Interaction of Cytochrome *c* and Cytochrome *c* Oxidase Studied by Spin-Label EPR and Site-Directed Mutagenesis

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Abstract : A thiol-specific spin label was attached to cysteine-102 of yeast cytochrome *c* and electron paramagnetic resonance (EPR) spectra were measured as a function of added cytochrome *c* oxidase concentration. The intensity decreased due to line broadening as cytochrome *c* formed a complex with cytochrome *c* oxidase and reached a minimum when the ratio of cytochrome *c* to cytochrome *c* oxidase became one. Replacement of either Lys-72 or Lys-87 of cytochrome *c* by Glu did not result in a significant change in binding affinity. Interestingly the K72E mutant, unlike K87E, had a much lower rate of electron transfer than the wild type. These results indicate that many positively charged residues as a group participate in complex formation but Lys-72 might be important for cytochrome *c* to be locked in an orientation for an efficient electron transfer. A stoichiometry of 1 was also confirmed by optical absorption of the cytochrome *c*-cytochrome *c* oxidase complex which had been run through a gel chromatography column to remove unbound cytochrome *c*. The EPR spectrum of this 1:1 complex, however, was a mixture of two components. This explains a biphasic kinetics for a single binding site on cytochrome *c* oxidase without invoking conformational transition.

Key words : cytochrome *c*, cytochrome *c* oxidase, EPR, mutagenesis

Cytochrome *c* (cyt *c*) is a globular protein with a *c*-type heme as the prosthetic group. Residing in the intermembrane space of a mitochondrion, cyt *c* accepts an electron from cyt *c* reductase and donates it to cyt *c* oxidase, where oxygen is reduced to water. As a basic protein carrying a net charge of +9 at neutral pH, it interacts electrostatically with its redox partners, cyt *c* reductase and cyt *c* oxidase. In yeast it can transfer an electron to another soluble enzyme, cyt *c* peroxidase. Cyt *c* may also be associated with the inner mitochondrial membrane which contains a large amount of anionic phospholipids.

The mechanism by which cyt *c* shuttles electrons is not clear. Two pieces of information, the binding stoichiometry and the structure of the binding site, are crucial for understanding the mechanism. From the results of differential chemical modification, cyt *c* appears to use the same domain on its surface to bind to all its redox partners, *i.e.*, cyt *c* oxidase (Rieder and Bosshard, 1978; Rieder and Bosshard, 1980; Koppenol and Mar-

goliash, 1982), cyt *c* reductase (Rieder and Bosshard, 1978; Bosshard *et al.*, 1979; Rieder and Bosshard, 1980; Koppenol and Margoliash, 1982) and cyt *c* peroxidase (Koppenol and Margoliash, 1982). Other workers reported slightly different residues at the binding interface. However, the region around Lys-72 and Lys-87 were identified in the binding domain in all cases.

High resolution X-ray crystal structure is available only for the cyt *c*-cyt *c* peroxidase complex (Pelletier and Kraut, 1992), where Lys-72 (or 73) and Lys-87 interact with negatively charged Asp and Glu of cyt *c* peroxidase. Since the same domain is used for the association with the electron donor and acceptor (Koppenol and Margoliash, 1982), cyt *c* should dissociate from the acceptor after the electron transfer before it binds to the donor. This can be accomplished if oxidized cyt *c* has a lower (higher) affinity toward the acceptor (donor) than reduced cyt *c*. This type of redox state-dependent change in affinity, however, has not been observed in the complexes between cyt *c* and its redox partners.

The number of binding sites on cyt *c* oxidase is also a matter of controversy. Based on a biphasic kinetics, Ferguson-Miller *et al.* (1976) proposed that cyt *c* oxidase has two binding sites for cyt *c* with different bind-

ing affinities. The idea was recently criticized by Ortega-Lopez and Robinson (1995), who showed that the biphasic kinetics may well be accounted for by slow reduction of cyt *c* by artificial reductants used in the kinetic measurements. Recently Lappalainen *et al.* (1995), using expressed Cu_A domain of cyt *c* oxidase, measured the electron transfer rates between cyt *c* and Cu_A domain to find a monophasic kinetics.

