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Binding and oxidation of mutant cytochromes *c* by cytochrome-*c* oxidase

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Mutation of conserved Phe-82 of yeast iso-1 cytochrome *c* to Tyr, Gly, Ser, Leu, or Ile affects binding to and reaction with cytochrome-*c* oxidase from beef heart. The observed changes of binding and kinetic constants reflect mutation-induced rearrangements in the heme vicinity brought about by the replacement of Phe-82. Such conformational rearrangements are also revealed by altered circular dichroism spectra of the oxidase-bound mutant cytochromes *c*. Variations in K_m for cytochrome *c* oxidation do not parallel variations in K_d, the dissociation constant for binding of cytochrome *c* to the oxidase. This observation does not support an enzymatic mechanism in which the rate of cytochrome *c* oxidation is governed by product dissociation.

Cytochrome-*c* oxidase; Site-specific mutagenesis; Steady-state kinetics; CD; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

Invariant Phe-82 of mitochondrial cytochrome *c* has been proposed to be involved in the electron transfer function of cytochrome *c* [1] and in controlling the polarity of the heme environment [2]. To assess the functional role of this residue, Phe-82 of yeast iso-1 cytochrome *c* was replaced by Tyr, Gly, Ser, Leu, and Ile through site-specific mutation [3,4]. These substitutions variably affect the reduction potential of the heme iron [3], the pH-dependent ligation state of the heme iron [5], the polypeptide conformation near the heme [6], and the reaction kinetics with cytochrome-*c* peroxidase [3,7]. Here we report on the equilibrium binding and oxidation of the five cytochrome *c* mutants by cytochrome-*c* oxidase from beef heart.

2. MATERIALS AND METHODS

Yeast iso-1 cytochrome *c* mutants were generated and purified as before [3,4]. In addition to the substitution at position 82, Cys-102 was replaced by Thr. This substitution does not alter the kinetic and spectroscopic properties of the protein [4,7]. Iso-1 cytochrome *c* with Thr-102 was used as the wild-type reference protein. Beef heart cytochrome-*c* oxidase was prepared as in [8]. Binding of cytochrome *c* to the oxidase was measured by spectrophotometric titration of the enzyme with cytochrome *c* [8]. The oxidation of cytochrome *c* was followed spectroscopically and the kinetic parameters deduced by numerical analysis of initial rate assays [8]. Difference circular dichroism (CD) spectra were measured as in [9]. Conditions for initial rate assays were 10 mM *N,N*-bis(2-hydroxyethyl)amino tris(hydroxymethyl)methane-HCl (bistris), pH 6.2, ionic strength 49 mM with NaCl, 0.05% (w/v) laurylmaltoside, 20°C. Optical binding mesurements and CD spectra were done in 10 mM Tris-HCl, pH 7.4, ionic strength 24 mM (with NaCl), 0.05% (w/v) laurylmaltoside, 20°C. Concentrations of heme *c* and heme aa_3 were determined as in [8].

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3. RESULTS AND DISCUSSION

3.1. *Binding of mutant cytochromes* c *to cytochrome-*c *oxidase*

Replacing Phe-82 by Tyr changes the dissociation constant, K_d, of the cytochrome *c*-cytochrome-*c* oxidase complex minimally. Removal of the side chain by substitution of a Gly residue increases the affinity 3.5-fold. Small changes are observed for the Ser and Leu mutants.

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Surprisingly, the Ile-82 mutant binds to the oxidase 50 × more strongly (table 1). These observations can be explained by assuming that the nature of residue 82 does not affect binding in a direct way but rather through different mutation-induced conformational rearrangements in the area of the solvent exposed heme edge where cytochrome-*c* oxidase binds. Indeed, replacement by Tyr alters the conformation of the reduced cytochrome minimally (Brayer, G.D., personal communication). On the other hand, serine at position 82 substantially increases the solvent accessibility of the heme group in ferrocytochrome *c* and induces conformational changes remote from residue 82 [6]. In the reduced Gly mutant, the polypeptide chain between residues 79 and 85 collapses toward the heme, closing the gap introduced by the loss of the aromatic side chain in position 82 (Louie, G.V. and Brayer, G.D., in preparation). The structural alterations in the Leu and the Ile mutants are not yet known, but it seems reasonable to assume that the much stronger binding of the Ile mutant is not due to the small change in side chain structure alone. As in a previous study [8], a second binding site for cytochrome *c* on cytochrome-*c* oxidase could not be detected (not shown).

3.2. *CD spectroscopy of the cytochrome* c-*cytochrome-*c *oxidase complex*

Binding of horse cytochrome *c* to beef oxidase induces a conformational change of the polypeptide chain, presumably around the exposed heme edge [9,10]. This change was inferred from a change in the CD spectrum of heme *c* when cytochrome *c* bound the oxidase. Fig.1 shows that this CD change in the Soret region is significantly smaller for the Ser, Leu, and Ile mutants. (The variation in the CD difference signal was not caused by incomplete binding of cytochrome *c* mutants to the oxidase, because the total concentration of heme aa_3 and cytochrome *c* was at least 15 × K_d.) It had been speculated that the CD change could reflect a movement of Phe-82 toward cytochrome-*c* oxidase [10], which in free cytochrome *c* is adjacent and almost parallel to the porphyrin plane. The lower CD differences in fig.1 are compatible with this explanation, except in the case of the Gly mutant which exhibits a large blue-shifted difference CD signal. There is an alternative explanation for the changed CD spectra of the mutant proteins. At pH 7.4 the mutants with a non-aromatic residue partially occupy the alkaline conformation state [5,11], which is characterized by a more intense positive CD band in the Soret region [12]. On binding to the oxidase, the mutant proteins could be forced to a native conformational state with a less intense CD signal in the Soret region. This, in turn, would yield a smaller difference CD signal between bound and free cytochrome *c*, as observed (fig.1). Whatever the correct explanation, the present data support a direct link between the CD change and the conformation of the polypeptide chain in the immediate vicinity of heme *c*.

