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Review

Interaction of cytochrome *c* with cytochrome oxidase: two different docking scenarios

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Abstract

Cytochrome *c* is the specific and efficient electron transfer mediator between the two last redox complexes of the mitochondrial respiratory chain. Its interaction with both partner proteins, namely cytochrome c_1 (of complex III) and the hydrophilic Cu_A domain (of subunit II of oxidase), is transient, and known to be guided mainly by electrostatic interactions, with a set of acidic residues on the presumed docking site on the Cu_A domain surface and a complementary region of opposite charges exposed on cytochrome *c*. Information from recent structure determinations of oxidases from both mitochondria and bacteria, site-directed mutagenesis approaches, kinetic data obtained from the analysis of isolated soluble modules of interacting redox partners, and computational approaches have yielded new insights into the docking and electron transfer mechanisms. Here, we summarize and discuss recent results obtained from bacterial cytochrome *c* oxidases from both *Paracoccus denitrificans*, in which the primary electrostatic encounter most closely matches the mitochondrial situation, and the *Thermus thermophilus* ba_3 oxidase in which docking and electron transfer is predominantly based on hydrophobic interactions.

Keywords: Electrostatic interaction; Docking complex; Electron transfer; Cu_A center; *Paracoccus denitrificans*; *Thermus thermophilus*

1. Introduction

With four electrons required for a full reaction cycle to reduce dioxygen, cytochrome *c* oxidase needs to interact, in a specific and catalytically efficient way, with its substrate in a series of four individual steps, transferring one electron at a time to the first acceptor, Cu_A, located within an extended hydrophilic domain on subunit (SU) II. The further pathway of electrons within the complex is the transfer to the redox centers located in SU I, heme *a* and the binuclear center heme a_3–Cu_B, accompanied by transmembrane proton pumping events and water formation [1].

The kinetics of interaction of both proteins have long been known to be strongly influenced by ionic strength (I) when assayed spectroscopically (reviewed in Ref. [2]). The resulting bell-shaped activity dependence indicates the formation of a stable complex in which turnover is rate-limited by the cytochrome *c* off-rate at low (I) and by the on-rate at high (I) as the complex is concomitantly destabilized by charge shielding, impeding fast electron transfer (ET) under either condition.

From a series of experiments modifying the mitochondrial cytochrome *c*, specifically in its numerous lysine residues clustered around its heme crevice, by individual charge reversal, by shielding approaches during complex formation, and by chemical cross-linking, an attempt was made to map the interaction domain on this cytochrome for its several physiological partner proteins (for reviews, see Refs. [3–6]). While the importance of the positively charged cluster was beyond doubt, a qualitative analysis revealed roughly the same pattern of lysines to contribute to the docking face to oxidase, to complex III, and others. From this observation, two conclusions became evident: (i) since the interaction face on cytochrome *c* is the same, at least a rotational, or an extended diffusional mobility is

Abbreviations: ET, electron transfer; SU, subunit; (I), ionic strength; TMPD, tetramethyl-*p*-phenylenediamine

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required for cytochrome *c* to mediate ET between complexes III and IV in mitochondria, posing some interesting problems in the context of non-freely diffusing supercomplex-integrated, membrane-anchored, or fused cytochromes *c*; (ii) the general dipole moment, or surface charge potential, may be the crucial criterion for interactions with partner proteins, rather than the individual charge pairs forming upon binding to any given reaction partner (see also Ref. [7]). Experimental support for this concept of a pseudospecific docking has been obtained in several cases recently (e.g. Refs. [8–11] and below). Under many experimental conditions reported, the reaction of cytochrome *c* with oxidase is described by non-hyperbolic steady-state kinetics, resulting in the so-called high- and a low-affinity reaction phases (see below). To account for this observation, various explanations have been given (reviewed in Ref. [12]), such as an additional regulatory site next to the catalytic one, a negative cooperativity regime based on steric hindrance in beef heart oxidase which is purified as a dimer, or by conformational transitions during oxidase cycling.

An alternative interpretation has been suggested by studying the ET kinetics of a caa_3 oxidase complex from *Bacillus subtilis* in which an additional cytochrome *c* domain, homologous with mitochondrial cytochrome *c*, is fused to the C-terminal end of its SU II [13]. In this system, the non-hyperbolic steady-state kinetics appear to be independent of exogenously added cytochrome *c* (but still observed with an artificial reductant such as tetramethyl-*p*-phenylenediamine (TMPD)) and therefore must originate from endogenous oxidase properties, such as changes in the redox kinetics within the metal centers.