A binding stoichiometry of 1 was obtained from most spectroscopic measurements (Michel and Bosshard, 1984; Weber *et al.*, 1987; Michel and Bosshard, 1989), indicating that cyt *c* oxidase has only one tight binding site or two binding sites one of which is spectroscopically silent. Based on the assumption that cyt *c* oxidase has only one binding site for cyt *c*, Michel and Bosshard (1989) proposed a conformational transition mechanism in which the biphasic kinetics originates from a transition between two different conformations of cyt *c* oxidase during turnover.

Yeast cyt *c* has a free cysteine at position 102 to which a spin label can be covalently attached. An electron paramagnetic resonance (EPR) spectrum of spin-labeled cyt *c* yields information about the environment of the spin label. This technique has been successfully applied in the study of cyt *c*-membrane interaction (Snel *et al.*, 1994; Min *et al.*, 1996) and protein folding of cyt *c* (Qu *et al.*, 1997). In the present study, the technique has been used to monitor the binding of cyt *c* to cyt *c* oxidase. As an effort to identify the binding domain, we prepared two mutants of cyt *c* in which positively charged Lys-72 and Lys-87 were replaced, one at a time, by negatively charged Glu. These residues seem to participate in complexation with all three redox partners. Both mutants had approximately the same binding affinity toward cyt *c* oxidase as the wild type but K72E mutation impaired the electron transfer from cyt *c* to cyt *c* oxidase.

Materials and Methods

Site-directed mutagenesis

Site-directed mutagenesis of yeast *CYC1* gene encoding iso-1-cyt *c* was performed by using an *in vitro* Mutagenesis System kit purchased from Promega. Briefly, plasmid pAB569 containing a cloned yeast *CYC1* and *Saccharomyces cerevisiae* B-7528 (MATa *cyc1-31 cyc 7-67 ura3-52 lys5-10*) lacking both iso-1-cyt *c* (due to *cyc1-31*) and iso-2-cyt *c* (due to *cyc7-67*) were kindly donated by Dr. Fred Sherman of the University of Rochester. The *CYC1* gene flanked by *Bam*HI and *Hind*III sites was transferred from pAB569 to pALTER-1 (included in the Promega kit) to generate a recombinant plasmid pKW200. Cyt *c* made from this *CYC1* gene re-
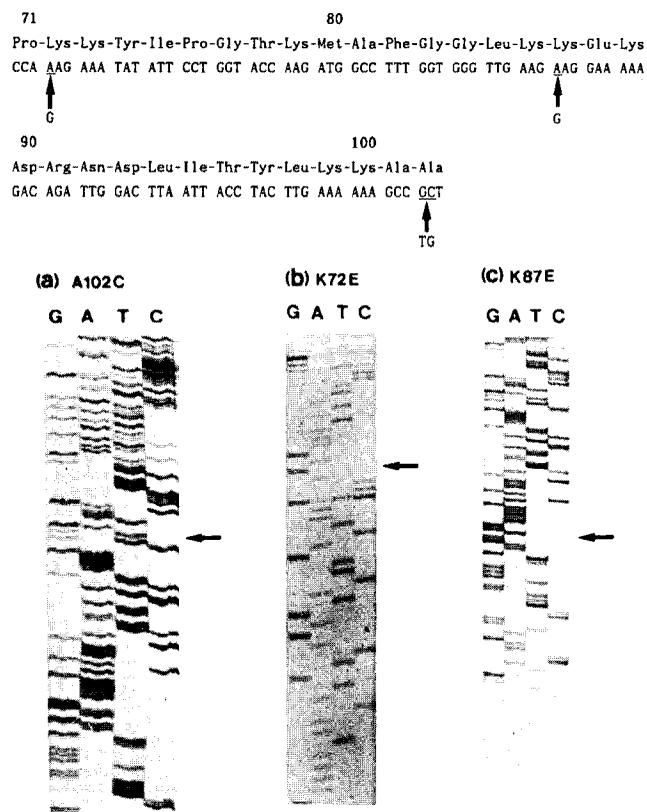

