Cytochrome *c* Is Cross-Linked to Subunit II of Cytochrome *c* Oxidase by a Water-Soluble Carbodiimide†

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ABSTRACT: Modification of beef heart cytochrome *c* oxidase with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) or 1-ethyl-3-[3-(trimethylamino)propyl]carbodiimide (CH_3EDC) has been found to significantly inhibit the high-affinity phase of the reaction of this enzyme with cytochrome *c*. Reaction conditions leading to a 70% inhibition of V_{max} resulted in a 16-fold increase in the K_m for cytochrome *c*. The loss in activity was accompanied by modification of subunit II to form a new species, II′, which migrated somewhat more rapidly than the unmodified subunit during sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis. This new species was the major site of radiolabeling when cytochrome *c* oxidase was treated with [^{14}C]CH_3EDC, indicating covalent incorporation of the carbodiimide. Equimolar concentrations of cytochrome *c* dramatically protected cytochrome *c* oxidase from inhibition by the carbodiimide and in approximately the same proportion shielded subunit II from modification to the labeled II′ species. In addition, cytochrome *c* was cross-linked to subunit II to form a new species migrating somewhat faster than subunit I during $NaDodSO_4$ gel electrophoresis. This cross-linked species was shown to contain subunit II by using subunit-specific antibodies. We propose that EDC or CH_3EDC reacts with one or more partially buried carboxyl groups on subunit II to form a positively charged *N*-acylurea which inhibits cytochrome *c* binding. In the presence of cytochrome *c*, EDC promotes formation of amide cross-links between lysine amino groups on cytochrome *c* and their complementary carboxyl groups on cytochrome *c* oxidase.

It is generally agreed that electron transfer from cytochrome *c* to cytochrome *c* oxidase involves the formation of a complex stabilized by electrostatic interactions. Extensive chemical modification studies have identified a group of seven or eight lysine residues on cytochrome *c* that are important for complex formation with cytochrome oxidase (Smith et al., 1977;Ferguson-Miller et al., 1978; Rieder & Bosshard, 1980), cytochrome c_1 (Ahmed et al., 1978; Speck et al., 1979), cytochrome *c* peroxidase (Kang et al., 1978; Smith & Millett, 1980), cytochrome b_5 (Stonehuerner et al., 1979), sulfite oxidase (Webb et al., 1980), and adrenodoxin (Geren & Millett, 1981). These highly conserved lysine residues form a ring of positively charged amino groups immediately surrounding the heme crevice on the front face of cytochrome *c* (Takano & Dickerson, 1980). The cytochrome *c* binding sites on cytochrome b_5 and cytochrome *c* peroxidase have been deduced from model-building studies based on the crystallographic structures. It was found that there is a ring of negatively charged carboxylates surrounding the heme crevice of each of these proteins that is complementary to the ring of lysines on cytochrome *c* (Salemme, 1976; Poulos & Kraut, 1980). Recent cross-linking studies have confirmed the proposed site for cytochrome *c* binding to cytochrome *c* peroxidase (Bisson & Capaldi, 1981).

It is reasonable to expect that the cytochrome *c* binding site on cytochrome *c* oxidase will also involve a ring of negatively charged carboxylate residues complementary to the cytochrome *c* lysines. Attempts to localize the cytochrome *c* binding site have focused so far on which subunits of cytochrome *c* oxidase are involved. Horse heart cytochrome *c* was cross-linked to subunit II of beef heart cytochrome *c* oxidase by dithiobis(succinimidylpropionate), an 11-Å homo-bifunctional reagent (Briggs & Capaldi, 1978). Cytochrome *c* specifically modified at lysine-13 with 4-fluoro-3-nitrophenyl azide also cross-linked to subunit II and blocked the high-affinity binding site for cytochrome *c* (Bisson et al., 1980). The site of insertion of the photoactivated azide was found to be His-161 (R. Bisson, unpublished results), which is close to several acidic residues in the sequence of subunit II. Yeast cytochrome *c* modified at Cys-107, on the backside of the protein, was cross-linked to subunit III of cytochrome *c* oxidase from both yeast (Birchmeier et al., 1976; Moreland & Dockter, 1981) and beef heart (Fuller et al., 1981). The above results suggest that cytochrome *c* binds at a cleft formed at the interface between the two monomers of the cytochrome oxidase dimer (Fuller et al., 1979). The ring of lysines surrounding the heme crevice of cytochrome *c* would interact with carboxylates on subunit II of one monomer, while the backside would be close to subunit III on the other monomer [for review, see Capaldi et al. (1982)].

In the present study, we have used a water-soluble carbodiimide to selectively modify carboxyl groups located at the cytochrome *c* binding site on cytochrome *c* oxidase. Seiter et al. (1979) have reported that 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC)[1] promoted the formation of amide cross-links between lysine amino groups on cytochrome *c* and carboxylate groups on cytochrome *c* oxidase. We have confirmed this result but find that the site of cross-linking is subunit II rather than subunit IV as previously reported. Three aspects of the reaction between EDC and cytochrome oxidase have been studied. First, we have found that EDC dramatically inhibits cytochrome *c* oxidase activity by modification of one or more carboxyl groups on subunit II. Second, cytochrome *c* binding was found to specifically protect these

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[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; [^{14}C]CH_3EDC, 1-ethyl-3-[3-[^{14}C](trimethylamino)propyl]-carbodiimide iodide; DCCD, dicyclohexylcarbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine dihydrochloride; Tris, tris(hydroxymethyl)aminomethane.

