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INTERACTION BETWEEN CYTOCHROME c AND UBIQUINONE-CYTOCHROME c OXIDOREDUCTASE: A STUDY WITH WATER-SOLUBLE CARBODIIMIDES

Heidi E. GUTWENIGER, Cristina GRASSI* and Roberto BISSON

Centro per la Fisiologia dei Mitocondri e Laboratorio di Biologia
e Patologia Molecolare, Istituto di Patologia Generale,
Università di Padova, Padova, ITALY

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SUMMARY: The role of carboxyl groups on the interaction between ubiquinone-cytochrome c oxidoreductase (Complex III) and cytochrome c has been probed using the two water-soluble carbodiimides EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and CMC (1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-p-toluensulphonate). The results suggest that: **1)** carboxyl groups present on both cytochrome c_1 and subunit VIII are modified. Some of these residues are shielded by cytochrome c. **2)** The enzyme activity decreases during the carbodiimide treatment and the extent of inhibition is larger in the presence of cytochrome c. **3)** Cytochrome c, equimolar with the enzyme, cross-links to cytochrome c_1 and subunit VIII via the carbodiimide-activated carboxyl groups. The two subunits appear to be in contact in the isolated enzyme.

Ubiquinone cytochrome c oxidoreductase (Complex III, EC 1.10.2.2) is the enzyme of the mitochondrial respiratory chain which catalyzes electron transfer from ubiquinone to cytochrome c coupled to proton translocation and ATP synthesis (1-4).

This protein complex appears composed of 8-10 different polypeptides of Mr ranging from 55000 to 4000 Da (5-7).Among them, the b cytochromes, cytochrome c_1 and the Fe-S protein bear prosthetic groups and are clearly involved in the electron transfer process. The role of the remaining polypeptide components is still unknown although some information about their location in the complex is now available (8-10).

The interaction of cytochrome c with this membrane enzyme has been studied with several techniques and general agreement exists on the involvement of cytochrome c_1 in the binding of the substrate (11-16). As well as for all the other physiological partners of cytochrome c this interaction is dominated by electrostatic forces (17,18). Since the cytochrome c binding site is mainly

*Present address: Istituto Sclavo, Via Fiorentina 1, SIENA (ITALY)

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defined by a ring of positively charged lysyl residues surrounding the heme crevice, the presence of a complementary cluster of negative residues on the surface of the interacting enzymes has been postulated (19). This hypothesis has been supported by model fitting (20,21) and later demonstrated by chemical modification and cross-linking experiments on cytochrome **c** peroxidase and oxidase (22-24).

However, a question which arises in the case of a multisubunit enzyme concerns the possibility that this cluster might be shared by different polypeptide components. This aspect is here investigated on complex III. Water soluble carbodiimides, reagents able to modify carboxylate groups specifically (25,26), have been used to probe the negatively charged domain interacting with cytochrome **c**. The data obtained, suggest that in addition to cytochrome c_1, there is a lower molecular weight component containing carboxyl groups which can bind cytochrome **c**.This smaller polypeptide also appears to contact cytochrome c_1 in the enzyme complex.

MATERIALS AND METHODS

Bovine heart ubiquinone-cytochrome c oxidoreductase has been prepared according to Rieske (27). A sample of this enzyme was the gift of Dr. Y.M. Galante (Recordati, S.p.A., Milan). Coenzyme Q-cytochrome c reductase activity was measured essentially according to Wan and Folkers (28). Coenzyme Q_1, kindly provided by Hoffmann La Roche (Basel), was used as substrate. Activities of different preparations ranged from 10 to 35 μmol cyt c/min/mg.EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide), from SERVA, Heidelberg and CMC(1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-p-toluensulphonate), from FLUKA, Buchs were reacted (final concentration 10 mM)with Complex III (3 μM) at 0° in 10 mM potassium phosphate, pH 7.0, 50 μM EDTA, 0.05% Triton X 100; alternatively Brij 35, Octylglucoside, Tween 80 were used.

Radioactive labeling was performed in the presence of 220 μM $[^{14}C]$ glycine ethyl ester (52.2 mCi/mmol, NEN, West Germany). When the reaction was carried out in the presence of cytochrome c, ratios of Complex III/cyt c of 1:1 and 2:1 were used.

