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Covalent Complex between Yeast Cytochrome *c* and Beef Heart Cytochrome *c* Oxidase Which Is Active in Electron Transfer†

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ABSTRACT: A covalently cross-linked complex of yeast cytochrome *c* and beef heart cytochrome *c* oxidase has been prepared by the method of Birchmeier et al. (1976) [Birchmeier, W., Kohler, C. E., & Schatz, G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4334–4338]. This complex is linked through cysteine-107 of yeast cytochrome *c* to a cysteine in subunit III of cytochrome *c* oxidase. The covalently bound yeast cytochrome *c* blocked the interaction of horse heart cytochrome *c* with the high-affinity binding site for substrate on the oxidase without affecting binding to the low-affinity site. These results along with the findings of Bisson et al. (1980) [Bisson, R., Jacobs, B., & Capaldi, R. A. (1980) *Biochemistry 19*, 4173–4178] indicate that cytochrome *c* in the high-affinity binding site occupies a cleft between subunits II and III on the cytochrome *c* oxidase complex. Covalent binding of cytochrome *c* in the high-affinity binding site inhibited cytochrome *c* oxidase activity with reduced cytochrome *c* as substrate. Complete inhibition was obtained with one cytochrome *c* covalently bound per oxidase monomer. Thus, the low-affinity site for cytochrome *c* does not function independently in electron transfer. Covalently bound cytochrome *c* was able to transfer electron from ascorbate and *N,N,N',N'*-tetramethylphenylenediamine to cytochrome *c* oxidase with an overall oxidase activity of around one-sixth of maximal. Binding of horse heart cytochrome *c* in the low-affinity site increased this electron-transfer activity to a level close to that of unmodified cytochrome *c* oxidase. Cytochrome *c* bound to the low-affinity site must increase the rate of electron transfer through the high-affinity site by some allosteric mechanism.

Cytochrome *c* oxidase (EC 1.9.3.1), the terminal member of the electron-transport chain in mitochondria, is an intrinsic membrane protein which spans the inner mitochondrial membrane. The enzyme catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen while conserving the energy released in the reaction as a proton gradient for the subsequent synthesis of ATP. The cytochrome *c* oxidase monomer contains two hemes (*a* and a_3) and two copper atoms as prosthetic groups in a complex of molecular weight between 140000 and 170000 [reviewed in Erecinska & Wilson (1978), Capaldi (1979), and Azzi & Casey (1979)].

Three-dimensional reconstruction of two different two-dimensional crystal forms has yielded the gross shape and approximate size of the cytochrome *c* oxidase monomer (Henderson et al., 1977; Fuller et al., 1979). It is a Y-shaped structure comprising three domains, two of which (M_1 and M_2) span the lipid bilayer, with the third or C domain extending from the cytoplasmic face of the inner mitochondrial membrane (Fuller et al., 1979). Beef heart cytochrome *c* oxidase has been shown to be a dimer in solutions of many nonionic detergents (Robinson & Capaldi, 1977; Bisson et al., 1980; Rosevear et al., 1980) and in membranes (Henderson et al., 1977).

The steady-state kinetics of the reduction of cytochrome *c* by cytochrome *c* oxidase are complicated, and most models intended to explain these data invoke two cytochrome *c* binding sites per cytochrome *c* oxidase monomer (Ferguson-Miller et al., 1976, 1978; Errede et al., 1976; Smith et al., 1979; Nicholls et al., 1980). Direct binding experiments show that there is a high-affinity ($K_d \simeq 10^{-8}$ M) and a low-affinity ($K_d \simeq 10^{-6}$ M) binding site for horse heart cytochrome *c* on beef heart cytochrome *c* oxidase (Ferguson-Miller et al., 1976). Eadie–

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Hofstee plots of cytochrome *c* oxidase show two phases, one predominating at low cytochrome *c* concentrations, with an apparent K_m very similar to the K_d for binding in the high-affinity site, the other seen at higher cytochrome *c* concentrations, with an apparent K_m close to the K_d for binding into the low-affinity site. This led Ferguson-Miller et al. (1978) to propose that the high- and low-affinity sites for cytochrome *c* on cytochrome *c* oxidase function as independent electron-transfer sites.

An added complication in studying the interaction of cytochrome *c* with cytochrome *c* oxidase is that since this substrate is positively charged it also reacts with negatively charged phospholipids, particularly cardiolipin (Birrell & Griffith, 1976), with affinities not much lower than that for the low-affinity site discussed above.

The aim of our studies is to map the high- and low-affinity binding sites for cytochrome *c* on beef heart cytochrome *c* oxidase and to evaluate the functional significance of each. Our first approach was to cross-link a preformed cytochrome *c*–cytochrome *c* oxidase complex with dithiobis(succinimidylpropionimidate), a lysine-modifying reagent (Briggs & Capaldi, 1978). Cytochrome *c* was found to be covalently cross-linked to subunit II in these experiments. Other workers using different cross-linking reagents obtained different results. Erecinska (1977) concluded that cytochrome *c* binds to subunits V and VI. Seiter et al. (1979) reported that subunit IV was involved.

A limitation of the above studies is that the site of modification in cytochrome *c* was not identified. Moreover, it was not determined if binding occurred at the high- or low-affinity site for cytochrome *c*.

More revealing cross-linking studies were undertaken by Bisson et al. (1978, 1980) and by Birchmeier et al. (1976). Bisson et al. (1978, 1980) used an arylazidocytochrome *c* derivative selectively modified at Lys-13 and showed that this derivative cross-linked to subunit II through interaction in the high-affinity site for cytochrome *c*. Birchmeier et al. (1976) used a yeast iso-1-cytochrome *c* activated by reaction with 5,5′-dithiobis(2-nitrobenzoate) (DTNB)[1] at a single cysteine, Cys-107. This derivative bound to yeast cytochrome *c* oxidase at subunit III. It is unclear whether this site corresponds to the high-affinity site as defined in studies of beef heart cytochrome *c* oxidase because the yeast enzyme binds two molecules of yeast cytochrome *c* with equal affinity (Dethmers et al., 1979).