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carboxyl groups from modification and the resulting loss in cytochrome *c* oxidase activity. Finally, cytochrome *c* was specifically cross-linked to subunit II of cytochrome oxidase by EDC.

Experimental Procedures

Materials. Beef heart cytochrome *c* oxidase was prepared according to Capaldi & Hayashi (1972). Enzyme preparations had a heme *a* content of 9.0–11.0 nmol/mg of protein and contained about 100 μg of phospholipid/mg of protein. Horse heart cytochrome *c* was obtained from Sigma (type VI) and used without further purification. EDC-HCl was obtained from Sigma.

Cytochrome Oxidase Activity. The cytochrome oxidase activity was measured spectrophotometrically by following the oxidation of 30 μM ferrocytochrome *c* at 550 nm in 50 mM sodium phosphate, pH 7.4, containing 0.3% Tween 80. The concentration of cytochrome oxidase in the assay was 10–30 nM. The first-order rate constant was calculated from a least-squares analysis of a log plot of absorbance vs. time. The activity was also measured polarographically with a Gilson Model KM Clark electrode cell in 50 mM sodium phosphate, pH 7.4, containing 12 mM ascorbate, 1 mM TMPD, and 0.3% Tween 80. Under these conditions, only the high-affinity phase of the cytochrome *c* reaction was observed (Smith et al., 1981). The turnover number of untreated cytochrome oxidase was typically about 100 s^{-1}, without asolectin activation. Cytochrome oxidase concentrations are expressed in terms of a complex containing two heme *a* groups.

Reaction of Cytochrome Oxidase with EDC. Beef heart cytochrome oxidase at a concentration of about 200 μM was passed through a Sephadex G-75 column (1.0 × 30 cm) equilibrated with 10 mM sodium phosphate, pH 7.0, containing 1% Tween 80 to remove sodium cholate and Tris-HCl. Cytochrome oxidase at a concentration of 5–30 μM in 10 mM phosphate–1% Tween 80 was then adjusted to the desired pH, and cytochrome *c* and EDC were added at 25 °C.

Gel Electrophoresis. Samples for electrophoresis were dissociated in 8 M urea, 5% $NaDodSO_4$, and 3% 2-mercaptoethanol, for 1 h at room temperature. One-millimeter-thick slab gels were run as described by Wilson et al. (1980) with a 5% polyacrylamide stacking gel and a 16% polyacrylamide separating gel, both containing 6 M urea and using the buffer system of Laemmli (1970). Staining and destaining were carried out as described by Downer et al. (1976). Densitometric traces were recorded at 560 nm on a Beckman DU spectrophotometer equipped with a linear-transport attachment. Quantitation of changes in subunit concentration was obtained by calculating the areas under the peaks by using a planimeter. The relative areas of subunits I, III, and IV changed very little throughout these experiments and were used as internal standards. The relative areas of the overlapping subunits II and II′ were estimated by integrating from the edge of the peak to the trough between the two peaks. The error in this procedure was estimated to be 15–20%.

Fluorescent Antibody Staining. Cytochrome oxidase subunits were purified on a Bio-Gel P-100 column in 3% $NaDodSO_4$. The purified subunit was passed through a P-4 column to remove most of the $NaDodSO_4$ and dissociated in 6 M urea. The protein (2 mg) was mixed with an equal volume of Freunds complete adjuvant and injected into a rabbit at multiple sites in the back. Booster injections of 1 mg of protein mixed in an equal volume of Freunds incomplete adjuvant were also injected in the back of the rabbit. Samples of blood were collected from the ear vein, allowed to clot at room temperature, and centrifuged at 15 000 rpm for 15 min. The IgG fraction was partially purified by ammonium sulfate fractionation and stored at −20 °C in 50 mM Tris-HCl, pH 7.4.

Cytochrome *c* oxidase was subjected to $NaDodSO_4$ gel electrophoresis and transferred electrophoretically to nitrocellulose paper by using a Bio-Rad transblot apparatus and buffer containing 0.19 M glycine, 0.02 M Tris, 0.1% $NaDodSO_4$ and 20% methanol (Towbin et al., 1979). Electrophoresis was carried out at 20 V for 2 h. The transfer efficiency was nearly 100% for subunits II–VII, about 50% for the subunit II–cytochrome *c* cross-linked product, and about 20% for subunit I. The nitrocellulose paper was soaked in 2% poly(vinylpyrrolidine) and 2% Ficoll for 30 min to block nonspecific binding and washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). The paper was then incubated with purified antibody in 5% bovine serum albumin for 2 h at room temperature, washed with phosphate-buffered saline, and incubated for 2 h with fluorescein isothiocyanate labeled goat anti-rabbit antibody. The fluorescent antibody labeled bands were visualized under UV light.

