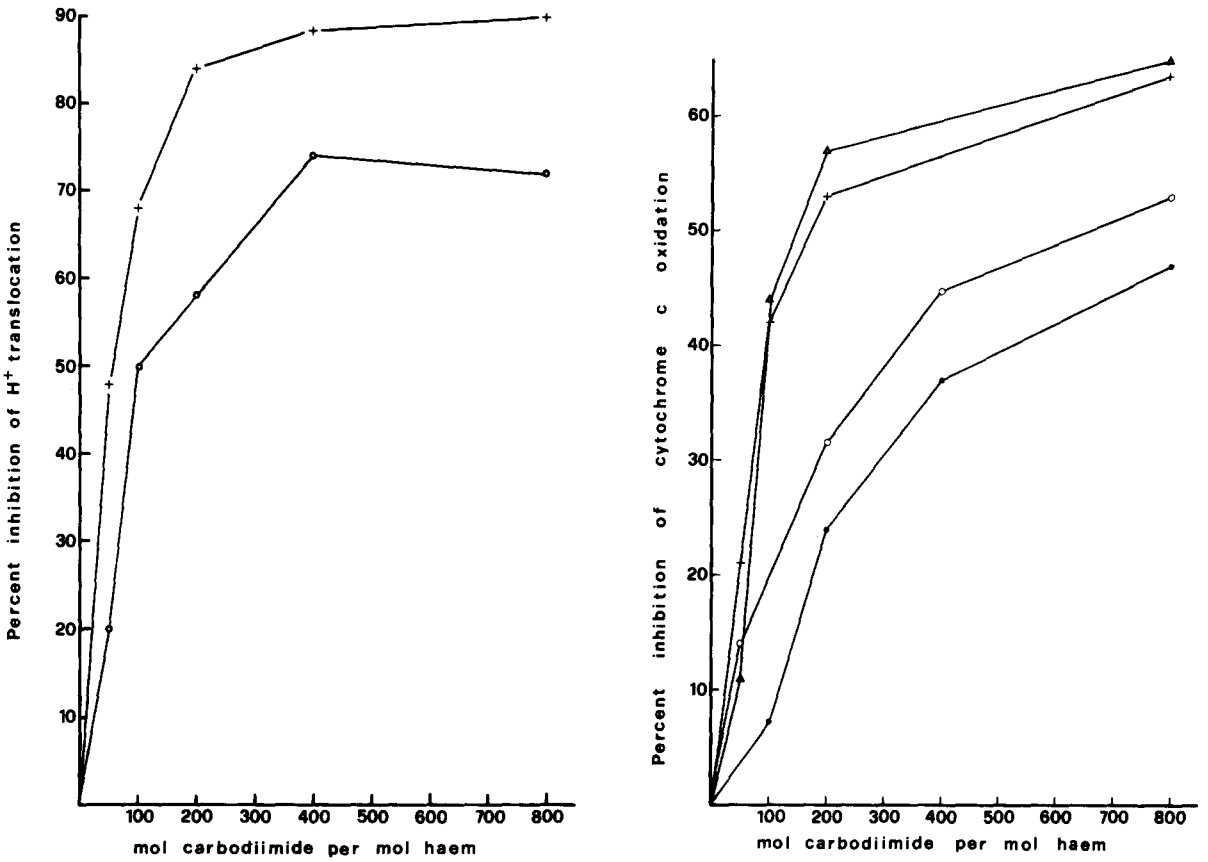

Fig. 3. Comparative inhibition of H^+ translocation and cytochrome *c* oxidation in reconstituted cytochrome *c* oxidase vesicles by NCCD and DCCD. 0.125 ml aliquots of the vesicles (0.7 nmol haem) were added to 1.75 ml of 75 mM choline chloride, 25 mM KCl, 50 μM phenol red, pH 7.4, and NCCD or DCCD from ethanolic solutions was added to give the amounts shown, the ethanol concentration being kept constant. The aliquots were then incubated for 22 h at 4°C. (a) Following incubation, the inhibition of H^+ translocation of the DCCD-containing (+) and NCCD-containing (○) samples was measured. (b) The inhibition of oxidative activity following the addition of 2.5 μl of 0.2 mM valinomycin and 10 μl of 1 mM CCCP (uncoupled: △, •) or no ionophores (coupled: +, ○) was also measured for the samples containing DCCD (△, +) and NCCD (○, •), respectively.

excluded [5,11,14]. Again, for the experiment of Fig. 3 careful measurements of the rate of back-flux of extruded protons showed that the H^+ permeability of the vesicles was not increased by DCCD or NCCD. In addition, and contrary to some of our previous findings [5], in the case of both NCCD and DCCD, inhibition of H^+ pumping by cytochrome *c* oxidase was accompanied by strong inhibition of cytochrome *c* oxidation under both coupled and uncoupled conditions. The inhibition of oxidation was, however, considerably less than that of the inhibition of H^+ translocation by either of the carbodiimides used.