In this work, we have repeated the experiments of Birchmeier et al. (1976) using beef heart cytochrome *c* oxidase. We have found that Cys-107-modified yeast cytochrome *c* cross-links to subunit III of beef heart cytochrome *c* oxidase and occupies the high-affinity binding site for horse heart cytochrome *c*. These results are discussed in terms of the emerging structure of the cytochrome *c* binding sites as well as from the point of view of the importance of high- and low-affinity sites in cytochrome *c* oxidase activity.

Materials and Methods

Preparation of the Thionitrobenzoate Derivative of Yeast Cytochrome c. Yeast cytochrome *c* was a generous gift from Professor Takashi Yonetani, Johnson Foundation, University of Pennsylvania. The protein was derivatized with DTNB (Sigma) by using the procedure of Birchmeier et al. (1976). Typically, modified preparations contained 0.85 mol of thionitrobenzoate (TNB) per *c* heme. This reflects complete derivitization of Cys-107 in the yeast iso-1-cytochrome *c* component of our yeast cytochrome *c* mixture. (Yeast iso-2-cytochrome *c* does not contain a reactive sulfhydryl group.)

TNB-yeast cytochrome *c* aggregates readily upon freezing and thawing or upon storage in low ionic strength buffers. Our preparations were kept at 4 °C in 0.5 M NaCl and 10 mM sodium phosphate (pH 7.2) and were chromatographed on CM-52 (Whatman) immediately before use to remove dimers and aggregates of yeast cytochrome *c* (Brautigan et al., 1978).

Reaction of Beef Heart Cytochrome c Oxidase with TNB-Cytochrome c. 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 approximately 100 μg of phospholipid/mg of protein. Cytochrome *c* oxidase (10 μM heme aa_3) was reacted with TNB-yeast cytochrome *c* (40–100 μM) in 2% (w/v) cholate and 25 mM Tris–acetate (pH 7.8) at 37 °C. Lower concentrations of cholate result in precipitation of the enzyme during incubation. After incubation for 1 h at 37 °C with 100 μM yeast cytochrome *c*, as much as 0.8 mol of cytochrome *c* was bound per mol of oxidase. A control sample, containing 10 μM beef heart cytochrome *c* oxidase and horse heart cytochrome *c* in the same concentration as the TNB-yeast cytochrome *c* concentration, was always incubated in parallel and used as the control for assays and gels.

Gel Electrophoresis of the TNB-Cytochrome c–Cytochrome c Oxidase Complex. Samples for electrophoresis were reacted with 10 mM *N*-ethylmaleimide (NEM) for 15 min at room temperature to block disulfide exchange reactions and then dissociated in 4 M urea and 2.5% $NaDodSO_4$ for 2 h at room temperature. One-dimensional electrophoresis on 5-mm diameter rod gels was performed with 12.5% or 15% polyacrylamide gels by the method of Downer et al. (1976). Slab gels (1 mm thick) 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). The system of Wilson et al. (1980), with the addition of 6 M urea, effected a complete separation of all the polypeptides of beef heart cytochrome *c* oxidase, including three peptides collectively labeled as VII's. These have been shown to be distinct polypeptides by sequencing (Buse et al., 1981). This good separation depends both on omitting disulfide reducing reagents from the dissociation buffer and on electrophoresing in the presence of urea (V. M. Darley-Usmar, unpublished results).

For two-dimensional gel electrophoresis, the yeast cytochrome *c*–cytochrome *c* oxidase complex was separated from noncovalently bound cytochrome *c* by chromatography on Sephadex G-75 (1 × 20 cm) in 2% cholate, 0.5 M NaCl, and 25 mM Tris–acetate (pH 7.8). The oxidase-containing fractions were dialyzed to remove salt, incubated with 10 mM NEM, and electrophoresed on a 1.0-mm diameter 15% polyacrylamide gel as described above. After electrophoresis, this gel was incubated at room temperature in a 10% solution (v/v in water) of 2-mercaptoethanol for 15 min and then sealed to the top of 2-mm slab gel prepared according to Wilson et al. (1980), but without urea.

Staining and destaining of all gels were as described in Downer et al. (1976). Densitometric traces of gels were made at 550 nm on a Beckman DU spectrophotometer equipped with a linear-transport attachment. Quantitation of the binding of cytochrome *c* to subunit III was performed by comparing the ratios of the areas corresponding to subunits II–IV between

[1] Abbreviations used: TMPD, *N,N,N′,N′*-tetramethylphenylenediamine dihydrochloride; TNB, thionitrobenzoate; NEM, *N*-ethylmaleimide; $NaDodSO_4$, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoate).

the control and reacted samples. All data used were taken from gels in which the change in the ratio of the area of subunit III to that of subunit II matched the change in the ratio of the area of subunit III to that of subunit IV to better than 10%.

Binding of Horse Heart Cytochrome c to High- and *Low-Affinity Sites.* The yeast cytochrome *c*–cytochrome *c* oxidase complex was separated from noncovalently bound cytochrome *c* by chromatography on Sephadex G-75 in the presence of 0.5 M NaCl, 25 mM Tris–acetate, and 2% (w/v) cholate (pH 7.8). Salt was then removed by dialyzing against several changes of 100 volumes of 25 mM Tris–acetate (pH 7.8), containing 0.25% (w/v) Tween 20. Complex or unmodified cytochrome *c* oxidase was mixed with a 10-fold molar excess of horse heart cytochrome *c* and chromatographed on Sephadex G-100 (1 × 20 cm) equilibrated with 25 mM Tris–acetate (pH 7.8), 0.25% (w/v) Tween 20, and either 0.64 or 20.5 μM horse heart cytochrome *c*. The peak of cytochrome *c* oxidase was always well separated from the peak of excess cytochrome *c*. The ratio of cytochrome *c* to cytochrome *c* oxidase was determined spectrophotometrically as described by Ferguson-Miller et al. (1976).

