Reaction of thionitrobenzoate-modified yeast cytochrome *c* with monomeric and dimeric forms of beef heart cytochrome *c* oxidase

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Thionitrobenzoate-modified yeast cytochrome *c* was shown to react with both monomeric and dimeric forms of beef heart cytochrome *c* oxidase through subunit III. This cytochrome *c* derivative was found to inhibit electron transfer in the dimer but not in the monomer. These results are interpreted to show that the high affinity binding site for cytochrome *c* is a cleft at the interface between monomers in the cytochrome *c* oxidase dimer.

Cytochrome c *Cytochrome* c *oxidase* *Thionitrobenzoate* *Electron transfer*

1. INTRODUCTION

Two different cytochrome *c* derivatives have been used to map the cytochrome *c* binding site on cytochrome *c* oxidase. Arylazidocytochrome *c*, with the photoaffinity group at lysine 13, was found to bind into high affinity binding site for substrate and cross-link to beef heart cytochrome *c* oxidase in subunit II [1,2], at around His_{161} in the sequence [3]. Yeast cytochrome *c*, modified at Cys_{107} with thionitrobenzoate, also bound in the high affinity binding site of both yeast [4,5] and beef [6] cytochrome *c* oxidase by covalently cross-linking to subunit III through Cys_{115} [7] (nomenclature for the beef enzyme).

The covalent cross-linking of these cytochrome *c* derivatives was found to inhibit electron transfer activity of beef heart cytochrome *c* oxidase when exogenous cytochrome *c* was used as substrate [1–7]. The covalently bound TNB-cytochrome *c* functioned in electron transfer when re-reduced with ascorbate and TMPD with as much as 15% of maximal activity [5,6].

The fact that both cytochrome *c* derivatives can covalently bind in the high affinity site has structural implications. Lysine 13 is on the front face of the cytochrome *c* while Cys_{107} is on the opposite side of the molecule [8]. Thus the binding site for cytochrome *c* must be a cleft of diameter close to that of the cytochrome *c* molecule.

All of the cytochrome *c* binding studies described above were conducted on preparations in which cytochrome *c* oxidase is a dimer. The cleft for cytochrome *c* binding could thus be within the monomer or a property of the dimer, i.e., at the interface between monomers and involving subunit II of one monomer and subunit III of the second. This study was undertaken to distinguish between the two possibilities.

2. EXPERIMENTAL

Beef heart cytochrome *c* oxidase purified as in [9] was used as the dimeric form of the enzyme. Monomeric enzyme was obtained as in [10] or by a modification of the procedure in [11] in making two-dimensional crystals as follows:

Beef heart mitochondria (23 mg/ml) were incubated with 0.3% deoxycholate, 1 M KCl, 10 mM Tris–HCl (pH 8.0) for 1 h on ice and a green pellet of crude cytochrome *c* oxidase, then separated from the red supernatant by centrifugation [12]. This pellet was treated with 1 mg deoxycholate/mg protein, 1 M KCl, 50 mM Tris–HCl (pH 8.0) on ice

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for 48 h. Cytochrome *c* oxidase was collected as a pellet and further purified by cholate solubilization and ammonium sulfate precipitation steps as in [12].

Yeast cytochrome *c* was a generous gift from Professor Takashi Yonetani, Johnson Foundation, University of Pennsylvania. The protein was derivatized with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) as in [4]. The reaction of yeast cytochrome *c* with beef heart cytochrome *c* oxidase was performed as in [6]. Cytochrome *c* oxidase activity was measured spectrophotometrically as in [13] using 1% Tween 80 as the solubilizing detergent.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as in [6]. Protein concentrations were determined as in [14].

Sedimentation velocity analysis was conducted as in [10,15].

3. RESULTS

Two preparations of cytochrome *c* oxidase were used, one monomeric as judged by sedimentation velocity measurements (7 S species), the other dimeric (13 S species) [10,15]. These contained approximately equal amounts of subunit III relative to subunit II as judged by SDS-PAGE.