Samples for electrophoresis were prepared after stopping the reaction with ammonium acetate pH 5.5 (final concentration 100 mM) and recovering the protein by centrifugation through a cushion of 5% Sucrose, 10 mM potassium phosphate pH 7.4. Pellets were dissolved in 4% SDS, 10 mM Tris-acetate pH 8.2, 0.1 mM EDTA and incubated at room temperature for 2 hours.

Electrophoresis was performed in a 20 cm long linear gradient 12-20% polyacrylamide gel with some modifications of the Laemmli procedure (29). The 2 cm stacking gel was composed of 4.5% Acrylamide, 0.12% Bis-acrylamide, 0.075 M Tris-HCl (pH 6.8), 0.1% SDS and 15% Sucrose. The running gel contained 0.4 M Tris-HCl (pH 8.8), 0.05% SDS and a 0-20% linear Sucrose gradient. The running buffer (pH 9.2) contained 0.033 M Tris, 0.038 M Glycine, 0.1% SDS. Gels were stained with Coomassie Blue or with Benzidine-H_2O_2 after fixing overnight in 45% Methanol, 9% Acetic Acid according to (30). Fluorographic images of the

radioactive gels were obtained according to Bonner and Laskey (31). Reconstituted Ubiquinone-cytochrome c reductase in asolectin vesicles having a respiratory control of 6, was kindly provided by Dr. M. Degli Esposti (University of Bologna).

RESULTS

The two water-soluble carbodiimides used in the present study are 1-ethyl-3-(3-dimethylaminopropyl carbodiimide (EDC) and 1-cyclohexyl-3-2-morpholinyl-4-ethyl)carbodiimide metho-p-toluensulphonate (CMC). In the presence of a carboxyl group, they form a highly reactive O-acyl isourea intermediate which may either rearrange to a stable N-acyl urea or react with a nucleophile to form a stable covalent adduct (25,26). Since the two reagents differ in their molecular dimensions, information on the relative accessibility of the modified acidic residues present on protein surfaces may be obtained.

The effect of the two carbodiimides on the activity of complex III is reported in Fig. 1. At 0°C they inhibit the enzyme activity to a similar degree. When the same reaction is performed in the presence of an equimolar amount of cytochrome **c** (which under the low ionic strength conditions used here, is supposed to reside mostly on the enzyme binding site (32)) the extent of inhibition is increased. This effect appears particularly pronounced in the enzyme treated with EDC. Higher temperatures (22 and 37°C) increase the rate of inhibition but also affect to some extent the stability of the control samples (data not shown). After protein modification no changes in the visible spectrum of the enzyme are detectable.

Two different, although not necessarily alternative hypotheses can be advanced to explain the loss of enzyme activity. Either some carboxyl groups important for the stability of Complex III are modified and/or a change of the negative charge distribution on the enzyme binding domain is induced by the carbodiimide treatment. In the latter case, the lysyl amino groups located on cytochrome **c** could act as nucleophiles toward the carbodiimide activated carboxyl groups of complex III producing a covalent, inactive enzyme-substrate complex (see below).

Fig. 2 analyzes the SDS-gel electrophoretic profiles of the enzyme modified with 10 mM EDC for 85 minutes. In agreement with recent results obtained using highly resolving SDS gel electrophoretic conditions, 5 polypeptides can be identified in the lower molecular weight region of our gel system, between 13 and 5 kDa (1,5-7). When compared with the Coomassie blue staining pattern of a

