Binding of Arylazidocytochrome *c* Derivatives to Beef Heart Cytochrome *c* Oxidase: Cross-Linking in the High- and Low-Affinity Binding Sites†

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ABSTRACT: Two arylazidocytochrome *c* derivatives, one modified at lysine-13 and the second modified at lysine-22, were reacted with beef heart cytochrome *c* oxidase. The lysine-13 modified arylazidocytochrome *c* was found to cross-link both to the enzyme and with lipid bound to the cytochrome *c* oxidase complex. The lysine-22 derivative reacted only with lipids. Cross-linking to protein was through subunit II of the cytochrome *c* oxidase complex, as first reported by Bisson et al. [Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Monteccuco, C., & Zanotti, A. (1978) *J. Biol. Chem. 253*, 1874]. Binding studies show that the cytochrome *c* derivative covalently bound to subunit II was in the high-affinity binding site for the substrate. Evidence is also presented to suggest that cytochrome *c* bound to the lipid was in the low-affinity binding site [as defined by Ferguson-Miller et al. [Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1976) *J. Biol. Chem. 251*, 1104]]. Covalent binding of the cytochrome *c* derivative into the high-affinity binding site was found to inhibit electron transfer even when native cytochrome *c* was added as a substrate. Inhibition was almost complete when 1 mol of the Lys-13 modified arylazidocytochrome *c* was covalently bound to the enzyme per cytochrome *c* oxidase dimer (i.e., ≃280 000 daltons). Covalent binding of either derivative with lipid (low-affinity site) had very little effect on the overall electron transfer activity of cytochrome *c* oxidase. These results are discussed in terms of current theories of cytochrome *c*–cytochrome *c* oxidase interactions.

Cytochrome *c* oxidase, the terminal member of the electron transport chain, is a protein of monomer molecular weight close to 140 000, containing two heme *a* groups (called *a* and a_3) and two copper atoms as electron acceptors [for recent reviews, see Erecinska & Wilson (1978), Capaldi (1979), and Mälmstrom (1979)]. Electron microscopy and image reconstruction studies show the enzyme as spanning the membrane and shaped like a lopsided Y, with two domains (the arms of the Y), each extending across the bilayer (Henderson et al., 1977; Fuller et al., 1979). The third domain extends from the bilayer on what would be the cytoplasmic side of the mitochondrial inner membrane (called the C domain) (Frey et al., 1978; Fuller et al., 1979).

The cytochrome *c* oxidase complex (cytochrome aa_3) appears to be composed of at least seven different polypeptides, ranging in molecular weight from 36 000 to <6000 (Sebald et al., 1974; Poyton & Schatz, 1975; Downer et al., 1976; Steffens & Buse, 1976). The three largest of these subunits are coded for on mitochondrial DNA and are made inside the mitochondrion [for a review, see Mason & Schatz (1973)]. Studies on the arrangement of the subunits in the cytochrome *c* oxidase complex indicate that both subunits II and III are exposed on the cytoplasmic side of the mitochondrial inner membrane (Eytan et al., 1975; Chan & Tracy, 1978; Ludwig et al., 1979), and are therefore part of the C domain.

The physiological electron donor for cytochrome *c* oxidase is cytochrome *c* (cyt *c*). The overall reaction of the terminal oxidase is $4\text{cyt } c^{2+} + 4H^+ + O_2 \rightleftarrows 4\text{cyt } c^{3+} + 2H_2O$. This electron transfer reaction yields free energy which is conserved, probably as a proton gradient (Wikstrom, 1977), for subsequent ion transport or ATP synthesis.

The site of binding of cytochrome *c* to cytochrome *c* oxidase has been examined in several studies, all involving the covalent cross-linking of this substrate to the oxidase. Thus, Birchmeier et al. (1976) modified yeast cytochrome *c* selectively at Cys-102 with 5,5′-dithiobis(2-nitrobenzoate) and showed that this derivative bound to yeast cytochrome *c* oxidase at subunit III. Bisson et al. (1978a,b) prepared derivatives of horse heart cytochrome *c* modified at selected lysines with 4-fluoro-3-nitrophenyl azide. The arylazidocytochrome *c* derivative modified at Lys-13 was shown to bind to subunit II in both beef heart and yeast cytochrome *c* oxidases. Briggs & Capaldi (1978) used a preformed complex of cytochrome *c* and cytochrome *c* oxidase and reacted this with the cleavable bifunctional reagent, dithiobis(succinimidylpropionate). In this study cytochrome *c* was again covalently cross-linked to subunit II.

Other workers have implicated different subunits of the beef heart enzyme in cytochrome *c* binding. Erecinska (1977) has proposed that one of the smaller subunits (V–VII) is the binding site of this substrate. Seiter et al. (1979) have concluded that subunit IV is involved.