To obtain further information on the environment of the NCCD-binding site, the effects on the spin-label signal of various reductants, predicted to have varying accessibility to the enzyme, were observed (Fig. 4). The rate of reduction of the spin label in the presence of ascorbate as the sole reducing agent was fairly low. The reduction was accelerated by TMPD but even then the rate was lower than that for reduction of free NCCD under the same conditions (not shown). The rate of spin-label reduction was also measured following the addition of cytochrome *c* in the presence of either ascorbate alone or ascorbate and TMPD. In order to prevent re-oxidation of the

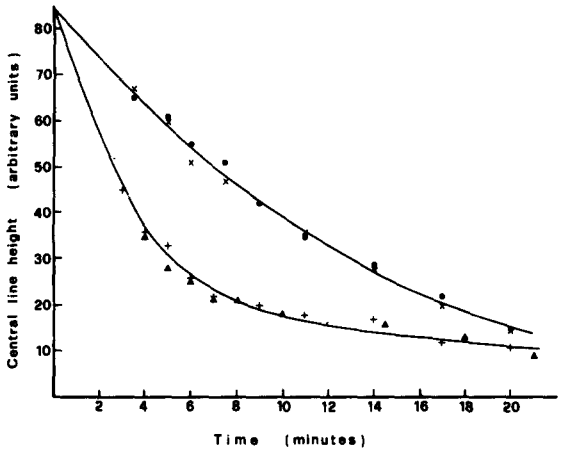

Fig. 4. Reduction of NCCD incorporated in cytochrome *c* oxidase. Cytochrome *c* oxidase reconstituted in vesicles was labelled with NCCD and processed as described in Materials and Methods except that 2 mM KCN was present during labelling and washing. The ESR spectra of aliquots of the labelled enzyme suspended in 1.5% sodium cholate, 50 mM sodium phosphate, 2 mM KCN, pH 7.4, were measured at 23°C at the times indicated following the addition of 1 mM sodium ascorbate (•), 1 mM sodium ascorbate and 200 μM TMPD (+), 1 mM sodium ascorbate and 25 μM cytochrome *c* (x) or 1 mM sodium ascorbate, 200 μM TMPD and 25 μM cytochrome *c* (△). Machine settings were as for Fig. 1.

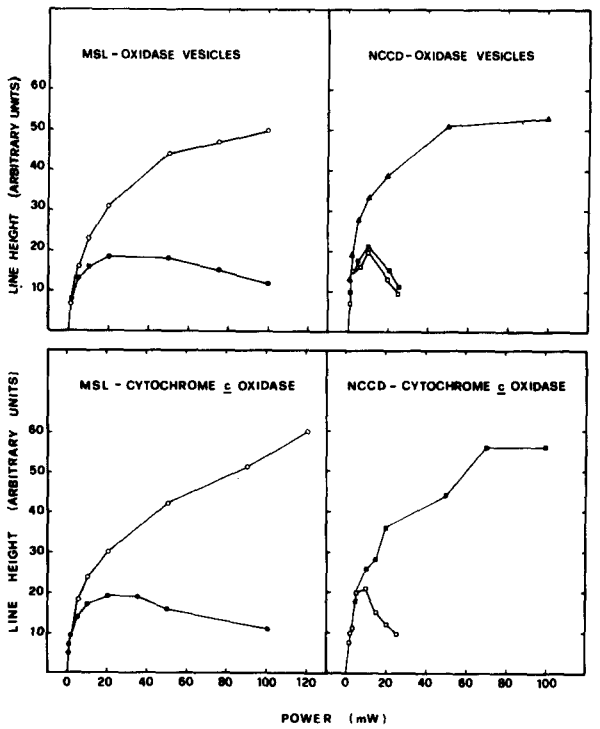

Fig. 5. Differential relief of signal saturation by Dy-EDTA with NCCD and MSL attached to cytochrome *c* oxidase. Reconstituted cytochrome *c* oxidase (approx. 10 nmol) was labelled with either NCCD or MSL and extracted from the vesicles and washed as described in Materials and Methods and then used directly or following re-incorporation into vesicles by the cholate dialysis procedure. The ESR spectrum of the enzyme labelled with MSL (•, ○) or NCCD (▪, □), either free or in vesicles as indicated, was then measured in the presence (○, ▪) or absence (•, □) of 5 mM $DyCl_3$, 50 mM EDTA, pH 7.4. In the case of the reconstituted enzyme, 1.5% sodium cholate was also present (+ Dy-EDTA) (△). The height of the central line in the spectrum is shown as a function of microwave power.

reduced redox centres of the enzyme, this experiment was carried out in the presence of KCN. The addition of cytochrome *c* clearly had no effect on the rates of spin-label reduction.