Fig. 1. Autoradiograms showing altered bases at the indicated positions of yeast *CYC1* gene by site-directed mutagenesis. A 102C (a), K72E (b), and K87E (c) mutations are indicated by horizontal arrows.

tained Ala-102 instead of wild type Cys-102. Thus, A 102C mutation was first introduced into pKW200 before generating K72E and K87E mutations, respectively, by using three appropriate mutagenic oligonucleotides (Fig. 1). The mutations were confirmed by DNA base sequencing (Sanger *et al.*, 1977; Fig. 1). The mutant *CYC1* genes were then transferred between the *Bam*HI and the *Hind*III sites on the multiple cloning sites of YEp357 (Myers *et al.*, 1986) which is an *E. coli*-yeast shuttle vector and contains *URA3* marker. Resulting plasmids YEp357-*CYC1*-K72E and YEp357-*CYC 1*-K87E were introduced, respectively, into B-7528 by selecting transformants growing on an uracil-deficient medium. Unlike B-7528, all the transformants can use a nonfermentable carbon source, either lactate or glycerol, indicating both mutant *CYC1* genes are functional.

Purification of cytochrome *c* and cytochrome *c* oxidase

Saccharomyces cerevisiae B-7528 transformants containing cloned *CYC1* genes were aerobically grown in 5 l of YPL medium (1% yeast extract, 2% peptone, 1% lactate) to the stationary phase at 30°C. The yeast cells were harvested and subjected to purification of cyt *c*

by the procedures of Sherman *et al.* (1968) and Koshy *et al.* (1992). Extinction coefficient ε_{550}=27.7 mM^{-1} cm^{-1} was used to determine the concentration of reduced cyt *c*. Cyt *c* oxidase was isolated according to Yonetani (1960). Enzyme concentration was determined spectrophotometrically by using extinction coefficient $\varepsilon_{605-630}$ (redox)=27 mM^{-1} cm^{-1}.

Polarographic assay

Twenty micromolar cyt *c* and 40 nM cyt *c* oxidase were mixed in 10 mM Hepes buffer (pH 7.4) containing 1% Tween 80 as a detergent. Immediately after the addition of 200 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and 5 mM ascorbate, the rate of oxygen consumption was measured by an oxygen electrode (Rank Brothers, Digital Oxygen System Model 10).

Spin-labeling of cyt *c*

Cyt *c* was spin-labeled at Cys-102 as previously described (Min *et al.*, 1996). Briefly, cyt *c* was incubated with 1.5 fold excess of 1-oxyl-2,2',5,5'-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSSL), a thiol-specific spin label. Unreacted spin label was removed by a small Sephadex G-15 column.

EPR spectroscopy

EPR measurements were performed at room temperature on a Bruker ER-200 X-band EPR spectrometer using a quartz flat cell. Spectral conditions: microwave frequency 9.76 GHz, modulation frequency 100 kHz.

Results and Discussion

S. cerevisiae expresses two isoforms of cyt *c*, iso-1- and iso-2-cyt *c*, the former being the majority in wild type cells. They are similar to each other in the primary as well as in the tertiary structure. It is not known if they have different functions in the cell. In the present system, however, the iso-2 gene was deleted and only iso-1-cyt *c* is expressed. Therefore the following discussion is concerned only with iso-1-cyt *c*. There was no striking difference between the mutants and the wild type in the growth rate of the cells and the yield of cyt *c*.

