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Biochimica et Biophysica Acta 1459 (2000) 533–539

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

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Proton transfer from glutamate 286 determines the transition rates between oxygen intermediates in cytochrome *c* oxidase

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Abstract

We have investigated the electron–proton coupling during the peroxy (P_R) to oxo-ferryl (F) and F to oxidised (O) transitions in cytochrome *c* oxidase from *Rhodobacter sphaeroides*. The kinetics of these reactions were investigated in two different mutant enzymes: (1) ED(I-286), in which one of the key residues in the D-pathway, E(I-286), was replaced by an aspartate which has a shorter side chain than that of the glutamate and, (2) ML(II-263), in which the redox potential of Cu_A is increased by ~100 mV, which slows electron transfer to the binuclear centre during the F→O transition by a factor of ~200. In ED(I-286) proton uptake during P_R→F was slowed by a factor of ~5, which indicates that E(I-286) is the proton donor to P_R. In addition, in the mutant enzyme the F→O transition rate displayed a deuterium isotope effect of ~2.5 as compared with ~7 in the wild-type enzyme. Since the entire deuterium isotope effect was shown to be associated with a single proton-transfer reaction in which the proton donor and acceptor must approach each other (M. Karpefors, P. Ådelroth, P. Brzezinski, Biochemistry 39 (2000) 6850), the smaller deuterium isotope effect in ED(I-286) indicates that proton transfer from E(I-286) determines the rate also of the F→O transition. In ML(II-263) the electron-transfer to the binuclear centre is slower than the intrinsic proton-transfer rate through the D-pathway. Nevertheless, both electron and proton transfer to the binuclear centre displayed a deuterium isotope effect of ~8, i.e., about the same as in the wild-type enzyme, which shows that these reactions are intimately coupled.

Keywords: Electron transfer; Proton transfer; Proton pumping; Cytochrome aa_3; Flash photolysis; Flow flash

1. Introduction

Cytochrome *c* oxidase is a membrane-bound protein complex which catalyses oxidation of four molecules of cytochrome *c* and the four-electron reduction of dioxygen to water. Electrons from cytochrome *c* are first transferred to the Cu_A centre, followed by consecutive, intramolecular electron transfer to haem *a* and the binuclear centre, consisting of haem a_3 and Cu_B, where oxygen binds. Part of the free energy released in this reaction is conserved by pumping of about one proton per electron across

Abbreviations: The deuterium isotope effect is defined as the ratio of the rates measured in H_2O and D_2O (k_H/k_D), respectively; P_M and P_R, peroxy intermediate formed with the mixed-valence and fully reduced enzymes, respectively; F, oxo-ferryl intermediate; O, fully oxidised enzyme; Amino-acid residue and mutant-enzyme nomenclature: E(I-286), denotes glutamate 286 of subunit I; ED(I-286), denotes a replacement of glutamate 286 by aspartate; Amino-acid residues are numbered according to the R. sphaeroides sequence

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0005-2728/00/$ – see front matter
PII: S0005-2728(00)00194-8

the membrane, which requires a tight coupling of the electron and proton-transfer reactions. On the basis of studies of site-directed mutants of terminal oxidases [1,2] and analyses of the X-ray crystal structures of the bovine [3], *Paracoccus denitrificans* [4] and *Rhodobacter sphaeroides* (Svensson-Ek et al., unpublished data) cytochrome *c* oxidases, proton-transfer pathways, consisting of 'wires' of protonatable amino-acid residues and bound water molecules, were identified. One of these pathways, the D-pathway, 'starts' with a conserved aspartate residue D(I-132), close to the surface of the protein on the proton-input side, and leads to another highly conserved residue glutamate (E(I-286)), located about 30 Å from the enzyme surface and about 10 Å from the binuclear centre.

The D-pathway is the only pathway used for proton uptake during reaction of the reduced enzyme with oxygen (see [5]) and therefore it is used for the uptake of both substrate and pumped protons. Consequently, to achieve efficient proton pumping the thermodynamics and/or rates of proton transfer through the D-pathway must be controlled.

During reduction of dioxygen by the fully reduced enzyme, proton uptake from solution is observed simultaneously with the $P_R \rightarrow F$ and $F \rightarrow O$ transitions [6,7], both being associated with proton pumping [8]. One of the protonatable amino-acid residues playing a central role in the D-pathway is E(I-286). A specific role for E(I-286) in regulating the proton transfer through the D-pathway has been proposed earlier on the basis of the examination of the three-dimensional structures of cytochrome *c* oxidase and theoretical calculations [4,9–11]. Depending on the (transient) conformation of the glutamate side chain the pathway may be connected either with the binuclear centre or with the propionates of haem a_3 on the proton output side.