Assay of Cytochrome c Oxidase Activity. After incubation at 37.0 °C, mixtures of beef heart cytochrome *c* oxidase and TNB-yeast cytochrome *c* or horse heart cytochrome *c* were brought to 10% (w/v) dodecyl β-D-maltopyranoside (Calbiochem) and allowed to sit at least 30 min on ice prior to assay to displace cholate from the enzyme. This material was used for both polarographic and spectrophotometric assays. [Some lots of dodecyl β-D-maltopyranoside contain a trace impurity which radically affected its solubility in water and must be removed by chromatography to obtain a clear solution at 0 °C (Rosevear et al., 1980)].

Polarographic assays were performed as described in Vik & Capaldi (1980). The concentration of cytochrome *c* oxidase in the assay was between 5 and 20 nM. The effect of polylysine upon activity was determined by comparing the activity of enzyme in the presence of 40 μM cytochrome *c* to that in the presence of 120 μM cytochrome *c* and 30 μg/mL polylysine (Sigma type VB, approximate molecular weight 30000).

The activity of the TNB-cytochrome *c*–cytochrome *c* oxidase complex was determined after separating the complex from noncovalently bound cytochrome *c* by gel filtration as described above. Samples were dialyzed to remove salt and then brought to 10% (w/v) in dodecyl β-D-maltopyranoside before assay. The ratio of cytochrome *c* to cytochrome *c* oxidase was determined spectrophotometrically. Activities were compared with samples of cytochrome *c* oxidase assayed in the presence of an equal amount of horse heart cytochrome *c*.

Samples of cytochrome *c* oxidase were assayed spectrophotometrically and results analyzed as described by Errede et al. (1976). Ferrocytochrome *c* was prepared by reduction of a stock solution of horse heart ferricytochrome *c* with sodium dithionite. The dithionite was then separated from the cytochrome *c* on a Sephadex G-25 column, and the ferrocytochrome *c* fractions were collected. The concentration of ferrocytochrome *c* was determined from its absorbance at 550 nm by using an extinction coefficient of 27600 M^{-1} cm^{-1} (Schejter et al., 1963). Samples were assayed over a range of ferrocytochrome *c* concentrations (11–30 μM) in 0.3% Tween 80, 2 mM EDTA, and 40 mM sodium phosphate, (pH 7.4). The concentration of cytochrome *c* oxidase was between 1 and 5 nM in the assay.

Results

TNB-Derivatized Yeast Cytochrome c Covalently Binds to Subunit III of Cytochrome c Oxidase. Yeast cytochrome *c* was reacted with DTNB to obtain a TNB-cytochrome *c* derivative selectively modified at Cys-107 (Birchmeier et al., 1976). This derivative was reacted with beef heart cytochrome *c* oxidase in a 4:1 molar ratio at 37 °C for various intervals of time. Samples were reacted with NEM to prevent disulfide exchange reactions and then subjected to $NaDodSO_4$–urea–polyacrylamide gel electrophoresis (Figure 1). The gel system used in these experiments effected a complete separation of all of the polypeptides of beef heart cytochrome *c* oxidase. These are labeled as in Downer et al. (1976).

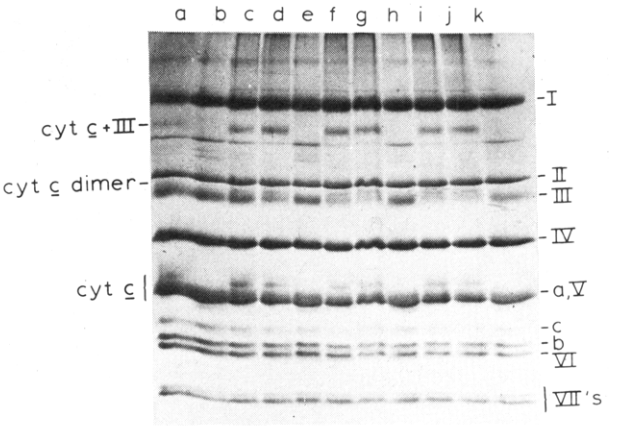

FIGURE 1: $NaDodSO_4$–urea–polyacrylamide gel electrophoresis of the TNB-yeast cytochrome *c* reaction with beef heart cytochrome *c* oxidase. Samples (10 μM cytochrome *c* oxidase) were incubated at 37 °C with either 40 μM TNB-yeast cytochrome *c* or 40 μM horse heart cytochrome *c* in 2% cholate and 25 mM Tris–acetate, pH 7.8, for various times (min): (a) 5, TNB-cyt *c*; (b) 5, cyt *c*; (c) 10, TNB-cyt *c*; (d) 20, TNB-cyt *c*; (e) 20, cyt *c*; (f) 30, TNB-cyt *c*; (g) 40, TNB-cyt *c*; (h) 40, cyt *c*; (i) 60, TNB-cyt *c*; (j) 50, TNB-cyt *c*; (k) 60, cyt *c*. The gel was run with a Laemmli buffer system as modified by Wilson et al. (1980).

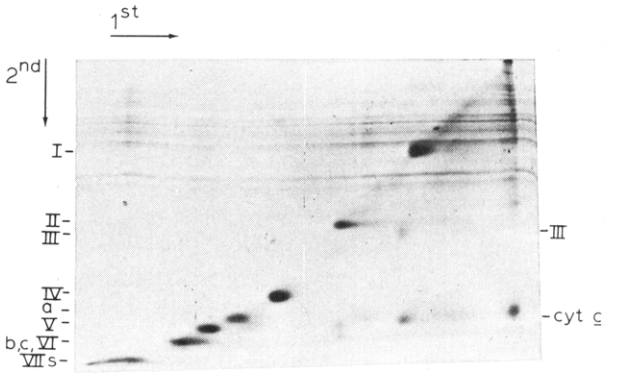

FIGURE 2: Two-dimensional $NaDodSO_4$–urea–polyacrylamide gel electrophoresis of disulfide-linked yeast cytochrome *c*–beef heart cytochrome *c* oxidase complex. Electrophoresis in the horizontal dimension was in the absence of 2-mercaptoethanol and was followed by electrophoresis in the vertical dimension after incubation of the rod gel (first dimension) in a 10% solution of 2-mercaptoethanol (v/v in water) for 15 min.