Two major α-helices in iso-1-cyt *c* are located at the N- and C-termini. The C-terminal helix extends from 87 to 103 and it has a free cysteine residue at position 102, which can readily be modified by MTSSL, a methanethiosulfonate spin label (Milhauser, 1992). Although the S_γ of Cys-102 in the native cyt *c* is pointing toward the interior of the protein (Louie and Brayer, 1990), it is readily available for the covalent attachment of the spin label. Spin labeling by MTSSL is almost quantita-
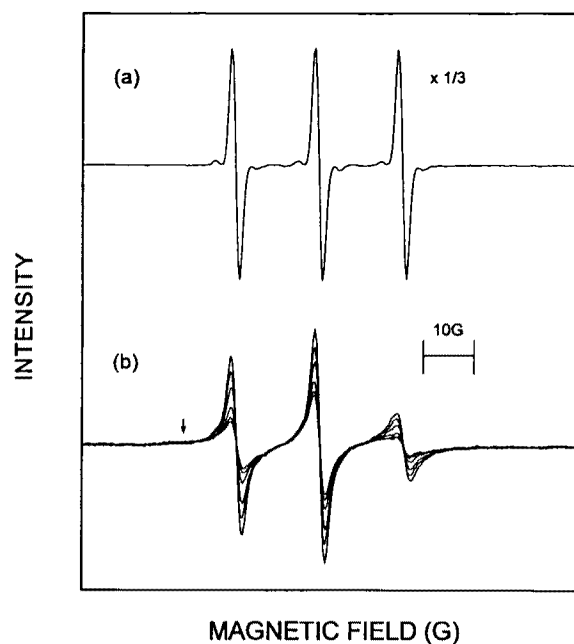

Fig. 2. Room temperature X-band EPR spectra of 20 μM free spin label (a) and 20 μM yeast cytochrome c spin labeled at Cys-102 (b). In (b) the intensity gradually decreased as the concentration of cytochrome c oxidase increased from 0 to 30 μM (0, 1, 5, 10, 20, and 30 μM). Arrow indicates an 'immobile' signal. The isotropic EPR spectrum of the spin label itself is shown in (a) for comparison. Spectral conditions: microwave frequency 9.76 GHz, power 64 mW, modulation 100 kHz.

tive and, importantly, specific to cysteine and has been successfully applied to many biological problems in conjunction with site-directed mutagenic introduction of cysteine residues (Hubbel *et al.*, 1996).

A conventional continuous wave EPR spectrum of a nitroxide spin label consists of three lines due to hyperfine coupling to the nuclear spin of nitrogen. The lineshape contains information about the dynamic tumbling processes on the 0.01-10 ns time scale (Milhauser *et al.*, 1995). Free MTSSL in buffer undergoes a rapid tumbling in isotropic environment and hence the three EPR lines are of equal intensity, as shown in Fig. 2a. The EPR spectrum of spin-labeled cyt *c* (the top spectrum of Fig. 2b) shows a large change in lineshape, reflecting significant motional restriction imposed on the spin label by the surrounding protein moiety. This is most evident in the reduced intensity of the high field line. Since the spin label on Cys-102 is, at least partially, embedded in the protein interior rather than exposed to the surface in the native structure (Louie and Brayer, 1990), one can expect motional restriction of the spin label due to the steric hindrance.

The rotational correlation time of the spin label of free cyt *c* in the buffer (Fig. 2b) was calculated to be ~0.7 ns within the motional narrowing regime (Milhauser *et*
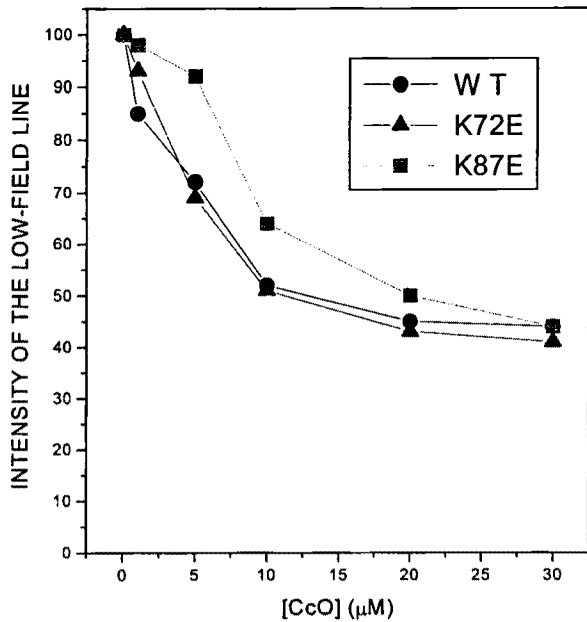
Fig. 3. Intensity of the low-field line of the EPR spectra as a function of the concentration of added cytochrome c oxidase. Square for wild type, triangle for K72E, and circle for K87E.