We have previously found that the $P_R \rightarrow F$ transition rate ($k \cong 8.5 \times 10^3$ s^{-1}) is determined by internal proton transfer from a group within the D-pathway to the binuclear centre [12,13]. Similarly, the rate of the following $F \rightarrow O$ transition ($k \cong 8.0 \times 10^2$ s^{-1}) is determined by a proton-transfer reaction that involves a structural change of the side chain of a group within the same pathway [14].

Mutation of E(I-286) to its non-protonatable analogue, glutamine, results in essentially total loss of the enzymatic activity and inhibition of the reaction of the reduced enzyme with oxygen at the level of the peroxy intermediate (P_R), i.e., before proton uptake from solution [15]. These results show that E(I-286) is directly or indirectly involved in proton transfer after formation of the P_R intermediate, but they do not provide information about the identity of the proton donor to the binuclear centre nor of the involvement of E(I-286) in later reaction steps. To address this problem, the $P_R \rightarrow F$ and $F \rightarrow O$ reactions in the E(I-286) $\rightarrow$ Asp (ED(I-286)) mutant enzyme were investigated. In addition, the deuterium isotope effect of the $F \rightarrow O$ transition rate was investigated in the ML(II-263) mutant enzyme in which the transfer of the fourth electron to the binuclear centre is dramatically slowed due to an increase of the Cu_A redox potential.

2. Materials and methods

The ED(I-286)-mutant enzyme from *R. sphaeroides* was constructed as described [2]. Bacteria were grown and the enzyme was purified as described earlier [16].

The catalytic activity of the enzyme was measured in 50 mM KH_2PO_4 (pH 6.5), 0.05% dodecyl β-D-maltoside as the initial rate of decrease in absorbance at 550 nm upon mixing reduced cytochrome *c* with cytochrome *c* oxidase (calculated using $\varepsilon^{550} = 21.1$ $mM^{-1}\,cm^{-1}$ for reduced minus oxidised cytochrome *c*). For a detailed description of the preparation of the fully reduced enzyme and the experimental set-up, see Refs. [6,14,15]. For proton-uptake measurements, buffer was removed on a PD-10 column (Pharmacia) equilibrated with 0.1 M KCl and 0.05% β-D-dodecyl maltoside at pH 7.8. The measurements were performed as described in detail in Refs. [6,15]. The enzyme sample and the O_2-containing solution was supplemented with the dye phenol red to a concentration of 40 μM. Calibration of the observed phenol-red absorbance changes to the number of protons taken up were done as described [6,15]. The amount of reacting enzyme was calculated from the CO-dissociation absorbance change at 445 nm using an absorption coefficient of 67 $mM^{-1}\,cm^{-1}$ [17]. The cuvette path length was 1.00 cm.

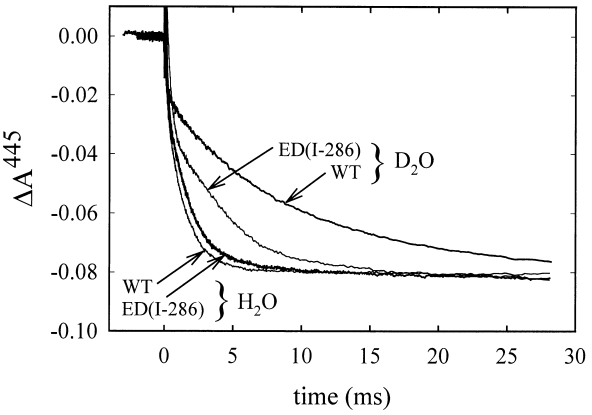

Fig. 1. Absorbance changes at 445 nm associated with the reaction of the fully reduced aa_3 wild-type and ED(I-286) mutant enzyme with dioxygen in H_2O and D_2O, respectively. The fully reduced, CO-bound enzyme equilibrated in H_2O or D_2O was mixed with an O_2-saturated H_2O/D_2O buffer solution. About 100 ms after mixing, the reaction was initiated by flash photolysis of CO from the enzyme. On the time scale shown in the graph, only the F→O transition can be resolved. Experimental conditions: 24°C, 0.1 M Hepes (pH-meter reading 7.8 (see Ref. [14])), 0.05% β-D-dodecyl maltoside, 1 mM O_2, 0.2 μM reacting enzyme. The traces have been normalised to 1 μM reacting enzyme.