The interaction of cytochrome *c* with cytochrome *c* oxidase is a complex one. Binding studies and kinetic measurements of cytochrome *c* oxidase activity indicate that there are both high- and low-affinity sites for cytochrome *c* on the oxidase complex (Ferguson-Miller et al., 1976, 1978; Errede et al., 1976; Smith et al., 1979). Ferguson-Miller et al. (1978) have proposed that both of these sites function separately if not independently to transfer electrons into cytochrome *c* oxidase. Another complication is that cytochrome *c* is strongly positively charged (p*I* > 9.5), and this protein binds quite tightly to negatively charged phospholipids, particularly cardiolipin molecules (Birrell & Griffith, 1976), which are present in large numbers in purified cytochrome *c* oxidase preparations. Thus, in addition to the high- and low-affinity sites described above, cytochrome *c* oxidase preparations may contain nonspecific sites for cytochrome *c* among the bulk lipids associated with the protein.

None of the cross-linking studies conducted to date have attempted to define whether covalently bound cytochrome *c* was in high- or low-affinity sites of the cytochrome *c* oxidase complex. To examine this question, we have extended the studies of Bisson et al. (1978a) with arylazidocytochrome *c* derivatives and have obtained quantitative data about the

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binding of these modified cytochrome *c*'s to beef heart cytochrome *c* oxidase. We have also examined the effect of covalent linkage of cytochrome *c* on the cytochrome *c* oxidase electron transfer activity. Arylazidocytochrome *c* derivatives were chosen for this study because they can, in principle, be used to map the binding site for cytochrome *c* on cytochrome *c* oxidase and on other proteins which interact with cytochrome *c* such as ubiquinone cytochrome *c* reductase, cytochrome b_5, and cytochrome *c* peroxidase. The derivative modified at Lys-13 should be particularly useful for this purpose because the chemical modification is near the center of the binding domain on cytochrome *c* for both oxidases and reductases (Ferguson-Miller et al., 1978; Rieder & Bosshard, 1978; Kang et al., 1978).

The results presented here show that the Lys-13 modified arylazidocytochrome *c* binds to cytochrome *c* oxidase through subunit II and that this is the high-affinity site for this substrate. Evidence is presented to suggest that the low-affinity site involves tightly bound phospholipids. Activity measurements are also presented which show "half of site" reactivity of beef heart cytochrome *c* oxidase in detergent solution.

Materials and Methods

Beef heart cytochrome *c* oxidase was prepared according to Capaldi & Hayashi (1972). Enzyme preparations had a heme *a* content of 9.0–11.7 nmol/mg of protein and contained from 84 to 120 μg of phospholipid/mg of protein. Lipid-depleted cytochrome *c* oxidase was obtained by the gel filtration in Triton X-100 exactly as described by Robinson & Capaldi (1977).

The preparation and characterization of arylazidocytochrome *c* derivatives were carried out essentially as described by Bisson et al. (1978a). The modification of cytochrome *c* by 4-fluoro-3-nitrophenyl azide should be similar to that by trinitrobenzenesulfonate which has been shown to involve mainly lysine-13 and lysine-22 (Wada & Okunuki, 1969). Pepsin digestion of the two major arylazidocytochrome *c* derivatives was used to generate the heme undecapeptides, and these were examined by thin-layer chromatography. This confirmed that the P_2 derivative was modified in lysine-13 and was at least 80% pure. The P_1 derivative was not altered at lysine-13 and by analogy with the experiments of Wada & Okunuki (1969) is most probably lysine-22-modified cytochrome *c*. Phospholipid was measured as inorganic phosphate as described by Chen et al. (1956). Cytochrome *c* oxidase activity was determined by the method of Vik & Capaldi (1977). Stock enzyme was diluted first with 1% Triton X-100 to disperse the protein and then assayed by using a Clarke-type oxygen electrode in a 2-mL water-jacketed chamber maintained at 25 °C. The assay buffer included 0.5% Tween-80, 0.05 M sodium phosphate (pH 7.4), 30 mM ascorbate, 40 μM cytochrome *c*, and 2.5 nM cytochrome *c* oxidase. In some experiments the enzyme was assayed in the presence of TMPD[1] and over a range of cytochrome *c* concentrations as described by Ferguson-Miller et al. (1976). Heme determinations were made as described by Williams (1964). Cytochrome *c* peroxidase was the kind gift of Dr. T. Poulos, University of California, San Diego.

Interaction of Cytochrome c Derivatives with Cytochrome c Oxidase. Cytochrome *c* oxidase (4 μM), dissolved in 0.3% Tween-80 and 0.5 mM Tris–EDTA, pH 6.5, was mixed with arylazidocytochrome *c* derivatives, and the samples were irradiated with a 3-W UV lamp (Ultraviolet Products, Inc., CA) at a distance of 1 cm and through a glass filter for 1 h on ice. The covalent cytochrome *c*–cytochrome *c* oxidase complex was separated from unbound cytochrome *c* by gel filtration through a Sephadex G-75 column (1 × 18 cm) in 0.25% Tween-80, 1 M KCl, and 50 mM sodium phosphate buffer, pH 7.8.