With the upcoming of structural information, and a closer look at bacterial systems providing simpler structural models of the complex mitochondrial enzyme and a better genetic accessibility to site-specific modifications in residues of interest, a much more precise definition of docking sites on either partner protein is now feasible. Moreover, a reduction in complexity may be achieved by separating the ET steps at the electron entry site of oxidase from later ET and energy transduction steps by engineering soluble modules such as the Cu_A domain of oxidase.

Here, we review recent approaches to study the interaction of oxidase with its electron donor for two bacterial systems, *Paracoccus denitrificans* and *Thermus thermophilus*. Terminal oxidase structures of both bacteria are known [14–16], as well as those of their interacting cytochrome *c* species or fragments thereof [17–19], but direct structural information on co-complexes is still not at hand. Interestingly, two widely differing modes of interaction seem to be operating, with the *Paracoccus* oxidase closely resembling the mitochondrial ET situation, whereas the *Thermus* couple largely relies on hydrophobic interaction.

2. Lessons from site-directed mutations along the presumed docking site on the *P. denitrificans* aa_3 oxidase complex

2.1. The cytochrome c binding site on heme aa_3 oxidase

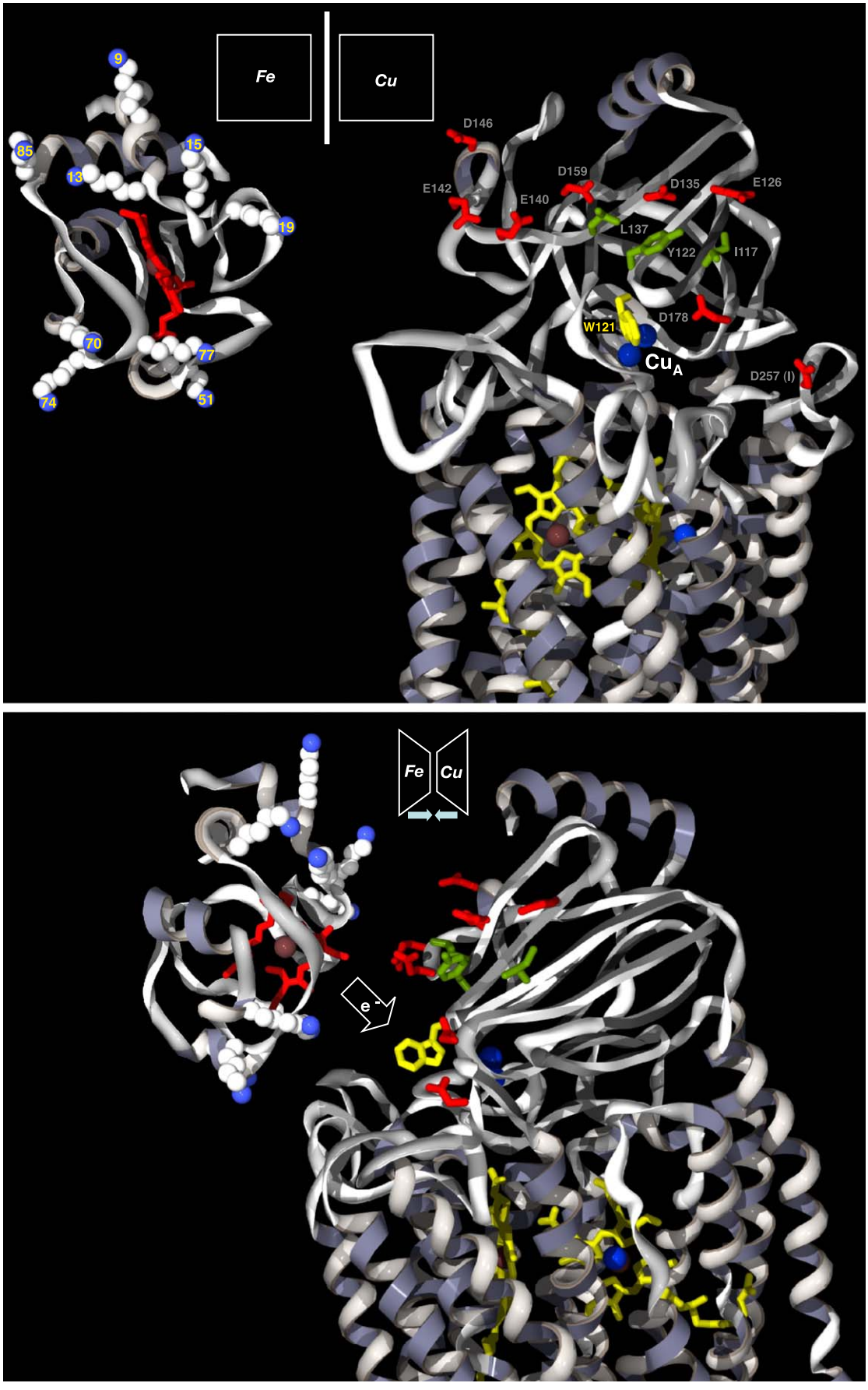
Interaction with cytochrome *c* has long been ascribed to presumed surface-exposed acidic residues [20–22] around the Cu_A center in subunit II, and such charged clusters were later confirmed both for the *Paracoccus* as well as the mitochondrial oxidase structures [14,23].