In order to investigate the depth of the NCCD-binding site beneath the enzyme surface, the paramagnetic complex Dy-EDTA was employed. This has been shown [18] to relieve differentially saturation of the ESR signal of redox centres in cytochrome *c* oxidase and consequently to provide information on their topographical arrangement within the enzyme. We compared the effects of Dy-EDTA on the ESR signals of bound NCCD and MSL. This comparison was made using both the enzyme which had been labelled in vesicles and then extracted (the 'free' enzyme) and that which had subsequently been re-incorporated into lipid vesicles (the 'reconstituted' enzyme). MSL has been shown to bind to cytochrome *c* oxidase at the surface facing outwardly in mitochondria [25]. As shown in Fig. 5, in both the reconstituted and free enzymes, the NCCD signal was saturated more clearly and at somewhat lower power than that of MSL. This might reflect the differential access of water to the binding sites of the two spin labels and its ability to facilitate magnetic relaxation [19]. In the case of the free enzyme, it is clear that Dy-EDTA exerted a strong relief of signal saturation of both spin labels and that this effect had a similar magnitude for the bound NCCD and MSL probes. In the case of the reconstituted enzyme, however, whilst Dy-EDTA exerted a relief of saturation of the MSL signal which was of a similar magnitude to that

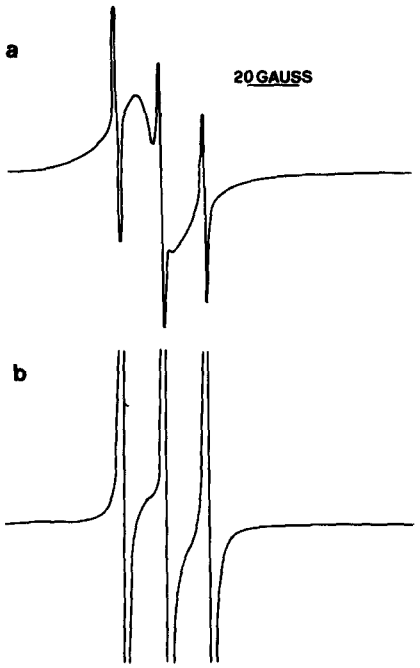

Fig. 6. Binding of NCCD to isolated cytochrome *c* oxidase. Isolated cytochrome *c* oxidase was labelled with NCCD as described in Materials and Methods. (a) ESR spectrum at 23°C of the NCCD-labelled oxidase suspended in 1.5% sodium cholate, 50 mM sodium phosphate, pH 7.4. (b) Spectrum of the enzyme under identical conditions except that SDS had been added to a final concentration of 2%. The modulation amplitude was 1 G, the time constant 0.128 s and the receiver gain 3200.

in the free enzyme, it had no effect on the signal from the bound NCCD, unless cholate was added to disrupt the vesicle membranes, when it again exerted a large relief of saturation.

We have observed [5] the interaction of DCCD with free cytochrome *c* oxidase differs from that with the reconstituted enzyme, in that with the former, both subunits III and IV are labelled. Fig. 6a shows that the ESR spectrum of NCCD incorporated into free cytochrome *c* oxidase is different from that bound when the enzyme is reconstituted, showing spin-spin exchange between the bound spin labels. The experiment of Fig. 6b shows, however, that when SDS was added to the enzyme suspension, causing dispersion of the subunits, spin-spin exchange was lost almost completely and the spectrum was dominated by three narrow lines. The consequences of these observations for the topographical relationship of subunits III and IV are discussed below.

