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Biochimica et Biophysica Acta 1230 (1995) 74–76

BBA Biochimica et Biophysica Acta

Rapid Report

Site-directed mutagenesis of cytochrome *c* oxidase reveals two acidic residues involved in the binding of cytochrome *c*

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Received 9 February 1995; revised 9 March 1995; accepted 28 March 1995

Abstract

Site-directed mutagenesis in subunit II of the cytochrome *c* oxidase (haem aa_3) from *Paracoccus denitrificans* reveals that two carboxylic residues, Glu-246 and Asp-206 (corresponding to 198 and 158 in the bovine subunit II), are involved in the binding of cytochrome *c*. Spectrophotometric and polarographic measurements with the isolated enzymes of both mutant strains show a strongly reduced activity compared to wild-type oxidase, with the overall catalytic capacity (k_{cat}/K_M) of both mutants decreased about 8-fold. EPR spectra reveal no significant differences between the wild-type and the mutant enzymes, indicating that neither residue contributes significantly to the structure of the Cu_A centre. We conclude that Glu-246 and Asp-206 constitute an essential part of the binding site for cytochrome *c*.

Keywords: Electron transfer; Electrostatic interaction; Respiratory chain; Steady-state kinetics; (*P. denitrificans*)

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal enzyme of the respiratory chain of mitochondria and many prokaryotes (for reviews see [1–3]). In the soil bacterium *Paracoccus denitrificans*, it is composed of three subunits which are related to the mitochondrially encoded subunits of the eukaryotic enzyme [3,4]. While haem *a* and the binuclear centre (haem a_3, copper B) reside in subunit I, subunit II carries the Cu_A site which presumably consists of two copper ions [5,6] and is believed to act as the entry point of electrons from cytochrome *c* [7].

Several studies identified subunit II of the oxidase as the binding site for cytochrome *c* [8–11]. Investigations of the catalytic centre where cytochrome *c* is supposed to donate electrons to the Cu_A site suggested the involvement of several carboxylate groups. Three such residues in the bovine heart enzyme (one of them corresponding to Glu-246 in *P. denitrificans* [1]) have been shown to be protected by cytochrome *c* against modification using a water-soluble carbodiimide [12]. Studies with the mammalian enzyme, using arylazidocytochrome *c*, indicated the involvement of an aspartate (D206 in *P. denitrificans*) in the binding of cytochrome *c* [13]. E246 and D206 are conserved among several species and are of particular interest due to their close vicinity to the putative copper ligands.

Interaction studies focussing on specific residues of the cytochrome *c* molecule used modified cytochromes *c*; experiments indicated that several lysine residues surrounding the haem are involved in binding to cytochrome *c* oxidase and other redox enzymes [14–16].

Here, we use the technique of site-directed mutagenesis on the prokaryotic cytochrome *c* oxidase to identify the role of two acidic residues in subunit II for binding cytochrome *c*. The oxidase from *P. denitrificans* has been characterized in great detail [3,4] and is easily accessible for gene deletion and mutagenesis studies [17,18]. In addition, it has long been known to interact efficiently with mammalian cytochrome *c* which we use as electron donor for the kinetic measurements. With the genuine electron mediator between complex III and oxidase in *P. denitrificans*, believed to be a membrane-bound cytochrome (*c*-552; see [19]; Turba, Jetzek and Ludwig, unpublished data), not available in reasonable amounts for kinetic measurements, the horse-heart cytochrome *c* has been used widely as a heterologous substitute providing turnover rates even supe-

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[1] If not stated otherwise, all residue numbering refers to *P. denitrificans* according to [27]

SSDI 0005-2728(95)00050-X

rior to the soluble cytochrome *c*-550 isolated from *P. denitrificans* [20,21].

In two individual mutations (E246Q and D206N) we analyze the steady-state kinetics of the isolated enzymes with horse-heart cytochrome *c* in the spectrophotometric and polarographic assay. We find K_M and k_{cat} to be affected by each mutation, resulting in a decreased overall catalytic efficiency (about eightfold) for both mutants.

Strains: *Paracoccus denitrificans* ST4 [22] carries a deletion in the *cta* operon and completely lacks expression of cytochrome aa_3. This strain is complemented in trans (as described earlier; [23]) by the *cta* operon conveying either the mutation E246Q or D206N. For enzyme isolation, the two mutant strains are grown on succinate medium [24] including streptomycin sulfate (25 μg/ml). To avoid interference of bound manganese in the EPR measurements [25], the final concentration of manganese is lowered to 0.5 μM in the growth medium.

Site-directed mutagenesis is done according to the altered sites mutagenesis protocol (Promega, Heidelberg).

Enzyme preparation: Membranes are isolated according to [23]. Cytochrome *c* oxidase is solubilized using dodecyl β-D-maltoside and purified as described earlier [26]. Protein determination, SDS PAGE, Western blotting and cytochrome *c* oxidase difference spectra were performed according to [22–24,27].

EPR spectra: X-band EPR spectra are recorded on a Bruker ER 200D spectrometer; *atomic absorption measurements* were performed on a Perkin-Elmer AAS 2380.

