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Tryptophan 121 of Subunit II Is the Electron Entry Site to Cytochrome-*c* Oxidase in *Paracoccus denitrificans*

INVOLVEMENT OF A HYDROPHOBIC PATCH IN THE DOCKING REACTION*

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To investigate the contribution of hydrophobic residues to the molecular recognition of cytochrome *c* with cytochrome oxidase, we mutated several hydrophobic amino acids exposed on subunit II of the *Paracoccus denitrificans* oxidase. K_M and k_{cat} values and the bimolecular rate constant were determined under steady- or presteady-state conditions, respectively. We present evidence that Trp-121 which is surrounded by a hydrophobic patch is the electron entry site to oxidase. Mutations in this cluster do not affect the binding of cytochrome *c* as the K_M remains largely unchanged. Rather, the k_{cat} is reduced, proposing that these hydrophobic residues are required for a fine tuning of the redox partners in the initial collisional complex to obtain a configuration optimal for electron transfer.

Electron transfer between redox proteins has been under extensive investigation during the last years. The protein surfaces involved in complex formation have been of particular interest as it became clear that electron transfer depends on specific recognition with an affinity low enough to allow rapid dissociation. Several studies with cytochrome *c* and its different redox partners demonstrated the involvement of electrostatic interactions mediated by lysines surrounding the heme edge on cytochrome *c* and acidic residues on the counterpart (1–6). Nevertheless, it became obvious from ionic strength dependence measurements that electrostatic interactions are not the only criterion governing optimal electron transfer (2). The bell-shaped dependence of activity on ionic strength lead to the proposal that at low ionic strength the redox partners lack the conformational flexibility to achieve a configuration optimal for electron transfer (7, 8) besides the fact that product dissociation becomes rate-limiting. At high ionic strength the rate of non-productive collisions increases due to the shielding of charges. Around the ionic strength optimum, when long range electrostatic forces have roughly aligned the reacting proteins, configurational freedom still prevails for short range forces such as hydrophobic interaction to contribute significantly to a configuration optimal for electron transfer.

Cytochrome-*c* oxidase (cytochrome aa_3; EC 1.9.3.1) (for reviews see Refs. 9–11) is one of the terminal enzymes in the respiratory chain of *Paracoccus denitrificans* besides a quinol oxidase (cytochrome ba_3 (12)) and an alternative cytochrome oxidase (cytochrome cbb_3 (11)). The three main subunits of cytochrome aa_3 show significant homology to the mitochondrially encoded subunits of the eukaryotic cytochrome oxidase. Heme a and the binuclear center (heme a_3·Cu_B) are located in subunit I, whereas the Cu_A center which is the primary electron acceptor resides in subunit II. As stated above, the involvement of electrostatic interactions in the reaction between cytochrome *c* and cytochrome-*c* oxidase mediated by acidic residues predominantly located on subunit II of the *P. denitrificans* oxidase has already been demonstrated (6).

To investigate the contribution of hydrophobic residues to the electron transfer reaction, we analyzed the interaction domain on subunit II of cytochrome-*c* oxidase for exposed residues. From the crystal structures of oxidase (13–15), we find tryptophan 121 exposed on the surface within a distance of 5 Å above the Cu_A center and several other hydrophobic residues protruding into the putative binding site (see also Fig. 3). We mutated residues in 4 positions and investigated the isolated mutant enzymes W121Q, Y122S, Y122K, I117Q, and L137Q by burst (stopped-flow) and steady-state kinetics. Results indicate that Trp-121 is the electron entry site to the oxidase. Comparison of the kinetic parameters reveals that the initial binding of cytochrome *c*, as reflected by K_M, is not affected by the mutations but the electron transfer is impaired, suggesting that an optimal configuration cannot be achieved in the mutant enzymes.

MATERIALS AND METHODS

P. denitrificans strain ST4 is a deletion mutant (16) lacking the heme aa_3 cytochrome oxidase; it is complemented in *trans* with the *cta* operon conveying the different mutations in subunit II. For enzyme purification, strains were grown on succinate medium (17) including streptomycin (25 μg/ml).

Site-directed mutagenesis was performed according to the altered sites mutagenesis protocol (Promega, Heidelberg, Germany).