When individual residues were mutated and their kinetic parameters under turnover and presteady-state conditions analyzed [24,27], shifts of the ionic strength dependency to lower (I) values, increased K_M and, to a varying degree, reduced k_{cat} values were reported. From this it was concluded that an extended area of negatively charged residues on subunit II (E126, D135, D178; and more peripherally E140, E142, D146, D159; *Paracoccus* numbering throughout; see Fig. 1A) plays a major role in the initial docking step between cytochrome *c* and oxidase. This docking area includes the four homologous acidic residues regarded most important for the *Rhodobacter sphaeroides* heme aa_3 oxidase as well [25]. Mutagenesis data for the *Paracoccus* enzyme also suggested a contribution of acidic residues on subunit I and III [24] which was in part confirmed by a shift in the ionic strength dependency for the two-subunit oxidase complex ([27], and see below).

2.2. Hydrophobic interactions are important for fine-tuning redox-partner interactions

To investigate the role of hydrophobic side chains on the surface of subunit II we mutated exposed residues (W121, Y122, I117, L137; see Fig. 1A). While K_M values for these mutants were largely unchanged, k_{cat} values were diminished, pointing out that these conserved residues acting via short-range hydrophobic forces are important for optimizing the orientation of both redox partners [10]. The encounter of both proteins may thus be described by a two-step model, as also suggested for other interprotein electron transfer complexes involving cytochrome *c*: initially, long-range interactions driven by the electrostatic surface potential preorient both reaction partners, and are followed by hydrophobic interactions to rearrange both components for a more productive ET complex.

Recently, structures of two membrane proteins in a co-complex with cytochrome *c* have been solved. Cytochrome c_2 and the reaction center from *R. sphaeroides* [11] are believed to primarily interact via regions of opposite charge, with most of the ionizable side chains around the central contact region in the crystal complex separated too far apart (more than 5 Å) to establish salt bridges. Short-range contacts at the interfaces of this complex are dominated by apolar interactions, mediated by van der Waals' contacts and a π-cation interaction. Thus it appears that by avoiding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salt bridges in the ET complex, the transient nature is efficiently maintained during interaction, supporting high electron transfer rates. A similar conclusion was drawn from the *Saccharomyces cerevisiae* bc_1–cytochrome *c* complex. Again, residues which are involved in this electron transfer couple are mainly non-polar side chains, forming only a small compact interaction site [26].

2.3. The electron entry point: tryptophan 121

Mutagenesis studies on the *Paracoccus* and *R. sphaeroides* enzyme indicated that the surface-exposed tryptophan 121 on subunit II [10,25,27] is essential for electron transfer from cytochrome *c*. This tryptophan residue is located approx. 5 Å above the Cu_A center. Extensive amino acid replacements in W121 and the neighbouring Y122 showed that W121 is the only electron entry point, strictly requiring a tryptophan, possibly for steric reasons, in this position which cannot even be substituted by other aromatic side chains [27].

