# The Cytochrome *c* Oxidase–Cytochrome *c* Complex: Spectroscopic Analysis of Conformational Changes in the Protein–Protein Interaction Domain†

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ABSTRACT: Binding to cytochrome *c* oxidase induces a conformational change in the cytochrome *c* molecule. This conformational change has been characterized by comparing the binding of native cytochrome *c* and chemically modified cytochrome *c* derivatives to bovine cytochrome *c* oxidase by using absorption, circular dichroism (CD), and magnetic circular dichroism (MCD) spectroscopy. The following derivatives were analyzed: (i) cytochrome *c* modified at all 19 lysine residues to yield the ($N^{\epsilon}$-acetimidyl)$_{19}$ cytochrome *c*, ($N^{\epsilon}$-isopropyl)$_{19}$ cytochrome *c*, and ($N^{\epsilon}$,$N^{\epsilon}$-dimethyl)$_{19}$ cytochrome *c*; (ii) cytochrome *c* in which Met$^{65}$ and Met$^{80}$ are converted to the methionine sulfoxide; (iii) cytochrome *c* with a single break in the polypeptide chain at Arg$^{38}$ or Gly$^{37}$. The derivatives bind to cytochrome *c* oxidase at a ratio of one heme *c* per heme $aa_3$. The association constants are similar to that of native cytochrome *c* except for ($N^{\epsilon}$-isopropyl)$_{19}$ and ($N^{\epsilon}$,$N^{\epsilon}$-dimethyl)$_{19}$ cytochromes *c*, which bind respectively four times and six times less strongly. The derivatives are good substrates for the cytochrome *c* oxidase reaction. The spectral changes accompanying the binding of the modified cytochromes *c* to cytochrome *c* oxidase are quite different from the spectral changes observed with native cytochrome *c*. The different optical absorption and MCD changes are explained by a polarity change around the exposed heme edge in the cytochrome *c*–cytochrome *c* oxidase complex. The CD changes indicate a conformational rearrangement restricted to the surface area surrounding the exposed heme edge. The rearrangement may involve a movement of the evolutionarily conserved Phe$^{82}$ out of the vicinity of the heme. The cytochrome *c* derivatives with a single break in the polypeptide chain are partially present in a conformational state characterized by an opened heme cleft and a changed ligation state of the heme iron, the so-called "alkaline state" of cytochrome *c*. On binding to the oxidase, these derivatives are forced into a conformation which is very similar to that of the oxidase-bound native cytochrome *c*.

Cytochrome *c* and cytochrome *c* oxidase form a tight complex at low ionic strength. The complex is similar to the transitory electron-transfer complex formed during the oxidation of reduced cytochrome *c* by cytochrome *c* oxidase in the last reaction step of the mitochondrial respiratory chain. The structural requirements for the formation of the complex and for cytochrome *c* oxidation in the steady-state reaction with the oxidase have been investigated in much detail (Nicholls, 1974; Ferguson-Miller et al., 1976, 1978; Smith et al., 1977; Errede & Kamen, 1978; Rieder & Bosshard, 1978; Margoliash & Bosshard, 1983).

Dynamic features of the electron-transfer complex may contribute to the efficiency and precision of the electron-transfer reaction. These dynamic features are much less well understood than the static features of the electron-transfer complex, though one may envisage a rearrangement of the heme cleft of cytochrome *c* when it binds to cytochrome *c* oxidase, cytochrome *c* peroxidase, cytochrome $b_5$, or other physiological or nonphysiological redox partners. The precise nature of the rearrangement is unknown, yet its purpose must be to maximize the conductance of the pathway between the two redox centers by optimizing the geometry of the redox centers within the electron-transfer complex and adjusting the protein matrix at the intermolecular interface (Mayo et al., 1986).

We recently presented spectroscopic evidence for a conformational rearrangement accompanying the formation of the cytochrome *c*–cytochrome *c* oxidase complex (Weber et al., 1987). The rearrangement occurs in the area of the heme of cytochrome *c*, and it was suggested that this movement optimizes the orientation between heme *c* and heme *a* of cytochrome *c* oxidase and rearranges the protein matrix between the hemes, thereby lowering the height of the electron tunneling barrier. We had noted that the CD[1] difference spectrum[2] of bound *minus* free cytochrome *c* was conspicuously similar to the CD difference spectrum of cytochrome *c* at alkaline pH *minus* cytochrome *c* at neutral pH [Figure 1 of Weber et al. (1987)]. In the binding of cytochrome *c* to the

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[1] Abbreviations: CD, circular dichroism; MCD, magnetic circular dichroism. Cytochrome *c* derivatives: acetimidyl cytochrome *c*, isopropyl cytochrome *c*, and dimethyl cytochrome *c*, cytochrome *c* derivatives with the N$^{\epsilon}$ amino group of all 19 lysine residues modified to $N^{\epsilon}$-acetimidyl, $N^{\epsilon}$-isopropyl, and $N^{\epsilon}$,$N^{\epsilon}$-dimethyl, respectively; cytochrome *c* 1–38:39–104, etc., cytochrome *c* with a single break in the polypeptide chain between the residues indicated; chloramine T cytochrome *c*, Met$^{65}$,Met$^{80}$-sulfoxide derivative of cytochrome *c*.