Discussion

The implication of subunit III of cytochrome *c* oxidase in H^+ translocation [5–7] leaves open the question of its precise role in enzyme function, i.e., whether it takes part simply in the electron-transfer function of the enzyme or is actually involved in H^+ translocation. We consider the latter the more likely function for the following reasons. First, the subunits most likely to contain the haem and copper atoms of the enzyme are subunits I, II and VI [20–22] and it is unlikely that a subunit lacking redox centres would be on the electron-transfer pathway. Second, subunit III can be depleted from the enzyme leaving enzymatic activity unaltered [6], also excluding the involvement of subunit III with H^+ channelling to the oxygen-reduction site. Third, we have found [5,11] that under some conditions H^+ translocation can be inhibited by DCCD through covalent binding to the enzyme whilst having only a minor effect on the oxidative activity. We thus consider it more proabable that subunit III is directly involved with the channelling of H^+ through the enzyme, which is consistent with the analogous effect of DCCD on the H^+-translocating ATPases [23,24].

In order that the data obtained here with NCCD attached to cytochrome *c* oxidase can be used to probe the H^+-channelling region of the enzyme, it must be established from the outset that this substance interacts with cytochrome *c* oxidase at the same site as does DCCD. Strong evidence in favour of this comes from the following two points. First, incubation of the enzyme with DCCD before adding NCCD reduces the extent of spin-label binding (Fig. 1). Second, at the same concentration relative to the oxidase at which DCCD blocks NCCD binding it binds almost exclusively to subunit III of the enzyme (Fig. 2) and this binding was greatly reduced by preincubation with NCCD. Third, NCCD induces a concentration-dependent inhibition of H^+ translocation similar to that induced by DCCD (Fig. 3). Furthermore, Steffens et al. [12] have recently reported that at a concentration (80 mol DCCD/mol enzyme) close to that used here, they too observe specific binding of DCCD to subunit III of the oxidase linked to inhibi-

tion of H^+ translocation. We conclude that under the conditions used here, both NCCD and DCCD bind at one site within subunit III of the enzyme.

We have proposed [5] that the DCCD-binding site in cytochrome *c* oxidase is apolar and this is supported here by two observations. First, the onset of inhibition of both H^+ translocation and oxidation by NCCD was slower than that with DCCD. This would be consistent with the NCCD molecule having more restricted access to the binding site than the less polar and smaller DCCD molecule. Second, only a slow loss of spin-label signal was observed when the polar ascorbate was used as reducing agent and this was accelerated by the apolar redox mediator TMPD. It is noteworthy, however, that even TMPD did not reduce bound NCCD as rapidly as it did the free spin label, indicating that also the small TMPD molecule does not have completely free access to the NCCD/DCCD-binding site (Fig. 4).

It is clear from these findings that the NCCD/DCCD-binding site cannot be located at the enzyme surface and it is of interest at this point to consider the depth of the binding site within the enzyme.

The paramagnetic complex of dysprosium and EDTA has already been used to derive information concerning the location of redox centres within cytochrome *c* oxidase [18]. A simple, quantitative relationship between relief of spin-label signal saturation and distance is lacking at present and consequently, to enable us to derive at least qualitative information about the binding site location, we compared the effects of the complex on NCCD saturation with that on the saturation of a spin label, MSL, which is believed to bind at the enzyme surface [25] (see Fig. 5). With the free enzyme, the Dy-EDTA complex exerted a releif of saturation of the ESR signal of the bound NCCD which was of a similar magnitude to that on the superficially bound MSL signal (Fig. 5). As the large and highly polar Dy-EDTA complex is unlikely to penetrate the enzyme, we can conclude that the binding site of NCCD, whilst being apolar, must be close to the enzyme surface. The observation that following reconstitution of the MSL- or NCCD-labelled oxidase into lipid vesicles, Dy-EDTA had no effect on the power of saturation of the bound NCCD signal unless the membrane was disrupted indicates that the NCCD-binding site either faces the vesicles interior or was protected from the paramagnetic complex by being coated with membrane lipid.

Labelling of free cytochrome *c* oxidase with NCCD results in a spectrum showing clear spin-spin interactions (Fig. 6) which indicates that the bound probe molecules are less than 20 Å apart [16]. It is conceivable that this interaction was between spin labels located on different enzyme molecules which were aggregated under the conditions used. This is unlikely, however, as the enzyme was suspended under the same conditions used for the enzyme extracted from vesicles (see Fig. 1) where no spin-spin interaction was observed. In addition, a similar spectrum to that of Fig. 6A was obtained using enzyme resuspended in sodium deoxycholate (not shown), in which cytochrome *c* oxidase exists largely as monomers [17]. We consider it, therefore, more likely that spin-spin interaction was between probe molecules on different subunits within the same enzyme molecule and this proposal is given strong support by the experiment of Fig. 6b. There it is clear that dissociation of the oxidase into its subunits results in almost complete loss of spin-spin interaction and the appearance of three fairly narrow lines. This would be expected if the spin label were attached to a free subunit, being much smaller and more mobile than the intact enzyme. On the basis of our previous findings [5] that DCCD binds to non-reconstituted cytochrome *c* oxidase at subunits III and IV with a maximal stoicheiometry of one molecule per subunit and assuming that, also in the non-reconstituted enzyme, the NCCD- and DCCD-binding sites are the same, the above results would imply that subunits III and IV are separated by a distance no greater than 20 Å.