3. The genuine electron donor: site-specificity and surface charge requirements for efficient interaction with the *P. denitrificans* oxidase

Kinetic experiments with cytochrome *c* oxidase from *P. denitrificans* were performed mainly with horse heart cytochrome *c* in the past, since the homologous electron donor had not been identified with certainty. Subsequently, several lines of evidence lead to the conclusion that a membrane-bound cytochrome termed c_{552} is the genuine electron donor for the aa_3 oxidase. This is supported by the finding that (i) it can be purified as an integral component of a bc_1/aa_3 oxidase supercomplex [28] with high electron transfer capacity, (ii) it is an obligatory mediator for NADH oxidation in intact membranes as shown by antibody inhibition and gene deletion [29,32], and (iii) that it is three times more efficient than cytochrome c_{550} [30] to deliver electrons to the soluble Cu_A domain (Maneg et al., 2003).

The heme-containing domain of cytochrome c_{552}, consisting of 100 amino acids, became available as a soluble protein expressed in *E. coli* [31] and the structure could be solved by X-ray [18] and NMR spectroscopy in both redox states [19]. This c_{552} fragment reveals all typical features of a class I *c*-type cytochrome, with a cluster of nine lysines surrounding its heme crevice [18]. We analyzed the electron donor properties of three different modules of this cytochrome: the minimal unit comprising 100 amino acids, a fragment with additional 40 amino acids of high polarity [32], and the full-size protein with its N-terminal membrane anchor [33].

In steady-state kinetics with the *Paracoccus* wild-type oxidase, each of the three electron donors showed a distinctly different pH optimum, and a severely lowered ionic strength optimum when compared to the mitochondrial cytochrome *c* [33]. Despite a much less pronounced strength in surface potential, the K_M values of the three c_{552} modules were determined to be in the low micromolar range, and k_{cat} values approached 1000 s^{-1} for the smallest fragment, making it a fully competent electron donor for oxidase.

Using a set of specific docking mutants in oxidase SU II (see above) for kinetic analysis, we were able to map the interaction domain for this 100-amino acid fragment of cytochrome c_{552}, and compare it to that of the horse heart donor. Both docking sites on oxidase share a central area, with some specific differences in peripheral regions [33]. To investigate whether the overall surface potential or specific localized charges on cytochrome c_{552} are responsible for interaction, we mutated eight lysines around the heme crevice in the minimum fragment, and determined kinetic parameters using the wild-type *P. denitrificans* oxidase [33]. Interestingly all lysine mutations showed a clear increase in K_M and a considerable decrease in k_{cat} when compared with the wild-type substrate. This uniform behaviour of all mutants indicated that it is more the general surface potential that drives the association of the cytochrome electron donor than individual, localized charges [7]. This finding contradicts earlier results from chemical modification and site-directed mutagenesis studies with horse heart cytochrome *c* which indicated pronounced effects for specific lysine residues [5,34].

4. Kinetics of interaction between soluble modules derived from the *P. denitrificans* partner proteins

To gain direct access to the initial electron transfer reaction, without spectroscopic and functional interference from other redox groups and energy transduction processes, we used the soluble Cu_A domain of subunit II in stopped-flow experiments with the smallest c_{552} module. As pioneered by Lappalainen [35] the *P. denitrificans* Cu_A protein was heterologously expressed in *E. coli*, reconstituted from inclusion bodies and metal insertion was performed. These fast kinetics experiments demonstrated that the reaction between the c_{552} and the Cu_A domain is diffusion controlled, as the apparent bimolecular rate constants exceed 10^7 $M^{-1}s^{-1}$ (extrapolated to I = 0) in either electron transfer direction (Maneg et al., 2003). On varying (I) of the buffer

Fig. 1. Presumed docking sites on the *P. denitrificans* aa_3 oxidase and its electron donor, cytochrome c_{552}, based on the coordinates for the oxidase complex (PDB: 1AR1) and the soluble fragment of c_{552} (PDB: 1I6D) and presented using the Swiss PDB Viewer program [54] and POVRay rendering [55]. (A) Docking sites of both proteins facing the viewer. Surface-exposed lysine residues clustered around the heme crevice of c_{552} are highlighted and numbered. Exposed acidic (red) and hydrophobic (green) residues relevant for cytochrome *c* interaction are specified on the solvent-exposed surface of subunits I and II; W121 (yellow) represents the electron entry site above the Cu_A center (blue spheres) in subunit II. Further redox centers within the transmembrane section of subunit I are colour-coded: hemes *a* and a_3, yellow; Cu_B, blue. (B) Each protein is rotated by 90° for their interaction sites to face each other. Relative positions and distance between the two partner proteins are arbitrary, and are solely meant to illustrate the electrostatically driven process during encounter.