Enzyme preparation was performed as described (18) with membranes solubilized with *n*-dodecyl-β-D-maltoside according to Ref. 19. Protein determination, SDS-polyacrylamide gel electrophoresis, Western blotting, and cytochrome oxidase spectra were carried out as described (5).

Steady-state activity of oxidase was assayed spectrophotometrically in 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.2 g/liter dodecyl maltoside, and either 20 or 40 mM KCl at 25 °C. The oxidation of reduced cytochrome *c* (horse heart, Sigma) was followed at 550 nm with the concentration varied between 0.5 and 40 μM after addition of purified enzyme (40–400 pM). The ionic strength dependence was assayed under the same conditions with the ionic strength adjusted to between 1.8 (2 mM Tris/HCl) and 296 mM by addition of KCl.

Stopped-flow kinetics were performed on a thermostated Applied

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TABLE I
Cytochrome c oxidation by cyanide-inhibited oxidase

In a stopped-flow experiment the oxidation of reduced cytochrome *c* by cyanide-inhibited oxidase at I = 140 mM was followed at 550 nm. The time course was fitted to the sum of two exponentials to determine k_{obs}. The second-order rate constant k_{on} was determined from the slope of the pseudo-first-order rate constant k_{obs} plotted *versus* the concentration of cytochrome *c*.

	k_{on}
	$\times 10^6\ M^{-1}\ s^{-1}$
Wild type	7.6
W121Q	(k_{obs} = 0.04 s^{-1})[a]
Y122S	6
Y122K	0.71
L137Q	1.75
I117Q	13.5

[a] No determination of the bimolecular rate constant was possible; the observed pseudo-first-order rate constant is given instead; see also Fig. 1.

Photophysics DX.17MV stopped-flow (Leatherhead, UK) at 20 °C in 20 mM KP_i (pH 8), 1 mM EDTA, 0.2 g/liter dodecyl maltoside and KCl to obtain a final ionic strength of 140 mM. Cytochrome oxidase (between 6 and 10 μM) was mixed 1:1 with reduced cytochrome *c* (4–32 μM) and the reaction followed as the oxidation of cytochrome *c* at 550 nm or the reduction of heme a at 605 nm. The average of three independent measurements was fitted to the sum of two exponentials, and the pseudo-first-order rate constant k_{obs} of the fast phase was plotted against the cytochrome concentration. From the slope, the bimolecular rate constant k_{on} was calculated.

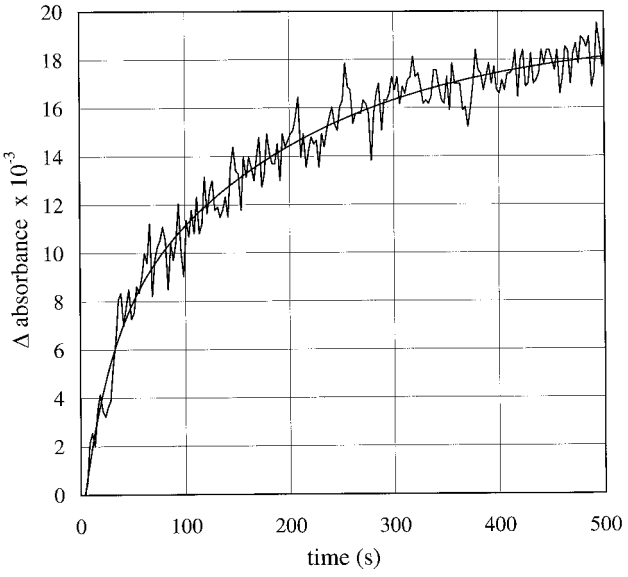

FIG. 1. **Oxidation of 100 μM cytochrome *c* by cyanide-inhibited mutant oxidase W121Q followed at 605 nm in the stopped-flow apparatus (for experimental details see "Materials and Methods").** The pseudo-first rate constant obtained from the fit to the sum of two exponentials is k_{obs} = 0.04 s^{-1} (fast phase). Under comparable conditions, the observed rate constant for the wild type enzyme is 760 s^{-1} (6).

RESULTS

By applying standard site-directed mutagenesis techniques, we constructed several mutants in subunit II of cytochrome oxidase to investigate the contribution of hydrophobic residues to the interaction with cytochrome *c*. The mutant enzymes W121Q, Y122S, Y122K, L137Q, and I117Q were isolated and investigated by steady-state and presteady-state kinetics. We also monitored the ionic strength dependence of the mutants in comparison to the wild type.