Table 1

Oxidation of reduced cytochrome *c* by cytochrome-*c* oxidase and binding of oxidized cytochrome *c* to cytochrome-*c* oxidase

Cytochrome *c*	K_{m1} (10^6 M)	TN_{max1} (s^{-1})	TN_{max1}/K_{m1} (10^6 $M^{-1} \cdot s^{-1}$)	K_{m2} (10^6 M)	TN_{max2} (s^{-1})	RMS[a]	K_d (10^6 M)	% noise[b]
Yeast wild-type	0.206	37.5	182	30	170	0.75	0.14	1.1
Tyr-82	0.117	16.0	137	13	53	0.51	0.17	2.0
Gly-82	0.640	46.5	72.7	17	100	1.69	0.04	4.2
Ser-82	0.362	4.49	12.4	310	1200	0.71	0.30	1.5
Leu-82	0.547	61.4	112	100	170	1.85	0.04	1.2
Ile-82	0.622	27.1	43.6	190	60	0.47	0.003	1.6
Horse[c]	1.90	334	176	–	–	7.4	0.07	1.2

[a] Root mean squares of best fit to $d[c]_f/dt \cdot [aa_3]_f = TN_{max1}[c]_f/(K_{m1} + [c]_f) + TN_{max2}[c]_f/(K_{m2} + [c]_f)$ [8]

[b] Noise of absorption difference in the spectroscopic binding titration from which K_d was calculated [8]

[c] Values from [8], no second kinetic phase observed with horse cytochrome *c* under the conditions used (see section 2)

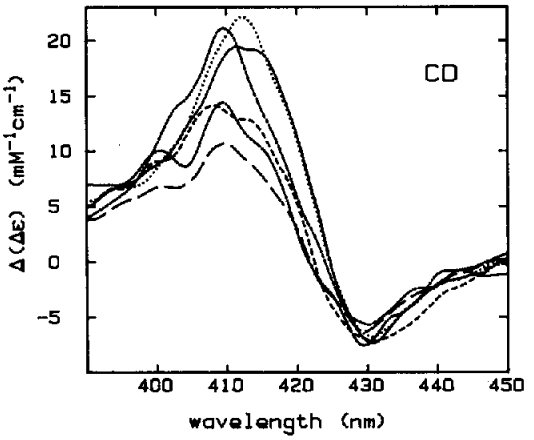

Fig.1. Binding of cytochrome *c* to cytochrome-*c* oxidase followed by circular dichroism. The spectra shown are difference spectra obtained by subtracting the spectra of free cytochrome *c* plus free cytochrome-*c* oxidase from the spectrum of the cytochrome *c*-cytochrome-*c* oxidase complex. Yeast iso-1 wild type (——), Tyr-82 mutant (·····), Gly-82 mutant (– – –), Ser-82 mutant (– – –), Leu-82 mutant (——), Ile-82 mutant (– – –).

3.3. *Cytochrome* c *oxidation kinetics*

Steady-state oxidation of the yeast cytochrome *c* variants is characterized by two kinetic phases at low (phase 1) and high (phase 2) cytochrome *c* concentration. This behavior is well known for many different cytochromes *c* ([8] and references therein). Differences between mutant cytochromes could be accurately measured for phase 1 (table 1). The second order rate constant of cytochrome *c* oxidation, TN_{max1}/K_{m1}, decreases in the order wild-type > Tyr > Leu > Gly > Ile > Ser mutant.

Clearly, replacement of Phe-82 by a non-aromatic residue does not eliminate the steady-state oxidation of the cytochrome by cytochrome-*c* oxidase. A similar observation was made for the reaction with cytochrome-*c* peroxidase, though in this case the order of reactivity was wild-type > Ser > Tyr > Gly. This difference in reactivity pattern may arise from subtle changes in the mode of interaction of cytochrome *c* with the two heme enzymes. Several productive modes of interaction between cytochrome *c* and peroxidase seem possible [13], so the observed oxidation rate may correspond to the average of several rates arising from different modes of interaction. Presumably, mutation of Phe-82 could alter the distribution among the various modes of interaction. Low rates of oxidation cannot be accounted for by perturbation of the cytochrome reduction potential as mutations at position 82 either have no effect on the potential or they lower it ≤50 mV [3]. A lower redox potential should increase the rate rather than lower it.

Correlation of the steady-state kinetic results with those obtained by Liang et al. [7] is currently not possible. In the latter study, it has been found that the rate of intramolecular electron transfer from ferrocytochrome *c* to Zn-cytochrome-*c* peroxidase porphyrin π-cation radical within the electrostatically-stabilized complex formed by the two proteins at low ionic strength is 10^4 times slower with a non-aromatic residue at position 82.

The mechanism of steady-state oxidation of cytochrome *c* by cytochrome-*c* oxidase is not clear (review in [8]). In one proposal, the two kinetic phases are believed to arise from binding of cytochrome *c* to a catalytic site and a regulatory site, respectively, with binding to the regulatory site increasing the rate of product dissociation from the catalytic site [14,15]. In this mechanism, K_{m1} must be proportional to the product dissociation constant and, hence, to K_d. The data in table 1 do not support this prediction. In a conformational transition mechanism the two parameters would not be related in a direct way [8,16].

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