[2] Difference spectra pertaining to the cytochrome *c*–cytochrome *c* oxidase complex are defined as the spectrum of the cytochrome *c*–cytochrome *c* oxidase complex *minus* the sum of the spectra of free cytochrome *c* and free cytochrome *c* oxidase.

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Table I: Derivatives of Horse Cytochrome c and Their Oxidation by Cytochrome c Oxidase

| derivative | steady-state oxidation by cytochrome c oxidase[a] | | $pK_a$ of alkaline transition[b] | % in alkaline state at pH 7.4 |
|---|---|---|---|---|
| | $K_m$ ($\mu$M) | $TN_{max}$ ($s^{-1}$) | | |
| native horse cytochrome c | 6.7 | 73.0 | 9.32 | negligible |
| acetimidyl cytochrome c | 18.3 | 61.4 | 9.98 | negligible |
| isopropyl cytochrome c | 32.6 | 89.7 | 9.78 | negligible |
| dimethyl cytochrome c | 71.6 | 26.9 | 10.59 | negligible |
| chloramine T cytochrome c | c | c | e | e |
| cytochrome c 1–38:39–104 | d | d | 7.10 | 67 |
| acetimidyl cytochrome c 1–38:39–104 | d | d | 8.15 | 15 |
| acetimidyl cytochrome c 1–37:38–104 | 26.6 | 36.5 | 8.10 | 17 |

[a] Determined spectrophotometrically in 10 mM Tris-HCl and 0.1% (v/v) dodecyl maltoside, pH 7.4, 25 °C, ionic strength adjusted to 150 mM with NaCl. [b] Wallace & Proudfoot, 1987; Wallace & Corthésy, 1987. [c] Indistinguishable from native horse cytochrome c in the polarographic assay at pH 7.8 (Pande et al., 1987). [d] No accurate measurement possible because the reduced derivative is autoxidizable. [e] No alkaline transition observed (Myer et al., 1987).

oxidase as well as in the transition from the so-called neutral state (state III) of ferricytochrome c to the alkaline state (state IV), the negative ellipticity centered at 417 nm of the CD spectrum of ferricytochrome c is lost [see Dickerson and Timkovich (1975) for nomenclature of the pH-dependent conformational states of cytochrome c]. Similarly, the 417-nm band is lost when cytochrome c binds to the nonphysiological electron donor flavodoxin (Tollin et al., 1987).

Ferricytochrome c in the alkaline state differs from the native conformation of cytochrome c prevalent at neutral pH by a more open heme cleft and by the presumed loss of the $Met^{80}$ sulfur–iron bond [Theorell & Åkesson, 1941; Myer, 1968; Myer & Pande, 1978; Osheroff et al., 1980; review by Dickerson and Timkovich (1975)].

The reversible transition from the neutral to the alkaline state is governed by a p$K$ value between 8.5 and 9.5, depending on the origin of the cytochrome c (Osheroff et al., 1979, 1980; Davis et al., 1974). Although the CD difference spectrum due to the binding of cytochrome c to the oxidase was found to be similar to the CD difference spectrum caused by the alkaline transition of native cytochrome c, the MCD difference spectrum was of opposite sign and of much smaller amplitude than expected for an alkaline transition. This apparent contradiction led to the present closer characterization of the conformational rearrangement of cytochrome c during the formation of the cytochrome c–cytochrome c oxidase complex. To this end, derivatives of cytochrome c with altered heme coordination and altered conformation were analyzed. The results, together with those obtained for the binding of three lysine-modified derivatives of horse cytochrome c, testify against an opening of the heme cleft as seen in the alkaline conformational state of cytochrome c. Rather, the conformational rearrangement which clearly distinguishes the oxidase-bound from the free cytochrome c seems to be confined to the "front face", that is, the surface area containing the exposed heme edge where binding of cytochrome c to its redox partners takes place (Capaldi et al., 1982; Margoliash & Bosshard, 1983).