Burst Kinetics—The electron entry to cyanide-inhibited oxidase, used to prevent turnover, was monitored by following the oxidation of reduced cytochrome *c*. The time course was fitted by the sum of two exponentials, and the derived pseudo-first-order rate constant k_{obs} of the fast phase was plotted against the cytochrome *c* concentration. The apparent bimolecular rate constant k_{on} was calculated from the slope, and the results are presented in Table I. Substitution of Tyr-122 by Ser does not affect the bimolecular rate constant, yielding a value comparable with the wild type, whereas mutation against Lys drastically reduces k_{on} to about one-tenth. This difference is most likely due to the introduction of a positively charged residue although sterical constraints cannot be excluded. The k_{on} of I117Q is slightly increased, whereas that of L137Q is decreased to about one-fourth compared with wild type. Strikingly, no electron entry at concentrations lower than 100 μM cytochrome *c* could be measured when Trp-121 was mutated to Gln, yielding strong evidence that Trp-121 is the actual electron entry site to cytochrome oxidase (Fig. 1). It seems that under these experimental conditions, the exchange of hydrophobic residues against hydrophilic ones has very different effects. Besides the fact that one of the mutated residues serves as the entry to the oxidase, the others obviously contribute to the interaction to different degrees.

Steady-state Kinetics—Steady-state measurements were performed at I = 36 mM and I = 56 mM to determine the kinetic parameters. When data were analyzed in a Lineweaver-Burk plot, two phases could be distinguished, each characterized by a K_M and a k_{cat} value resulting in a high and a low affinity phase as observed earlier (5, 20). The parameters for the high affinity phase are presented in Table II. The absolute K_M values for the wild type are 1.3 μM at I = 36 mM and 4.5 μM at I = 56 mM. K_M values for the mutants are given as relative Michaelis constants $(K_M)_{mutant}/(K_M)_{wild\ type}$.

The K_M can be taken as an apparent dissociation constant under certain conditions, and the work of several groups during the last few years has shown that K_M is equivalent to K_D for oxidase (21, 22). We suppose from our earlier investigations that this holds at least for the condition of I = 56 mM (6). At lower ionic strength, product dissociation seems to be one of the rate-limiting factors, and therefore interpretation of K_M, although less straightforward, nevertheless provides a good indication of the affinity. It is obvious that the dissociation constants (Michaelis constants) of Y122S, L137Q, and I117Q are virtually identical to that of the wild type, indicating that the affinity of the oxidase for cytochrome *c* has not changed. In contrast, the K_M of Y122K is significantly increased demonstrating a reduced affinity. As already indicated by the burst experiments, this can probably be attributed to the introduction of a bulky and positively charged residue.

The different contributions to the interaction with cytochrome *c* are evident from the comparison of the catalytic constant k_{cat}. All mutants and the wild type show an increased catalytic constant at I = 56 mM compared with I = 36 mM. W121Q displays a drastically reduced k_{cat} compared with wild type (around 1% at I = 56 mM) just like Y122K, although these results may be attributed to different effects. The three remaining mutants (with unaltered affinity) show a varying decrease in their catalytic constants. Again, like in the burst experiment, L137Q is affected to the highest degree, followed by Y122S and I117Q revealing the smallest deviation from wild type.

Ionic Strength Dependence of the Turnover Number of Oxidase—The interaction between several redox proteins was shown to be ionic strength-dependent as a result of the electrostatic forces driving complex formation. Introduction of a charged residue on the interacting surface should be experimentally detectable, whereas mutation of hydrophobic ones against uncharged hydrophilic ones should not. Fig. 2 presents

TABLE II
Kinetic parameters of the steady-state oxidation of cytochrome c by cytochrome oxidase

Kinetic parameters were obtained under two different ionic strength conditions (I). For the high affinity phase the relative Michaelis constant $(K_M)_{mutant}/(K_M)_{wild\ type}$, the maximal turnover number (k_{cat}) as calculated from Lineweaver-Burk plots, and the specificity constants (k_{cat}/K_M) are listed.