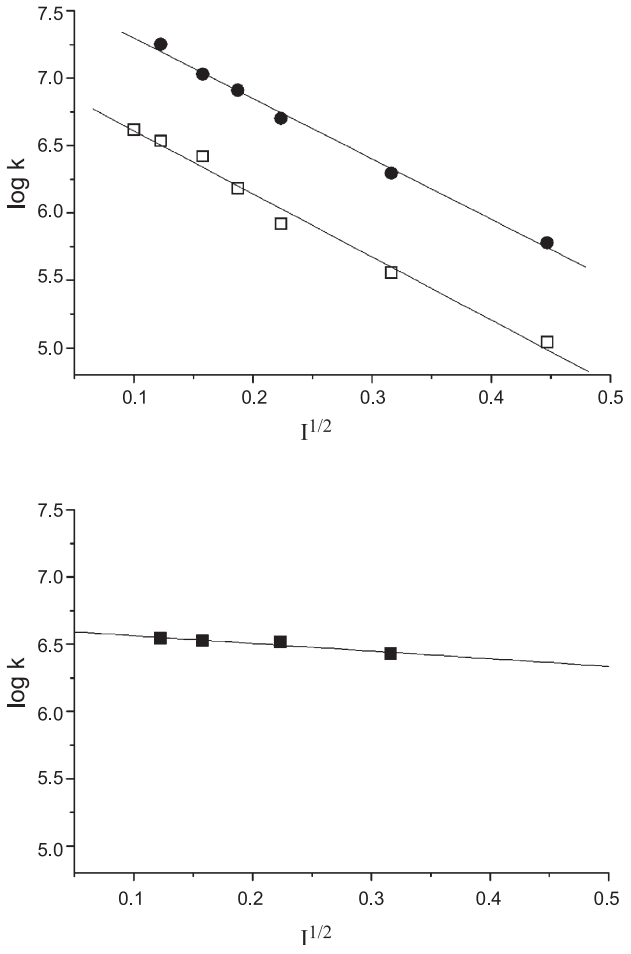

Fig. 2. Ionic strength dependencies of the electron transfer kinetics (Brønsted plot) for the cytochrome c_{552}–Cu_A couple from *P. denitrificans* (A) in the physiological (□) and reverse direction (●) and *T. thermophilus* (B). The logarithms of the apparent bimolecular rate constants are plotted versus the square root of the ionic strength. From the slope the product of interacting charges ($z_a.z_b$) is determined.

the association process of this electron transfer reaction was further investigated and represented in a Brønsted diagram (Fig. 2), where the logarithm of the bimolecular rate constant is plotted against the square root of (I). This indicates that two to three effective charges on each protein are involved in the interaction. This raises the question of which residues are involved in direct contacts upon complex formation between both partner proteins.

5. Interactions between redox protein fragments as observed by solution NMR

Attempts to map docking site structures of interacting electron transfer proteins by co-crystallization or in solution have been numerous, e.g. Refs. [8,11,36–40,42]. The first structure of an interprotein electron transfer complex [8], obtained by co-crystallization of yeast iso-1-cytochrome *c* with cytochrome *c* peroxidase revealed only a small contact area formed by hydrophobic and van der Waals' interactions. One hydrogen bond and two salt bridges were identified, though on both proteins, charged residues of opposite sign are abundant. The same holds true for two recently solved docking structures involving membrane protein complexes (see above).

To meet the criticism of a 'static' view gained from the co-crystals and to acknowledge the dynamic nature of such a complex, redox-protein interactions have been studied with NMR solution spectroscopy by chemical shift perturbation mapping, using isotopically enriched ^{15}N cytochrome *c* [40,42]. From this technique, no direct side chain information is gained, but the affected chemical shifts of respective amide protons give information about the amino acids influenced upon binding. In a recent work on the interaction of ^{15}N-labeled cytochrome c_{552} with the isolated Cu_A domain from *P. denitrificans* [41], a cluster of non-charged amino acid residues around the heme cleft was identified to experience the strongest chemical shift perturbations.

Only one of the nine lysine residues found to be responsible for the electrostatic long-range interaction seems to be involved in these direct contacts. In the reduced and the oxidized form of the protein similar amino acids are affected, suggesting that the docking complex is not dependent on the redox state, nor do the individual solution structures differ in their redox states [19].

