Effects of mutation of residue I67 on redox-linked protonation processes in yeast cytochrome *c* oxidase

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We describe effects of a mutation, Ile-67 → Asn, in subunit I of yeast cytochrome *c* oxidase on redox-linked protonation processes within the protein. The mutation lowers the midpoint potential of haem *a* and weakens its pH dependency, but has little effect on the potential of haem *a*₃. The residue is close to a conserved glutamate (Glu-243) in the crystal structure. We

propose that protonation of Glu-243 is redox-linked to haem *a*, that Asn-167 perturbs its pK and that redox-linked protonation in this location is essential for the catalytic reactions of the binuclear centre. These proposals are discussed in terms of a 'glutamate trap' mechanism for proton translocation in the haem/copper oxidases.

INTRODUCTION

The structures of the cytochrome *c* oxidase from *Paracoccus denitrificans* [1] and from beef heart mitochondria [2] have been solved by X-ray crystallography to 0.28 nm (2.8 Å) resolution and co-ordinates for the latter are available. Possible protonation sites and routes in subunit I can be identified [3,4]. Candidates for stable sites of charge-linked protonation include the histidine ligands to the metal centres, the haem propionates and residues close to them, and conserved protonatable residues such as Lys-319, Glu-243 and Tyr-245 (numbering refers to the yeast sequence [5]). Analyses of mutant enzymes provides one method of assessing the roles of these residues in the protonation processes.

We have isolated and partly characterized a mutant form of yeast which has a single mutation of Ile-67 → Asn in cytochrome *c* oxidase subunit I (equivalent to Ile-66 in the bovine sequence)

[6]. The residue Ile-67 is located between haem *a* and Glu-243 (E243) (Figure 1). The mutant enzyme has a low catalytic centre activity compared with the wild-type form and the redox titration curve of haem *a*, as observed in the cyanide-ligated enzyme, is lowered by around 60 mV at pH 7.5. No significant effects were found on the ligand reactivity of the binuclear centre [7].

In this paper, we show that the mutation Ile-67 → Asn has no effect on the redox properties of haem *a*₃. Analysis of the pH dependency of the midpoint potential of haem *a* indicates a weakening of the redox-linked protonation(s) associated with haem *a*. The results are discussed in the light of the crystal structure and catalytic cycle of the enzyme, and we propose that the Ile-67 → Asn mutation effects are most likely mediated through a modulation of the protonation properties around the conserved glutamate residue (E243), a residue which is close to the Ile-67 position and which we propose may be central to the protonmotive mechanism.

MATERIALS AND METHODS

Production of the mutant and enzyme purification

The production, selection and genetic characterization of the Ile-67 → Asn mutant in yeast [6,8], and large-scale growth and enzyme purification [7,9] have been described previously.

Spectrophotometric and kinetic measurements

Difference spectra were monitored with a single-beam scanning/kinetic instrument built in-house.

Potentiometric titrations

Potentiometric titrations were performed anaerobically in 2 ml of 50 mM Hepes or Mes/0.05% (w/v) lauryl maltoside containing 200 units/ml catalase, at different pH values. Purified cytochrome *c* oxidase was added to around 3 μM. Mediators and electrode system were as described previously [7]. The cyanide- and formate-ligated enzymes were prepared by addition of 1.5 mM cyanide or 100 mM formate respectively. The redox state of haem A (i.e. haem *a* in the cyanide-ligated enzymes and

Figure 1 Location of residue I67 in the structure of the subunit I of cytochrome *c* oxidase

The structure has been drawn from the co-ordinates of the bovine enzyme published by Tsukihara et al. [2]. Numbering of residues is according to the yeast subunit I sequence. The Figure is a top view from the cytochrome *c* side of the membrane.

Abbreviations used: E_m , midpoint potential; E_h , ambient potential.

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haems a plus a_3 in the unligated or formate-ligated enzymes) was monitored by a triple wavelength measurement at $445-(435+455)/2$ nm. Titrations were performed in both oxidative and reductive directions with 10 mM potassium permanganate or 20 mM sodium ascorbate respectively, and no significant hysteresis was detected, indicating that equilibration with mediators was occurring. Each titration was performed at least twice and derived midpoint potentials for identical conditions were within 10 mV.