## Materials and Methods

*Materials.* Cytochrome c (horse) was Sigma type VI and was used without further purification. Beef heart cytochrome c oxidase was prepared according to Hartzell and Beinert (1974). The specifications of the enzyme were as before (Weber et al., 1987). Acetimidyl cytochrome c (Wallace & Harris, 1984), the alkylated derivatives isopropyl and dimethyl cytochrome c (Wallace & Corthésy, 1987), cytochrome c 1–38:39–104 (Proudfoot et al., 1984), acetimidyl cytochrome c 1–38:39–104 (Proudfoot et al., 1984), acetimidyl cytochrome c 1–37:38–104 (Proudfoot et al., 1986), and chloramine T cytochrome c (Pande et al., 1987) were prepared as described. The reaction of chloramine T with cytochrome c produces two isomeric forms that differ in the chirality of the sulfur of the $Met^{80}$ sulfoxide (Myer et al., 1987). Only the results obtained with the form having the same chirality as native horse cytochrome c are reported. This form was called FIII in Pande et al. (1987).

*Methods.* Photometric titration of the binding of cytochrome c and its derivatives to cytochrome c oxidase and oxidation by cytochrome c oxidase were measured as described elsewhere (Michel & Bosshard, 1988). Absorption spectra were recorded on a photodiode array spectrophotometer (Hewlett-Packard 8451) and CD and MCD spectra on a JASCO (Tokyo) spectropolarimeter (Model 500C), as described in detail before (Weber et al., 1987). To obtain the difference spectra shown in Figures 1–3, cytochrome c and its derivatives were added in 10% excess over heme $aa_3$. The concentration of heme $aa_3$, determined as in Michel and Bosshard (1984), was at least five times higher than the $K_d$ of the complex under study. Under these conditions, the single cytochrome c binding site per heme $aa_3$ was virtually saturated.

CD and MCD are expressed as the difference absorption coefficient for left versus right circularly polarized light ($\Delta\epsilon$, $mM^{-1}$ $cm^{-1}$), normalized to an applied magnetic field of 1 T in the case of MCD ($\Delta\epsilon/H$, $mM^{-1}$ $cm^{-1}T^{-1}$). Difference CD and MCD are expressed as $\Delta(\Delta\epsilon)$ and $\Delta(\Delta\epsilon/H)$. Absorption coefficients are based on the concentration of heme $aa_3$.

## Results

*Characterization of the Derivatives.* Three types of derivatives of horse cytochrome c have been used in this study (Table I). The first differs from native cytochrome c in having all the 19 lysine residues modified. The substituents chosen render the lysine side chains slightly bulkier but preserve the positive charge. The steady-state rates of oxidation of acetimidyl, isopropyl, and dimethyl cytochromes c by cytochrome c oxidase, measured in the spectrophotometric assay at 150 mM ionic strength, are not very different from that of native cytochrome c, dimethyl cytochrome c being the least reactive derivative. At 25 mM ionic strength the reaction of the three derivatives with the oxidase is biphasic, as observed for native cytochrome c (Ferguson-Miller et al., 1976), and the kinetic constants differ little from those of native cytochrome c (not shown; Michel, 1988).

The second derivative is the product of the reaction of cytochrome c with chloramine T (*N*-chloro-4-toluenesulfonamide). In this derivative $Met^{65}$ and $Met^{80}$, the only two methionines of horse cytochrome c, are oxidized to the sulfoxide. As a consequence the $Met^{80}$ sulfur–iron bond that characterizes the native conformational state of cytochrome

Table II: Photometric Titration of Bovine Cytochrome *c* Oxidase with Cytochrome *c* Derivatives[a]

| derivative | $K_d$[b] ($\mu$M) | $n$[b] (heme *c*/heme $aa_3$) | maximum of absorption difference[c] (nm) | $\Delta\epsilon$[c] ($mM^{-1}$ $cm^{-1}$) |
|---|---|---|---|---|
| native cytochrome *c* | 0.15 | 0.95 | 411.5 | 9.0 |
| acetimidyl cytochrome *c* | 0.21 | 0.94 | 412.5 | 7.0 |
| isopropyl cytochrome *c* | 0.60 | 0.90 | 409 | 5.6 |
| dimethyl cytochrome *c* | 0.93 | 0.80 | 408 | 4.8 |
| chloramine T cytochrome *c* | 0.29 | 0.95 | 412 | 6.2 |
| cytochrome *c* 1–38:39–104 | 0.21 | 1.04 | 414 | 16.8 |
| acetimidyl cytochrome *c* 1–38:39–104 | 0.16 | 0.92 | 414 | 8.8 |
| acetimidyl cytochrome *c* 1–37:38–104 | 0.18 | 0.98 | 412.5 | 10.6 |

[a] Determined in 10 mM Tris-HCl, 0.2% (v/v) Tween-80, and 15 mM NaCl, pH 7.4, 25 °C, ionic strength 24 mM. [b] Values for the dissociation constants $K_d$ and stoichiometries $n$ were obtained from a fitting procedure of the absorption difference $\Delta A$ at $\lambda_{max}$ to the binding equation $[\text{cyt } c]_{bound}/[aa_3]_{total} = K_d[\text{cyt } c]_{total}/(1 + K_d[\text{cyt } c]_{total})$, as described elsewhere (Michel & Bosshard, 1988). [c] Obtained from the difference spectrum of a 1:1 complex.