In a NMR study involving the ferric and the ferrous form of ^{15}N-labeled yeast iso-1-cytochrome *c* and peroxidase, a patch of hydrophobic and several polar residues was identified around the heme cleft [40]. Overall, the residues identified to be involved in the contact interface are the same for both redox-states, although the size of the differences in the chemical shifts varies and in the ferric form more amide protons are affected. For this reaction it has been suggested that electrostatic interactions stabilize an ensemble of several 'loose' complexes, enhancing the probability for an electron transfer event.

In an analogous analysis, the interaction of ^{15}N-labeled plastocyanin with its physiological acceptor cytochrome *f* from the thermophilic, photosynthetic cyanobacterium *Phormidium laminosum* was studied [42]. Again a hydrophobic contact area surrounding an exposed histidine ligand (experiencing the largest chemical shifts) of the copper atom, on the plastocyanin surface was identified in the interaction with the cytochrome. Complex formation is unaffected by increasing salt conditions, confirming the hydrophobic character of this interaction and the lack of electrostatic contributions in this complex formation. A similar situation is encountered for the interaction of the ba_3 cytochrome *c* oxidase from the extremely thermophilic eubacterium *T. thermophilus* with its substrate, see below.

6. Computational approaches to complex formation

Using the published coordinates of the *P. denitrificans* cytochrome c_{552} fragment and both the 4-SU and the 2-SU

oxidase complex (see below), docking programs were applied in a two-step approach (i) to account for the mutual complementarity of interacting protein surfaces, and (ii) to energy-minimize interprotein interactions, based on selected, experimentally determined input parameters obtained from mutagenesis studies (see above). Hypothetical complexes were further screened for their cytochrome heme edge to tryptophan-121 (SU II) ET distance, yielding two suggestions differing clearly in heme orientation for the two oxidase structures considered. Both feature three ion charge pairs (lysine/carboxylate side chains) [43], matching the Brønsted plot data (see Fig. 2).

In a similar study [44], possible interaction surfaces between beef heart oxidase and horse heart cytochrome *c* were calculated by a comprehensive search routine: again it provides evidence for a salt bridge-stabilized complex, primarily involving two lysine residues on the electron donor molecule, and a central hydrophobic region surrounding the heme cleft. This prediction is consistent with results from site-directed mutagenesis, steady-state and rapid kinetics using bacterial oxidases and horse heart cytochrome *c*, e.g. Refs. [25,27].

7. The *T. thermophilus* ba_3 oxidase—an alternative mode of interaction

In the respiratory chain of *T. thermophilus*, a different situation is encountered for the interaction of the ba_3 cytochrome *c* oxidase and its electron donor, a soluble cytochrome identified as c_{552}.

Almost no charged residues are found in the presumed interaction interfaces as shown by the crystal structures of both proteins [16,17,45], rendering this interaction mainly hydrophobic.

The Brønsted plot for the electron transfer reaction between cytochrome c_{552} [46] and the Cu_A fragment [47] from *T. thermophilus* strongly supports this idea: from the slope, almost independent of ionic strength, a value of only 0.57 for the product of interacting charges is determined (Fig. 2B), indicating that less than one charge on each protein interface is involved in the reaction. This points at the strong hydrophobic character of the interaction mode in the thermophilic system, which should be an advantage at high temperatures when electrostatic interactions are weakened under these conditions.

8. The enigmatic non-linear kinetic behaviour of cytochrome *c* oxidation

An intriguing and reproducible experimental finding, since the initial observation [48–50], is still lacking a more definitive explanation, namely the non-hyperbolic steady-state kinetic behaviour of cytochrome *c* oxidase as discussed above. Deviation from the Michaelis–Menten law has been empirically described by two steady-state cytochrome *c*-dependent kinetic regimes: one with low apparent K_M and k_{cat} values (the so-called high-affinity low-turnover phase) and a second with numerically higher Michaelis–Menten parameters (the so-called low-affinity high-turnover phase). Several models have been proposed to account for the observed non-hyperbolic steady-state behaviour of cytochrome oxidase (see Introduction).