Fig. 4. The optical absorption (a) and EPR (b) spectra of the purified 1:1 complex of cytochrome c and cytochrome c oxidase. The EPR spectral conditions are same as in Fig. 2.

al., 1995). Using the Stokes-Einstein equation which relates the rotational correlation time to molecular volume and viscosity of the medium, a correlation time of ~5 ns was calculated for the tumbling motion of the protein as a whole. Therefore the spin label, though attached covalently to the protein, has local freedom of motion due primarily to free rotation about five intervening single bonds between the C_α of Cys-102 and the proxyl group of the spin label.

Addition of cyt c oxidase to cyt c resulted in additional decrease in the heights of the EPR lines (Fig. 2b), which indicates a further restriction in the rotational motion of the spin label as cyt c binds to cyt c oxidase. At the saturating concentration of cyt c oxidase, the EPR spectrum appears to contain two components: an 'immobilized' (marked by an arrow) and a 'mobile' signal. This is more evident in Fig. 4b, where pure cyt c-cyt c oxidase complex was isolated from any unbound cyt c and cyt c oxidase. The mobile component is not a signal arising from the unbound cyt c since this component can not be subtracted out by the EPR spectrum of the free cyt c. The motion of the spin label in this mobile state is still more restricted than the free cyt c. The 'immobile' component, characterized by a large anisotropy in hyperfine splitting, is similar to that of spin label embedded in phospholipid membranes. Both signals can be reversed to that of free cyt c by the addition of salt or unlabeled cyt c suggesting that the binding is electrostatic and reversible (data not shown).
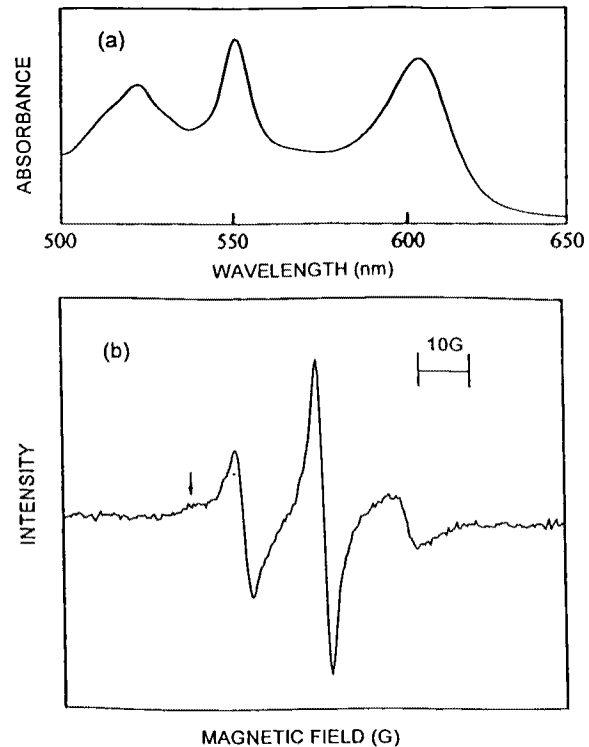
Similar two-component spectra were obtained in cyt

c complexed with acidic membranes (Min et al., 1996) and with cyt c reductase (Park and Han, unpublished data). The major driving force of binding is the electrostatic interaction between positively charged cyt c and negatively charged membranes or cyt c reductase. The same electrostatic interaction is involved in the cyt c-cyt c oxidase complex. The mobile component reflects minor decrease in mobility compared to free cyt c. Crystal structure of yeast cyt c (Louie and Brayer, 1990) shows that Cys-102 is located on the surface opposite to the proposed binding domain which includes basic residues at positions around 13, 27, 72, and 87. It is difficult to rationalize the decrease in mobility of the spin label attached to Cys-102 which is located far away from the binding domain. However, the spin label and Lys-87 are positioned at opposite ends of the C-terminal helix. Therefore it is not surprising to see structural perturbation around the spin label which can be triggered by the binding through Lys-87.