Binding of Native Cytochrome c to Covalently Linked Cytochrome c–Cytochrome c Oxidase Complexes. The binding of native cytochrome *c* with cytochrome *c* oxidase and with preformed arylazidocytochrome *c*–cytochrome *c* oxidase complexes was measured by using the gel filtration procedures of Ferguson-Miller et al. (1976). Irradiated samples were concentrated by dialysis against solid sucrose for 3–5 h at 0 °C, to ~10 times the starting concentration. This solution, containing 4–5 nmol of cytochrome *c* oxidase, was then mixed with native cytochrome *c* in a 10-fold molar excess, and the samples were applied to a Sephadex G-75 column (1.5 × 60 cm) equilibrated in 0.25% Tween-80, 0.05% cholate, and 25 mM Tris–acetate buffer (pH 7.4), containing 2 μM cytochrome *c*. The sample was eluted from the column in this same buffer, and the cytochrome *c* and cytochrome *c* oxidase concentrations of the eluant were measured spectrophotometrically [as pyridine hemochromagens according to Williams (1964)].

$NaDodSO_4$–polyarylamide gel electrophoresis was performed with 15% polyarylamide gels (1:30 cross-linker) by using the procedure of Swank & Munkres (1971) as modified by Downer et al. (1976).

Results

Two derivatives of horse heart cytochrome *c* were used in this study, one modified at lysine-13 (called P_2 derivative) and the second derivative most probably altered in lysine-22 (P_1 derivative) (Bisson et al., 1978a). Each was reacted with beef heart cytochrome *c* oxidase at low molar ratios of the substrate (cyt *c*) to oxidase (aa_3) and at concentrations (10^{-6} M) and ionic strength conditions (10 mM acetate buffer) where essentially all of the derivatives would be bound to either high- or low-affinity sites on the cytochrome *c* oxidase complex (Bisson et al., 1978a; Ferguson-Miller et al., 1978). The covalent cytochrome *c*–cytochrome *c* oxidase complex was separated from unbound cytochrome *c* by gel filtration through a column of Sephadex G-75, and the ratio of heme *c* to heme *a* in this complex was determined spectrally. The reaction with both derivatives was very efficient. Thus, in experiments in which 2 mol of the cytochrome *c* derivatives was reacted per mol of aa_3, as much as 20% of the P_1 and ~50% of the P_2 added were covalently bound to the complex, and this was with a preparation of cytochrome *c* oxidase containing only 55 μg of phospholipid/mg of protein [mainly cardiolipin; see Robinson & Capaldi (1977)]. This lipid-depleted enzyme was used in most experiments to avoid binding of cytochrome *c* to nonspecific sites on the bulk lipid. As shown in the next sections, both protein and lipid are involved in this covalent binding.

Binding of Cytochrome c to Protein Examined by $NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The binding of cytochrome *c* to protein, as opposed to lipid, could be monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis. Figure 1 shows the polypeptide profile of both P_1- and P_2-treated cytochrome *c* oxidase as well as that of untreated enzyme. The gel profile of the P_1-treated oxidase was very similar to that of the untreated enzyme, except for the presence of peaks of monomeric and dimeric cytochrome *c*. The cytochrome *c* dimer was generated during the photolysis reaction and is seen in cytochrome *c* samples without cytochrome *c* oxidase present (result not shown). Both monomer and dimer cytochrome *c* were also seen in the P_2-treated oxidase. In

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $NaDodSO_4$, sodium dodecyl sulfate.