RESULTS

General properties and homogeneity of the mutant enzyme

We have found previously that some mutant oxidase preparations display considerable heterogeneity of properties, and this enormously hinders detailed analyses and interpretation of mutation effects. However, the Ile-67 $\rightarrow$ Asn mutant was specifically chosen for a detailed study because, besides being a locus of particular interest to us, it is expressed at fairly high levels in an intact state and can be purified in a relatively homogeneous and stable form. Some tests for structural integrity and homogeneity have been described previously [7]. In native PAGE, followed by staining with Coomassie Blue, of the enzyme preparation, no partially assembled cytochrome c oxidase was observed and Tricine-SDS/PAGE showed a subunit composition identical with that of the wild-type.

Even for intact mutant enzymes, structural heterogeneity in some cases is evident in heterogeneous recombination kinetics of photolysable ligands such as CO and cyanide with the reduced enzyme. However, in the purified Ile-67 $\rightarrow$ Asn mutant enzyme the kinetics of recombination with these ligands are monophasic and essentially identical to those of the wild-type. A range of other optical features are also very similar to those of the wild-type.

The maximum catalytic-centre activity of the Ile-67 $\rightarrow$ Asn enzyme is around 25 s^{-1} at pH 7.4, less than 2% of that of the wild-type [7]. This low activity is unlikely to arise from poor reactivity with substrates, since a reaction between reduced cytochrome c and haem a can be observed which is far too fast to account for lack of enzyme activity and reaction of the reduced enzyme with O_2 is close to the wild-type rate.

In summary, the Ile-67 $\rightarrow$ Asn mutant form appears to be assembled in an intact state, which is relatively homogeneous in terms of the above tests. Furthermore, the low catalytic activity appears to originate in a malfunction of internal catalytic processes rather than in an impediment to substrate reactivities.

Redox titrations of the unligated, cyanide-ligated and formate-ligated enzymes

Comparison of the titration curves of the wild-type and mutant enzymes are presented in Figure 2. In the unligated wild-type enzyme, the three redox centres, haem a , haem a_3 and Cu_B (which is optically invisible), are expected to titrate at similar potentials and to interact anti-co-operatively [10], which has been observed in more detailed studies of the bovine enzyme [11,12]. The behaviour of the unligated yeast wild-type enzyme was similar to that of the bovine enzyme, whereas the unligated form of Ile-67 $\rightarrow$ Asn was shifted, in part, to lower potentials (Figure 2A).

Because of the complexity of the titration data in the absence of ligands, which is caused by multiple redox interactions, a detailed analysis of mutation effects is difficult. However, in the cyanide-ligated enzyme the E_m of haem a_3 is lowered so that haem a can be titrated whilst haem a_3 remains oxidized. Comparison of the titrations of the cyanide-ligated forms showed

Figure 2 Redox titration of the unligated, cyanide-ligated and formate-ligated enzymes

Redox titrations were performed at pH 7.5 as described in the Materials and methods section. At least two titrations for each condition were performed and the values are from a single representative experiment. The redox state of haem A was monitored at $445-(435+455)/2$ nm. The absorbance changes are plotted versus potential for the wild-type (●) and Ile-67 $\rightarrow$ Asn (I67N) (■). (A) Unligated enzyme. For clarity of comparison, curves composed of two $n = 1$ curves have been overlaid with midpoints of 310 and 250 mV for the wild-type and 295 and 195 mV for the mutant, although the detailed redox behaviour is far more complex (see the text). (B) Cyanide-ligated enzyme. The simulations overlaying the titration curves of the cyanide-ligated enzymes are described in the text. (C) Formate-ligated enzyme. For comparison, curves composed of two $n = 1$ curves have been overlaid with midpoints of 340 and 210 mV for the wild-type and 260 and 210 mV for the mutant, although, as for the unligated enzyme in (A), the detailed redox behaviour is more complex than this.

clearly that the potential of haem a was lower in the mutant (Figure 2B).

Formate binds to haem a_3 and lowers its midpoint potential, and in this case both haems can be titrated separately in the potential range that we have applied (Figure 2C). In the wild-type enzyme, at pH 7.5, haems a and a_3 titrated at 340 mV and 210 mV respectively, values very similar to those obtained with the bovine enzyme [11,12]. In the mutant enzyme, the potential of formate-ligated haem a_3 was relatively unaffected whilst that of haem a was clearly shifted to a lower value.

