Different polypeptides of bovine heart cytochrome *c* oxidase are in contact with cytochrome *c*

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Two water-soluble carbodiimides, differing in molecular dimensions, have been used to characterize the cytochrome *c* binding site of bovine heart cytochrome *c* oxidase. Several polypeptide components of the enzyme contain acidic residues which are modified by these reagents. Carboxyl groups present in subunit II, VII and polypeptide c, are protected from modification when cytochrome *c*, equimolar to oxidase, is added and they can cross-link to the substrate once activated by the carbodiimide. Comparison of the modification patterns suggest that the most reactive residues are located on subunit II and VII, the former being also more exposed. The data obtained indicate that eventhough subunit II plays the major role in binding cytochrome *c*, at least two other lower M_r polypeptides contribute to the cytochrome *c* binding domain.

Cytochrome c *oxidase* *Cytochrome* c *Cross-linking* *Protein modification* *Carbodiimide*

1. INTRODUCTION

Cytochrome *c* oxidase catalyzes the transfer of electrons from cytochrome *c* to oxygen coupled to proton translocation across the inner mitochondrial membrane [1]. The interaction of cytochrome *c* with this enzyme as well as with all its physiological partners is mainly based on electrostatic forces [2]. A ring of positive charges, surrounding the heme crevice on the protein 'front face', has been implicated in this binding [3] suggesting that a corresponding set of negatively charged amino acid residues is present on a defined domain of the cytochrome *c* redox enzyme [4].

Model fitting, supported by cross-linking experiments, suggests that this is the case for the cytochrome *c*–cytochrome *c* peroxidase couple [5,6]. Water-soluble carbodiimides, specific modifying reagents of the carboxylic group, have been used to identify the negative residues of cytochrome *c* peroxidase involved in the interaction with cytochrome *c* [7].

The definition of the cytochrome *c* binding site on cytochrome *c* oxidase is more difficult since this is a membrane-bound enzyme composed of several different polypeptides of M_r 57000–5400 [8,9]. However, it has been shown by cross-linking experiments that subunit II of the oxidase binds cytochrome *c* on its 'front face' while subunit III interacts with its 'back face' [10–12]. A subunit II peptide fragment involved in the interaction with cytochrome *c* has been identified by amino acid sequence of a photo-cross-linked cytochrome *c*–subunit II derivative [13].

Here, we present some evidence that carboxylic groups located on more than one cytochrome *c* oxidase subunit are involved in the binding of cytochrome *c* at the high affinity site.

2. MATERIALS AND METHODS

Bovine heart cytochrome *c* oxidase was prepared as in [14] omitting the final dialysis step. Activity was measured polarographically in 0.5% Tween 80, 50 mM P_i (pH 7.4) and ranged from 150 −190 mol cytochrome $c.s^{-1} \cdot mol aa_3^{-1}$ in different

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preparations. Reaction with 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) was performed at 0°C, 25°C and 37°C by adding the reagent (from a freshly prepared stock solution; final concentration in figure legend) to a 5 μM cytochrome *c* oxidase solution in 10 mM P_i, 0.1% lauroyl-β-maltoside (pH 6.5). Radioactive labelling was performed by adding to the above reaction mixture [^{14}C]glycine ethyl ester (52.2 mCi/mmol, NEN) to 500 μM final conc. Protection against EDC or CMC modification and cross-linking to cytochrome *c* were performed in the presence of cytochrome *c* (type VI, Sigma MO) at enzyme/substrate ratios of 1:1 and 2:1, respectively. At different times, aliquots were assayed for oxidase activity. The reaction was stopped by addition of ammonium acetate (100 mM final conc.). The oxidase was recovered by centrifugation on 10% sucrose, 100 mM P_i (pH 7.4). Pellets were dissolved in 3% SDS. SDS–polyacrylamide gel electrophoresis was performed in 15% polyacrylamide, 0.5% bis-acrylamide as in [15]. After staining with Coomassie blue and recording of the densitometric trace the gels were sliced in 1 mm thick slices and incubated in Lumasolve–Lipoluma–Water (Lumac System AG, Basel). After 24 h at 40°C they were counted in a Packard Tri Carb 300C scintillation counter. Heme staining was performed as in [16]. Lauroyl-β-maltoside was prepared essentially as in [17].

3. RESULTS AND DISCUSSION

Water-soluble carbodiimides are protein-modifying reagents specific for glutamate and aspartate residues [18]. They form a highly reactive *O*-acyl isourea intermediate which, in the presence of an amino group, can form a stable amide derivative [19]. The two most used are CMC and EDC, which differ in dimension. The reactivity of protein carboxyl groups toward water-soluble carbodiimides depends on their accessibility, on their pK_a and on the size of the modifying reagent. CMC is bulkier and will react with buried residues more difficultly than the smaller EDC.