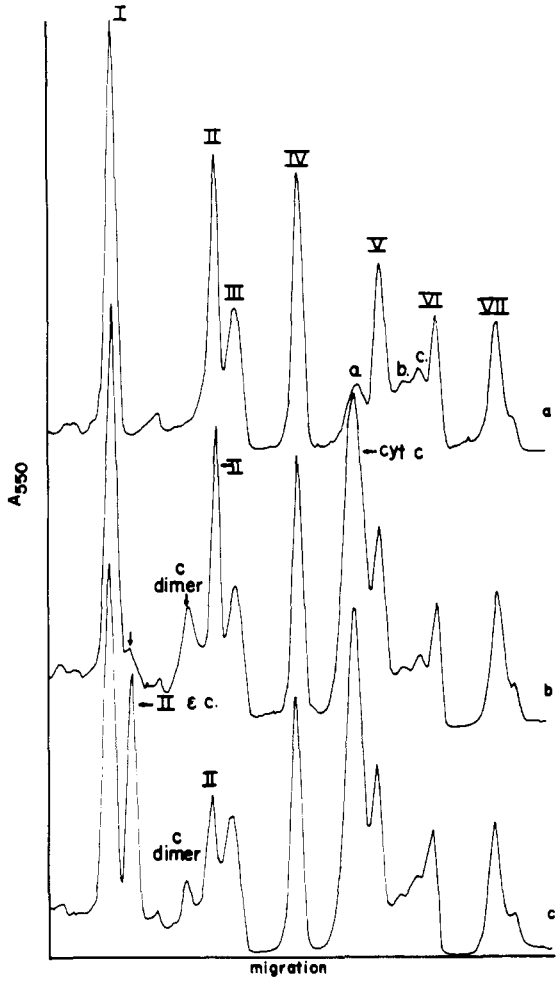

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of cytochrome *c* oxidase before and after reaction with the arylazidocytochrome *c* derivatives. Densitometric trace a shows untreated cytochrome *c* oxidase. The seven subunits of the enzyme are numbered in order of decreasing molecular weight. Bands a–c are impurities common to most preparations of enzyme. Trace b shows P_1-reacted cytochrome *c* oxidase. The sample contained 2.5 mol of P_1/mol of aa_3. Trace *c* shows P_2-reacted cytochrome *c* oxidase. Again the sample contained 2.5 mol of P_2/mol of aa_3. Samples in (b) and (c) were examined directly without separating the cytochrome *c*–cytochrome *c* oxidase complex from noncovalently bound cytochrome *c* derivative. IIεc is the product formed by cross-linking of the cytochrome *c* derivative to subunit II.

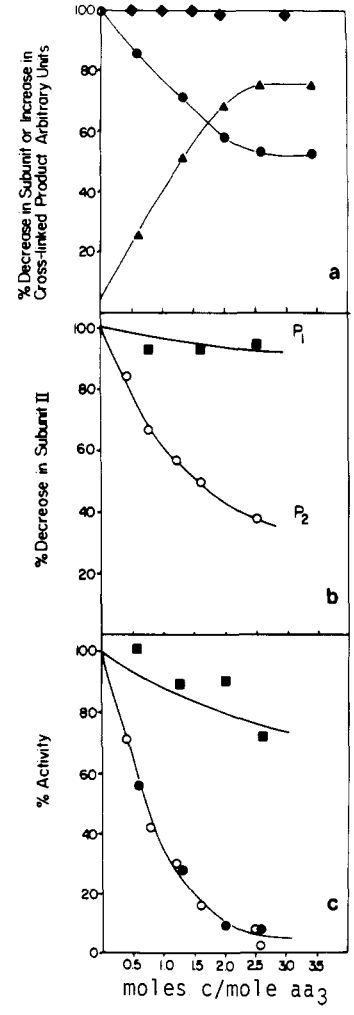

FIGURE 2: Quantitation of the binding of cytochrome *c* derivatives with cytochrome *c* oxidase. (a) Interaction of the P_2 derivative with lipid-depleted oxidase quantitated by measuring the decrease in area of the band containing subunit II (●) and the appearance of cross-linked product (▲). The change in peak area of subunit III is shown for comparison (♦). (b) Interaction of P_1 and P_2 derivative with stock cytochrome *c* oxidase containing 110 μg of phospholipid/mg of protein. (c) Inhibition of cytochrome *c* oxidase activity by reaction with P_1 or P_2 derivatives. (○) shows the results for stock enzyme. (●) shows data for lipid-depleted enzyme. (■) (for P_1) shows the results with lipid-depleted enzyme.

addition, there was a partial disappearance of subunit II and the appearance of a new band of molecular weight 33 000 (cytochrome *c* + subunit II) in the gel profile. The results in Figure 1 fully confirm the original findings of Bisson et al. (1978a) that P_2 but not P_1 covalently binds to cytochrome *c* oxidase and that this reaction is through subunit II.

The gel conditions used here are an improvement over those used previously (Bisson et al., 1978a,b) in that they separated subunit II of the oxidase from the cytochrome *c* dimer and resolved subunit I from the cross-linked product of cytochrome *c* plus subunit II. This allowed us to quantitate the reaction between the substrate and the oxidase. Figure 2 shows an analysis of the binding of the P_2 derivative to cytochrome *c* oxidase over a range of molar ratios of cytochrome *c* to oxidase. Figure 2a shows the reaction of P_2 with lipid-depleted cytochrome oxidase. Both the disappearance of subunit II and the appearance of the cross-linked product are plotted. Also, the change in peak area of subunit III is shown to confirm that it is not involved in cytochrome *c* binding, at least in beef heart cytochrome *c* oxidase. Part b of Figure 2 shows the reaction of both P_1 and P_2 with our standard cytochrome *c* oxidase preparation (containing 110 μg of phospholipid/mg of protein), and Figure 2c shows the effect of covalent binding of cytochrome *c* on cytochrome *c* oxidase activity (discussed later).

In the experiments shown in Figure 2a,b, the amount of cytochrome *c* bound to the protein increased as the ratio of cytochrome c/aa_3 was increased, but in neither case did the amount bound approach one molecule of *c*/molecule of aa_3. Several factors can be expected to affect this cross-linking reaction. First, the extent of cross-linking will depend on the efficiency of the insertion reaction between the activated nitrene group and the protein. Second, the kinetics of photolysis will be important. In addition, the cytochrome *c* to cytochrome *c* oxidase ratio is clearly important as is the preparation of cytochrome *c* oxidase being used (compare parts a and b of Figure 2).