Reaction of TNB-cytochrome *c* with dimeric cytochrome *c* oxidase led to cross-linking of this cytochrome *c* derivative to subunit III and concomitant inhibition of electron transfer activity, as described previously. This interaction has been shown to involve the high affinity binding site for substrate. Fig. 1 shows that the inhibition of electron transfer is proportional to the extent of covalent cross-linking to subunit III as reported in [6].

Reaction of TNB-cytochrome *c* with monomeric cytochrome *c* oxidase was less efficient than with the dimeric enzyme. For example, incubation of monomeric enzyme with a 4-fold molar excess of TNB-cytochrome *c* for 1 h at 37°C, as in [6], resulted in the covalent binding of 0.1–0.2 mol of cytochrome *c*/mol aa_3 as measured by the disappearance of subunit III from the gel profile. This compares with covalent binding of 0.4–0.5 mol of cytochrome *c*/mol aa_3 with the dimeric enzyme under the same conditions. Efficient complexing of TNB-cytochrome *c* with monomeric cytochrome *c* oxidase (i.e., 0.5–0.8 mol of cytochrome *c*/mol aa_3) required high ratios of TNB cytochrome *c* to oxidase (10:1) and prolonged incubation times (4–6 h at 20°C).

Reaction of TNB-cytochrome *c* with monomeric cytochrome *c* oxidase gave one major cross-linked product (fig.2) under all conditions, identified by two-dimensional gel electrophoresis as being a covalent complex between cytochrome *c* and subunit III. Under conditions of high excess of cytochrome *c* and with prolonged incubations at room temperature, additional cross-linked products were generated in small amounts between TNB-cytochrome *c* and polypeptides a and c.

In marked contrast to the result with the dimeric form of cytochrome *c* oxidase, the covalent cross-linking of the TNB-cytochrome *c* derivative to monomers had no effect on electron transfer activity. This is shown in fig. 1. Further, TNB-cytochrome *c* covalently bound to monomeric

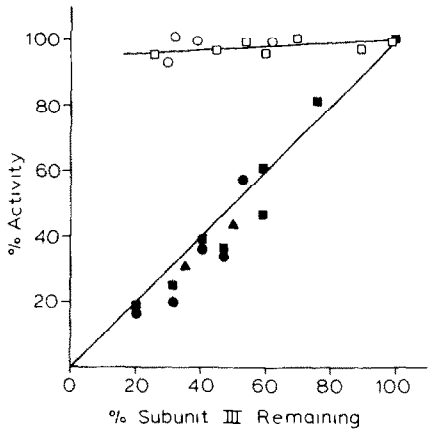

Fig. 1. Inhibition of cytochrome *c* oxidase activity as a function of TNB-cytochrome *c* covalently bound to subunit III. Electron transfer activity was measured spectophotometrically with reduced cytochrome *c* as substrate. The amount of cross-linking of TNB-cytochrome *c* to subunit III was estimated by SDS-PAGE from the percentage of subunit III unmodified (or remaining) after the reaction. The estimate of subunit III remaining was measured in relation to subunit II (□,■,○,●) or subunit IV (▲) in different experiments depending on the sharpness of the peaks for these two subunits in any set of gels. (●,■,▲) Dimeric enzyme, (○,□) monomer cytochrome *c* oxidase. The straight line for the dimeric enzyme shows the expected plot for a 1:1 relationship between loss of activity and cross-linking to subunit III.