	I = 36 mM			I = 56 mM		
	Relative K_M	k_{cat}	k_{cat}/K_M	Relative K_M	k_{cat}	k_{cat}/K_M
		s^{-1}	$\times 10^8\ M^{-1}\ s^{-1}$		s^{-1}	$\times 10^8\ M^{-1}\ s^{-1}$
Wild type	1	357	2.75	1	769	1.71
W121Q	1.5	11	0.06	0.9	8.3	0.02
Y122S	0.9	260	2.17	1	303	0.64
Y122K	3.1	40	0.1	4.8	43	0.02
L137Q	0.8	139	1.26	1.1	167	0.33
I117Q	0.5	204	2.91	0.9	476	1.2

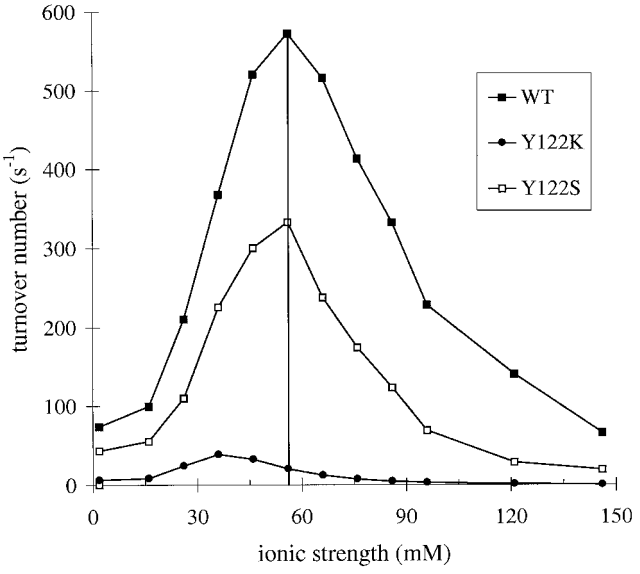

FIG. 2. **Ionic strength dependence of wild type and two Tyr-122 mutant oxidases.** Plot of the turnover number at 20 μM cytochrome *c versus* ionic strength (for experimental details see "Materials and Methods").

the effects of ionic strength on the turnover number of the Tyr-122 mutants. As expected, Y122S reveals a similar profile as wild type but with a decreased rate over the whole range. In contrast, the optimum of Y122K is significantly shifted toward lower ionic strength, and at the same time this mutant displays a drastically reduced activity. The profile of I117Q is virtually identical to wild type with reduced activity in the low ionic strength region up to the optimum and only slightly lower turnover numbers at higher ionic strength. Likewise, no shift of the optimum, but a reduced activity is seen for L137Q, whereas W121Q lacks an actual optimum between 36 and 56 mM (data not shown).

DISCUSSION

Previous investigations of the interaction between cytochrome *c* and oxidase primarily focused on charged residues on either protein. Nevertheless, from several investigations and structural data derived from co-crystals with other redox proteins (see Introduction), the involvement of additional factors such as hydrophobic interactions was postulated.

Analysis of the docking site on subunit II of oxidase (6, 13) revealed several surface-exposed hydrophobic residues. For mutagenesis, we chose four positions because of their location within the acidic patch; in addition, two of them are highly conserved aromatic amino acids. Ile-117, Trp-121, and Leu-137 were mutated to Gln, whereas Tyr-122 was exchanged against Ser and Lys. The purified enzymes containing single mutations were investigated by burst experiments and steady-state kinetics.

It is obvious from the burst experiment that electrons enter the W121Q mutant oxidase only very slowly under the given conditions (I = 140 mM). Determination of the apparent bimolecular rate constant k_{on} was impossible as no electron entry was detectable below 100 μM cytochrome *c*. We therefore conclude that Trp-121 is the electron entry site to cytochrome oxidase. This is in accordance with theoretical simulations.[1] An electron was placed on Cu_A and allowed to reach a surface residue. The rate of transfer was calculated and was highest for Trp-121, although, with a much slower rate, residues in close proximity could also be reached. This could explain the residual activity we observe for W121Q in the steady-state experiments, when we assume different tunneling paths for the electron to arrive at Cu_A although with drastically different probabilities. There is no indication that an aromatic residue at this position is an essential prerequisite for enzyme function as has been postulated previously (23); however, from a theoretical point of view, non-aromatic residues are capable of accepting electrons as well. We exclude any serious sterical constraints due to the exchange of Trp against Gln in particular since the molecular volume of Gln is even slightly smaller than that of Trp (24). At present, we cannot decide whether in the W121Q mutant enzyme electron transfer through this residue is only diminished or whether it is completely blocked and a neighboring residue such as Tyr-122 takes over this function, accounting for the 1% residual activity. In addition, the burst experiments employed cyanide-inhibited oxidase, whereas for the steady-state measurements the enzyme was in turnover. If the inhibition at the binuclear site was sensed by Cu_A, the barrier for an electron to enter the oxidase should be higher under these conditions.