*c* at neutrality is missing in chloramine T cytochrome *c* (Pande et al., 1987; Myer et al., 1987). In this respect, then, the conformation of chloramine T cytochrome *c* is similar to that of cytochrome *c* in the alkaline conformational state. In both forms of cytochrome *c* the sixth heme ligand is replaced, by an unknown strong-field ligand in the alkaline state (Bosshard, 1981; Gadsby et al., 1987; Wallace & Corthêsy, 1987) or, possibly, by the oxygen of $Met^{80}$ sulfoxide in chloramine T cytochrome *c* (Myer et al., 1987). In CD, both chloramine T cytochrome *c* and cytochrome *c* in the alkaline state lack the negative ellipticity centered at 417 nm. This CD band is diagnostic of a closed heme pocket (Myer & Pande, 1978). Chloramine T cytochrome *c* is indistinguishable from native cytochrome *c* in the steady-state reaction with cytochrome *c* oxidase, but not with ubiquinone–cytochrome *c* reductase (Pande et al., 1987).

The third type of derivative has a single break in the polypeptide chain between $Arg^{38}$ and $Lys^{39}$ or $Gly^{37}$ and $Arg^{38}$. The derivatives are noncovalent complexes forming spontaneously upon mixing of the appropriate peptides, obtained from cytochrome *c* by limited proteolysis and semisynthesis (Harris & Offord, 1977; Proudfoot et al., 1984, 1986). The single break in the polypeptide chain results in a destabilization of the native structure as revealed by a significantly lower $pK_a$ for the transition to the alkaline conformational state (Wallace & Proudfoot, 1987; Table I). In the case of cytochrome *c* 1–38:39–104 and acetimidyl cytochrome *c* 1–38:39–104 the destabilization is also evident from the autoxidizability of the derivatives in air-saturated solution. The cytochrome *c* oxidase catalyzed oxidation could therefore not be accurately followed in the spectrophotometric assay. In a polarographic assay where the substrate is continuously reduced, the derivatives are somewhat less active than native cytochrome *c* (Harris & Offord, 1977; Proudfoot et al., 1986). This assay employs cytochrome *c* depleted mitochondria, however, and the rate-limiting step is thought to be the reductase to cytochrome *c* electron transfer (Wallace & Proudfoot, 1987). Acetimidyl cytochrome *c* 1–37:38–104 is more stable and a good substrate for cytochrome *c* oxidase in the photometric (Table I) and in the polarographic assay with cytochrome *c* depleted mitochondria (Proudfoot et al., 1986).

*Photometric Binding Titration.* Binding of cytochrome *c* to cytochrome *c* oxidase perturbs the optical absorption spectrum of cytochrome *c* in the Soret band ($\gamma$ band) of ferric and ferrous cytochrome *c* (Michel & Bosshard, 1984). The difference absorption at varying heme *c*/heme $aa_3$ ratios can be used to determine the stoichiometry and dissociation constant of the cytochrome *c*–cytochrome *c* oxidase complex (Michel & Bosshard, 1984, 1988). The spectral difference signal saturates at 1 heme *c* per heme $aa_3$, i.e., per functional unit of cytochrome *c* oxidase. The results of the photometric titration of bovine cytochrome *c* oxidase with the cytochrome *c* derivatives of Table I are shown in Table II. Each derivative binds to cytochrome *c* oxidase at a stoichiometry of 1 heme *c* per heme $aa_3$. The dissociation constants are within a fairly narrow range. The derivatives with a break in the polypeptide chain bind to cytochrome *c* oxidase with the same affinity as native cytochrome *c*. The small variation in $K_d$ is within the limit of error of analysis. The other derivatives bind more weakly, the lowest affinity being observed for dimethyl cytochrome *c*, but none of the derivatives differs very strongly from native cytochrome *c* in equilibrium binding to cytochrome *c* oxidase. In contrast, however, the profiles of the difference absorption, CD, and MCD spectra differ considerably for the seven derivatives of Tables I and II.