In conclusion, the above findings establish that NCCD is a useful probe in the characterization of the structural aspects of the cytochrome *c* oxidase proton pump. Further studies using NCCD may provide a clearer insight into the question of the spatial relationship of the proton-transferring region of the enzyme to the cytochrome *c*-binding site and the redox centres.

Acknowledgements

This work was supported by Grant 3.228.077 from Schweizerischen Nationalfonds and by the E. Barrell Stiftung, the Clark Joller Fund and the Schweizerische Gesellschaft für Chemische Industrie. We thank Valerie Riess for technical assistance.

References

1 Wikström, M.K.F. and Krab, K. (1979) Biochim. Biophys. Acta 549, 177–222
2 Azzi, A. and Casey, R.P. (1979) Mol. Cell. Biochem. 28, 169–184
3 Mitchell, P. (1980) Ann. N.Y. Acad. Sci. 341, 564–584
4 Lorusso, M., Capuano, F., Boffoli, D., Steffanelli, R. and Papa, S. (1979) Biochem. J. 182, 133–147
5 Casey, R.P., Thelen, M. and Azzi, A. (1980) J. Biol. Chem. 255, 3944–4000
6 Saraste, M., Pentila, T. and Wikström, M.K.F. (1981) in Proceedings of Symposium on Interaction of Iron and Proteins in Oxygen in Electron Transport (Ho, C., ed.), Elsevier, New York, in the press
7 Ludwig, B. (1980) Biochim. Biophys. Acta 594, 177–189
8 Azzi, A., Bragandin, M.A., Tamburro, A.M. and Santato, M. (1973) J. Biol. Chem. 248, 5520–5526
9 Casey, R.P., Chappell, J.B. and Azzi, A. (1979) Biochem. J. 182, 149–156
10 Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88
11 Casey, R.P., Thelen, M. and Azzi, A. (1979) Biochem. Biophys. Res. Commun. 87, 1044–1051
12 Steffens, G.C.M., Prochaska, L. and Capaldi, R.A. (1980) First European Bioenergetics Conference Short Reports, 95,–96
13 Coin, T. and Hinkle, P.C. (1979) in Membrane Bioenergetics (Lee, C.P., Schatz, G. and Ernster, L., eds.), pp. 405–411, Addison Wesley, Reading, MA
14 Azzi, A., Casey, R.P. and Thelen, M. (1980) First European Bioenergetics Conference Short Reports, 21–22
15 Schairer, H.U., Fridl, P., Schmid, B.I. and Vogel, G. (1976) Eur. J. Biochem. 66, 257–268
16 Träuble, H. and Sackmann, E. (1972) J. Am. Chem. Soc. 94, 4499–4510
17 Robinson, N.C. and Capaldi, R.A. (1977) Biochemistry 16, 375–380
18 Ohnishi, T., Blum, H., Leigh, J.S. and Salermo, J.C. (1979) in Membrane Bioenergetics (Lee, C.P., Schatz, G. and Ernster, L., eds.), pp. 21–30, Addison Wesley, Reading, MA
19 Symons, M. (1978) Chemical and Biochemical Aspects of Electron-Spin Resonance Spectroscopy, Van Nostrand Reinhold Co., New York
20 Steffens, G.J. and Buse, G. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 613–619
21 Steffens, G.C.M., Steffens, G.J. and Buse, G. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1641–1650
22 Winter, D.B., Bruyninckx, W.J., Foulke, F.G., Grinich, N.P. and Mason, H.W. (1980) J. Biol. Chem. 255, 11408–11414
23 Beechey, R.B., Robertson, A.M., Holloway, C.T. and Knight, I.G. (1967) Biochemistry 6, 3867–3879
24 Nelson, N., Eytan, E., Notsani, B.-E., Sigrist, H., Sigrist-Nelson, K. and Gitter, C. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2375–2378
25 Kornblatt, J.A., Baraff, G.A. and Williams, G.R. (1973) Can. J. Biochem. 51, 1417–1427