Fig. 1 shows the time course of EDC and CMC inhibition of cytochrome *c* oxidase activity at 0°C. Their efficiency in lowering enzymic activity

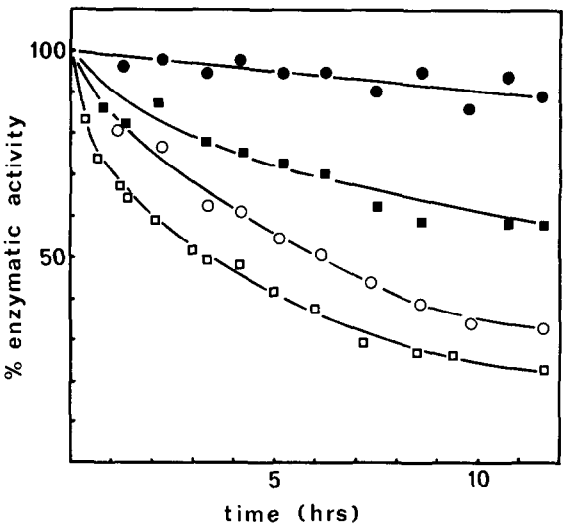

Fig. 1. Carbodiimide inhibition of cytochrome *c* oxidase activity. Inhibition of cytochrome *c* oxidase in the absence of cytochrome *c* by EDC (□) and CMC (○) and in the presence of cytochrome *c* by EDC (■) and CMC (●). Cytochrome *c* oxidase 5 μM, CMC and EDC 2 mM, cytochrome *c* 5 μM in P_i 0.1 M, 0.1% lauroyl maltoside, pH 6.5 at 0°C. Assays as in section 2 except for the substitution of Tween 80 with 0.025% lauroyl maltoside.

decreases with time. As expected the smaller EDC is more effective than CMC. Their reaction with oxidase does not result in any modification of its absorption spectrum (not shown). Fig. 1 also shows that cytochrome *c*, equimolar to the oxidase, is very effective in protecting the enzyme from carbodiimide modification and inhibition particularly with CMC. It is noteworthy that under these conditions only the high affinity cytochrome *c* binding site is occupied [20,21]. Higher temperatures increase the rate of reaction without changing the inhibition patterns and the extent of cytochrome *c* protection (see below). These results support the idea that some carboxyl residues of the oxidase are directly involved in the interaction with cytochrome *c*.

To identify the cytochrome *c* oxidase subunit(s) containing the carbodiimide-reactive carboxyl groups we have used the radioactive nucleophile [^{14}C]glycine ethyl ester, which forms a stable amide derivative [19]. The pattern of labelling after 90 min reaction (fig. 2 inset) shows that the most reactive carboxyl residues are located on sub-

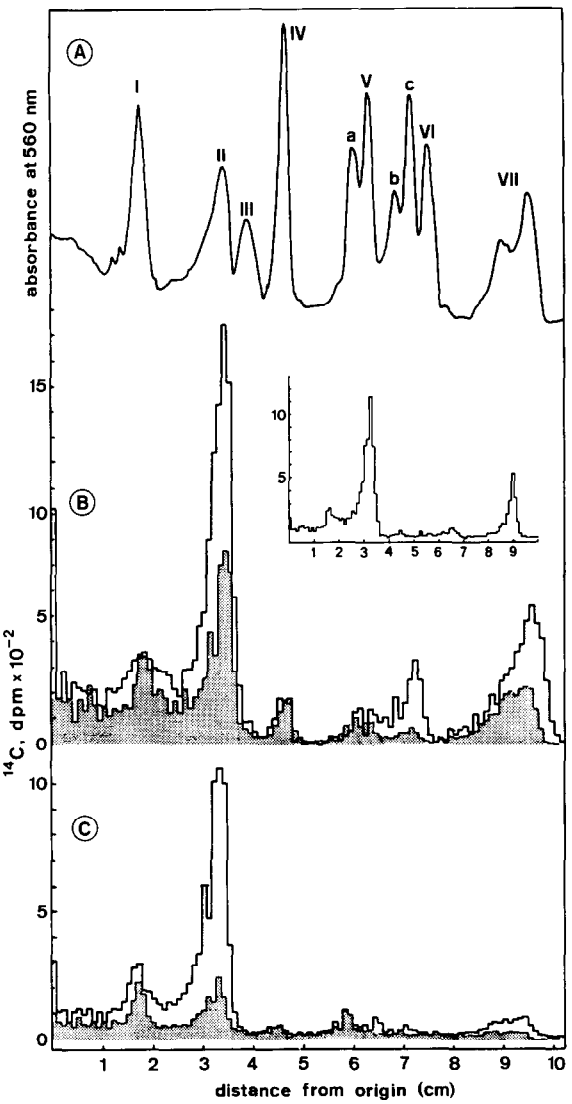

Fig. 2. Cytochrome *c* oxidase subunits modified by carbodiimide. (A) Coomassie blue-staining profile of cytochrome *c* oxidase after electrophoresis as in [15]. Subunit nomenclature as in [22]. The pattern of labelling of cytochrome *c* oxidase is reported in the presence of [^{14}C]glycine ethyl ester with EDC (B) and CMC (C) after 12 h at 0°C. The shaded areas show the labelling in the presence of cytochrome *c*. The inset reports the result of labelling with EDC after 90 min at 0°C. Reaction conditions are as in fig. 1 legend with 500 μM [^{14}C]glycine ethyl ester.