*Difference Absorption Spectra of Lysine-Modified Cytochrome c.* The difference absorption spectrum in the Soret region of native cytochrome *c* peaks at 412 nm, 3 nm to the red of the absorption maximum of ferricytochrome *c* (Figure 1A; spectra of native cytochrome *c* are indicated by dotted line in Figures 1–3). Since the difference absorption spectrum is positive, it reflects both a red shift and an increase of absorption when cytochrome *c* binds to fully oxidized resting oxidase. The individual contribution by heme *c* and heme $aa_3$ to the absorption difference spectrum cannot be distinguished unequivocally. However, a major contribution by heme *c* is implied by the closely adjacent maxima of the absolute spectrum of ferric heme *c* and the maximum of the difference spectrum of the cytochrome *c* oxidase–cytochrome *c* complex, 409 and 412 nm, respectively. In contrast, the Soret maximum of oxidized heme *a*, the first electron acceptor of ferrocytochrome *c* (Antalis & Palmer, 1982), is at 427 nm (Vanneste, 1966). A contribution to the absorption difference spectrum by heme $a_3$ whose absorption maximum is at 414 nm (Vanneste, 1966) cannot be ruled out, yet must be small as discussed by Weber et al. (1987). In MCD the contributions from heme *c* and heme $aa_3$ can be better distinguished, and cytochrome *c* is the almost exclusive contributor to the difference MCD spectrum of the cytochrome *c*–cytochrome *c* oxidase complex, as shown before (Weber et al., 1987). In the following, therefore, we regard the spectral changes as being due to molecular changes of the cytochrome *c* molecule, even though this assumption may be valid only as a first approximation.

The three lysine-substituted derivatives exhibit approximately the same difference absorption spectra as native cytochrome *c* upon complexation with the oxidase (Figure 1A). The extinction coefficient at the maximum of the absorption difference decreases in the order native, acetimidyl, isopropyl, and dimethyl cytochrome *c*. The affinity to cytochrome *c*

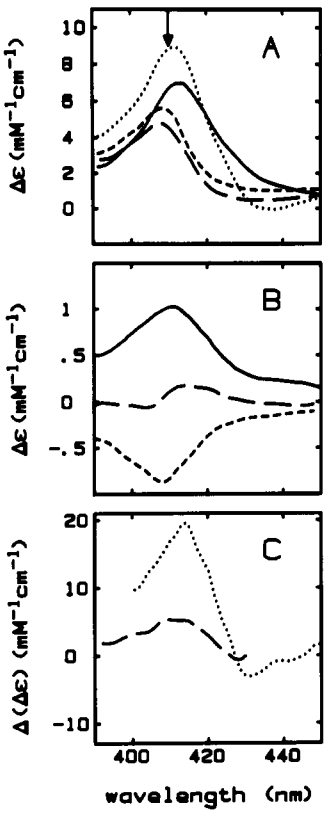


FIGURE 1: Binding to cytochrome c oxidase of cytochrome c derivatives modified at lysine residues. (A) Absorption difference spectra of acetimidyl cytochrome c (—), isopropyl cytochrome c (---), dimethyl cyrochrome c (−−), and, for comparison, native cytochrome c (···). (B) Absorption difference spectrum obtained by adding to native cytochrome c in 10 mM Tris-HCl, pH 7.4, 5% (v/v) ethylene glycol (—), 50 mM glycine (---), or 100 mM NaCl (−−). (C) CD difference spectra of dimethyl cytochrome c (−−) and native cytochrome c (···). The arrow in (A) marks the maximum of the absolute absorption spectrum of ferricytochrome c.

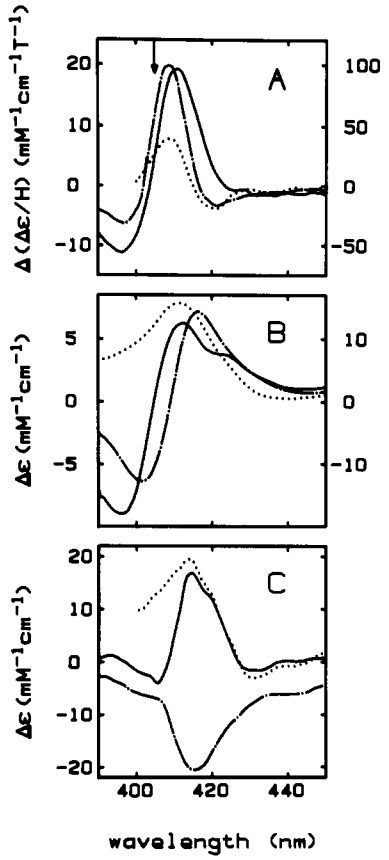


FIGURE 2: Binding to cytochrome c oxidase of chloramine T cytochrome c. (A) MCD difference spectra of chloramine T cytochrome c (—) and, for comparison, of native cytochrome c (···). MCD difference spectrum of native cytochrome c in the neutral minus the alkaline conformational state (−·−) (right ordinate). The arrow indicates the zero-line crossing of the S-shaped absolute MCD spectrum of chloramine T cytochrome c. (B) Absorption difference spectra. Line type and assignment of ordinates as in (A). (C) CD difference spectra. Line type as in (A).

oxidase decreases in the same order (Table II). This correlation is underscored by an increase in the same order of the Michaelis constants (Table I). The fit between cytochrome c and cytochrome c oxidase seems to get poorer in going from native to dimethyl cytochrome c without, however, destroying the overall dipole–dipole interaction between the strongly positive surface area around heme c and a complementary negative domain of cytochrome c oxidase (Koppenol & Margoliash, 1982). The dimethylamino group may obstruct ionic interaction more than a single isopropyl group, perhaps because the latter can be bent aside more easily.