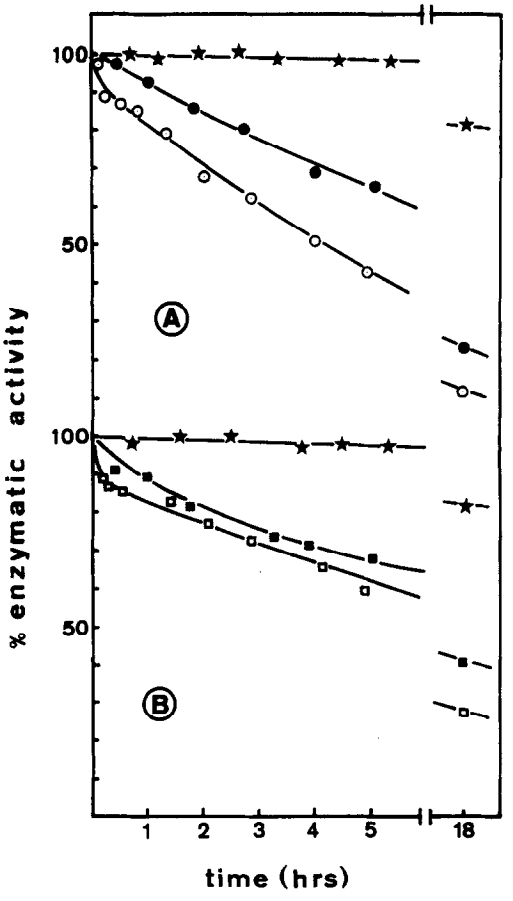

Fig. 1 - Effect of EDC and CMC on the ubiquinone cytochrome c reductase activity.
Complex III (3 μM) in 10 mM phosphate buffer pH 7.0, 50 μM EDTA, 0.05% w/v Triton X100 was reacted at 0°C with 10 mM EDC (A) or 10 mM CMC (B) in the absence (closed symbols) or in the presence (open symbols) of equimolar amount of cytochrome c. At different times aliquots of the enzyme (4 μl) were added to 1 ml of a solution containing 25 mM Phosphate pH 7.4, 50 μM EDTA, 0.05% Tween 80, 8 μM cytochrome c and the activity measured at 20°C spectrophotometrically according to (28).

control sample, the EDC treated enzyme (Fig. 2A, shaded area) shows a decrease in the content of cytochrome c_1 (Mr 27 kDa) and of a polypeptide at lower molecular weight, namely subunit VIII (Mr 9 kDa).A new band is detectable at a molecular weight (37.5 kDa) which approximates that expected for c_1 + VIII (27 + 9 = 36 kDa).

A similar decrease in cytochrome c_1 and subunit VIII is also found after modification of the enzyme with EDC in the presence of cytochrome c (Fig. 2 B). However, in this case, the densitometric trace shows two new peaks with an apparent Mr of 38.5 and 20.5 kDa. The new band at higher molecular weight does

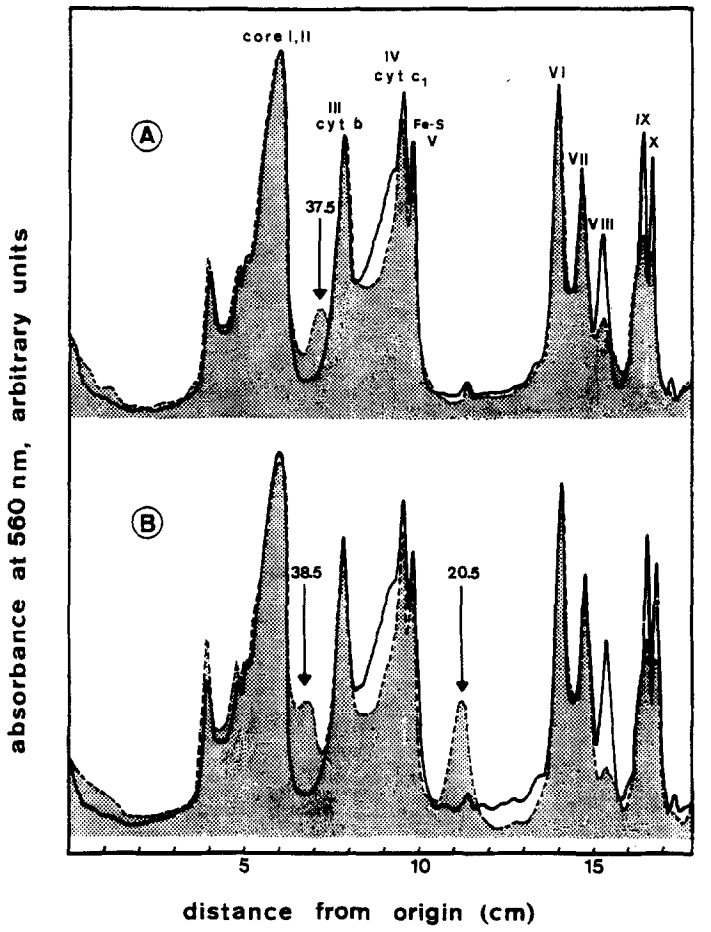

Fig. 2 - SDS gel electrophoresis of carbodiimide modified Complex III.
Enzyme samples were treated with EDC in the presence and in the absence of cytochrome c, in the same experimental conditions reported in Fig. 1. Reactions were stopped after 85 minutes by adding 100 mM ammonium acetate. Protein samples were recovered and electrophoretized on a 12-20% gradient slab gel as reported in the Experimental section. The figure shows the Comassie blue densitometric traces of the EDC treated enzyme (shaded areas) in the absence (panel A) and in the presence (panel B) of equimolar cytochrome c. Solid line shows a control sample for comparison.Cross-linked polypeptides are indicated by the molecular weight.

not coincide with that found in the absence of cytochrome c which is undetectable in this gel. The Mr's of the two new polypeptides closely approximate that of c + c_1 (12.5 + 27 = 39.5) and c + VIII (12.5 + 9 = 21.5).