3. Results

The ED(I-286) mutant enzyme displayed an activity of about 50% of that of the wild-type enzyme and in the *E. coli* cytochrome bo_3 the mutant enzyme it is able to pump protons [18].

3.1. The F→O transition

The flow-flash technique was used to investigate the reaction of the fully reduced ED(I-286) mutant enzyme with dioxygen. Fig. 1 shows absorbance changes associated with the F→O kinetic phase after flash-induced dissociation of CO from the fully reduced wild-type and ED(I-286) mutant enzymes after addition of O_2. In H_2O at pH 7.5 the F→O-transition rates were found to be 550 ± 50 s^{-1} and 800 ± 50 s^{-1} for the mutant and wild-type enzymes, respectively. With both enzymes the reaction was slower

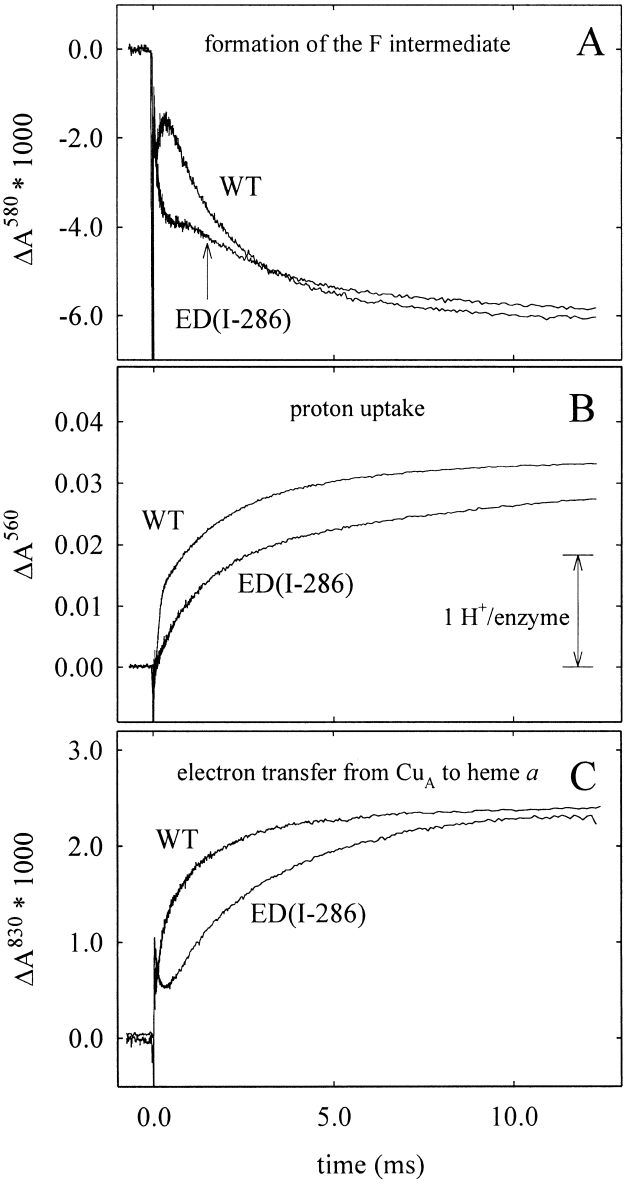

Fig. 2. Absorbance changes associated with the reaction of the fully reduced wild-type and ED(I-286) mutant enzyme with dioxygen after flash photolysis of CO. (A) In the wild-type enzyme, at 580 nm the oxo-ferryl intermediate appears as an increase in absorbance with a time constant of 120 μs and the following decay is associated with formation of the oxidised enzyme. (B) Proton uptake from the bulk solution was measured at 560 nm as a change in the absorbance of the pH-indicator phenol red. Data at 560 nm were collected in both buffered and in non-buffered solutions and the trace in B is the difference between these signals. (C) Oxidation of Cu_A measured at 830 nm. Experimental conditions: 24°C, 0.1 M Hepes (pH 7.8) (except in B where the buffer was exchanged for 0.1 M KCl), 0.05% β-D-dodecyl maltoside, 1 mM O_2, 3.3 μM reacting enzyme. All traces have been normalised to 1 μM reacting enzyme.

in D_2O than in H_2O. However, the deuterium isotope effect was only ~2.5 with the ED(I-286) ($k_D \cong 220 \pm 40$ s^{-1}) as compared with ~7 with the wild-type ($k_D \cong 115 \pm 15$ s^{-1}) enzyme.