The microenvironment around the exposed heme edge of cytochrome c is expected to be less polar in the cytochrome c–cytochrome c oxidase complex. This could account for the red shift and absorbance increase in the absorption spectrum of the complex. Two experiments in agreement with this simple explanation of the observed spectral change are shown in Figure 1B. Ethylene glycol (1,2-ethanediol) added to a solution of free ferricytochrome c elicits a difference absorption spectrum similar in shape, though much smaller in amplitude, to that caused by binding of ferricytochrome c to cytochrome c oxidase. The small amount of ethylene glycol was not denaturing since the 695-nm absorption band characterizing the native structure at neutrality remained intact (not shown). A difference absorption spectrum of opposite sign and with a blue-shifted maximum is induced by 50 mM glycine. This amino acid has a strong dipole moment and presumably renders the microenvironment around heme c more polar. The spectral change cannot be due simply to a change of ionic strength since 100 mM NaCl does not perturb the absorption spectrum of cytochrome c.

That the binding of cytochrome c to cytochrome c oxidase is accompanied by a rearrangement of the polypeptide chain in the heme c area follows from a pronounced change of the CD of cytochrome c in the cytochrome c–cytochrome c oxidase complex (Weber et al., 1987). If lysine substituents interfere with binding of cytochrome c to the oxidase, such interference might lead to a smaller CD difference signal. Indeed, binding of dimethyl cytochrome c to the oxidase gives a much smaller CD maximum (Figure 1C). This result is remarkable since it indicates that the conformational rearrangement is induced through the front surface of cytochrome c where binding takes place and where the modified lysine residues are encircling the exposed heme edge.[3] The importance of lysines on the front surface of cytochrome c to the formation of an electrostatically stabilized complex with cytochrome c oxidase has been well documented by numerous kinetic and protein-chemical studies with lysine-modified cytochrome c derivatives [review by Margoliash and Bosshard (1983)].

*Difference Spectra of Chloramine T Cytochrome c.* As mentioned above, chloramine T cytochrome c is similar to cytochrome c at alkaline pH. When chloramine T cytochrome

[3] Cytochrome c oxidase was saturated with the cytochrome c derivatives under the conditions where the difference spectra were measured. Hence the lower CD signal was not caused by incomplete binding of those derivatives which had a higher $K_d$.

*c* binds to the oxidase, MCD and absorption difference spectra are observed that are larger than those of the native or lysine-modified protein (Figure 2A,B). The MCD difference spectrum has a maximum at 414 nm. The shape of the spectrum indicates a red shift (Figure 2A, solid line). The absolute S-shaped MCD spectrum of chloramine T cytochrome *c* is indeed blue-shifted by 4 nm with respect to the absolute MCD spectrum of native cytochrome *c* (arrow in Figure 2A). Therefore, the red shift indicated by the MCD difference spectrum of oxidase-bound chloramine T cytochrome *c* might reflect a transition toward the conformation of native cytochrome *c*.[4] In other words, oxidase may force chloramine T cytochrome *c* into a conformation that is similar to that of oxidase-bound native cytochrome *c*.

This interpretation is supported by the absorption difference spectrum of bound chloramine T cytochrome *c* which features a large trough below 400 nm (Figure 2B, solid line). This trough is again indicative of a red shift upon binding to the oxidase. In accordance, the absolute absorption spectrum of chloramine T cytochrome *c* is blue-shifted by 3 nm in comparison to native cytochrome *c* (not shown). Also, both the absolute absorption and MCD spectra of cytochrome *c* in the alkaline state are blue-shifted by 3–4 nm in comparison to the spectra of cytochrome *c* in the neutral state (Vickery et al., 1976), further supporting the notion that free chloramine T cytochrome *c* is somehow related to cytochrome *c* in the alkaline state and becomes native-like when bound to the oxidase. Thus, it is satisfying to see that the shapes of the MCD and absorption difference spectra for the alkaline to neutral transition of native horse cytochrome *c* (Figure 2A,B, dash–dot lines) are conspicuously similar to the MCD and absorption difference spectra for the transition from the free to the oxidase-bound chloramine T cytochrome *c* (Figure 2A,B, solid lines).

The CD difference spectrum for the binding of chloramine T cytochrome *c* to the oxidase is shown in Figure 2C. The CD difference spectra for the binding of native and chloramine T cytochrome *c* peak at 414 nm (Figure 2C). The lower amplitude of the CD difference spectrum of chloramine T cytochrome *c* and the trough around 410 nm again testify to a conformational transition from an alkaline-like to a native-like state upon binding to the oxidase. This follows from the large negative peak in the CD difference spectrum of neutral minus alkaline cytochrome *c*, shown for comparison in the bottom panel of Figure 2C.