The amount of P_2 covalently bound to cytochrome *c* oxidase could be increased above that shown in Figure 2 if photolyzed but noncovalently bound derivative was removed by ion-exchange chromatography (see Materials and Methods) and then the sample was subjected to a second cycle of reaction with

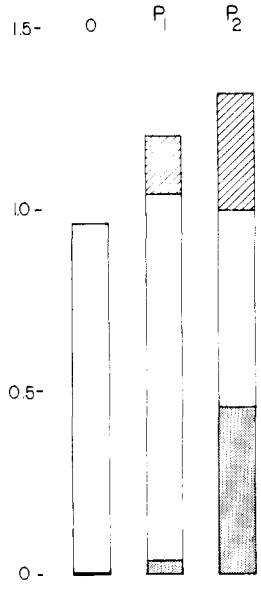

FIGURE 3: Binding of native cytochrome *c* to covalent cytochrome *c*–cytochrome *c* oxidase complexes. The shaded area is covalent binding to the protein; the hatched area is covalent binding to the lipid. Unshaded areas represent the amount of native cytochrome *c* bound to the complexes.

a fresh aliquot of the P_2 derivative. In a typical experiment, 0.75 mol of cytochrome *c*/molecule of cytochrome *c* oxidase was bound to the protein, and this amount could be increased further by repeated reactions.

Binding of Cytochrome c Derivatives to Lipid. That fraction of cytochrome *c* covalently bound to the complex but not inserted into the protein must be bound to phospholipid (although a small amount may be bound to detergent). The extent of covalent linking of P_1 or P_2 to lipid was difficult to determine accurately due in part to the dissociation of the cytochrome *c*–lipid adduct from the complex in the column procedures needed to remove noncovalently bound derivatives. However, in all experiments, there was a significant amount of covalent cross-linking of both P_1 and P_2 to lipids. For example, in experiments where 2 mol of the cytochrome *c* derivatives were reacted per mol of aa_3, as much as 0.3 mol of P_1 and 0.6 mol of P_2 became covalently linked to the phospholipid, and this was for the low-lipid enzyme containing only 6 or 7 molecules of the most tightly bound lipid, out of a boundary layer containing 40–54 molecules [depending on how much cardiolipin (four-chain lipid) is present]. This binding to lipid was seen not only at low molar ratios of cytochrome *c* to aa_3 but also at concentrations in which essentially all of the derivative present should be bound to the high- or low-affinity site on the oxidase.

Binding of Cytochrome c in Relation to High- and Low-Affinity Sites. It has been shown that cytochrome *c* oxidase can bind two cytochrome *c* molecules/monomer, one with a high affinity (10^{-8} M) and the second with a lower affinity (10^{-6} M). The question arises, therefore, whether covalently bound P_1 or P_2 occupies one or both of these sites. Figure 3 shows the results of experiments to examine the binding in high-affinity sites. Samples of lipid-depleted cytochrome *c* oxidase were reacted with P_1 or P_2 and aliquots of the reaction mixture used to determine the total amount of cytochrome *c* bound to the complex (from gel filtration experiments at high ionic strength) and how much derivative was bound to the protein (from $NaDodSO_4$–polyacrylamide gel electrophoresis). The amount of cytochrome *c* bound to lipid was taken as the difference between these two numbers. A third sample was concentrated (as described under Materials and Methods) and then mixed with native cytochrome *c* in a 10-fold molar excess (with respect to aa_3) and eluted through a Sephadex G-75 column equilibrated with 25 mM Tris–acetate and containing 2 μM cytochrome *c*. These are conditions which favor the binding of cytochrome *c* to the high-affinity binding site and which preclude binding to the low-affinity site (Ferguson-Miller et al., 1976). The total amount of cytochrome *c* bound (covalently bound P_1 or P_2 plus native cytochrome *c*) was determined on samples eluting from the column. Untreated cytochrome *c* oxidase was found to bind 1 mol of cytochrome *c*/mol of aa_3 after elution from the column (Figure 3). Similarly, P_1-reacted cytochrome *c* oxidase bound 1 mol of cytochrome *c*/mol of aa_3, indicating that cytochrome *c* bound to the lipid did not block interaction of the substrate with the high-affinity site. Samples of P_2-treated cytochrome *c* oxidase gave a different result. Figure 3 shows the data for an experiment using a P_2-treated oxidase preparation containing 0.5 mol of cytochrome *c*/mol of aa_3 covalently bound to the protein. In this case, the amount of native cytochrome *c* eluting with the oxidase was 0.5 mol/mol of aa_3, i.e., half of the high-affinity sites were apparently occupied by the P_2 derivative. Other P_2-treated cytochrome *c* oxidase preparations were used in which the amount of covalently bound derivative was <0.5. In all experiments, the total amount of cytochrome *c* bound to the oxidase protein (i.e., covalently bound P_2 and native cytochrome *c*) was 1 mol/mol of aa_3.