Figure 1 shows that incubation of TNB-cytochrome *c* with the beef heart cytochrome *c* oxidase complex leads to a time-dependent disappearance of subunit III and the concomitant appearance of a single new band running below subunit I. The relative staining intensities of other subunits were unaltered through the experiment. The new band had an apparent molecular weight of 35000, which is close to that expected for a cross-linked product of cytochrome *c* and subunit III. Direct evidence that the 35000-dalton band contained subunit III + cytochrome *c* was obtained by two-dimensional gel electrophoresis (Figure 2). In this experiment, the cytochrome *c* oxidase–cytochrome *c* complex was separated

from noncovalently bound cytochrome *c* by gel exclusion chromatography on Sephadex G-75 in high ionic strength buffer. This complex was reacted with NEM as before, dissociated in $NaDodSO_4$ and urea, and then electrophoresed on a 15% polyacrylamide stick gel by using gel conditions described by Swank & Munkres (1971). The cross-linked product and unlinked subunits separated in this gel (first dimension) were dissociated by incubation in 10% (v/v) 2-mercaptoethanol, electrophoresed out of the first-dimensional gel into a slab gel made with 16% polyacrylamide, and run as described under Materials and Methods under the Laemmli (1970) buffer conditions. Unlinked subunits of cytochrome *c* oxidase ran as a function of their monomer molecular weight in the second dimension, forming a diagonal across the gel. The 35 000-dalton cross-linked product was cleaved by mercaptoethanol and separated into subunit III and cytochrome *c*, each of which ran off the diagonal and to the position of their monomer molecular weights. Three other off-diagonal spots of cytochrome *c* were seen. The major product ran at the top of the first-dimensional gel. This represents the cytochrome *c*–subunit III complex which had aggregated during treatments prior to the gel electrophoresis step. The amount of aggregate was much less if the gel filtration step was omitted (as in the gels in Figure 1). There was a small amount of cytochrome *c* dimer resolved on the gel migrating close to subunit II. Also, a small amount of a complex between cytochrome *c* and impurity *c* was generated. This sulfhydryl-rich impurity (Darley-Usmar & Wilson, 1981) is found only in mammalian cytochrome *c* oxidases and so was not involved in cytochrome *c* binding to the yeast enzyme (Birchmeier et al., 1976).

Yeast Cytochrome c Is Covalently Bound in the High-Affinity Site for Horse Heart Cytochrome c on Cytochrome c Oxidase. Beef heart cytochrome *c* oxidase contains high- (10^{-8} M) and low-affinity (10^{-6} M) binding sites for horse heart cytochrome *c* which can be distinguished experimentally by gel filtration experiments at low ionic strength (Ferguson-Miller et al., 1976). Yeast cytochrome *c* has been found to bind to beef heart oxidase at two sites with equal affinity ($K_D \simeq 10^{-7}$ M) under similar binding conditions (Ferguson-Miller et al., 1976). Covalently bound yeast cytochrome *c* could therefore be bound at one or both of these sites. To identify which site was involved, we mixed the disulfide-linked yeast cytochrome *c*–cytochrome *c* oxidase complex, separated from noncovalently bound yeast cytochrome *c*, with a 10-fold excess of horse heart cytochrome *c* and chromatographed on Sephadex G-100 in the presence of 0.64 μM horse heart cytochrome *c*. Under these conditions, unmodified cytochrome *c* oxidase eluted from the column with 1 mol of cytochrome *c* tightly bound (in the high-affinity site). Figure 3 shows that the covalent yeast cytochrome *c*–cytochrome *c* oxidase complex, run through the column under identical conditions, also eluted with 1 mol of cytochrome *c* bound per mol of cytochrome *c* oxidase, 0.72 mol of which is the covalently bound derivative, the other 0.28 mol being horse heart cytochrome *c*. This is direct evidence that the yeast cytochrome *c* is essentially all bound in the high-affinity binding site for horse cytochrome *c* on the cytochrome *c* oxidase complex.

The results of eluting a disulfide-linked yeast cytochrome *c*–cytochrome *c* oxidase complex through the gel filtration column in the presence of 20.5 μM horse heart cytochrome *c* is also shown in Figure 3. These are conditions which favor binding of horse heart cytochrome *c* into both the high- and low-affinity sites. The same proportion of the low-affinity site was occupied in the covalent yeast cytochrome *c*–beef heart

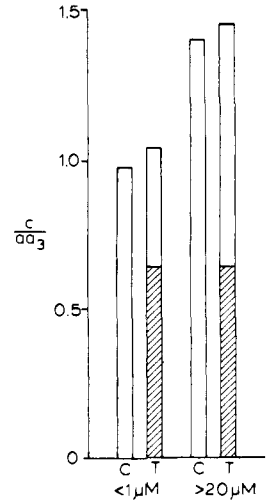

FIGURE 3: Binding of horse heart cytochrome *c* to disulfide-linked cytochrome *c*–cytochrome *c* oxidase complex. Either the disulfide-linked yeast cytochrome *c*–cytochrome *c* oxidase complex (T) or the unmodified cytochrome *c* oxidase (C) was mixed with a 10-fold excess of horse heart cytochrome *c* and chromatographed on a Sephadex G-100 column equilibrated with either 0.64 or 20.5 μM horse cytochrome *c*. The cross-hatched area indicates the ratio of covalently bound cytochrome *c* per cytochrome *c* oxidase monomer. The total area indicates the total ratio of cytochrome *c* to cytochrome *c* oxidase monomer coeluting with the enzyme.

cytochrome *c* oxidase complex as in unmodified cytochrome *c* oxidase. This shows that the low-affinity site is still accessible to cytochrome *c* in complexes containing the yeast cytochrome *c* covalently bound in the high-affinity site.