Observation of the immobile signal is even more puzzling. According to a resonance Raman study (Hildebrandt et al., 1990b), the complex formation between cyt c and cyt c oxidase causes a major structural change in which ~50% of the heme is in more open conformation. Iron-methionine bond is broken in ~5% of the bound cyt c. A similar change in conformation was observed when cyt c interacts with negatively

charged surfaces (Hildebrandt et al., 1990a). Therefore it seems to be a common phenomenon that cyt c has more open heme crevice when bound to negatively charged membranes or proteins. The heme crevice is open, however, toward the proposed binding domain so that immobilization of the spin label is hardly expected. Since immobilized signals were observed when the spin label penetrates membrane bilayer, we suggest that the C-terminal helix is in a close contact with cyt c oxidase in the complex that yields the immobile signal. However, this does not necessarily mean that cyt c uses two completely separate binding domains or cyt c oxidase has two binding sites for which cyt c can use the same domain. Slightly different orientations of the bound cyt c on cyt c oxidase might be enough to account for the two components in the EPR spectrum. A similar conclusion has been drawn in the cyt c-membrane interaction (Snel et al., 1994; Min et al., 1996).

To test the possibility of two or more binding sites on cyt c oxidase, the intensity of the low field line was plotted as a function of the cyt c oxidase concentration. As shown in Fig. 3, the change in the intensity of 20 μM cyt c levels off at around 20 μM of cyt c oxidase. This clearly shows the ratio of cyt c to cyt c oxidase is one. Cyt c oxidase as isolated from bovine heart mitochondria sometimes contains residual phospholipids, which makes the ratio larger than one (Park and Han, unpublished results). After removal of these lipids by gel chromatography, the ratio consistently yielded a value of one, indicating that only one cyt c is bound per cyt c oxidase monomer.

Various methods have been employed to identify the amino acid residues of cyt c that are involved in binding to its redox partners. Although there are discrepancies among the methods used, lysine residues in the vicinity of position 72 and 87 appear to be part of the binding domain. Using site-directed mutagenesis techniques, we prepared two mutants, namely K72E and K 87E, in which a positively charged lysine residue was replaced by a negatively charged glutamate residue. EPR spectral titration of these mutants are shown in Fig. 3. To our surprise, binding affinities of the mutants were very similar to that of the wild type. Our result indicates that a single mutation within the binding domain does not severely impair the electrostatic interaction between cyt c and cyt c oxidase. Kim and King (1991) modified Lys-79, Lys-86, and a few others simultaneously with pyridoxal phosphate and found that the modified cyt c does not bind to either cyt c oxidase or cyt c reductase. From these results it can be concluded that a number of lysine residues as a group participate in the interaction. This hypothesis can be tested by a multiple mutagenesis.

In order to determine the stoichiometry more accurately, a large access of spin-labeled cyt c was added to cyt c oxidase and the mixture was run through a gel chromatography column to remove unbound cyt c. The EPR spectrum of the eluent (Fig. 4b) is clearly a mixture of two components. Since the eluent does not contain unbound cyt c, the mobile signal must represent a bound species. The stoichiometry can be accurately determined by the optical absorption spectrum (Fig. 4a), which was taken after dithionite was added to reduce both cyt c and cyt c oxidase. The absorption maxima at 550 nm and 605 nm arise from reduced cyt c and cyt c oxidase, respectively. Since the extinction coefficients of cyt c at 550 nm and cyt c oxidase at 605 nm are very similar, the stoichiometry of the cyt c-cyt c oxidase complex estimated from Fig. 4a is close to 1. This provides a concrete proof that cyt c and cyt c oxidase form a tight 1:1 complex in two (or more) different configurations. A 1:1 complex was also observed in a reconstructed electron microscope image (Frey and Murray, 1994).