unit II and on one of the subunits VII (nomenclature as in [22]). With longer incubations, polypeptides I, IV and C are also significantly labelled (fig. 2B). CMC only modifies carboxyl groups present on subunit II and, at very low extent, subunit I (fig. 2C). The presence of cytochrome *c* drastically alters the patterns of labelling. In the enzyme treated with EDC it lowers specifically the amount of radioactivity associated with polypeptides II, VII and C. In the case of CMC, while the labelling of subunit II is greatly reduced, that of subunit I is almost not affected. The patterns of labelling obtained at higher temperatures are similar except for the larger proportion of radioactivity associated with subunit I and for a slight change in the electrophoretic pattern due to some aggregation of the smaller oxidase subunits (not shown). The higher and different incorporation of radioactivity in the EDC with respect to the CMC-treated enzyme (at a similar extent to inhibition of oxidase activity) can be explained by the fact that additional carboxyl groups are modified by the more reactive EDC.

These data provide further evidence that subunit II plays the major role in the formation of the binding site for cytochrome *c*. As shown by the experiments with CMC, this subunit contains the most reactive and exposed carboxyl groups responsible for the binding of cytochrome *c*. However, the results of EDC modification suggest that also some carboxyl residues of polypeptides VII and C contribute to the electrostatic interaction with cytochrome *c*.

An alternative possibility for the different patterns of labelling in the presence of cytochrome *c* is that its binding induces a conformational change of the oxidase altering the reactivity of some carboxyl residues. However, this is unlikely in the light of the cross-linking experiments in fig. 3. If the glutamate and aspartate residues of the oxidase, shielded by cytochrome *c*, are facing lysine residues they should be able, once activated by a carbodiimide, to form amide linkages and hence a covalent cytochrome *c*-cytochrome *c* oxidase complex. Fig. 3 shows clearly that cross-linking does occur with the carbodiimide-modified polypeptides, shielded by cytochrome *c*. The heme staining procedure used here is very sensitive and in SDS-gels it detects only cytochrome *c* cross-linked polypeptides because its heme is covalently bound to the protein [16]. Out of the 5 subunits modified by EDC only 3 cytochrome *c* cross-linked products could be identified after SDS–polyacrylamide gel

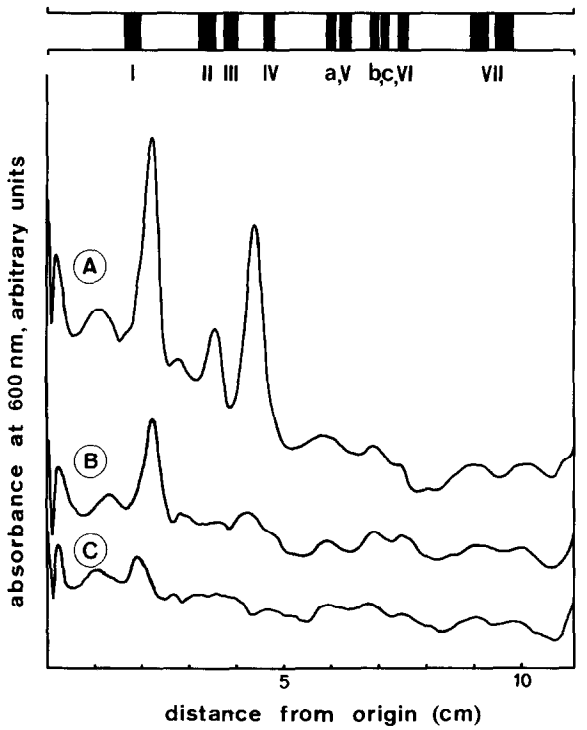

Fig. 3. Carbodiimide-mediated cytochrome *c* cross-linking to cytochrome *c* oxidase. The figure shows the densitometric trace of benzidine-stained gels of cytochrome *c* oxidase (5 μM) reacted in the presence of cytochrome *c* (10 μM) with EDC (2 mM, A) or CMC (10 mM, B) at 0°C for 12 h. An EDC-treated cytochrome *c* oxidase sample not containing cytochrome *c* is shown (C). Only cytochrome *c* is stained by benzidine because its heme is covalently linked to the protein, however traces of cytochrome *c* oxidase heme unspecifically absorbed, especially on subunit I, are detected (see C). Top panel is the Coomassie blue-staining pattern of cytochrome *c* oxidase.