Steady-state kinetics of cytochrome c and cytochrome oxidase: Cytochrome oxidase activity is assayed at 25° C in 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA and 0.2 g/l dodecyl maltoside. The spectrophotometric assay is performed using a Kontron Uvikon 941 photometer. The reaction is started by the addition of the purified enzyme (40 pM), and the oxidation of reduced cytochrome *c* is followed at 550 nm. The polarographic assay is performed in the same buffer supplemented with sodium ascorbate and TMPD to a final concentration of 10 mM and 100 μM, respectively. After addition of the enzyme (40 nM) the oxygen consumption in the presence of various cytochrome *c* concentrations is followed. In both assays the cytochrome *c* concentration is varied between 0.5 and 80 μM, and the reaction rates are presented as turnover numbers for cytochrome *c* (s^{-1}).

Chemicals: Horse-heart cytochrome *c* and TMPD are obtained from Sigma (Deisenhofen), ascorbate from Merck (Darmstadt) and dodecyl maltoside from Biomol (Hamburg).

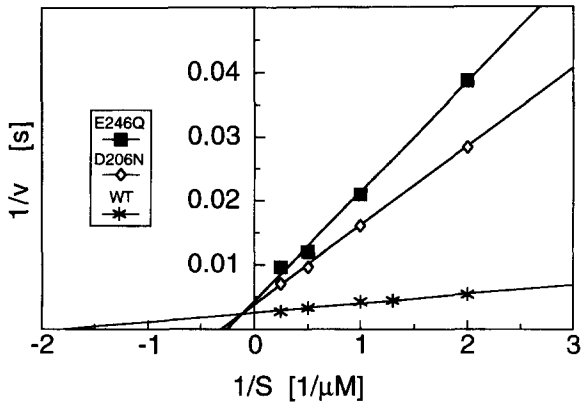

Fig. 1. Lineweaver-Burk plot of the spectrophotometrically determined high affinity phase comparing wild type (WT) and mutants (for details, see text).

In this study, two independent mutants in subunit II of cytochrome *c* oxidase from *P. denitrificans* are constructed by site-directed mutagenesis. Glutamate 246 is changed to glutamine and aspartate 206 to asparagine. Using reduced horse-heart cytochrome *c* (see above), steady-state kinetics are measured spectrophotometrically.

Commonly, analysis of kinetic data on the interaction between cytochrome *c* and oxidase reveals non-linear behaviour in a Lineweaver-Burk plot, leading to the definition of two pairs of K_M and k_{cat} values, which describe a high- and a low-affinity phase of the reaction (e.g., [28–30]). We observe the same general kinetic pattern in our present study with the isolated *P. denitrificans* enzymes.

When compared to kinetic parameters of the wild type enzyme (see Table 1 and Fig. 1), both mutations clearly affect the interaction with cytochrome *c*. This is reflected most significantly by the increase of the K_M value (about 4- to 5-fold) for the high-affinity phase in both mutants. Taking the Michaelis constant as a measure of the affinity of the enzyme for its substrate, the increased values of the mutants indicate a decreased affinity for cytochrome *c* at the catalytic centre. In contrast, the K_M of the low-affinity phase remains essentially unchanged (see Table 1).

However, both turnover numbers of the mutated enzymes are affected as well, although to a lesser extent (see Table 1), confirmed also by the polarographic assay (data not shown). So we obviously face a more complex situa-

Table 1
Kinetic parameters for the purified enzymes in the spectrophotometric assay for the high (K_{M1}, k_{cat1}) and the low (K_{M2}, k_{cat2}) affinity phases

	K_{M1} (μM)	k_{cat1} (s^{-1})	k_{cat1}/K_{M1}	K_{M2} (μM)	k_{cat2} (s^{-1})
Wild type	0.59	363	615	5.3	543
E246Q	2.5	200	80	6.7	299
D206N	2.8	247	88	8.2	317

tion where not only the binding of substrate, reflected by the K_M value, is impaired (see below).

In order to exclude effects of the mutations on the integrity of the Cu_A centre leading to an impeded electron transfer, EPR spectra are recorded for the isolated wild type and mutant enzymes. They reveal no remarkable differences for the mutants in comparison to the wild type (data not shown). Furthermore, quantitation of the copper content in the oxidase preparations by atomic absorption spectroscopy (wild type, 2.9; D206N, 3.2; E246Q, 2.8 mol/mol aa_3) suggests that both amino acids have no structural impact on the Cu_A centre.

With the copper centre obviously left intact, we can only speculate at present why the turnover numbers are diminished in both mutants. An explanation may be given by assuming that, apart from the decreased binding constant for the substrate molecule, a disturbed orientation of the electron donor at the (mutated) contact site on subunit II would also lead to an obstruction in the optimal transfer pathway for the electron. To solve this question, a more detailed analysis of the individual redox steps between reduced cytochrome *c* and oxygen will be required.