Ferguson-Miller and Margoliash (1976) observed a biphasic kinetics for the oxidation of cyt c by cyt c oxidase and interpreted the data in terms of two binding sites on cyt c oxidase. Most of the binding experiments (Michel and Bosshard, 1984; Weber et al., 1987; Michel and Bosshard, 1989) including the present work, however, consistently yielded a 1:1 stoichiometry. In order to account for both single binding site and the biphasic kinetics, which appear to be contradictory to each other, Michel and Bosshard (1989) proposed a model in which cyt c oxidase undergoes a conformatinal transition. Recently Ortega-Lopez and Robinson (1995), arguing against this model, suggested that the biphasic kinetics is primarily due to slow reduction of cyt c by artificial reductants. The finding of this work provides a simplest explanation that the biphasic kinetics results from two different orientations of the bound cyt c on a single binding site of cyt c oxidase.

Cyt c oxidase reduces oxygen to water with the four electrons transferred from cyt c. Therefore the rate of electron transfer between cyt c and cyt c oxidase can be measured conveniently by an oxygen electrode. The reaction, like a typical enzyme reaction, stays in the steady-state most of the time except the very early and late stages (Fig. 5). The electron transfer rate was not affected when Lys-87 was replaced by Glu. Mutation of Lys-72 to Glu, however, slowed down the rate significantly. Ionic strength of the medium is known to have a profound effect on the rate of oxygen consumption, suggesting that the formation of the subtrate-enzyme (cyt c-cyt c oxidase) complex strongly affects the rate. Therefore a large decrease in the rate for K72E is some-
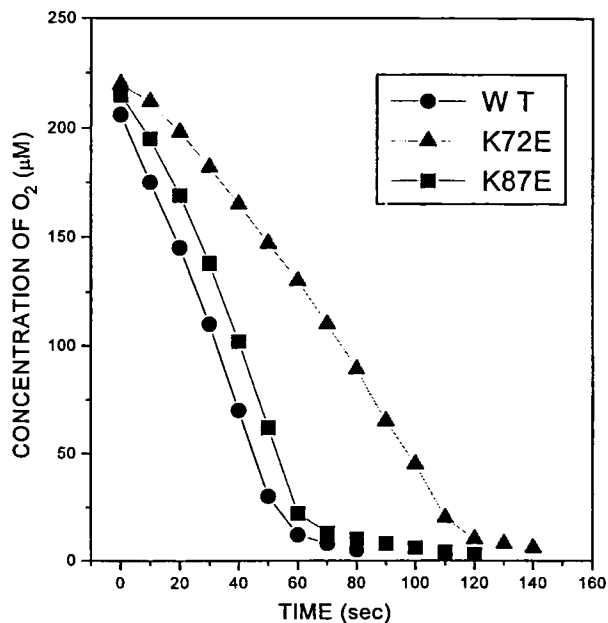
Fig. 5. Rate of oxygen consumption by cytochrome c oxidase. Two hundred micromolar TMPD was added to a buffer (Hepes, pH 7.4, 1% Tween 80) containing 20 µM cytochrome c and 40 nM cytochrome c oxidase. After 5 mM ascorbate was injected into the reaction mixture, consumption of oxygen was monitored using a Clark-type oxygen electrode. Square for wild type, triangle for K72E, and circle for K87E.

what unexpected since the binding affinity of this mutant was almost the same as that of the wild type. These results strongly suggest that the binding domain on cyt c consists of multiple lysine residues so that a single mutation can hardly affect the binding affinity. A proper orientation of cyt c on the surface of cyt c oxidase, however, may be important for an efficient electron transfer. Lys-72 but not Lys-87 appears to be important for cyt c to lock in that orientation. As Northrup et al. (1988) pointed out, cyt c may move around on the surface of cyt c oxidase before it finds a correct orientation.

In summary, cyt c forms a 1:1 complex with cyt c oxidase but there are two modes of binding that account for the observed biphasic kinetics. Neither K72E nor K 87E mutation altered the binding affinity indicating the binding involves a group of lysine residues. Lys-72 unlike Lys-87 appears to be important for cyt c to assume an orientation on cyt c oxidase which facilitates an efficient electron transfer.

Acknowledgements

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