Radiolabeling Techniques. ^{14}C-Methylated cytochrome *c* was prepared by treating 20 mg of horse heart cytochrome *c* with 100 μCi of [^{14}C]formaldehyde (57 mCi/mmol; Research Products International Corp.) and 1 mg of $NaCNBH_3$ in 1 mL of 50 mM sodium phosphate, pH 7.0 (Jentoft & Dearborn, 1979). The specific activity of the resulting product was 8 mCi/mmol, and the small extent of methylation allowed the cytochrome *c* to be cross-linked to cytochrome *c* oxidase. [^{14}C]CH_3EDC was synthesized according to the procedure of Sheenan et al. (1961) by treating the free base EDC with [^{14}C]CH_3I (53 mCi/mmol; Research Products International Corp.). The resulting product had a specific activity of 40 ± 10 mCi/mmol and caused the same extent of inhibition of cytochrome oxidase as EDC. Unlabeled CH_3EDC was synthesized by the same method. $NaDodSO_4$ gels containing radiolabeled protein were fractionated into 0.7-mm slices on a Mickel gel slicer. These were then dissolved in 1.5 mL of H_2O_2 at 60 °C for 10 h, mixed with 8.5 mL of Omnifluor (New England Nuclear) (2.66 g/L) in 2:1 toluene:Triton X-100, and counted on a Packard scintillation counter. Radioactive gels were also dried and subjected to autoradiography with XAR-5 Kodak film.

Results

Inhibition of Cytochrome Oxidase Activity by EDC Is Prevented by Cytochrome c Binding. Addition of 2 mM EDC to cytochrome oxidase at pH 7.0 resulted in the loss of electron transfer activity (Figure 1). The effect on the Michaelis constant for the high-affinity phase of cytochrome *c* oxidation was even more dramatic (Figure 2). Reaction conditions leading to a 70% inhibition of V_{max} resulted in an increase in the K_m for cytochrome *c* from 1.6 μM to 27 μM. Cytochrome *c* dramatically protected cytochrome *c* oxidase from inactivation by EDC (Figure 1). This protection was essentially complete at a molar ratio of one cytochrome *c* per cytochrome *c* oxidase (Figure 3), indicating that inactivation by EDC involved the high-affinity cytochrome *c* binding site. Very high concentrations of nucleophiles such as taurine partially prevented the loss of cytochrome *c* oxidase activity (Figure 1). Treatment of cytochrome *c* oxidase with CH_3EDC led to nearly the same rate of inactivation as EDC, and this inactivation was protected in a similar fashion by cytochrome *c* binding (data not shown).

EDC Alters the Mobility of Subunit II of Cytochrome c Oxidase on $NaDodSO_4$ Gel Electrophoresis. $NaDodSO_4$ gel

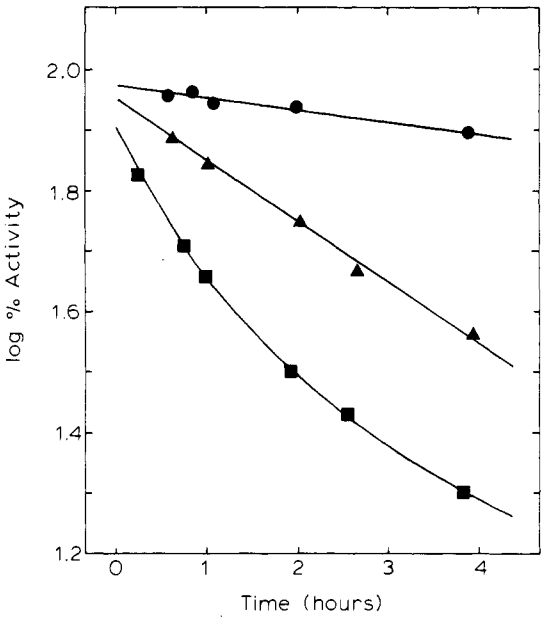

FIGURE 1: Inactivation of cytochrome *c* oxidase by EDC. Cytochrome *c* oxidase (5 μM) in the absence of added nucleophile (■), in the presence of 0.4 M taurine (▲), or in the presence of 20 μM cytochrome *c* (●) was treated with 2 mM EDC in 10 mM phosphate, pH 7.0, and 1% Tween 80, at 25 °C. The activities were measured spectrophotometrically at the indicated times in 50 mM phosphate, pH 7.4, and 0.3% Tween 80 by using 30 μM ferrocytochrome *c*. The activities are presented as the percent of an untreated control measured at the same time. The control activity decreased linearly by 10% after 4 h.