Similar results, except for a lower cross-linking efficiency, were obtained when the bulkier carbodiimide CMC was used (not shown). Some decrease in the content of polypeptides IX and X was also detectable in these experiments, particularly at higher temperatures and when long reaction times were used. When

the enzyme was treated under different conditions (as after reconstitution in asolectin vesicles, or in the presence of Tween 80 (0.05%), octylglucoside or Brij 35) similar results were obtained. However the protein/detergent ratio is an important parameter. In the standard experiments we have used 0.7 g of Triton X100 per gram of enzyme (0.05% w/v), an amount well in excess of that bound by complex III (0.2-0.3 g/g enzyme) (33,34). Modification in the presence of 0.14 g/g of protein (0.01% w/v) induced some protein aggregation.

Fig. 3 shows the benzidine staining profile of the carbodiimide-modified enzyme samples. With this procedure only polypeptides containing covalently bound heme are detectable (30) so that cytochrome c_1 is the only subunit of complex III which appears on the gels (Fig. 3, A). As has been frequently observed, this polypeptide appears as a broad peak. This broadening is even more pronounced here since β-mercaptoethanol was not used in the dissociation buffer (35) so as to allow better staining with the benzidine reagent and better resolution between subunits VII and VIII. The heme staining of the EDC treated enzyme (Fig. 3, panel B shaded area) reveals that part of cytochrome c_1 is shifted to higher molecular weight. This result suggests a cross-linking to c_1 of another polypeptide and considering the data of Fig. 2, this is likely to be subunit VIII.

Modification in the presence of cytochrome **c** gave a qualitatively similar pattern for the distribution of cytochrome c_1. However the staining intensity of the new peak and its molecular weight, slightly higher when compared with that found in the absence of the substrate, indicates that cross-linking of cytochrome c to c_1 occurs in this case. Cytochrome c is also responsible for the benzidine staining of the lower molecular weight cross-linked product. As suggested by Fig. 2, it is component VIII which is most probably the bound enzyme subunit.

In summary these experiments indicate that some carboxyl groups located on cytochrome c_1 and subunit VIII of Complex III can make contact with cytochrome **c** and when activated by water-soluble carbodiimides, this leads eventually to a covalently linked enzyme-substrate complex.

However the possibility that negative charged residues present in other subunits of Complex III might be modified causing the inhibition of the enzyme activity cannot be excluded. To gain more information on this point we have performed the modification experiments in the presence of the radioactive