An additional argument for the role of Trp-121 is the lack of a genuine ionic strength optimum. Although a precise orientation of cytochrome *c*, which is critically dependent on the salt concentration, is of great importance for activity in wild type and the other mutants, this criterion seems to be negligible for W121Q.

Trp-121 is located about 5 Å above the Cu_A site and, interestingly, is conserved not only among cytochrome oxidases (mitochondrial and bacterial, with the exception of *Bacillus subtilis* where it is replaced by Tyr) but also among quinol oxidases. A role in electron transfer of these aromatic residues has already been proposed long ago (25, 26).

Tyr-122 protruding from the surface close to Trp-121 is also highly conserved among cytochrome oxidases but is replaced by Lys in quinol oxidases. However, exchanging Tyr-122 against Lys in the cytochrome oxidase has drastic consequences concerning the activity as well as the affinity for cytochrome *c* visualized by the increased K_M. The initial docking reaction of oxidase with cytochrome *c* relies on electrostatic interaction which is mediated by several glutamates and aspartates clustered in negatively charged patches on subunits I and II. Introduction of a positively charged residue not only disrupts the electrostatic potential but probably leads to repulsion of the positively charged patch on cytochrome *c* and thereby prevents the association of the redox partners; in addition, steric effects of the lysine residue may have to be considered which could increase the redox center distance. Moreover, the positive charge in close proximity to the electron entry site could have some influence on the electron transfer. Mutation against Ser at this position does not affect the initial binding reaction (K_M

[1] L. Dutton, personal communication.