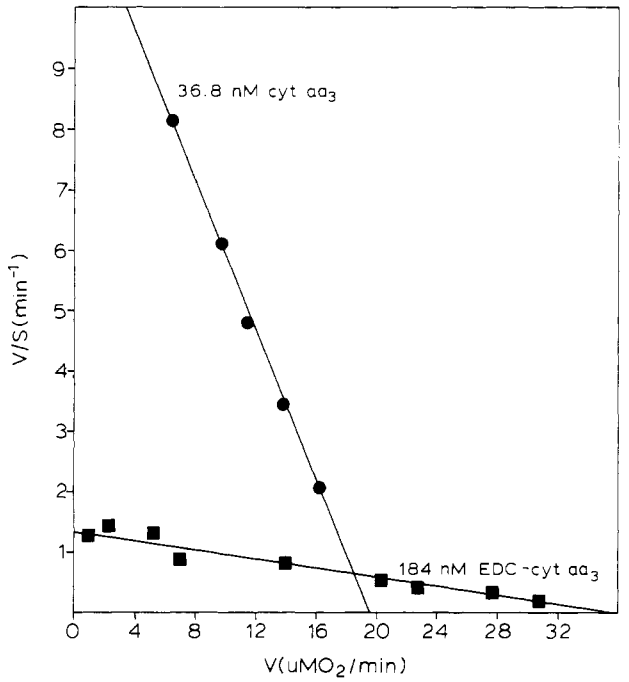

FIGURE 2: Effect of EDC modification on the enzyme kinetics of cytochrome oxidase. 23 μM cytochrome oxidase was treated with 2 mM EDC for 6 h at 25 °C in 10 mM phosphate, pH 6.7, containing 1% Tween 80. The cytochrome oxidase activities were measured polarographically at 25 °C in 50 mM phosphate, pH 7.4, containing 0.3% Tween 80, 10 mM ascorbate, 1 mM TMPD, and 0.1–20μM cytochrome *c* (*S*). (■) 184 nM EDC–cytochrome oxidase; (●) 36.8 nM untreated cytochrome oxidase control.

electrophoresis was used to determine whether modification of cytochrome *c* oxidase with EDC or CH_3EDC led to intersubunit cross-linking or changes in subunit mobility. Comparison of the densitometric trace of a gel of EDC-

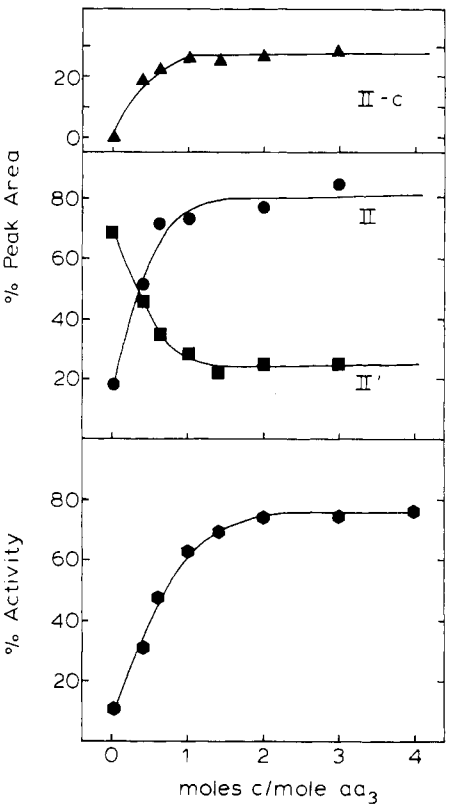

FIGURE 3: Effect of cytochrome *c* on the modification of cytochrome oxidase by EDC. 5 μM cytochrome oxidase was treated with 2 mM EDC for 8 h at 25 °C in 10 mM phosphate, pH 7.0, containing 1% Tween 80 and the indicated concentration of cytochrome *c*. (Bottom panel) Cytochrome oxidase activities measured spectrophotometrically in 50 μM phosphate, pH 7.4, and 0.3% Tween, 80, 25 °C, as a percent of an untreated control. (Middle and top panels) Samples treated as described above were subjected to $NaDodSO_4$ gel electrophoresis, and the Coomassie blue stained gels were scanned at 560 nm. The areas of the indicated peaks are given as a percent of the area of subunit II in an untreated control.

modified cytochrome *c* oxidase with that of an untreated control showed that the subunit II band decreased in amplitude and three new bands appeared, II′ between subunits II and III and a faint doublet, II″, between subunits I and II (Figure 4B). These new bands were identified by electrophoretic transfer to nitrocellulose paper, followed by treatment with subunit-specific antibodies. Both II′ and II″ bound antibody specific for subunit II (Figure 5B). The slightly increased electrophoretic mobility of subunit II′ relative to II is not without precedent. Substitution of a single amino acid residue from glutamate to lysine led to a similar increase in the mobility of a mutant subunit II in the yeast enzyme (Fox, 1979). The significantly decreased electrophoretic mobility of II″ indicates that these are probably species in which subunit II is cross-linked to one of the smaller subunits. Figure 4C shows that cytochrome *c* protected subunit II from modification by EDC and inhibited the formation of II′ and II″. This protection was essentially complete at a molar ratio of one cytochrome *c* per cytochrome *c* oxidase (Figure 3).