Covalently Bound Yeast Cytochrome c Inhibits Cytochrome c Oxidase Activity in the Spectrophotometric Assay. Cytochrome *c* oxidase activity can be measured spectrophotometrically by monitoring the oxidation of reduced cytochrome *c* through its absorbance at 550 nm. Figure 4 correlates the covalent binding of the TNB-yeast cytochrome *c* derivative, as measured by the disappearance of subunit III and the appearance of the cross-linked product, with inhibition of electron-transfer activity measured spectrophotometrically. Activity of the yeast cytochrome *c*–cytochrome *c* oxidase complex is reported as a percentage of the activity of an identically treated mixture of horse heart cytochrome *c* and cytochrome *c* oxidase in the same molar ratio as the experimental sample. The concentration of cytochrome *c* used in the assay had no effect on the degree of inhibition between 11 and 30 μM.

The data in Figure 4A,B are taken from a time course of the reaction of TNB–yeast cytochrome *c* with cytochrome *c* oxidase. This reaction displays pseudo-first-order kinetics. The half-times for the appearance of the subunit III–*c* complex, the disappearance of subunit III, and the inhibition of cytochrome *c* oxidase activity are all equal (approximately 25 min at 37 °C with a c/aa_3 ratio of 4:1), indicating that all three phenomena reflect the same reaction.

Extrapolation of the data in Figure 4A shows that complete inhibition would occur at a disulfide-linked yeast cytochrome *c* to aa_3 ratio of 1 to 1. This demonstrates that one cytochrome *c* bound in the high-affinity site completely inhibits electron transfer through either the low- or the high-affinity site in the spectrophotometric assay.

Yeast Cytochrome c–Beef Heart Cytochrome c Oxidase Complex Retains Electron-Transfer Activity. Visible spectra of the purified yeast cytochrome *c*–cytochrome *c* oxidase complex freed of noncovalently bound cytochrome *c*, in the absence and presence of artificial reducing agents, are shown in Figure 5. These spectra show the steady-state levels of

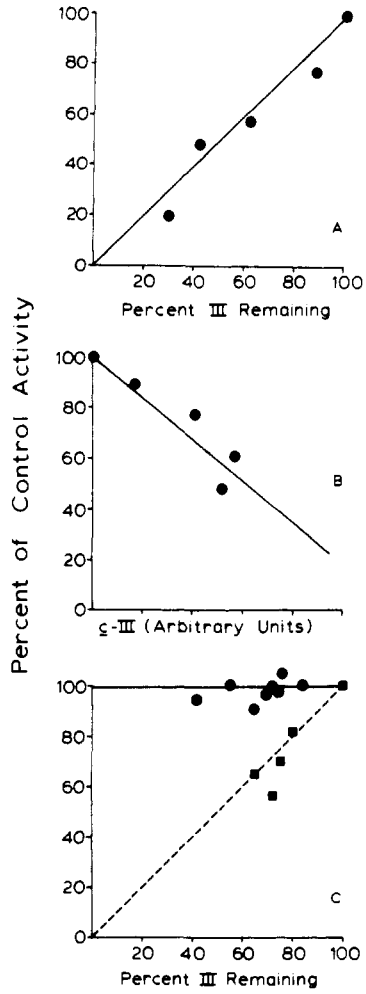

FIGURE 4: Correlation of the amount of yeast cytochrome *c*–cytochrome *c* oxidase complex with steady-state activity. Steady-state activity of the yeast cytochrome *c*–cytochrome *c* oxidase complex was calculated as a percentage of control samples. The amount of covalent complex formed between yeast cytochrome *c* and subunit III of cytochrome oxidase was estimated from gel electrophoresis as described under Materials and Methods. Figure 4A depicts the variation in activity, measured spectrophotometrically, with the amount of complex measured as the disappearance of the band corresponding to subunit III. Similarly, Figure 4B shows the same activity measurements plotted against the amount of complex measured as the intensity of the cytochrome *c*–subunit III band (*c*-III) after $NaDodSO_4$ gel electrophoresis. In contrast, the activity measurements in Figure 4C were determined polarographically in the absence (●) and the presence (■) of polylysine. The amount of complex formed was determined as described above for Figure 4A. [Control activities in the spectrophotometric assay were typically 70 mol of *c* (mol of aa_3)$^{-1}$ s^{-1} and in the polarographic assay 150 mol of *c* (mol of aa_3)$^{-1}$ s^{-1} at 30 μM *c*]. The lines corresponding to a 1:1 ratio between inhibition and complex formation are drawn in (A) and in the polylysine portion of (C).

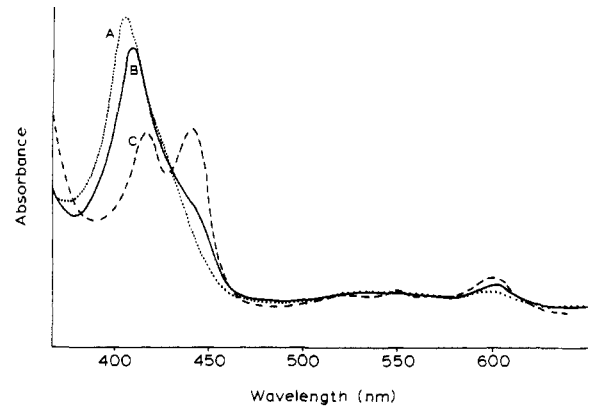

FIGURE 5: Reduction of disulfide-linked yeast cytochrome *c*–beef heart cytochrome *c* oxidase complex. The complex was purified by chromatography in the presence of 0.5 M NaCl on Sephadex G-75 to remove noncovalently bound cytochrome *c*. (A) No additions; (B) 30 mM ascorbate; (C) 0.5 mM TMPD, 30 mM ascorbate in 2% cholate, and 25 mM Tris–acetate, pH 7.8.