Figure 3 Effect of pH on midpoint potentials of the cyanide-ligated enzymes

Redox titrations were performed as described in the Materials and methods section. (A) Percentage of haem *a* reduction plotted versus potential, at pH 6.5 (□, ■) and pH 8.8 (○, ●) for the wild-type (open symbols) and mutant (I67N) (solid symbols). For each titration curve, the points represent data from two independent experiments, and indicate the reproducibility of the measurements. The dotted lines are fitted simulations of the wild-type titration curves for a model in which haem *a* interacts anti-co-operatively with Cu_B (see the text). The mutant titration curves are overlaid with simple Nernstian $n = 1$ curves (dashed lines) (see the text for explanation). (B) Plots of the observed midpoints of the titration curves versus pH.

Effect of pH on the midpoint potential of haem *a*

Redox titrations of cyanide-ligated forms of the wild-type and mutant enzymes were performed at pH 6.5, 7.5, 8 and 8.8 in order to probe mutation effects on protonation processes linked to redox changes of haem *a*. The data obtained at pH 6.5 and 8.8 are presented in Figure 3(A) and the midpoints of the titration curves (observed E_m values) are plotted as a function of pH in Figure 3(B).

For the wild-type form, the curves were distorted from an $n = 1$ shape, presumably due to the interaction between haem *a* and the other redox centres, as observed in the bovine enzyme. Simulations were fitted as described [13], using a model for anti-co-operative interaction between haem *a* and Cu_B , but without taking account of any weak interaction with Cu_A . Microscopic E_m values of haem *a* (i.e. E_m values of haem *a*, either when Cu_B is reduced or when Cu_B is oxidised) derived from these fits are very similar to those reported for the mammalian enzyme [13].

$E_{ma}(Cu_{B_{ox}})$ was estimated to be 390 ± 10 mV and 315 ± 10 mV at pH 6.5 and 8.8, respectively, and $E_{ma}(Cu_{B_{red}})$ to be 300 ± 10 mV and 255 ± 10 mV, again at pH 6.5 and 8.8 respectively. Since the distortions of the haem *a* titrations, which are caused by the redox state change of Cu_B , are quite prominent in these wild-type data, the microscopic midpoint potentials for Cu_B are also given quite reliably by the simulations, and these E_m values of Cu_B when haem *a* is reduced or when Cu_B is oxidised are estimated to be within 10 mV the equivalent E_{ma} values.

The Ile-67 → Asn mutation clearly lowered the observed midpoints of the titration curves at all pH values. It appeared that the E_m of Cu_B was not lowered in the mutant to the same extent as that of haem *a*, since splitting of the titration of haem *a* into two waves [10,13] was far less pronounced than in the wild-type. Because of this, it was not possible to determine, with confidence, the E_m values of Cu_B from the weak distortions of these haem *a* titration curves. For simulation of the redox behaviour of haem *a* in the Ile-67 → Asn mutant enzyme shown in Figure 3(A), we instead used simple $n = 1$ Nernstian curves so that no assumptions were required regarding the redox properties of Cu_B .

Because of the inability to assess redox properties of Cu_B in the Ile-67 → Asn mutant, and of the different simulation models therefore required, a comparison of the pH-dependencies of redox behaviour of haem *a* in wild-type and mutant was made by simply plotting the ambient potential at the midpoint of the titration curves as a function of pH (Figure 3B). For the wild-type enzyme, a slope of around -35 mV/pH unit was observed. This pH-dependency was clearly weakened to around -15 mV/pH unit in the Ile-67 → Asn mutant.

DISCUSSION

We have already reported that the Ile-67 → Asn mutant enzyme, which appears to be assembled intact and in a relatively homogeneous form, has very little catalytic activity when compared with the wild-type enzyme. However, the mutation has no major effect on ligand and oxygen reactions of the binuclear centre or on reactivity with cytochrome *c* [7], and it therefore appears that malfunction of a critical internal process is affected. We show, in the present study, that the redox potential of haem *a*₃ appears to be unaffected. In contrast, the redox titration curves of haem *a* are lowered by around 60 mV at pH 7.5 (Figure 2). However, if this were the sole effect of the mutation, it seems unlikely that this shift could, in itself, be sufficient to account for the decrease in activity, since cytochrome *c* (E_m of +255 mV) should still provide a sufficiently reducing potential to drive reduction of most of the haem *a*, and the cytochrome *c*–haem *a* equilibration is extremely fast. Furthermore, onward electron transfer from haem *a* to the binuclear centre might be expected to be increased by the lower potential of the donor.