Steady-state cytochrome oxidase activity is usually measured according to two different experimental protocols, both of which use very low oxidase concentrations (nM) as required to achieve experimental conditions which allow to determine accurate initial velocity data. In the first protocol, cytochrome *c* oxidase is mixed with ferrocytochrome *c* (obtained following reduction with dithionite and removal of the excess reductant) and the turnover followed at appropriate wavelengths (generally 550 nm). In this type of experiment ferrocytochrome *c* is continuously oxidized by the oxidase, provided that excess dioxygen is present, and the initial rate determined from the extrapolated slope of the time course at time zero. In this protocol ferricytochrome *c* continuously accumulates during the assay. In the second protocol, dioxygen consumption is monitored polarographically. In this assay, the ferro- and ferricytochrome *c* concentrations are constant in the steady-state time window since an artificial reducing system (usually TMPD-ascorbate) is added to the reaction mixture which regenerates ferrocytochrome *c* oxidized by cytochrome oxidase. In both experimental protocols the extrapolated initial rates (v_c or v_{O_2}) display a non-hyperbolic ferrocytochrome *c* concentration dependence and due to the stoichiometry of the cytochrome *c* oxidase reaction it follows and is demonstrated that $v_c = 4v_{O_2}$. The observed experimental data may be described by the empirical equation [50]:

$$v = \frac{k_{cat1}E_0[S]}{K_{M1} + [S]} + \frac{k_{cat2}E_0[S]}{K_{M2} + [S]}$$

where the k_{cat} and K_M values represent the apparent catalytic efficiencies and affinities for the high and low affinity kinetic steady-state phases, and E_0 the total cytochrome oxidase concentration. Although this equation indeed describes the experimental data, it lacks a physical explanation accounting for the observed deviation from the Michaelis–Menten equation.

The interaction of cytochrome *c* with the oxidase is clearly electrostatic as discussed above. However, several experimental situations [27] yield or modulate conditions for observing hyperbolic kinetics including: (i) high ionic strength, which has long been known to affect both pre-steady-state and steady-state behaviour [48,51,52]; (ii) subunit composition, addressing the question of a potential second binding site for cytochrome *c*; (iii) charged-to-neutral subunit II amino acid replacements; and (iv) binding of an F_v antibody fragment directed to an epitope on subunit II opposite to the cytochrome *c* docking site [27].

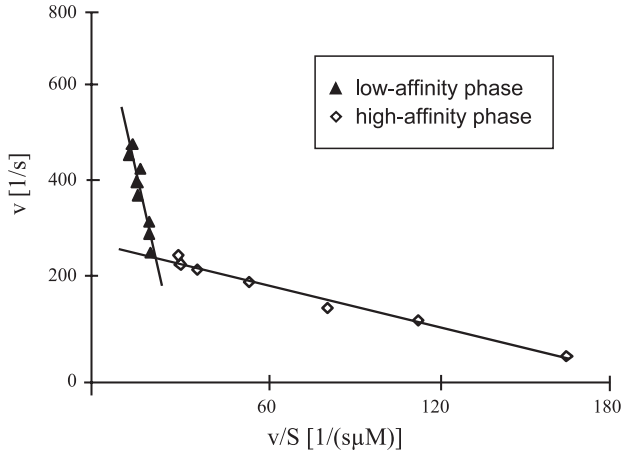

Fig. 3. Eadie–Hofstee plot representing horse heart cytochrome *c* oxidation by *Paracoccus* oxidase at low ionic strength (36 mM), experimental conditions as in Ref. [27].

Nevertheless, all the above artificially induced "hyperbolic" experimental situations do change to a non-hyperbolic behaviour if only the ionic strength is sufficiently decreased (see biphasic kinetics presented in an Eadie–Hofstee plot, Fig. 3). This discussion suggests, in line with the observations of Ref. [13] that the non-hyperbolic steady-state kinetics must arise from properties intrinsic to the protein which include only one cytochrome *c* binding site on the oxidase and ionic strength and cytochrome *c*-dependent shifts in the population of oxidase turnover intermediates. Simulations carried out by numerical integration and steady-state approximation of a "bona fide" cytochrome oxidase mechanism (F. Malatesta, A. Giuffrè, P. Sarti, O. Maneg, B. Ludwig, M. Brunori, manuscript in preparation) indeed show that non-hyperbolic steady-state kinetics may be obtained if the O-to-E or O-to-R kinetic transitions rate limit the subsequent O_2 reaction and the ensuing population of P and F intermediates of the cytochrome oxidase turnover cycle [52,53].

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