electrophoresis in the position corresponding to II + cytochrome *c*, VII + cytochrome *c* and C + cytochrome *c*. With CMC exclusively subunit II cross-links to cytochrome *c*. Hence, only the cytochrome *c*-protected subunits are able to form covalent derivatives with the substrate. These results rule out the possibility of unspecific cross-linking in agreement with the considerable evidence showing that cytochrome *c* encounters its oxidase only in a defined orientation and only at its binding site [23]. Similar patterns were found at higher temperatures except for a greater efficiency of cross-linking in the presence of CMC with respect to EDC (not shown).

On the base of kinetic studies it has been suggested that more than one binding site for cytochrome *c* exist on isolated cytochrome *c* oxidase [20,21]. Only the so-called 'high affinity binding site' is involved in these experiments. Carbodiimide modification of this site leads to a decrease of >80% of electron transfer activity. These data and our previous findings [11,24] support the idea that only the 'high affinity site' is functional in electron transfer [12]. Polypeptides, II, VII and C appear to contribute to the cytochrome *c* binding domain present on cytochrome *c* oxidase. Among them, subunit II plays the major role. While this subunit is present in all cytochrome *c* oxidases so far isolated, the smaller subunits appears to be lacking in the bacterial enzymes [25]. However, polypeptides VII and C could fulfill a regulatory role in the interaction with cytochrome *c* in eukaryotic oxidases.

We are currently investigating which of the 3 subunits VII is cross-linked to cytochrome *c* and which glutamate and aspartate residues are modified by EDC and CMC.

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REFERENCES

[1] Wikstrom, M., Krab, K. and Saraste, M. (1981) Cytochrome *c* oxidase: a synthesis, Academic Press, London, New York.
[2] Rieder, R. and Bosshard, H.R. (1980) J. Biol. Chem. 255, 4732–4739.
[3] Dickerson, R.E. and Timkovich, R. (1975) The Enzymes 3rd edn, 11, 397–547.
[4] Salemme, F.R., Kraut, J. and Kamen, M.D. (1973) J. Biol. Chem. 248, 7701–7716.
[5] Poulos, T.L. and Kraut, J. (1980) J. Biol. Chem. 255, 10322–10330.
[6] Bisson, R. and Capaldi, R.A. (1981) J. Biol. Chem. 256, 4362–4367.
[7] Waldmeyer, B., Bechtold, R., Bosshard, H.R. and Poulos, T.L. (1982) J. Biol. Chem. 257, 6073–6076.

[8] Fuller, S., Capaldi, R.A. and Henderson, R. (1979) J. Mol. Biol. 134, 305–327.
[9] Kadenbach, B. and Merle, P. (1981) FEBS Lett. 135, 1–11.
[10] Birchmeyer, W., Kohler, C.E. and Schatz, G. (1976) Proc. Natl. Acad. Sci. USA 73, 4334–4338.
[11] Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Montecucco, C. and Zanotti, A. (1978) J. Biol. Chem. 253, 1874–1880.
[12] Capaldi, R.A., Darley-Usmar, V., Fuller, S. and Millet, F. (1982) FEBS Lett. 138, 1–7.
[13] Bisson, R., Steffens, G.C.M., Capaldi, R.A. and Buse, G. (1982) FEBS Lett. 144, 359–363.
[14] Steffens, G.J. and Buse, G. (1976) Hoppe-Seyler's Z. Physiol. Chem. 329, 1125–1137.
[15] Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462–477.
[16] Trumpower, B.L. and Katki, A. (1975) Biochemistry 14, 3635–3642.
[17] Rosevear, P., Van Aken, T., Baxter, J. and Ferguson-Miller, S. (1981) Biochemistry 19, 4108–4115.
[18] Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Protein, Holden Day, San Francisco CA.
[19] Hoare, D.G. and Koshland, D.E. (1967) J. Biol. Chem. 242, 2447–2453.
[20] Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E. (1976) J. Biol. Chem. 251, 1104–1115.
[21] Errede, B., Haight, G.P. and Kamen, M.D. (1976) Proc. Natl. Acad. Sci. USA 73, 113–117.
[22] Downer, N.W., Robinson, N.C. and Capaldi, R.A. (1976) Biochemistry 15, 2930–2936.
[23] Koppenol, H.W. and Margoliash, E. (1982) J. Biol. Chem. 257, 4426–4437.
[24] Bisson, R., Jacobs, B. and Capaldi, R.A. (1980) Biochemistry 19, 4173–4178.
[25] Ludwig, B. (1981) Biochim. Biophys. Acta 594, 177–189.