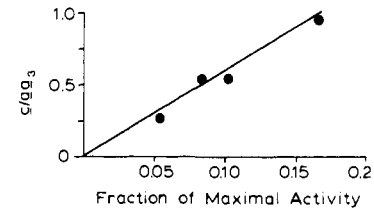

FIGURE 6: Activity of isolated yeast cytochrome *c*–beef heart cytochrome *c* oxidase complex. Yeast cytochrome *c*–beef heart cytochrome *c* oxidase was separated from noncovalently bound cytochrome *c* and assayed polarographically in the presence of TMPD and ascorbate without added cytochrome *c* and then in the presence of 30 μM cytochrome *c* (see Materials and Methods). The rate in the presence of TMPD and ascorbate alone is expressed as a fraction of the rate in the presence of 30 μM cytochrome *c* and is plotted vs. the ratio of covalently bound *c* to aa_3. Typical activities in the presence of 30 μM cytochrome *c* were 100 mol of *c* (mol of aa_3)$^{-1}$ s^{-1}. The rate of electron transfer for a mixture of unmodified cytochrome *c* oxidase and horse heart cytochrome *c* in the same ratio as the covalent complex was essentially the background rate and was subtracted from the complex rate before the data were plotted. The line drawn corresponds to the activity of the complex being one-sixth of the maximal activity.

reduction of cytochrome *c* and of cytochrome *c* oxidase. Spectrum A is for the complex prior to reduction. Spectrum B was taken in the presence of ascorbate alone. The steady-state level of reduction of cytochrome *c* and of cytochrome *c* oxidase is small. In contrast, when both TMPD and ascorbate were added (spectrum C), cytochrome *c* and cytochrome *c* oxidase were substantially reduced. This shows that electron transfer from ascorbate alone to cytochrome *c* is slow relative to the rate of transfer from cytochrome *c* to cytochrome *c* oxidase. The presence of TMPD increases the rate of electron transfer to the bound cytochrome *c*, resulting in an almost total reduction of cytochrome *c* oxidase, although the steady-state level of cytochrome *c* reduction remains less than 50%. Hill & Nicholls (1980) have reported similar results for the relative abilities of ascorbate and TMPD to reduce cytochrome *c* bound to cytochrome *c* oxidase. We conclude that reduction of covalently bound cytochrome *c* with artificial electron donors leads to reduction of cytochrome *c* oxidase.

The electron transfer from ascorbate and TMPD through cytochrome *c* to cytochrome *c* oxidase was measured polarographically (Figure 6). The activity of the covalent complex in the absence of free cytochrome *c* was approximately 16% of the maximal activity of the complex in the presence of 30 μM cytochrome *c* (Figure 6). The electron-transfer activity of a control containing cytochrome *c* oxidase and free cytochrome *c* in the same molar ratio was essentially zero.

In the presence of free horse heart cytochrome *c* (30 μM), the electron-transfer activity of the covalent yeast cytochrome *c*–cytochrome *c* oxidase complex was as high as that of untreated enzyme (Figure 4C). This high activity could not be a consequence of cleavage of the complex because the assay conditions do not cleave the labile disulfide in DTNB and are unlikely to cleave the disulfide bond holding the complex together. Dissociation of the complex would be expected to abolish electron transfer from ascorbate and TMPD through covalently bound cytochrome *c*, but this is not the case (see Figure 6).

Although the maximal activities of the yeast cytochrome *c*–cytochrome *c* oxidase complex and unmodified enzyme were

the same, their inhibition by polylysine was very different (Figure 4C). Polylysine acted as an inhibitor in both the complex and control oxidase samples. As expected, addition of high concentrations of horse heart cytochrome *c* relieved the inhibition of the unmodified cytochrome *c* oxidase completely. However, the activity of the yeast cytochrome *c*–cytochrome *c* oxidase complex was not recovered even after addition of a very large excess of cytochrome *c*, the inhibition being proportional to the amount of complex present (Figure 4C). We interpret this to indicate that free cytochrome *c* cannot displace polylysine bound to the yeast cytochrome *c*–cytochrome *c* oxidase complex.

Discussion

The present studies using TNB-yeast cytochrome *c* complement our previous studies using arylazidocytochrome *c* derivatives (Bisson et al., 1980) and lipid depletion experiments (Vik et al., 1981) in defining the substrate binding sites on beef heart cytochrome *c* oxidase.

It is now generally agreed that there are two binding sites for cytochrome *c* per cytochrome *c* oxidase monomer. These are distinguishable in direct binding experiments by their relative affinity for substrate, one being a high-affinity site ($K_d = 10^{-8}$ M), the second a low-affinity site ($K_d = 10^{-6}$ M) (Ferguson-Miller et al., 1976). It has been inferred that the two phases of activity seen in Eadie–Hofstee plots of cytochrome *c* oxidase activity (obtained in the presence of TMPD and ascorbate) are related to electron transfer through these two sites independently (Ferguson-Miller et al., 1978). The two sites must be structurally distinct, given that the cytochrome *c* oxidase monomer has no 2-fold symmetry and that it contains one copy only of each subunit. However, it is possible that the distinction between sites in terms of affinities is operational, both sites having high affinity in the absence of cytochrome *c* with binding of substrate in one site converting the second site into a low-affinity site.

Our previous experiments with arylazidocytochrome *c* derivatives have provided some detail of the two cytochrome *c* binding sites (Bisson et al., 1980). An arylazidocytochrome *c* derivative selectively modified at Lys-13, which is in the binding domain on cytochrome *c* for oxidase and reductases, was found to bind to cytochrome *c* oxidase through subunit II. This interaction occurred at a very low cytochrome *c* concentration where binding of substrate would be limited to the (operational) high-affinity site. The covalently bound cytochrome *c* was found to block binding of horse heart cytochrome *c* in the high-affinity site as indicated by direct binding experiments, while binding of native cytochrome *c* into the low-affinity site was unimpaired. Most importantly, arylazidocytochrome *c* covalently bound in the high-affinity site was found to inhibit electron transfer completely: i.e., there was no independent electron transfer through native cytochrome *c* in the low-affinity site.

Experiments were also conducted with a second arylazidocytochrome *c* derivative, this one probably modified at Lys-22, on the back side of the cytochrome *c* molecule [terminology of Takano & Dickerson (1980)] and away from the heme cleft. This derivative was bound to cytochrome *c* oxidase through tightly bound cardiolipin, rather than with protein, and was in the low-affinity site for substrate. The presence of covalently bound cytochrome *c* in the low-affinity site did not inhibit activity when cytochrome *c* oxidase was assayed in the presence of native cytochrome *c*.