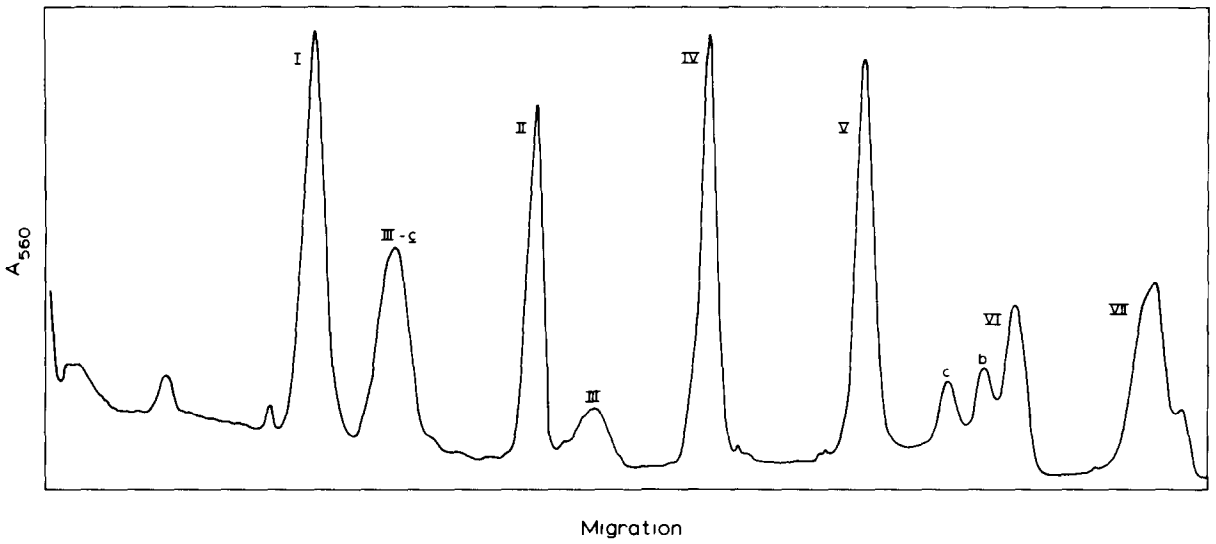

Fig. 2. SDS-PAGE showing the cross-reaction of TNB-cytochrome *c* with the monomeric form of beef heart cytochrome *c* oxidase. A 10-fold molar excess of yeast TNB-cytochrome *c* was reacted with cytochrome *c* oxidase and the covalent complex was purified by DEAE chromatography. The subunit III-cytochrome *c* adduct is indicated by III-*c* and the cytochrome *c* oxidase subunits are numbered as in [6].

cytochrome *c* oxidase did not function to transfer electrons from ascorbate and *N,N,N*^{1},N^1-tetramethylphenylenediamine (TMPD) to cytochrome *c* oxidase. TNB-cytochrome *c* covalently bound to dimeric cytochrome *c* oxidase supported a small but significant cytochrome *c* oxidase activity with ascorbate and TMPD as substrate (see [6]).

The possibility that binding of TNB-cytochrome *c* causes release of subunit III from monomers but not dimers was examined as this could explain the different properties of the substrate derivative when bound to the two aggregation states of the oxidase. Fig. 3 shows the sedimentation patterns of the TNB-cytochrome *c*–cytochrome *c* oxidase complex through a sucrose density gradient. The sedimentation patterns of monomeric and dimeric cytochrome *c* oxidase are shown for comparison. The TNB-cytochrome *c* derivative monitored by A_{420}, sedimented as a peak very close to the position of monomeric cytochrome *c* oxidase. SDS-PAGE of this peak showed a full complement of subunits of cytochrome *c* oxidase as well as the presence of cytochrome *c*.

4. DISCUSSION

The important finding of the present study is that TNB-cytochrome *c* cross-links to both monomeric and dimeric forms of beef heart cytochrome *c* oxidase predominantly through subunit III, but only with the dimer is there any inhibition of electron transfer activity. This is taken to indicate that the high affinity binding site for cytochrome *c* is at the interface between monomers in the dimer. It has been clearly established that electron transfer from cytochrome *c* to both oxidases and reductases occurs through the front face of the cytochrome *c* molecule [16–18] and that this face of cytochrome *c* binds to subunit II of cytochrome *c* oxidase [1,2,19,20].