Experiments were also performed in an effort to determine whether covalently bound P_1 or P_2 affected the binding of native cytochrome *c* in the low-affinity sites. Thus, cytochrome *c* oxidase and P_1- or P_2-treated enzyme were subjected to gel filtration as described above but in 3 mM Tris–acetate buffer (instead of 25 mM). According to Ferguson-Miller et al. (1976), a 2:1 complex of *c* to aa_3 is obtained in very low ionic strength buffer. However, in our hands such conditions gave variable results with from two to three cytochrome *c* molecules bound/mol of cytochrome *c* oxidase. With either P_1- or P_2-reacted cytochrome *c* oxidase, the results were equally variable, but the total amount of cytochrome *c* (covalent plus native cytochrome *c*) was always in the same range as that for untreated enzyme. However, these results are ambiguous and are not considered further.

Effect of Covalently Bound Cytochrome c on Cytochrome c Oxidase Activity. Beef heart cytochrome *c* oxidase assayed in the presence of Tween-80 (see Materials and Methods) showed an activity of 200–250 s^{-1}. P_1 or P_2 which had been covalently bound to the cytochrome *c* oxidase complex supported an activity of <5 s^{-1}. The activity of P_1-reacted enzyme could, however, be restored by the addition of native cytochrome *c*. The maximal inhibition of cytochrome *c* oxidase activity by P_1 was 20% (at the highest ratios of P_1 to aa_3 used). At least half of this inhibition can be accounted for by a small contamination of the P_1 preparation with P_2. It may also be due in part to alteration of cytochrome *c* oxidase by byproducts of the photolysis such as hydroxyl free radicals, and there may be a small amount of inhibition of electron transfer by the binding of cytochrome *c* to the lipids. The small effect with P_1 is to be contrasted with the results of binding P_2 to the oxidase. As shown in Figure 2c, covalent linkage of P_2 strongly inhibited cytochrome *c* oxidase activity. This inhibition was proportional to the amount of cytochrome *c* covalently bound to the protein through subunit II. Almost complete inhibition was obtained when 0.5 mol of *c* was bound per mol aa_3. Cytochrome *c* oxidase is a dimer in nonionic detergents at least down to a concentration of 0.1 mg/mL (Robinson & Capaldi,

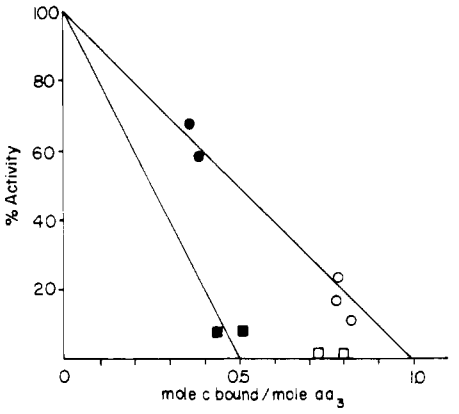

FIGURE 4: Relation between covalent binding of cytochrome *c* and inhibition of cytochrome *c* oxidase and cytochrome *c* peroxidase activities. Square symbols show data for cytochrome *c* oxidase; circles are data for cytochrome *c* peroxidase. Solid symbols are for one cycle of reaction (see text); open symbols are for a second cycle of reaction. The molar ratio of P_2/aa_3 was 2.5:1 for each cycle of reaction. The molar ratio of P_2 to cytochrome *c* peroxidase was 3:1 for each cycle of reaction.

1977). The activity data present here can only be explained if cytochrome *c* oxidase is a dimer under the assay conditions, with binding of one cytochrome *c*/dimer giving essentially complete inhibition of electron transfer. This is "half of site" reactivity at least in an operational sense [see Seydoux et al. (1974)].

The exact nature of the chemical reaction of the arylazidocytochrome *c* with cytochrome *c* oxidase is not well understood. Therefore, in order to be certain that the apparent half of site reactivity was not an artifact, we did parallel experiments on cytochrome *c* peroxidase. This enzyme uses cytochrome *c* as an electron donor for the reduction of H_2O_2 to H_2O but under conditions where cytochrome *c* peroxidase is a monomer (Yonetani, 1976). The reaction of arylazidocytochrome *c* derivatives with cytochrome *c* peroxidase will be described in detail elsewhere (R. Bisson and R. A. Capaldi, unpublished experiments). However, for comparison, the inhibition of this enzyme with covalent binding of P_2 is shown in Figure 4. For cytochrome *c* peroxidase, one cytochrome *c* molecule (P_2) was needed per mol of enzyme to completely inhibit activity. Thus, the half of site effect seen with cytochrome *c* oxidase is not an artifact of the chemical reaction of aryl azides.

Discussion

The studies described here are an extension of the original experiments of Bisson et al. (1978a) with arylazidocytochrome *c* derivatives. These workers purified two arylazidocytochrome *c* derivatives, one selectively modified at lysine-13 and the other probably altered in lysine-22, and showed that the former but not the latter derivative would covalently bind to beef heart cytochrome *c* oxidase through subunit II (Bisson et al., 1978a). These findings are confirmed in the present study. The binding of the lysine-13 modified derivative has now been examined under conditions where up to 0.75 mol of cytochrome *c* was bound per mol of cytochrome *c* oxidase and where there was additional covalent binding of cytochrome *c* to the lipid which is associated with the complex. No evidence was seen for the binding of cytochrome *c* to subunits other than subunit II. Moreover, there was no more than one cytochrome *c* bound to any single subunit II polypeptide.