In other studies, we have progressively removed tightly bound cardiolipin from beef heart cytochrome *c* oxidase and monitored the effect on activity (Vik et al., 1981). This work showed that the enzyme required one or two molecules of cardiolipin for maximal electron-transfer activity. Removal of the last few lipids led to the disappearance of the low-affinity site for cytochrome *c* binding (as measured in direct binding experiments) and the low-affinity phase of activity (as resolved in Eadie–Hofstee plots). Lipid-free cytochrome *c* oxidase retained 15–20% of maximal activity although the low-affinity binding site for cytochrome *c* was missing. The above experiments show that the high- and low-affinity binding sites for cytochrome *c* can be differentiated both structurally and functionally.

In the present study, TNB yeast cytochrome *c* has been reacted with beef heart cytochrome *c* oxidase and shown to bind to subunit III. These results confirm the findings of Birchmeier et al. (1976) with yeast cytochrome *c* oxidase. Covalent binding of the yeast cytochrome *c* derivative blocked binding of horse heart cytochrome *c* in the high-affinity site (as determined by direct binding experiments) but did not affect binding of substrate to the low-affinity site. It effected the complete inhibition of electron transfer (at 1 mol of cytochrome *c* bound/mol of aa_3), which from the foregoing discussion is additional evidence that the derivative is indeed in the high-affinity site. The covalent yeast cytochrome *c*–beef cytochrome *c* oxidase complex was able to transfer electrons from TMPD and ascorbate to molecular oxygen. This cytochrome *c* oxidase activity was 15–20% of maximal, the same activity seen for cytochrome *c* oxidase from which the low-affinity site of cytochrome *c* has been removed by depletion of cardiolipin (Vik et al., 1981).

Implications for the Structure of the High-Affinity Binding Site. Our studies have shown that an arylazidocytochrome *c* derivative modified at Lys-13 and yeast cytochrome *c* modified by DTNB at Cys-107 (homologous to Thr-102 in horse heart cytochrome *c*) both covalently link into the high-affinity site for substrate on cytochrome *c* oxidase, one to subunit II and the other to subunit III of the oxidase complex [see Bisson et al. (1980) and this study].

Examination of the three-dimensional structure of cytochrome *c* indicates an important feature of the high-affinity binding site. Lys-13 is close to the heme edge and on the front face of the molecule, as shown in Figure 5 of Takano & Dickerson (1980). This residue is one of a ring of lysines important for activity. Cys-107, in contrast, is on the opposite side of the cytochrome *c* molecule. This is a separation of approximately 20 Å, almost the diameter of the protein. Therefore, there must be a large pocket for cytochrome *c* on the cytochrome *c* oxidase complex, at least 20 Å across. This pocket could be at the interface between subunits II and III in a single cytochrome *c* oxidase monomer. Alternatively, the cleft could be between monomers because cytochrome *c* oxidase is a dimer under the conditions in which the cytochrome *c* binding experiments are done (Robinson & Capaldi, 1977; Bisson et al., 1980). In this case, cytochrome *c* covalently bound at subunit III in one monomer would interact through its front face with subunit II of the second monomer of the dimer. The three-dimensional reconstruction of the dimer in the $P22_12_1$ crystal form of cytochrome *c* oxidase shows that a cleft of the appropriate size does indeed exist in the dimer (Deatherage et al., 1981).

Two-dimensional arrays of beef heart cytochrome *c* oxidase ($P12_1$ crystal form) have recently been reacted with arylazidocytochrome *c* without disordering the crystals. These are now being examined by electron microscopy and image reconstruction to identify the binding site for cytochrome *c* on the cytochrome *c* complex directly.

The orientation of cytochrome *c* in the high-affinity site places the heme edge closest to subunit II. This region of the cytochrome *c* molecule is the more highly conserved through evolution. Modifications of horse heart cytochrome *c* on its back face (near the 102) produce negligible effects on the cytochrome *c*–cytochrome *c* oxidase interaction [reviewed in Takano & Dickerson (1980)]. Thus, subunit II probably contains the entry point for electrons in the cytochrome *c* oxidase complex.

Implications for the Steady-State Activity of Cytochrome c Oxidase. Both yeast cytochrome *c* and arylazidocytochrome *c* inhibit electron transfer completely when covalently bound in the high-affinity binding site for substrate, even though the low-affinity site is able to bind cytochrome *c*. Therefore, the low-affinity site cannot act as an independent electron-transfer site as has been proposed (Ferguson-Miller et al., 1978).

However, the binding of cytochrome *c* in the low-affinity site is necessary for maximal cytochrome *c* oxidase activity as indicated by single-turnover and steady-state kinetic studies (Ferguson-Miller et al., 1976, 1978; Errede et al., 1976; Smith et al., 1979; Nicholls et al., 1980; Veerman et al., 1980; Wilms et al., 1981) and by the activity measurements on the covalent yeast cytochrome *c*–beef heart cytochrome *c* oxidase complex described here. The key observation is that electron-transfer activity of the covalent complex is inhibited when measured spectrophotometrically, and yet the complex is fully active when observed polarographically.

In the spectrophotometric assay, cytochrome *c* oxidase is mixed with ferrocytochrome *c* and the subsequent oxidation of cytochrome *c* monitored by the change in absorbance at 550 nm. No other reducing agents besides cytochrome *c* are present in the assay. As a consequence, yeast cytochrome *c* covalently bound to cytochrome *c* oxidase remains in its oxidized form. In the polarographic assay, ascorbate and TMPD are present along with cytochrome *c* as reducing agents, and the rate of oxygen consumption rather than the oxidation of cytochrome *c* is monitored. Under these conditions, the bound cytochrome *c* can be rereduced, leading to a steady-state transfer of electrons to the oxidase complex. This occurs with the covalent yeast cytochrome *c*–cytochrome *c* oxidase complex as evidenced by its significant activity in the polarographic assay in the absence of free cytochrome *c*.