We also investigated in detail the dependence of the kinetics of the F→O transition in the ED(I-286) mutant enzyme on the fraction of D_2O in solution (not shown, proton-inventory technique, see [14,19]). In principle, this approach can provide information on the number of protonatable sites involved in the rate-limiting step(s) of the reaction. However, the maximum deuterium isotope effect of 2.5 was too small to determine the number of protonatable sites involved in proton transfer in the mutant enzyme (see [14,19]).

Proton uptake measurements were done using the dye phenol red in the bulk solution. Absorbance changes at 560 nm of the dye are shown in Fig. 2B. As seen in the figure, proton uptake during the F→O transition displayed about a factor of 1.5 slower rate in the ED(I-286) mutant as compared with the wild-type enzyme.

In addition, in some enzyme preparations, at 560 nm and 830 nm, we observed a slower component with a rate of ~100 s^{-1} and an amplitude of up to 20% of the total change.

In the wild-type enzyme, the relatively large deuterium isotope effect of 7 for the F→O transition indicates that the rate of this transition is determined by proton transfer. To investigate the details of the coupling between the electron and proton transfer during this transition, we investigated the deuterium isotope effect of the transition rate in the ML(II-263) mutant enzyme [20]. The residue M(II-263) is one of the Cu_A ligands and the mutation results in an increase of the Cu_A-redox potential, which has as a consequence a dramatic retardation of the transfer of the fourth electron to the binuclear centre, thereby slowing the F→O transition. Fig. 3 shows absorbance changes at 445 nm associated with the F→O transition in the ML(II-263) mutant enzyme in H_2O and D_2O, respectively. As seen in the figure, even though in the ML(II-263) mutant enzyme the F→O transition is slowed by a factor of ~200 as compared with the wild-type enzyme ($k_H \cong 6.7$ s^{-1}), the deuterium isotope effect is about the same as with the wild-type enzyme, i.e., ~8 ($k_D \cong 0.8$ s^{-1}). This indicates that during the F→O reaction the electron- and proton-transfer reactions are tightly coupled (see Section 4).

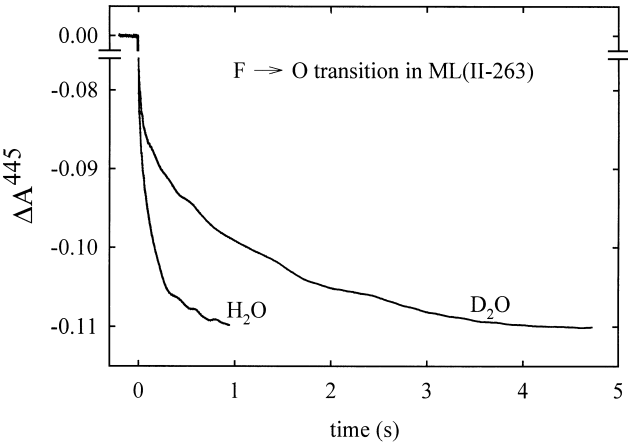

Fig. 3. Reaction of the fully reduced ML(II-263) mutant enzyme with O_2 in H_2O and in D_2O, respectively, monitored at 445 nm. The reaction was initiated with a laser flash about 100 ms after mixing the fully reduced, CO-bound enzyme (equilibrated in H_2O or D_2O) with an O_2-saturated H_2O/D_2O buffer solution. On this time scale only the last kinetic phase of the oxidative reaction (the F→O transition) is resolved. Conditions after mixing: 24°C, 0.1 M Hepes (pH-meter reading 7.5), 0.05% β-D-dodecyl maltoside, 1 mM O_2, 2.0 μM reacting enzyme.