Because the CD difference spectra for native and chloramine T cytochrome *c* are so similar, it is unlikely that the conformational rearrangement, signaled by the CD change, is taking place around the $Met^{80}$ sulfur–heme iron bond, since it is in lacking this bond that chloramine T cytochrome *c* differs from native cytochrome *c*. Instead, the CD signal of bound chloramine T cytochrome *c*, as well as the lowered CD signal of dimethyl cytochrome *c* described above, points to the surface area around the exposed heme edge as the area where the conformational adaptation of the protein is taking place, though some change in the area of the modified sixth heme ligand of chloramine T cytochrome *c* cannot be rigorously excluded.

In conclusion, we propose that the binding of chloramine T cytochrome *c* to the oxidase involves two conformational

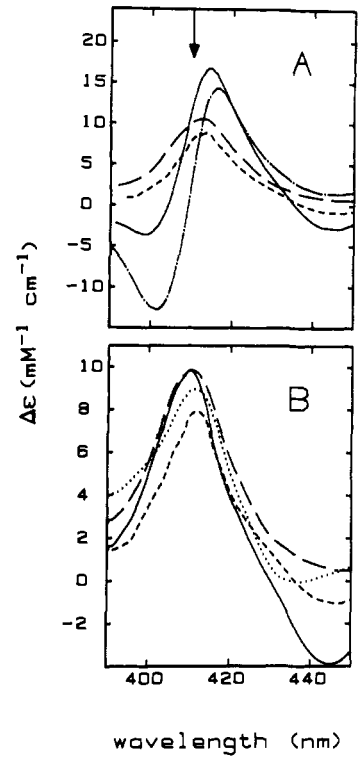


FIGURE 3: Binding to cytochrome *c* oxidase of cytochrome *c* derivatives with a break in the polypeptide chain. (A) Absorption difference spectra of cytochrome *c* 1–38:39–104 (—), acetimidyl cytochrome *c* 1–38:39–104 (---), and acetimidyl cytochrome *c* 1–37:38–104 (– –). Absorption difference spectrum of native ferricytochrome *c* in the neutral conformational state minus the alkaline conformational state (–·–). (B) Absorption difference spectra shown in (A) corrected for the fraction of the derivative in the alkaline state at pH 7.4. The correction for cytochrome *c* 1–38:39–104 was made by subtracting from the solid line spectrum shown in (A) a fraction of 0.67 of the neutral minus alkaline difference spectrum of native cytochrome *c* [dash–dot spectrum in (A)] to yield the solid-line spectrum in (B). Correction for the other derivatives was done accordingly, by using the percentage alkaline state cited in Table I. Absorption difference spectrum for native cytochrome *c* shown for comparison (···). Note the different ordinate scales in (A) and (B). Arrow indicates the maximum of the absolute absorption spectrum of ferricytochrome *c*.

transitions. The first is a change from an alkaline-like state to a native-like state, similar to the pH-dependent transition of horse cytochrome *c* from the alkaline to the neutral state. The second is a transition from free native-like chloramine T cytochrome *c* to oxidase-bound chloramine T cytochrome *c*. The latter transition is best seen in the CD difference spectrum. Unlike the case of the cytochrome *c* derivatives with a break in the polypeptide chain to be discussed next, it is not possible to calculate the percentage contribution of the two conformational events to the overall spectral changes in CD, MCD, and absorption.

*Difference Spectra of Derivatives with a Break in the Polypeptide Chain.* The difference absorption spectra of these derivatives are quite different from the difference absorption spectrum of native cytochrome *c* (Figure 3A). In particular, the difference absorption spectrum of cytochrome *c* 1–38:39–104 is large in comparison to that of native cytochrome *c* and shows a negative trough around 400 nm (compare solid line of Figure 3A with dotted line of Figure 3B). This difference between native cytochrome *c* and cytochrome *c* 1–38:39–104 must reflect a more pronounced change for cytochrome *c* 1–38:39–104. The change can be explained by considering that 67% of cytochrome *c* 1–38:39–104 is in the alkaline conformational state at the pH of the binding ex-

[4] A red shift of the S-shaped absolute MCD spectrum should result in a difference MCD spectrum with a peak flanked by two troughs. That the trough at longer wavelengths is not well developed in the solid line spectrum of Figure 2A could be due to a contribution by heme $aa_3$ to the difference spectrum.