Interaction of reduced cytochrome *c* with the low-affinity site occurs in both assays. When cytochrome *c* in the high-affinity site is oxidized, there is no transfer of electrons through the low-affinity site as indicated by the results of the spectrophotometric assay. When cytochrome *c* in the high-affinity site is reduced, as in the polarographic assay, binding of reduced cytochrome *c* in the low-affinity site increases electron transfer to the level of unmodified cytochrome *c* oxidase. This result can be explained by at least three mechanisms.

(i) Binding of cytochrome *c* to the low-affinity site could increase activity by speeding up the rate of electron transfer through the cytochrome *c* oxidase complex, e.g., by increasing the rate of intramolecular transfer of electrons from the heme *a*–Cu_a couple to the heme a_3–C_{a_3} couple. This would require that cytochrome *c* bound in the low-affinity site in some way alters the conformation of cytochrome *c* oxidase.

(ii) Electron transfer could proceed through the low-affinity site but be dependent on the functioning of the high-affinity site. For example, binding of reduced cytochrome *c* to the high-affinity site could open up the pathway of electron transfer through the low-affinity site.

(iii) Cytochrome *c* bound in the low-affinity site could speed up electron transfer through the high-affinity site by increasing the dissociation constant (off rate) of the substrate from this site.

We favor the last of these three possibilities. The off rate of cytochrome *c* from the high-affinity site has been measured directly recently and shown to be about 1 s^{-1} (Wilms et al., 1981). According to Wilms et al. (1981), this off rate is increased by at least 50-fold by binding of a positively charged molecule in the low-affinity site. These observations are entirely consistent with mechanism iii but are hard to explain by mechanisms i or ii.

In summary, structural and functional studies of the binding of a TNB derivative (at Cys-107) of yeast cytochrome *c* with beef heart cytochrome *c* oxidase indicate that the high-affinity binding site for cytochrome *c* on the oxidase complex is a cleft, probably in the interface between oxidase monomers in the stable enzyme dimer. Activity measurements are interpreted in favor of a mechanism in which electron transfer from cytochrome *c* to cytochrome *c* oxidase occurs only at the high-affinity site. Cytochrome *c* bound at the low-affinity site is thought to increase the off rate of ferricytochrome *c* from the high-affinity site. This might occur through electrostatic repulsion between the two cytochrome *c* molecules or by a change in conformation of cytochrome *c* oxidase induced by binding of cytochrome in the low-affinity site.

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Semisynthesis of Insulin: Specific Activation of the Arginine Carboxyl Group of the B Chain of Desoctapeptide-(B23–30)-insulin (Bovine)†

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ABSTRACT: The semisynthesis of insulin through specific activation, by a combination of enzymatic and chemical means, of the arginine carboxyl of the B chain of desoctapeptide-(B23–30)-insulin (DOI) is described. During this synthesis the amino functions of insulin were protected by the *tert*-butyloxycarbonyl (Boc) group. A1,B1,B29-$(Boc)_3$-insulin (tri-Boc-insulin) was prepared by reaction of di-*tert*-butyl dicarbonate with zinc-free insulin in dimethyl sulfoxide. Digestion of the tri-Boc-insulin with trypsin at pH 8.5 yielded A1,B1-$(Boc)_2$-DOI (di-Boc-DOI) and Gly-Phe-Phe-Tyr-Thr-Pro-(ε-Boc)Lys-Ala (ε-Boc-octapeptide) which were separated by gel chromatography. The trypsin-catalyzed coupling of the arginine carboxyl group of di-Boc-DOI with phenylhydrazine to yield di-Boc-DOI-phenylhydrazide in 87% yield was effected at pH 6.5 in an aqueous organic solvent consisting of dimethyl sulfoxide–2,4-butanediol–water (35:35:30 v/v). A favorable thermodynamic situation for this synthesis of a peptide bond is attributed, in part, to the increase in the pK_a value of the carboxyl group in organic solvents and to the low pK_a value (5.2) of phenylhydrazine. At pH 6.5, this combination of factors allows the un-ionized carboxyl group and the unprotonated amino group to be substantial components of the reaction mixture. Treatment of the di-Boc-DOI-phenylhydrazide with various oxidizing agents yielded the phenyldiimide derivative which was coupled with ε-Boc-octapeptide to resynthesize tri-Boc-insulin. Removal of the Boc groups with trifluoroacetic acid gave a semisynthetic insulin which was indistinguishable from native insulin by several criteria including hormonal activity and susceptibility to trypsin digestion at pH 8.5. This latter point indicates that the chemical coupling through the diimide took place without substantial racemization.

The action of trypsin on insulin has been the subject of several studies from this laboratory (Carpenter & Young, 1959; Young & Carpenter, 1961; Carpenter & Baum, 1962; Wang & Carpenter, 1965, 1967, 1969). When bovine insulin is treated with trypsin at pH values 8–9.5, alanine, a heptapeptide (Gly-Phe-Phe-Tyr-Thr-Pro-Lys), and desoctapeptide-insulin (DOI)[1] are formed. The latter compound, which is missing the C-terminal octapeptide on the B chain, exhibits less than 1% of the hormonal activity of insulin (Young & Carpenter, 1961). Results of the action of carboxypeptidase A on insulin revealed that the C-terminal alanine of the B chain of bovine insulin was nonessential for hormonal activity (Slobin & Carpenter, 1963). Therefore, the presence of some component of the above-mentioned heptapeptide was implicated as a substantial contributor to the hormonal activity. The hepta-

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[1] Abbreviations used: BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolinine; Boc-, *tert*-butyloxycarbonyl; ε-Boc-octapeptide, Gly-Phe-Phe-Tyr-Thr-Pro-(ε-Boc)Lys-Ala; Boc-ON, 2-[[(*tert*-butyloxycarbonyl)oxy]imino]-2-phenylacetonitrile; OBu^t, *tert*-butyl ester; DEAE-, diethylaminoethyl; di-Boc-DOI, A1,B1-$(Boc)_2$-DOI; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; DOI, desoctapeptide-(B23–30)-insulin; $NaDodSO_4$, sodium dodecyl sulfate; tri-Boc-insulin, A1,B1,B29-$(Boc)_3$-insulin; F_3CCOOH, trifluoroacetic acid.

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