TNB-cytochrome *c* can only inhibit electron transfer sterically when exogenous cytochrome *c* is used as substrate [4–6], here) if subunit III provides a back face to the cytochrome *c* binding site. This implies that substrate cytochrome *c* binds in a cleft on the molecule. Such a cleft between subunits II and III could occur within the monomer, but electron transfer within the cytochrome *c*–cytochrome *c* oxidase complex, as well as inhibition of electron transfer by TNB-cytochrome *c* would be independent of the aggregation state of cytochrome *c* oxidase, which is not the case. With the cleft formed at the interface between monomers in the dimer, the inhibition

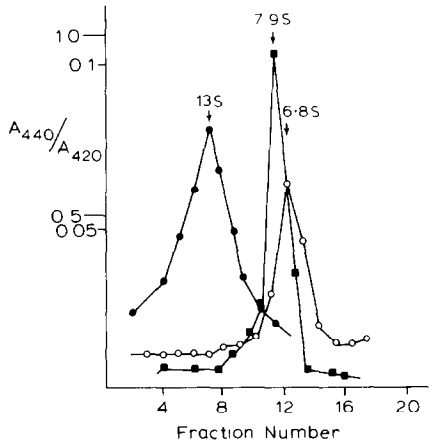

Fig. 3. Sedimentation studies of cytochrome *c* oxidase and the cross-linked product of TNB-cytochrome *c* and cytochrome *c* oxidase. Control cytochrome *c*-oxidase (●—●) and the chymotrypsin digested, subunit III-less enzyme (○—○) were suspended in 10 mM Tris-HCl (pH 7.4), 0.1% Triton X-100 and loaded onto linear (10–40%) sucrose density gradients buffered with 10 mM Tris-HCl, 0.1% Triton X-100 (pH 7.4). Yeast TNB-cytochrome *c* complex with cytochrome *c* oxidase (■—■) was suspended in 10 mM Tris-HCl (pH 8.5), 0.1% Triton X-100 and loaded onto a 10–40% sucrose density gradient buffered with 10 mM Tris-HCl (pH 8.5) at 0.1% Triton X-100. Gradients were centrifuged in the Beckman VTi80 rotor (at 5°C) at 80 000 rev./min for 1.5 h. The sedimentation coefficients were determined in a Beckman-Spinco Model E analytical ultracentrifuge.

result can be explained by cytochrome *c* linked to one monomer blocking the subunit II site of the associated monomer. When the dimer is dissociated into monomers, TNB-cytochrome *c* will be well away from the substrate cytochrome *c* binding site. It would not be expected to inhibit approach of exogenous cytochrome *c* to the substrate site or be active in electron transfer to the oxidase. Binding of this derivative did not release subunit III from the monomer as is evident from sedimentation studies. The possibility that TNB-cytochrome *c* reacts with subunit III in both monomer and dimer but at different sites is very unlikely. There are only two cysteines for reaction with the derivative in this subunit. Cys_{115} is by far the most reactive sulfhydryl in the cytochrome *c* oxidase molecule [7,21]. This may explain the relative specificity on binding of the TNB-cytochrome to subunit III in the monomer where binding through the front face is not involved. Cys_{218} does not react with sulfhydryl reagents such as DTNB and iodacetamide and is in a sequence of hydrophobic amino acids which probably form one of the bilayer intercalated parts of this subunit [22].

The inhibition studies are thus consistent with our previous proposal [22,23] that the high affinity binding site for cytochrome *c* is in a cleft between monomers in the dimeric enzyme. A cleft of the appropriate size is clearly evident in the structure of the cytochrome *c* oxidase dimer obtained recently by electron microscopy and image reconstruction studies [23].

As outlined, the covalent complex of TNB-cytochrome *c* with dimeric cytochrome *c* oxidase from yeast or beef, retains electron transfer activity in the polarographic assay with TMPD and ascorbate as electron donors to the bound cytochrome *c* molecule [5,6]. Essentially maximal activity was observed for the beef heart enzyme when exogenous cytochrome *c* was added along with TMPD and ascorbate [6]. This result was taken to support the proposal in [24] that (exogenous) cytochrome *c* in the low affinity site for substrate increased the off rate of substrate (in this case covalently bound cytochrome *c*) from the high affinity site (for re-reduction with artificial electron donors in the above experiment). Our findings offer an alternative explanation. It is possible that beef heart cytochrome *c* oxidase dissociates into monomers under the condition of the polarographic assay (i.e., low concentration of enzyme, laurylmaltoside as detergent, low ionic strength) when high concentrations of cytochrome *c* are present. This possibility is now being tested.

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