3.2. The P_R→F transition

During the P_R→F transition (8500 s^{-1} in the wild-type enzyme) in the reaction of the fully reduced enzyme with O_2, three coupled reactions take place: the transition between the P_R and F intermediates at the binuclear centre; proton uptake from the bulk solution; and electron transfer from Cu_A to haem *a*. Consequently, in order to fully understand the effect of the ED(I-286) mutation, all these events must be investigated. Fig. 2A shows absorbance changes at 580 nm, a wavelength at which in the wild-type enzyme the P_R→F transition is associated with an increase in absorbance with a time constant of 120 μs. The rate is limited by an internal transfer of a proton from a group within the D-pathway to the binuclear centre [12]. In the ED(I-286)-mutant enzyme the formation of F was slower and the absorbance increase is only seen as a plateau since the following F→O transition, which has an opposite sign to that of the P_R→F transition, is not retarded to the same extent as P_R→F. Also proton uptake (Fig. 2B) and electron transfer from Cu_A to haem

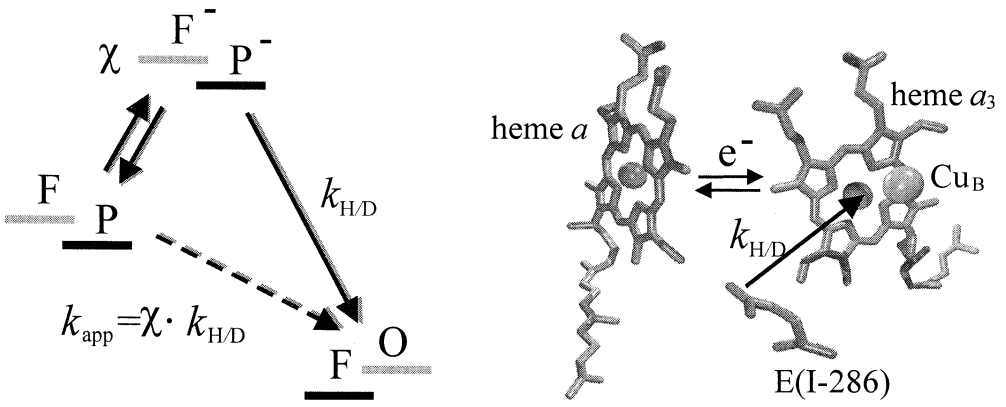

Fig. 4. An electron from haem *a* is in rapid equilibrium with an oxygen intermediate at the binuclear centre (P or F). The 'reduced' intermediate (P^- or F^-) is a thermodynamically unfavourable state and therefore only populated to a low extent (χ). The electron is trapped by a proton transfer from E(I-286) which results in formation of F from P_R or O from F. Consequently, the apparent rate constant (k_{app}) for the transition between the intermediates is determined by the product of the fraction (χ) of P^- or F^- and the rate of proton/deuteron transfer (k_H or k_D) from E(I-286) to the binuclear centre. In the ML(II-263) mutant enzyme the electron equilibrium for the fourth electron is shifted away from the binuclear centre because of the higher redox potential of Cu_A (smaller χ), which results in a slower apparent $F \rightarrow O$ transition rate.

a (Fig. 2C) were slowed. From a global fit of the data measured at 445, 560, 580, and 605 nm ($<10\%$ residuals at all wavelengths) a rate of $\sim 1800\ s^{-1}$ was found for all events during the $P_R \rightarrow F$ transition in the ED(I-286) enzyme, i.e., a factor of ~ 5 slower than with the wild-type enzyme.

4. Discussion

4.1. The $F \rightarrow O$ transition

In the *R. sphaeroides* wild-type enzyme the rate constant for the $F \rightarrow O$ transition is about $800\ s^{-1}$. This transition is associated with both electron and proton transfer to the binuclear centre where the electron is transferred from the haem *a*/Cu_A equilibrium and the proton is taken from the bulk solution through the D-pathway. Even though the proton is transferred a distance of 30 Å through the protein, the total deuterium isotope effect of ~ 7 is associated with a single protonatable site [14]. In the ED(I-286) mutant enzyme the kinetic deuterium isotope effect was much smaller (~ 2.5) than in the wild-type enzyme, which indicates that E(I-286) is the proton donor to the binuclear centre. In the ED(I-286)-mutant enzyme, the side chain of the residue is one methyl group shorter than in the wild-type enzyme. This structural modification is likely to result in a cavity, large enough to contain a water molecule. Thus, the smaller isotope effect with the mutant enzyme may be explained in terms of a more completely coupled hydrogen-bonding structure in the D-pathway.

We have here assumed that the proton transfer from E(I-286) occurs to the oxygen intermediate, but it should be noted that the transition also involves proton pumping. Thus the rate-limiting step of the reaction may be proton transfer from E(I-286) to the a pump element, which may be distinct from the binuclear centre (see also below).