Elevated K_M values for the high-affinity phase are not observed with the polarographic assay (not shown); values are in the same range for wild type and mutants. The polarographic assay provides a continuous reduction by the TMPD/ascorbate couple for the substrate cytochrome *c*, whether in solution or bound to the enzyme. Product dissociation, which is the rate-limiting step in the spectrophotometric assay [29], is not required under these conditions. Therefore, the spectrophotometric assay is the more discriminating test in steady-state kinetics for our purposes.

The differences between wild type and mutants can also be highlighted if the k_{cat}/K_M criterion is considered. As this should be a measure of the overall catalytic efficiency, the decreased values of the mutants (about 8-fold) indicate that both mutations affect the catalytic centre. These results lead to the conclusion that glutamate 246 and aspartate 206 provide part of the cytochrome *c* binding site on oxidase; further mutational studies will be needed to investigate the role of other conserved residues possibly contributing to this site as well.

We thank Stefan Anemüller and Eckard Bill (Medical University of Lübeck) for recording EPR spectra. We are also grateful to Doris Bergmann-Dörr (Institute of Geography, Frankfurt) for performing the atomic absorption measurements.

References

[1] Gennis, R.B. and Trumpower B.L. (1994) Annu. Rev. Biochem. 63, 675–716.
[2] Capaldi, R.A. (1990) Annu. Rev. Biochem. 59, 569–596.
[3] Saraste, M. (1990) Q. Rev. Biophys. 23, 331–366.
[4] Ludwig, B. (1987) FEMS Microbiol. Rev. 46, 41–56.
[5] Kelly, M., Lappalainen, P., Talbo, G., Haltia, T., Van der Oost, J. and Saraste, M. (1993) J. Biol. Chem. 268, 16781–16787.
[6] Kroneck, P.M.H., Antholine, W.E., Kastrau, D.H.W., Buse, G., Steffens, G.C.M. and Zumft, W.G. (1990) FEBS Lett. 268, 274–276.
[7] Hill, B.C. (1991) J. Biol. Chem. 266, 2219–2226.
[8] Bisson, R., Gutweniger, H., Colonna, R., Zanotti, A. and Azzi, A. (1977) FEBS Lett. 81, 147–150.
[9] Ludwig, B. and Reichhardt, J. (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri et al., eds.), pp. 3–10, Elsevier, Amsterdam.
[10] Millett, F., Darley-Usmar, V. and Capaldi, R.A. (1982) Biochemistry 21, 3857– 3862.
[11] Taha, T.S.M. and Ferguson-Miller, S. (1992) Biochemistry 31, 9090–9097.
[12] Millett, F., De Jong, C., Paulson, L. and Capaldi, R.A. (1983). Biochemistry 22, 546–552.
[13] Bisson, R., Steffens, G.C.M., Capaldi, R.A. and Buse, G. (1982) FEBS Lett. 144, 359–363.
[14] Staudenmayer, N., Ng, S., Smith, M.B. and Millett, F. (1977) Biochemistry 16, 600–604.
[15] Ferguson-Miller, S., Brautigan, D. and Margoliash, E. (1978) J. Biol. Chem. 253, 149–159.
[16] Rieder, R. and Bosshard, H.R. (1980) J. Biol. Chem. 255, 4732–4739.
[17] Steinrücke, P., Gerhus, E., Jetzek, M., Turba, A. and Ludwig, B. (1991) J. Bioenerg. Biomembr. 23, 227–239.
[18] Van der Oost, J., Haltia, T., Raitio, M. and Saraste, M. (1991) J. Bioenerg. Biomembr. 23, 257–267.
[19] Berry, E.A. and Trumpower, B.L. (1985) J. Biol. Chem. 260, 2458–2467.
[20] Bolgiano, B., Smith. L. and Davies H.C. (1988) Biochim. Biophys. Acta 933, 341–350.
[21] Smith, L. and Davies, H.C. (1991) J. Bioenerg. Biomembr. 23, 303–319.
[22] Steinrücke, P., Gerhus, E. und Ludwig, B. (1991) J. Biol. Chem. 266, 7676–7681.
[23] Gerhus, E., Steinrücke, P. and Ludwig, B. (1990) J. Bacteriol. 172, 2392–2400.
[24] Ludwig, B. (1986) Methods Enzymol. 126, 153–159.
[25] Seelig, A., Ludwig, B., Seelig, J. and Schatz, G. (1981) Biochim. Biophys. Acta 636, 162–167.
[26] Hendler, R.W., Pardhasaradhi, K., Reynafarje, B. and Ludwig, B. (1991) Biophys. J. 60, 415–423.
[27] Steinrücke, P., Steffens, G.C.M., Panskus, G., Buse, G. and Ludwig, B, (1987) Eur. J. Biochem. 167, 431–439.
[28] Ferguson-Miller, S., Brautigan, D. and Margoliash, E. (1976) J. Biol. Chem. 251, 1104–1115.
[29] Speck, S.H., Dye, D. and Margoliash, E. (1984) Proc. Natl. Acad. Sci. USA 81, 347–351.
[30] Michel, B. and Boshard, H.R. (1989) Biochemistry 28, 244–252.