[^{14}C]CH_3EDC Is Incorporated into Cytochrome c Oxidase. The effect of EDC or CH_3EDC on cytochrome *c* oxidase activity and the change in electrophoretic mobility of subunit II could result from incorporation of carbodiimide as the *N*-acylurea or from the EDC-initiated cross-linking of amino and carboxyl groups on the protein (Timkovich, 1977; Carraway & Koshland, 1972). Incorporation of carbodiimide into the enzyme was monitored by using [^{14}C]CH_3EDC. As shown in Figure 6, reaction conditions leading to 70% inhibition of

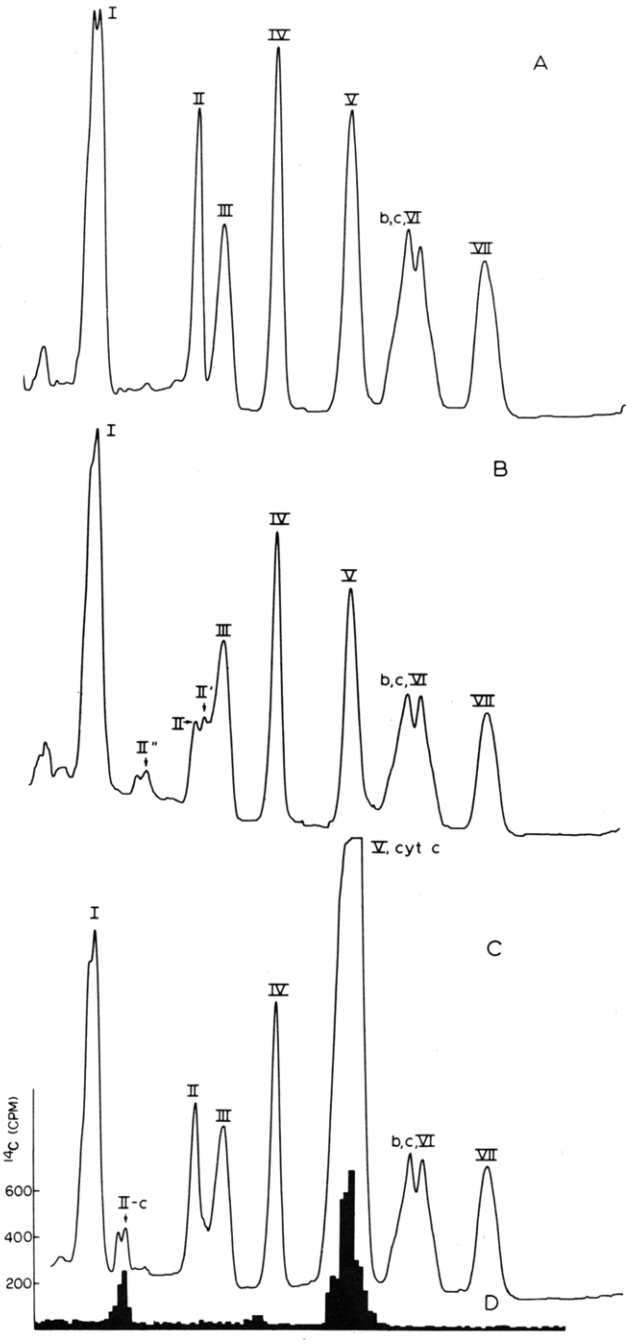

FIGURE 4: $NaDodSO_4$–urea–polyacrylamide gel electrophoresis of cytochrome *c* oxidase modified with EDC in the presence and absence of cytochrome *c*. Samples containing 23 μM cytochrome *c* oxidase were incubated for 6 h at 25 °C in 1% Tween 80–10 mM phosphate, pH 6.7. (A) No additions; (B) 2 mM EDC; (C) 2 mM EDC and 130 μM cytochrome *c*; (D) 2 mM EDC and 20 μM ^{14}C-methylated cytochrome *c*. A 30-μL sample of (D) was subjected to $NaDodSO_4$ gel electrophoresis, and the sliced gel was analyzed for ^{14}C by scintillation counting. 10-μL samples of (A–C) were subjected to $NaDodSO_4$ gel electrophoresis and stained with Coomassie blue, and densitometric traces were recorded at 560 nm.

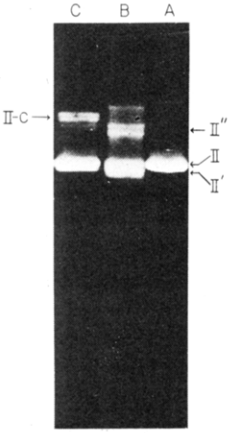

FIGURE 5: Identification of EDC-modified cytochrome oxidase subunits by using antibody specific for subunit II. The following samples were prepared as described in Figure 4: (A) unmodified cytochrome *c* oxidase; (B) EDC-modified cytochrome *c* oxidase; (C) EDC-modified cytochrome *c*–cytochrome *c* oxidase. The samples (10 μL) were subjected to $NaDodSO_4$ gel electrophoresis, electrophoretically transferred to nitrocellulose paper, and incubated with rabbit antibody specific for subunit II and then with fluorescein isothiocyanate labeled goat anti-rabbit antibody. The fluorescent bands were photographed under far-UV light.