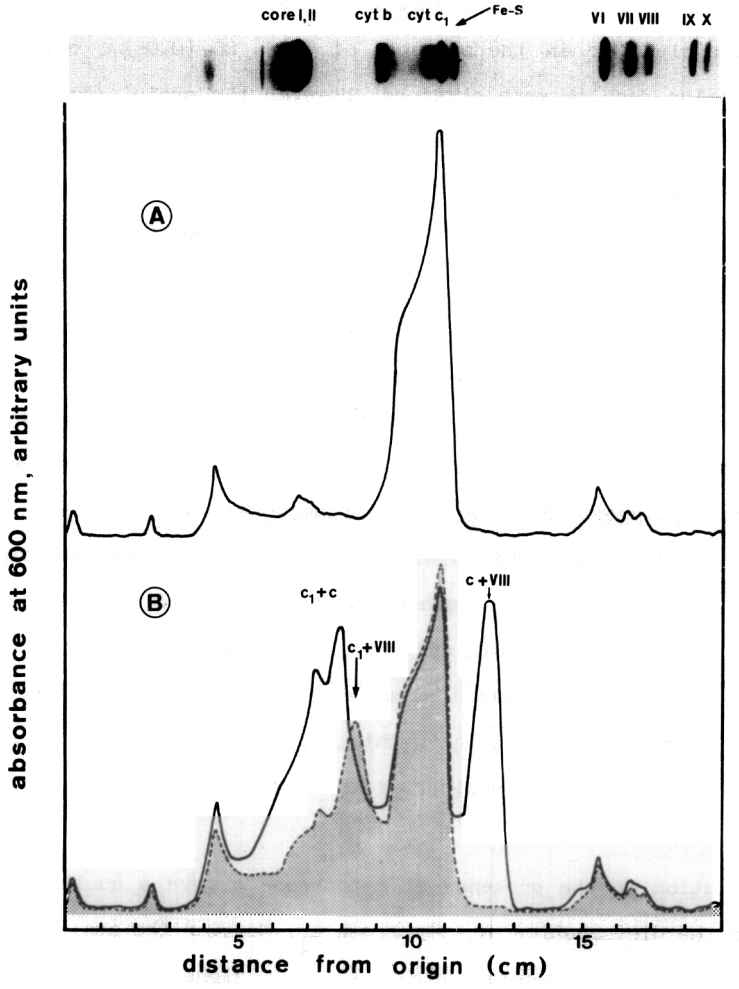

Fig. 3 - Heme staining profiles of carbodiimide modified Complex III. SDS gels of the protein samples obtained as in Fig. 2 were fixed and stained with benzidine and H_2O_2 according to ref. 30. In panel A is the densitometric trace of a control sample of Complex III. Comparison with the Comassie blue staining pattern reported on top figure shows that only cytochrome c_1, which bears a heme covalently bound, can be stained. Traces of free heme unspecifically absorbed by other enzyme subunits are low. In panel B are the profiles of the enzyme treated with 10 mM EDC in the absence (shaded area) and in the presence (solid line) of an equimolar amount of cytochrome **c**. Similar results were obtained with CMC.

nucleophile $[^{14}C]$-glycine ethyl ester which can label the carbodiimide activated residues by formation of an amide bond.

Fig. 4 shows the radioactivity distribution among the subunits of the enzyme treated with EDC or CMC in the absence or in the presence of cytochrome **c**. More than 90% of the radioactivity is incorporated in cytochrome c_1,

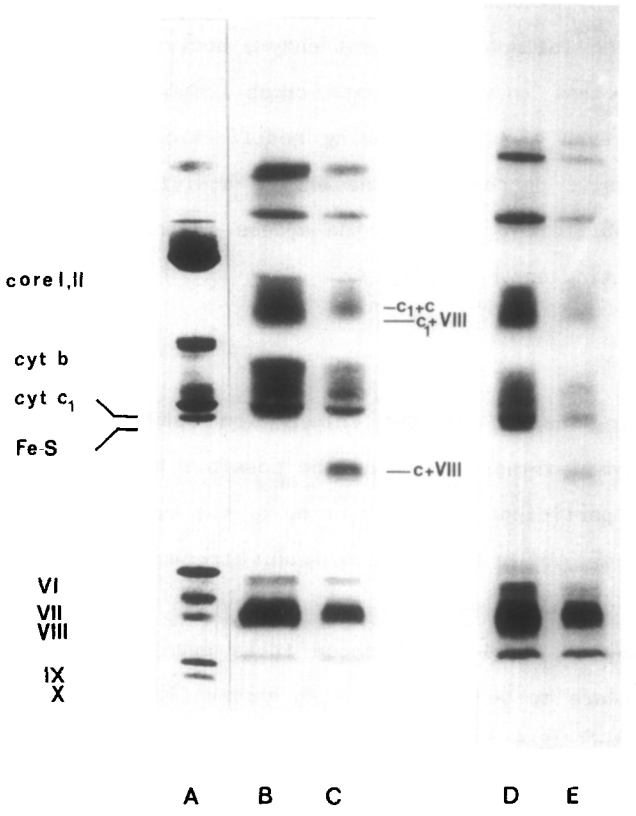

Fig. 4 - Autoradiographs of Complex III modified with EDC and CMC in the presence of [^{14}C] glycine ethyl ester.
Enzyme samples (3 μM) in 10 mM phosphate buffer pH 7.0, 50 μM EDTA, 0.05% Triton X100, 220 μM [^{14}C] glycine ethyl ester were reacted with 10 mM EDC or CMC at 0°C for 150 minutes and 4 hrs respectively, in the presence or in the absence of cytochrome **c**. After addition of ammonium acetate (100 mM), protein samples were recovered and electrophoretized as described in M % M. Autoradiography was performed according to ref. 31. A is the Comassie blue staining pattern of the enzyme. Trace B and C show the results obtained for the EDC treated enzyme in the absence and in the presence of the substrate respectively. The results obtained in a different gel for the CMC treated enzyme in the absence (D) and in the presence (E) of cytochrome **c** are also reported.

polypeptide VIII and their cross-linked products. The presence of cytochrome **c** decreases the radioactivity of both enzyme subunits and this protection is more effective when the bulkier CMC is used instead of EDC. Moreover CMC allows better discrimination between the reactive carboxyl groups in the two subunits. Clearly the carbodiimide modified residues of cytochrome c_1 are better protected by cytochrome **c** than those located on subunit, VIII indicating that they probably play a major role in the interaction with the substrate.