Under all of the reaction conditions used, P_2 and P_1 were covalently linked to phospholipid. This was true of preparations of cytochrome *c* oxidase which contained only five to seven molecules of phospholipids/mol of enzyme.

Recent studies have indicated that there are at least two binding sites for cytochrome *c* on isolated cytochrome *c* oxidase (Ferguson Miller et al., 1976, 1978; Errede et al., 1976). One is of high affinity ($K_d > 10^{-8}$ M) and the second is of lower affinity ($K_d = 10^{-6}$ M). Under the appropriate assay conditions, cytochrome *c* oxidase activity shows two phases (Errede et al., 1976; Ferguson-Miller et al., 1978) which Ferguson-Miller et al. (1978) attribute to electron transfer through the high- and low-affinity sites separately. It is important then to know whether covalent binding of cytochrome *c* to subunit II seen in the studies of Bisson et al. (1978a) and Briggs & Capaldi (1978) was in the high-affinity site, the low-affinity site, or both sites. Data presented here show clearly that this binding is exclusively through the high-affinity sites. The evidence is as follows: (1) Covalent binding occurred with the Lys-13 modified derivative where the reactive group is in the domain for high-affinity binding with cytochrome *c* oxidase. (2) Covalent binding to the protein inhibited electron transfer activity nearly completely. Moreover, full inhibition was achieved when one cytochrome *c* was bound per cytochrome *c* oxidase dimer. Such a half of site effect has been reported before only in systems where there was covalent modification of an important functional site in the enzyme [see Seydoux et al. (1974)]. (3) Covalent binding and the resulting inhibition occurred with the Lys-13 derivative when present in low molar ratios with respect to the oxidase (0.2–0.5 mol/mol of aa_3) and at low concentrations of cytochrome *c* when most of the derivative would be in the high-affinity site. (4) Direct binding studies show that covalently bound cytochrome *c* (on subunit II) prevents native cytochrome *c* from binding in the high-affinity site.

The above evidence is taken to show that the high-affinity binding site for cytochrome *c* on cytochrome *c* oxidase is in subunit II. This is of immediate significance for the activity of the enzyme because of the conclusions that have been drawn from the sequencing of subunit II from yeast and beef heart cytochrome *c* oxidase. It has been argued by Steffens & Buse (1979) that the sequence of subunit II shows homologies with two well-known copper binding proteins (azurin and plastocyanin), particularly in the segments of the chain containing the copper binding ligands. It is possible then that a copper atom (low-potential copper) rather than heme *a* is a primary acceptor of electrons from cytochrome *c*. In this connection, it will be interesting to see if the site of covalent cross-linking of the Lys-13-modified cytochrome *c* is anywhere close to the presumed ligand sites for copper. This information should come from our current experiments to determine the point(s) of interaction of the cytochrome *c* derivative within the sequence of subunit II.

If subunit II provides the high-affinity site for cytochrome *c*, the question now is where is the low-affinity site on the cytochrome *c* oxidase molecule? There was no linkage of cytochrome *c* to other subunits of cytochrome *c* oxidase under conditions where there should have been interaction with both high- and low-affinity sites. Also, there was no indication of more than one site for the binding of cytochrome *c* on subunit II (although this possibility will be clearer when an analysis of the cytochrome *c*–subunit II product is completed). The only other site of binding of the cytochrome *c* derivatives is with lipid molecules and in particular with the small number of tightly bound lipids, mainly cardiolipins, which are not exchanged for detergent during the preparation of the lipid-depleted enzyme (Robinson & Capaldi, 1977). This suggests that these tightly bound lipids are in fact involved in the

low-affinity site, a possibility also indicated by recent experiments on the role of phospholipids on cytochrome *c* oxidase (S. B. Vik and R. A. Capaldi, unpublished experiments). It has proved possible to remove all but one of the highly bound cardiolipins from cytochrome *c* oxidase by centrifuging the enzyme through a glycerol gradient equilibrated with high concentrations of Triton X-100 (Robinson, 1979; S. B. Vik and R. A. Capaldi, unpublished experiments).[2] The removal of these tightly bound cardiolipin molecules has been found to cause a loss of the low-affinity phase of cytochrome oxidase activity, without much effect on the high-affinity phase. Addition of cardiolipin to the enzyme from the glycerol gradient was found to regenerate the low-affinity phase of activity, while phosphatidylcholine or phosphatidylethanolamine had very little effect. Taken together, the binding studies reported here and the above-mentioned lipid depletion experiments provide strong evidence that phospholipids are important components of the low-affinity binding site on cytochrome *c* oxidase.

The observation that full inhibition of cytochrome *c* oxidase is obtained when only one cytochrome *c* is bound with high affinity per dimer is significant for several reasons. Sedimentation equilibrium experiments (Robinson & Capaldi, 1977) and electron microscopy and image reconstruction studies (Henderson et al., 1977) show cytochrome *c* oxidase as a dimer in nonionic detergent but at 0.1 mg/mL and higher concentrations. The observed half of site reactivity seen in the present studies is clear evidence that cytochrome *c* oxidase is a dimer under assay conditions, that is, at 1–2 μg/mL in Tween-80 or in lysolecithin–asolectin mixed micelles.