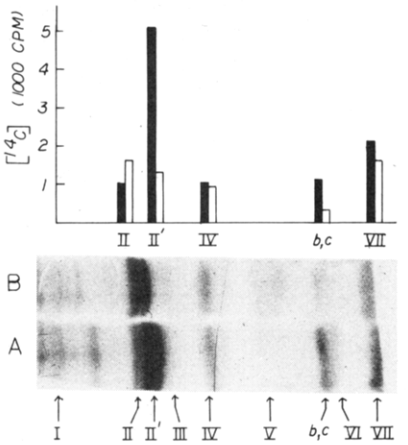

FIGURE 6: Incorporation of [^{14}C]CH_3EDC into cytochrome *c* oxidase subunits. Samples containing 20 μM cytochrome *c* oxidase alone (autoradiograph A and closed bars of histogram) or with 64 μM cytochrome *c* (autoradiograph B and open bars) were incubated for 10 h at 25 °C with 0.44 mM [^{14}C]CH_3EDC and 1.6 mM EDC in 10 mM phosphate, pH 7.0, and 1% Tween 80. 10-μL aliquots were subjected to $NaDodSO_4$ electrophoresis on 14% polyacrylamide gels. Autoradiographs A and B were exposed for 10 days at −70 °C. The incorporation of ^{14}C into the individual subunits shown in the histogram was obtained by scintillation counting of sliced gels.

electron transfer activity resulted in heavy labeling of subunit II′, along with incorporation of smaller amounts of radioactivity into subunits IV, b or c, and VII. The band at the position of unmodified subunit II was also labeled but to a much smaller extent than II′. In the presence of a 4-fold molar excess of cytochrome *c*, the labeling of subunit II′ was dramatically reduced, from 1.5 ● 0.5 to 0.4 ± 0.1 mol/mol of subunit II (Figure 6). This 4-fold decrease was in proportion to the protection of electron transfer activity in the presence of cytochrome *c* (from 70% inhibition to 15% inhibition). The labeling of polypeptides b or c and VII was also reduced in the presence of cytochrome *c*, but to a much smaller extent. Subunit III was not detectably labeled by [^{14}C]CH_3EDC.

Cytochrome c Is Cross-Linked to Subunit II by EDC. Cytochrome *c* not only protects specific carboxyl groups on subunit II from labeling by EDC but also is cross-linked to the oxidase through subunit II. $NaDodSO_4$ gel electrophoresis revealed the presence of a new doublet, II-c, formed when cytochrome *c* oxidase was treated with EDC in the presence of cytochrome *c* (Figure 4C). II-c was the only detectable cross-linked species containing radioactivity when ^{14}C-methylated cytochrome *c* was used in the reaction system (Figure 4D). Antibodies made against subunit II bind to II-c (Figure 5C), but antibodies elicited against the smaller subunits IV, V, and VI do not (results not shown). The extent of cross-linking between cytochrome *c* and subunit II was complete at a molar ratio of one cytochrome *c* per cytochrome

oxidase (Figure 3) and never exceeded about 30%. Addition of higher concentrations of EDC simply resulted in more extensive conversion of subunit II to II′ without increasing the yield of cross-linked product. Cytochrome *c* was cross-linked to subunit II at all pHs from 5.5 to 8.5, with an optimum at about pH 7.0. Higher concentrations of EDC were required to produce optimum cross-linking at pH values above 7.5, however. High concentrations of taurine (0.4 M) prevented cross-linking, providing further evidence that amide bond formation was involved.

Discussion

The results presented show that EDC and CH_3EDC react to an equal extent with cytochrome *c* oxidase to inhibit electron transfer from cytochrome *c*. The evidence summarized below strongly suggests that this inhibition is caused primarily by modification of several carboxyl groups on subunit II. Reaction of cytochrome *c* oxidase with the water-soluble carbodiimide leads to a modification of subunit II to a new species, II′, with slightly increased migration in $NaDodSO_4$–polyacrylamide gel electrophoresis. This new species is the major site of radiolabeling when [^{14}C]CH_3EDC is used as the modifying reagent. The presence of equimolar concentrations of cytochrome *c* protects cytochrome *c* oxidase from inhibition by carbodiimide and in approximately the same proportion shields subunit II from modification to the labeled II′ species. There is no altered migration of any of the other subunits, or shielding of polypeptides from radiolabeling, except for the components b or c and VII. However, the extent of modification of these polypeptides is too small to contribute significantly to the inhibition of cytochrome *c* oxidase activity. It is possible that b or c is the same component that is cross-linked to subunit II to form II″ in the absence of cytochrome *c*. It is interesting to note that the water-soluble carbodiimide [^{14}C]CH_3EDC does not radiolabel subunit III. This is in marked contrast to the hydrophobic carbodiimide DCCD which has been shown to react primarily with glutamic acid-90 in subunit III and inhibit both proton pumping and electron transfer activity of cytochrome *c* oxidase (Prochaska et al., 1981; Casey et al., 1980).