The shielding by cytochrome **c** of the carboxyl groups of Complex III does not correlate with the failure to protect enzyme activity. A possible explanation may be the efficient enzyme-substrate cross-linking which is found in this system. In fact even though increasing modification of the negative residues leads to progressive decrease in the enzyme activity, a single cross-linking event might be sufficient to block the access of the substrate to the binding site, thus inhibiting enzyme activity.

DISCUSSION

An intriguing aspect of the interaction between cytochrome **c** and its multisubunit enzyme partners concerns the possibility that separate polypeptide components might participate in the binding of the substrate, either at the same or different sites, in order to accomplish structural or possibly regulatory roles.

In the case of Complex III these ideas seem improbable since a unique binding site appears to be present on the enzyme (32) and the polypeptide known to act as electron donor to cytochrome **c**, namely cytochrome c_1, can be isolated and still be capable of interaction and electron transfer with the substrate (36,37). Moreover kinetic studies with selectively modified cytochrome **c** derivatives suggest that almost identical amino acid domains on cytochrome **c** are involved in the interaction with the enzyme or isolated c_1 (12,15,32). Differential chemical labelling of cytochrome **c** also supports this view even though some differences are present in the data obtained from the two interacting systems (11).

However, until recently the isolation of pure cytochrome c_1 was a difficult task (37-40) and several laboratories report the presence of a smaller polypeptide component associated with cytochrome c_1 derived from different sources (35, 41-44). Kim et al. (45) first proposed that this component may have a structural role in the formation of the complex between cytochrome **c** and c_1. In this respect, its polypeptide chain which shows a high density of negatively charged groups with an unsual cluster of 8 consecutive glutamyl residues (46) is provocative.

Photoaffinity labelling of complex III performed in intact mitochondria also indicates that some low molecular weight subunits could be involved together with cytochrome c_1, in the interaction with cytochrome c (13).

The present results suggest that cytochrome **c** can make electrostatic contact with both cytochrome c_1 and subunit VIII. It is likely that subunit VIII (9000 kDa from gel) corresponds to the cytochrome c_1 associated polypeptide (9175 kDa derived by sequence analysis (46)) initially described by King (42).

Whether this subunit actually forms part of the enzyme binding site for the substrate is more questionable. In fact the definition of a quantitative correlation between loss of enzyme activity and extent of subunit modification was a difficulty of this investigation. Carbodiimides have been found to alter polypeptide migration on SDS-polyacrylamide gel electrophoresis (47) and therefore broadening and/or aggregation can be expected, particularly in the case of very acidic components such as subunit VIII. This effect may be an explanation for the large decrease in the amount of subunit VIII which was associated with rather a marginal loss of enzyme activity. An alternative interpretation is that this polypeptide might not be part of the enzyme binding site at all. However Fig. 4 B and D show that both, broadening of polypeptide VIII and higher molecular weight radioactive bands (possibly due to aggregates) are present in the gel of the modified enzyme.

The high negative charge density of subunit VIII could favour efficient cross-linking to cytochrome c even under conditions of low frequency protein-protein collisions. On the other hand random collisions appear to be improbable on the basis of recent observations which suggest that cytochrome **c** contacts its enzyme partners along a defined trajectory having an appropriate orientation (17,48). Moreover, the protection against modification by cytochrome **c**, supports the idea of participation of the subunit VIII negative residues in the enzyme binding domain. This conclusion may also be correlated with the carbodiimide-mediated cross-linking of subunit VIII to cytochrome c_1, which indicates the proximity of the two separate polypeptides. The absence of such cross-linking, when cytochrome **c** interacts with the enzyme might indicate possible conformational changes induced by the substrate.

Hopefully, isolation of the modified and cross-linked polypeptides and their fragments will perhaps clarify these aspects and will allow the identification of the specific residues which, in complex III, are responsible for the binding of cytochrome **c**.

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