periment and assuming that cytochrome c oxidase is able to shift the equilibrium from the alkaline to the neutral conformational state in bound cytochrome c 1–38:39–104. In this way, the difference absorption spectrum may be regarded as composed of a contribution from the "alkaline to neutral" transition and a contribution to be assigned to the actual binding of cytochrome c 1–38:39–104 to the oxidase. Indeed, when a contribution by the alkaline to neutral transition of 67% for cytochrome c 1–38:39–104 and one of 15% and 17% for acetimidyl cytochrome c 1–38:39–104 and acetimidyl cytochrome c 1–37:38–104, respectively, are subtracted, one obtains difference spectra that are very similar to one another and, most important, also very similar to the difference absorption spectrum of oxidase-bound native horse cytochrome c (Figure 3B). Cytochrome c oxidase may shift the equilibrium toward the neutral conformational state in two ways. The enzyme may bind preferentially to cytochrome c 1–38:39–104 in the neutral state. Alternatively, cytochrome c 1–38:39–104 may bind in both conformational states, and the enzyme may then change the conformation of the bound alkaline form. In the first case, we have to assume that cytochrome c 1–38:39–104 in the neutral state binds more strongly than native cytochrome c since the $K_d$, which averages over both conformation states, is only slightly larger for cytochrome c 1–38:39–104 than for native cytochrome c (Table II). For the same reason, in the second case, only little energy would be necessary to accomplish the transition to a conformation of bound cytochrome c 1–38:39–104 which features the spectral properties of the neutral conformational state. There is no way to distinguish between the two possibilities.

A comparison of the MCD difference spectra of the nicked cytochrome c derivatives with the MCD difference spectrum of native cytochrome c reveals a similar difference in shape and amplitude as just described for the absorption difference spectrum. Again, the MCD difference spectra of the nicked derivatives can be corrected for the contribution from the alkaline to neutral transition, to yield spectra that are highly similar to that of oxidase-bound native cytochrome c (not shown).

## Discussion

The results presented in this paper establish conclusively that binding to cytochrome c oxidase changes the conformation of cytochrome c in the area of the heme c. There is strong evidence for a rearrangement of the polypeptide conformation in the surface area of cytochrome c contacting the oxidase, while a change in the vicinity of the $Met^{80}$ sulfur–heme iron bond, tentatively suggested before (Webet et al., 1987), is not supported by the present data.

The nature of the conformational rearrangement in oxidase-bound cytochrome c is unknown, and it is also not known if the same or a similar rearrangement accompanies the formation of the actual electron-transfer complex of which the complex composed of ferricytochrome c and resting oxidase studied here is only an approximate model. The evolutionarily invariant $Phe^{82}$ may be a candidate for involvement in a structural rearrangement. The parallel position to the porphyrin ring of $Phe^{82}$ may be responsible for the negative CD band at 417 nm of native cytochrome c (Pielak et al., 1986; Hsu & Woody, 1971). A movement of $Phe^{82}$ on binding to the oxidase could contribute to the change of the CD spectrum. Recent calculations of the molecular dynamics of the cytochrome c–cytochrome $b_5$ complex predict a movement of $Phe^{82}$ towards $b_5$ (Wendoloski et al., 1987). Independent evidence for a rearrangement in the heme area of oxidase-bound cytochrome c was provided by NMR spectroscopy. The $^1H$ NMR resonances of heme methyl groups and several amino acid side chains are shifted when cytochrome c binds to the oxidase (Falk & Ångström, 1983; Satterlee et al., 1987). We would also like to suggest that binding to the oxidase could break the ionic bond between $Lys^{13}$ and $Glu^{90}$ located above the exposed edge of heme c. Following this rupture, $Glu^{90}$ may swing out, pulling the loop 80–90 toward the oxidase and thereby moving $Phe^{82}$ away from its location close to the heme plane (Satterlee et al., 1987).

An opening of the heme cleft in the oxidase-bound cytochrome c comparable to the opening characterizing the transition from the neutral to the alkaline conformational state is not supported by the present analysis. On the contrary, cytochrome c oxidase enforces the transition from the open alkaline state to a bound state somewhat similar to the neutral conformational state. We suggest that binding to the oxidase of a cytochrome c molecule in the alkaline state rearranges the electronic structure around the heme iron to a native state while the conformation around the exposed heme edge, possibly including $Phe^{82}$, remains in a more alkaline-like state, viz., no negative CD band at 417 nm. This then explains why the difference CD signal is small in the case of the derivatives that are partially in the alkaline state, whereas the difference absorption and MCD signals are much larger than observed with native cytochrome c. The remaining absorption and MCD change of oxidase-bound native cytochrome c may be accounted for by a change of polarity in the microenvironment around the exposed heme edge. The change arises by the extrusion of water from the contact area between cytochrome c and cytochrome c oxidase and by the neutralization of the charges surrounding the heme.

**Registry No.** Cytochrome c oxidase, 9001-16-5; cytochrome c, 9007-43-6.

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