The labeling experiments show that inhibition of cytochrome *c* oxidase by EDC or CH_3EDC under the very mild conditions used here probably involves the modification of only a small number of carboxyl groups in partially buried environments (Timkovich, 1977). A hydrophobic environment would favor rearrangement of the intermediate *O*-acylurea to the stable *N*-acylurea rather than hydrolysis back to the free carboxyl. The conversion of negatively charged carboxylates on subunit II to bulky, positively charged *N*-acylurea groups could account for the 16-fold increase in the K_m for cytochrome *c* binding. Under highly resolving gel conditions, subunit II′ can actually be resolved into two bands (results not shown), raising the possibility that at least two different carboxyls are involved. An additional carboxyl was modified by [^{14}C]CH_3EDC without affecting the electrophoretic mobility of subunit II, but it was not shielded from modification by cytochrome *c* binding. We cannot at present rule out the possibility that EDC also promotes formation of intrasubunit cross-links between neighboring amino and carboxyl groups which could contribute to the inhibition of electron transfer. This, however, can be assessed by fragmentation studies of subunit II now in progress.

It was also found that cytochrome *c* was specifically cross-linked to subunit II of cytochrome *c* oxidase by EDC. Our results confirm the finding of Seiter et al. (1979) that EDC can promote the formation of amide cross-links between lysine amino groups on cytochrome *c* and their complementary carboxyl groups on cytochrome *c* oxidase. However, we have used subunit-specific antibodies to definitively identify the site of cross-linking to be subunit II, rather than IV as previously reported. It is interesting to speculate that the two bands observed for the II-c species reflect two different sites of cross-linking.

The hypothetical models for the interaction of cytochrome *c* with cytochrome b_5 (Salemme, 1976) and cytochrome *c* peroxidase (Poulos & Kraut, 1980) lead us to expect a ring of five or more carboxylates at the cytochrome *c* binding site on subunit II. The electrostatic interaction between cytochrome *c* and its redox partners has been quantitatively described in a new semiempirical theory recently developed by Smith et al. (1981). They assume that specific complementary charge-pair interactions between lysine amino groups on cytochrome *c* and carboxylate groups on the partner dominate the total electrostatic interaction. The data on the interaction between cytochrome *c* and cytochrome *c* oxidase are best interpreted by the involvement of seven or eight complementary charge-pair interactions. There are a total of 25 aspartate and glutamate residues in the primary sequence of subunit II from beef heart cytochrome oxidase (Steffens & Buse, 1979). However, a comparison of the sequences of subunit II from beef, human (Barrell et al., 1979), mouse (Bibb et al., 1981), yeast (Fox, 1979), and maize (Fox & Leaver, 1981) cytochrome *c* oxidase reveals that only 11 acidic residues are fully conserved. These are located at residues 11, 62, 88, 109, 112, 137, 139, 158, 173, 198, and 212. The seven or eight carboxylates forming the cytochrome *c* binding site are probably to be found among these highly conserved acidic residues.

Fragmentation studies of subunit II isolated from [^{14}C]-CH_3EDC-modified cytochrome *c* oxidase and of the subunit II–cytochrome *c* cross-linked product are now in progress. These experiments should help localize the high-affinity binding site for cytochrome *c* on cytochrome *c* oxidase more precisely than has previously been possible.

Acknowledgments

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References

Ahmed, A. J., Smith, H. T., Smith, M. B., & Millett, F. S. (1978) *Biochemistry 16*, 4971.

Barrell, B. G., Bankier, A. T., & Dronin, J. (1979) *Nature (London) 282*, 189.

Bibb, M. I., Van Etten, R. A., Wright, C. T., Walberg, M. W., & Clayton, D. A. (1981) *Cell (Cambridge, Mass.) 26*, 167–180.

Birchmeier, W., Kohler, C. E., & Schatz, G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4334.

Bisson, R., & Capaldi, R. A. (1981) *J. Biol. Chem. 256*, 4362.

Bisson, R., Jacobs, B., & Capaldi, R. A. (1980) *Biochemistry 19*, 4173.

Briggs, M. M., & Capaldi, R. A. (1978) *Biochem. Biophys. Res. Commun. 80*, 553.

Capaldi, R. A., & Hayashi, H. (1972) *FEBS Lett. 26*, 261.

Capaldi, R. A., Darley-Usmar, V. M., Fuller, S., & Millett, F. (1982) *FEBS Lett. 138*, 1.

Carraway, K. L., & Koshland, P. E. (1972) *Methods Enzymol. 25*, 616.

Casey, R. P., Thelen, M., & Azzi, A. (1980) *J. Biol. Chem. 255*, 3994–4000.

Downer, N. W., Robinson, N. C., & Capaldi, R. A. (1976) *Biochemistry 15*, 2930.