We show here that the mutation causes an additional effect, namely that the redox potentials of haem *a* have become less pH dependent. A slope of around -35 mV/pH unit was observed with the wild-type enzyme but this was decreased to around -15 mV/pH unit in the Ile-67 → Asn mutant. The shapes of the titration curves for haem *a* in the wild-type are complex, and their pH dependency is weaker than the -60 mV/pH unit slope found in simpler systems, in which one proton is bound per electron. These features are caused by redox and protonation sites which are close enough to interact electrostatically. In a more detailed analysis of titrations of the cyanide-ligated beef heart enzyme, we have suggested that the titrations can be modelled by redox interaction between haem *a* and Cu_B (and to a weaker extent with Cu_A), and with both metal centres redox-

Figure 4 'Glutamate trap' model for proton coupling in cytochrome oxidase

In this model, proposed in general form in [18] and developed in the light of the atomic structure of cytochrome oxidase in [19], electron transfer to haem *a* and from haem *a* to haem *a*₃ is linked to the protonation-deprotonation of Glu-243. The 'translocated protons' (H_t^+) enter through 'pore A' [1] from the N phase (A) and are relocated to a site that is redox-linked to the binuclear centre in response to the change in electrostatic fields caused by electron transfer to haem *a*₃ (B). This relocation site may be the haem *a*₃ propionates or nearby residues, such as His-368 or Asp-364. This electron transfer-linked relocation of the protons could provide the gating or trapping mechanism by which the P-phase exit route is favoured (C). Because of the position of these sites, their protons are not available for water formation. When oxides are formed during oxygen reduction, additional 'substrate' protons (H_s^+) must have access to the binuclear centre (C) in order to form water. This route may overlap part of the 'trapped' protons route or may be quite separate, e.g. through a putative 'pore B' [1].

linked to at least two acid/base groups with different p*K* values [13]. If the p*K* values of one of these groups is altered, then the redox potential of the metal centre can be lowered and its pH dependency of midpoint potential can be weakened. We suggest that this is the case for the Ile-67 → Asn mutation. In the structure of the yeast enzyme, Ile-67 (Ile-66 in beef heart) is located between haem *a* and the conserved glutamate residue Glu-243 (Glu-242 in beef heart) (Figure 1) [2]. This Glu-243 residue is a good candidate for a redox-linked protonation site. In the case of the Ile-67 → Asn mutant, we propose that this substitution may perturb the protonation properties of Glu-243, which in turn alters the redox properties of haem *a*. Specifically, the introduction of an asparagine group in place of isoleucine could raise the p*K* values of the glutamate residue, perhaps by hydrogen bonding to it, and this in turn could lower the redox potential of the haem *a*. This interaction might also affect the mobility of the glutamate residue, and this mobility may also be an essential component of the proton-conduction mechanism.

In our 'glutamate-trap' model for proton translocation in the haem/copper oxidases (Figure 4), protonation of the conserved glutamate, in response to reduction of haem *a*, is essential for the catalytic reaction of the binuclear centre. Movement of the

electron from haem *a* to the binuclear centre is likely to be linked with an associated relocation of the glutamate-bound proton to another site, and this movement can act essentially as the gate to prevent the proton being released through the same pathway by which it was taken up. Evidence for internal proton rearrangement associated with electron transfer from binuclear centre to haem *a* has been reported [14,15]. It is proposed that it is perturbation of these critical processes that leads to the dramatic decrease in catalytic activity observed for the Ile-67 → Asn mutant. The site(s) for proton transfer from the glutamate would have to be redox linked to the binuclear centre processes, but unable to react with the oxide products of oxygen reduction; the haem propionates and nearby protonatable residues such as Asp-364 and His-368 would be reasonable candidates, particularly since there is a precedence for redox linkage in this region in other haem systems [16,17]. We are currently examining this model further by looking at the effects of mutation of the glutamate residue on protonation processes associated with the catalytic-reaction cycle and its associated redox-linked protonations.

This work was funded by a grant from the International Human Frontier Science Program (RG0464/1995-M) to both laboratories and by a Wellcome Trust Project Grant (049722) to P.R.R. B.M. holds a Medical Research Council Career Development Award.

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