To investigate the electron–proton coupling in the $F \rightarrow O$ transition we studied these reactions in the ML(II-263) mutant enzyme in which one of the Cu_A ligands, methionine 263 in subunit II, was replaced by a leucine. As a result, the redox potential of Cu_A increased by about 100 mV and the $F \rightarrow O$ transition rate decreased by about two orders of magnitude [21]. Despite the extremely slow $F \rightarrow O$ transition rate in the ML(II-263) mutant enzyme the deuterium isotope effect of this transition was ~ 8, i.e., about the same as in the wild-type enzyme. This result indicates a very tight coupling between electron and proton/deuteron transfer since electron transfer in the ML(II-263) mutant enzyme and deuteron transfer in the wild-type enzyme are both in themselves much faster than the apparent $F \rightarrow O$ rate in D_2O in the mutant enzyme. Earlier studies

of the ML(II-263) mutant enzyme showed that the mutation at the Cu_A site does not perturb the environments of the other redox sites [20,21].

A model describing electron and proton transfer to the binuclear centre during the F→O transition is shown in Fig. 4. The F state is in rapid equilibrium with an intermediate in which the binuclear centre is reduced. This intermediate is thermodynamically unfavourable and therefore only populated to a low extent. Once at the binuclear centre, the electron is trapped by a proton. Consequently, the apparent rate constant (k_{app}) for the F→O transition is determined by the product of the fraction (χ) of reduced F at the binuclear centre and the rate of proton/deuteron transfer. The inherent ratio of 7–8 between proton (k_H) and deuteron (k_D) transfer through the D-pathway is therefore independent of the apparent rate of the F→O transition. In the ML(II-263) mutant enzyme electron transfer to the binuclear centre is less favourable because the higher redox potential of Cu_A (χ in Fig. 4 is smaller), which results in a slower apparent F→O transition rate.

4.2. The P_R→F transition

As discussed above, the P_R→F reaction step is composed of three events that occur sequentially: internal proton transfer from the D-pathway to the binuclear centre; electron transfer from Cu_A to haem *a*; and proton uptake from the bulk solution. In the wild-type enzyme the rate of all these events is controlled by the internal proton transfer from the D-pathway to the binuclear centre [12,21]. As shown in Fig. 2A–C, in the ED(I-286) mutant enzyme all three events during the P_R→F transition were slowed by a factor of ∼5, which indicates that E(I-286) is the proton donor to the binuclear centre during the P_R→F transition.

Thus, both the P_R→F and F→O transitions are rate limited by internal proton transfer from E(I-286) to the binuclear centre. Still, their rates differ by one order of magnitude. One phenomenological difference between these two transitions is that while the F→O transition requires transfer of an electron to the binuclear centre, the P_R→F transition does not. Thus, assuming that the model in Fig. 4 is applicable also to the transition from P_R to F, the difference in formation rates of F (from P_R) and O (from F) can be attributed to the fractional (cf. χ in Fig. 4) electron transfer to the centre. In P_R the third electron, which is necessary to form F, is found at the binuclear centre (corresponds to $\chi = 1$, see Fig. 4) and the P_R→F transition only involves proton transfer to the binuclear centre. In the F intermediate the fourth electron is mainly distributed between Cu_A and haem *a*, resulting in a much smaller fraction of reduced binuclear centre, which results in a slower transition rate of F→O than of P_R→F.

An observation in support of this model was made by Siletsky et al. [22], who found that flash-induced reduction of the pre-formed P_M intermediate (formed by reaction of the two-electron reduced enzyme with O_2), i.e., the P_M→F transition, was significantly slower than the P_R→F transition. Since in the P_M intermediate there is one electron less at the binuclear centre as compared with the P_R intermediate, during rapid electron injection to the enzyme, the fraction reduced binuclear centre is smaller, which results in the slower P_M→F transition.

5. Conclusions

In conclusion, the results indicate that proton transfer from E(I-286) to the binuclear centre (or the pumping element, see above) determines the transition rates of both the P_R→F and F→O transitions, and during the F→O transition electron transfer to the binuclear centre and proton uptake are intimately coupled.

Acknowledgements

Supported by grants from the Swedish Natural Science Research Council and The Swedish Foundation for International Co-operation in Research and Higher Education (STINT). We would like to thank Peter Rich, University College London, for valuable discussions.

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