The second important conclusion from the inhibition studies relates to the low-affinity sites. Inhibition of electron transfer was here shown to be correlated with covalent binding of cytochrome *c* to the high-affinity sites and not to low-affinity sites. Therefore, either there is some cooperative effect, such that modification of one high-affinity site alters both low-affinity sites of the dimer (i.e., quarter-site reactivity), or the low-affinity sites do not function to transfer electrons to heme *a* or copper directly but instead have a regulatory role. The half of site reactivity observed in this study is intriguing, but the physiological importance of this result is not immediately clear. One reason is that it is by no means certain that cytochrome *c* oxidase is a dimer in the mitochondrial inner membrane [see Hackenbrock & Hammon (1975)]. Moreover there are several lines of evidence to indicate that cytochrome *c* oxidase can function as a monomer (Brunori et al., 1979), albeit at a lower turnover rate (Robinson & Capaldi, 1977). Clearly then, the possible role of the cytochrome *c* oxidase dimer in the activity of the terminal oxidase warrants a more detailed study.

References

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

Birrell, G. B., & Griffith, O. H. (1976) *Biochemistry 15*, 2925.

Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Montecucco, C., & Zanotti, A. (1978a) *J. Biol. Chem. 253*, 1874.

Bisson, R., Gutweniger, H., & Azzi, A. (1978b) *FEBS Lett. 92*, 219.

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

Brunori, M., Colosimo, A., Rainoni, G., Wilson, M. T., & Antonini, E. (1979) *J. Biol. Chem. 254*, 10769.

Capaldi, R. A. (1979) *Membr. Proteins 2*, 201–231.

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

Chan, S. H. P., & Tracy, R. P. (1978) *Eur. J. Biochem. 89*, 595.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1956.

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

Erecinska, M. (1977) *Biochem. Biophys. Res. Commun. 76*, 495.

Erecinska, M., & Wilson, D. F. (1978) *Arch. Biochem. Biophys. 188*, 1.

Errede, B., Haight, G. P., & Kamen, M. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 113.

Eytan, G. D., Carroll, R. C., Schatz, G., & Racker, E. (1975) *J. Biol. Chem. 250*, 8598.

Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1976) J. *Biol. Chem. 251*, 1104.

Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1978) *J. Biol. Chem. 253*, 140.

Frey, T. G., Chan, S. H. P., & Schatz, G. (1978) *J. Biol. Chem. 253*, 4389.

Fuller, S. D., Capaldi, R. A., & Henderson, R. (1979) *J. Mol. Biol. 134*, 305.

Hackenbrock, C. R., & Hammon, K. H. (1975) *J. Biol. Chem. 250*, 8598.

Henderson, R., Capaldi, R. A., & Leigh, J. S. (1977) *J. Mol. Biol. 112*, 631.

Kang, C. H., Brautigan, D. L., Osheroff, N., & Margoliash, E. (1978) *J. Biol. Chem. 253*, 6502.

Ludwig, B., Downer, N. W., & Capaldi, R. A. (1979) *Biochemistry 18*, 1401.

Mälmstrom, B. (1979) *Biochim. Biophys. Acta 549*, 281.

Mason, T. L., & Schatz, G. (1973) *J. Biol. Chem. 248*, 1355.

Poyton, R. O., & Schatz, G. (1975) *J. Biol. Chem. 250*, 752.

Rieder, R., & Bosshard, H. R. (1978) *J. Biol. Chem. 253*, 6045.

Robinson, N. C. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 577.

Robinson, N. C., & Capaldi, R. A. (1977) *Biochemistry 16*, 375.

Sebald, W., Machleidt, W., & Otto, J. (1974) *Eur. J. Biochem. 38*, 311.

Seiter, C. H. A., Margalit, R., & Perreault, R. A. (1979) *Biochem. Biophys. Res. Commun. 86*, 473.

Seydoux, F., Malhotra, O., & Bernhard, S. A. (1974) *CRC Crit. Rev. Biochem. 2*, 227.

Smith, L., Davies, H. C., & Nava, M. E. (1979) *Biochemistry 18*, 3140.

Steffens, G., & Buse, B. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1125.

Steffens, G. J., & Buse, G. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 613.

Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462.

Vik, S. B., & Capaldi, R. A. (1977) *Biochemistry 16*, 5755.

Wada, K., & Okunuki, K. (1969) *J. Biochem. (Tokyo) 66*, 263.

Wikstrom, M. K. F. (1977) *Nature (London) 266*, 271.

Williams, J. R. (1964) *Arch. Biochem. Biophys. 107*, 537.

Yonetani, T. (1976) *Enzymes, 3rd Ed. 12*, 345–362.

[2] The presence of large amounts of Triton X-100, which is hard to remove from the enzyme, precludes, as yet, using this essentially lipid-free preparation for cytochrome *c* binding experiments such as those described here.