Ferguson-Miller, S., Brautigan, P. L., & Margoliash, E. (1978) *J. Biol. Chem. 253*, 149.
Fox, T. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6534.
Fox, T. D., & Leaver, C. I. (1981) *Cell (Cambridge, Mass.) 26*, 315–323.
Fuller, S. F., Capaldi, R. A., & Henderson, R. (1979) *J. Mol. Biol. 134*, 305.
Fuller, S. D., Darley-Usmar, V. M., & Capaldi, R. A. (1981) *Biochemistry 20*, 7046–7053.
Geren, L. M., & Millett, F. S. (1981) *J. Biol. Chem. 256*, 4851.
Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359–4365.
Kang, C. H., Brautigan, D. L., Osheroff, N., & Margoliash, E. (1978) *J. Biol. Chem. 253*, 6502.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Moreland, R. N., & Dockter, M. E. (1981) *Biochem. Biophys. Res. Commun. 99*, 339.
Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 10, 322.
Prochaska, L. I., Bisson, R., Capaldi, R. A., Steffens, G. L. M., & Buse, G. (1981) *Biochim. Biophys. Acta 637*, 360–373.
Rieder, R., & Bosshard, H. R. (1980) *J. Biol. Chem. 255*, *4732*.
Salemme, F. R. (1976) *J. Mol. Biol. 102*, 563.
Seiter, C. H. A., Margalit, R., & Ferreault, R. A. (1979) *Biochem. Biophys. Res. Commun. 86*, 473.
Sheenan, J. C., Cruickshank, P. A., & Boshart, G. L. (1961) *J. Org. Chem. 26*, 2525–2528.
Smith, H. T., Staudenmayer, N., & Millett, F. (1977) *Biochemistry 16*, 4971.
Smith, H. T., Ahmed, A. J., & Millett, F. (1981) *J. Biol. Chem. 256*, 4984.
Smith, M. B., & Millett, F. (1980) *Biochim. Biophys. Acta 626*, 64.
Speck, S. H., Ferguson-Miller, S., Osheroff, N., & Margoliash, E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 155.
Steffens, G., & Buse, G. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 613.
Stonehuerner, J., Williams, J. B., & Millett, F. (1979) *Biochemistry 18*, 5422.
Takano, T., & Dickerson, R. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6371.
Thomas, P. E., Ryand, D., & Levin, W. (1976) *Anal. Biochem. 75*, 168–176.
Timkovich, R. (1977) *Anal. Biochem. 79*, 135–143.
Towbin, H., Staehelin, T., & Gorden, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Webb, M., Stonehuerner, J., & Millett, F. S. (1980) *Biochim. Biophys. Acta 543*, 290.
Wilson, M. T., Lalla-Maharajh, W., Darley-Usmar, V. M., Bonaventura, J., Bonaventura, C., & Brunori, M. (1980) *J. Biol. Chem. 255*, 2722.

Proteolytic Dissection of Turnip Crinkle Virus Subunit in Solution†

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ABSTRACT: Turnip crinkle virus (TCV) can be dissociated under mild conditions, yielding dimers of the protein subunit, as shown by chemical cross-linking and by gel filtration. These dimers have defined regions of proteolytic sensitivity, corresponding to the arm and hinge of the folded polypeptide chain. The results are discussed in terms of the known high-resolution structure of TCV and with regard to implications for the pathway of viral assembly.

Determination of the complete three-dimensional structure of tomato bushy stunt virus (TBSV) has revealed a number of remarkable features with implications for mechanisms of assembly (Harrison et al., 1978; Harrison, 1980). The related turnip crinkle virus (TCV) has also been visualized by high-resolution X-ray diffraction, and the structure of its protein shell is, as expected, substantially the same as that of TBSV (J. Hogle and S. C. Harrison, unpublished results). Success in reversible disassembly of TCV makes it a better choice than TBSV for mapping an assembly pathway. As an initial step toward this mapping, we have carried out chemical cross-linking and proteolytic dissection studies of the isolated TCV subunit.

The rationale of these experiments depends on the general architecture of TBSV and TCV (shown diagrammatically in Figure 1). The particle contains 180 coat protein subunits (M_r ~40000), probably one chain of a M_r 80000 protein, and a molecule of single-stranded RNA (4800 nucleotides) (Ziegler et al., 1974). The coat subunit, containing about 390 amino acids, folds into distinct regions: a projecting domain (P), a domain forming a tightly connected shell (S), an arm (a), and an internal domain (R). The three symmetrically distinct environments for this subunit are labeled A, B, and C. The polypeptide accommodates to these three packing modes by flexion at the hinge (h) between S and P and by an ordering or disordering of the arm, a. Units at positions A and B (60 of each) have one hinge configuration, and the entire N-terminal region (R and a) appears to be spatially disordered. Subunits at positions C (60 in all) have another hinge position, and the arm is folded in an ordered way along the inner side of the S domain. The R region is spatially disordered in C subunits as well as in A and B. "Spatially disordered" is used here to mean merely that the segment is not fixed with respect to the outer shell. The R domain itself may be precisely folded but flexibly tethered to the rest of the subunit such that it can adopt a variety of positions vis-à-vis the S domain and hence present no strong features in a high-resolution electron density map. The entire RNA molecule is spatially disordered in this sense.

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