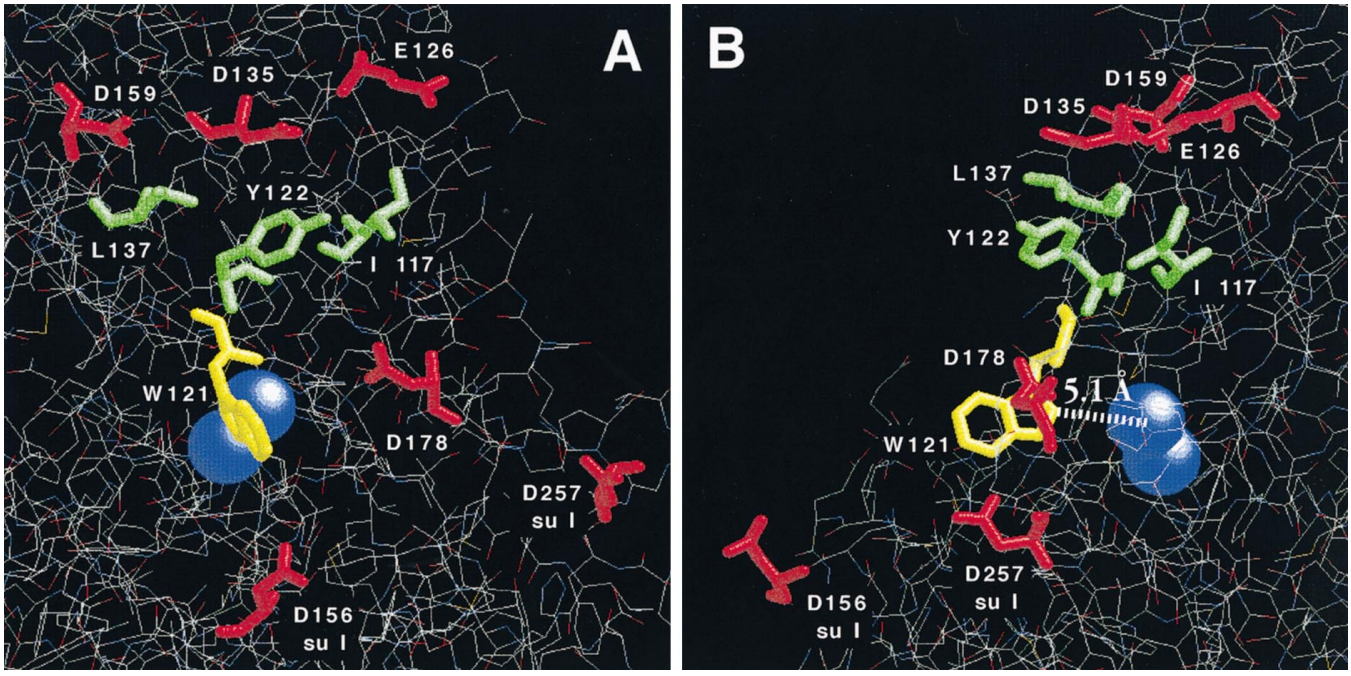

FIG. 3. **Docking site for cytochrome *c* on *P. denitrificans* cytochrome oxidase subunits I and II in a frontal (*A*) and side view (*B*).** Residues important for interaction with cytochrome *c* are highlighted and numbered: acidic amino acids, *red*; hydrophobic, *green*; Trp-121 *yellow*; the *two blue spheres* represent the Cu_A center in subunit II. Figures were drawn using the program Rasmol and based on the coordinates of the 2-subunit enzyme complex structure (32).

unchanged) but decreases the activity. We therefore assume this residue to play a role in the fine tuning of both partners necessary for optimal electron transfer. The interesting question still remains why this position is occupied by Lys in quinol oxidases whereas other aromatic side chains in this area, Trp-121, Trp-123, Tyr-125, and Tyr-127, are strictly conserved; however, the three latter residues are not surface-exposed. With the vastly different nature of both substrates in the two different classes of oxidases, substrate interaction and electron entry pathways must be drastically different in quinol and cytochrome-*c* oxidases, as evidenced by the complete loss of acidic, cytochrome *c* binding residues in quinol oxidases during evolution (27).

Ile and Leu have an almost identical side chain volume and no difference in hydrophobic free energy (24); replacement of Ile-117 and Leu-137 by Gln from this point of view should lead to similar effects. This is actually not the case; L137Q displays a strongly reduced activity whereas I117Q is in the range of wild type. The differences of both mutations can most likely be attributed to their different spatial position. Like Y122S, the affinity of the mutant enzymes for cytochrome *c* remains unchanged, arguing against a function in the initial docking reaction which we showed to be mediated through long range electrostatic forces (6). The lowered electron transfer rate is evidence that in the subsequent step following initial recognition, these residues contribute to the rearrangement of the proteins as a prerequisite for efficient electron transfer. This fine tuning means that hydrophobic residues stabilize an energetically favorable conformation representing a more specific complex than that formed in the initial encounter. The conformational equilibrium between this initial encounter complex and the subsequent electron transfer complex might be described by an equilibrium binding constant, as already postulated in general (28) and for other proteins (29). This constant contributes to the electron transfer rate; assuming that the various mutations alter the equilibrium between the initial encounter complex and the electron transfer complex, this would be reflected in a change of this equilibrium constant thereby reducing the rate constant to different degrees.

Conclusion—We have demonstrated that hydrophobic residues play an important role in the docking reaction between cytochrome-*c* and cytochrome oxidase, in addition to the contribution of charged amino acids. We suggest a two-step model for oxidase, in which long range electrostatic forces (varying with $1/r^2$) dominate the diffusional encounter, followed by a short range hydrophobic interaction for closer apposition (7, 30). Our results on the structure of the interaction domain are in line with the analysis of docking surfaces on electron transfer proteins by Williams *et al.* (31) who proposed "pseudo-specific" surface patches comprised of hydrophobic and charged residues. However, the sequence of events is at variance with their model which implies promiscuity in the primary docking mediated by hydrophobic residues requiring further stabilization by charged residues of a complex efficient in electron transfer (31). The pseudo-specificity would also account for the fact that a single redox protein is capable of reacting with several other partners.

The results presented here together with those from a preceding paper (6) define the docking site for cytochrome *c* on the oxidase. The acidic residues are organized in an extended, negatively charged cluster interspersed with hydrophobic residues (Fig. 3). We also conclude that our results are valid for other oxidases, including the mitochondrial enzyme, where these acidic and hydrophobic residues are either identical or exchanged conservatively.

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