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Cytochrome *bo* from *E. coli* does not exhibit the same proton transfer characteristics as the bovine cytochrome *c* oxidase during oxygen reduction

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The reaction where fully reduced cytochrome *bo* from *E. coli* partially reduces dioxygen has been characterized with respect to the kinetics of the associated proton uptake, and with respect to the pH- and D_2O-sensitivity of the electron transfer reactions. A monophasic proton uptake with a rate constant of about 8×10^3 s^{-1} and a stoichiometry of 0.8 H^+/*bo* were recorded, using the indicator dye, Cresol red, at pH 8.2. The electron transfer reactions were independent of pH in the range 6.0–9.5 and were not affected by exchanging H_2O to D_2O as solvent. Comparison of these results with those obtained in an earlier investigation of the bovine cytochrome *c* oxidase [(1992) Biochemistry 31, 11853–11859], indicates differences between the two oxidases with respect to the role of protons in oxygen reduction and/or the mechanism of proton uptake from the medium.

Cytochrome *bo*; Cytochrome *c* oxidase; Proton uptake; Isotope effect; Electron transfer; Flow flash

1. INTRODUCTION

Cytochrome *bo* (ubiquinol:O_2 oxidoreductase) of *E. coli* and bovine cytochrome *c* oxidase (ferrocytochrome *c*:O_2 oxidoreductase) both belong to the superfamily of terminal oxidases. These enzymes catalyse the reduction of dioxygen and couple this redox reaction to vectorial transport of protons across a lipid membrane, i.e. they are redox-linked proton pumps [2–5]. Despite differences with respect to the number of subunits, type of heme groups, and reducing substrate, they both have a similar functional core. This consists of a low-potential centre, accepting electrons from the reducing substrate, and a heme-copper binuclear centre that binds and reduces dioxygen. The amino acid sequence of the largest subunit of these enzymes, subunit I, which in the bovine cytochrome *c* oxidase binds three of the four redox centres, and in cytochrome *bo* all three metals, have been compared and shown to match in approximately 40% of the amino acid residues [6]. Using site-directed mutagenesis, six conserved histidines in subunit I have been identified as metal ligands in both types of oxidase [6–8]. It has therefore been suggested that these enzymes may also have closely related mechanisms for redox-driven proton pumping [9].

The oxygen binding site of the terminal oxidases is situated in the interior of the protein, and therefore specific pathways for both electrons and protons from the surrounding medium into the binuclear centre are required. In addition, a proton link facilitating proton translocation over the membrane is obligatory. There are examples of energy-converting proteins, e.g. bacteriorhodopsin and bacterial photosyntetic reaction centres, that are critically dependent on specific internal protonatable groups for their proton-transfer reactions. These groups have been identified by using combinations of site-directed mutagenesis and kinetic methods (see [10,11]).

Using the flow–flash approach [12], our group has focussed on the fast electron- and proton-transfer reactions occurring during the oxidative part of the cytochrome *c* oxidase catalytic cycle, i.e. when the enzyme reduced to different degrees is oxidized by molecular oxygen [1,13,14]. Results from these investigations [1] indicate that proton transfer is rate limiting in two of the steps of the oxygen reduction process. The present characterisation of the proton transfer reactions accompanying partial reduction of dioxygen by fully reduced cytochrome *bo* indicates, however, that proton transfer does not limit the rate for the structurally and functionally related ubiquinol oxidase. This suggest that the acid–base chemistry accompanying oxygen reduction in the two enzymes is different.

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2. MATERIALS AND METHODS

2.1. *Materials*

Cytochrome *bo* was prepared from *E. coli*, strain RG145, using the protocol of [15]. Prior to experiments with the pH indicator dye, where a low buffering capacity is necessary, the enzyme stock solution was dialysed against 3 × 100 vols. of 0.1 M KCl and 0.05% sodium N-dodecyl sarcosinate adjusted to pH 8. This was also the medium used in the proton uptake measurements. All other experiments, except those in D_2O, were performed in 0.1 M MES-KOH (pH < 7.0), 0.1 M HEPES-KOH (7.0 ≤ 8.0) or 0.1 M Tris-HCl (pH > 8.0), with 0.1% lauryl maltoside as detergent. The D_2O experiments were done in 0.1 M HEPES-KOD and at pH* = 7.5 (pH meter reading). To accomplish complete exchange of H_2O to D_2O, the enzyme stock solution was diluted with 2 vols. of D_2O buffer and reconcentrated. This procedure was repeated 3 times during a period of 24 h. D_2O (99.9%) and the sodium salt of Cresol red were from Sigma, and all other chemicals used were of the purest grade available. The pK_a value of Cresol red was determined by spectrophotometric titration under the conditions used in the present work, and a value of 8.3 was found.

*At this pH, pD becomes 7.9, due to the equilibrium effect on the glass electrode.

2.2. *Methods*

Formation of the fully reduced CO complex of cytochrome *bo* was accomplished as described earlier [16]. The flow–flash kinetics, both with respect to electron-transfer and proton-uptake kinetics, were performed as in [1]. Concentrations of cytochrome *bo* in the electron-transfer and proton-uptake experiments were 1.5 μM and 5 μM, respectively, and the oxygen concentration was 1.0 mM in both. The proton uptake reaction was detected using 30 μM of the pH indicator dye, Cresol red. Calibration of the indicator signal was made by 15 μl additions of 0.5 mM H_2SO_4 to 2.5 ml of the exhausted reaction mixture, collected at the flow cell outlet and transferred to a stirred cuvette. All experiments were performed at 22°C. Curve fitting was done as described previously [1] and also using a program based on the Levenberg–Marquardt algorithm, written by Dr. Örjan Hansson at this department.

3. RESULTS

3.1. *pH indicator experiments*

The choice of observation wavelength for these experiments was dictated by the need for minimal interference from absorbance changes due to the cytochromes. We found that at 580 nm, the rapid CO photodissociation is visible, whereas the two phases of the oxygen reaction are not seen. In Fig. 1 trace A, the experiment is performed in the absence of buffer, and changes in the protonation state of the indicator is recorded together with the enzyme contribution at this wavelength. Fig. 1, trace B shows the course of the reaction detected in the presence of buffer and indicator. Under these conditions, only the enzyme contribution to the absorbance changes is detectable. The difference between these traces, shown in Fig. 1 trace C, then gives only the indicator contribution. Since it is the alkaline form that absorbs at 580 nm, the increasing absorbance seen in Figure 1C corresponds to proton uptake. The size of the absorbance change corresponds to the uptake of about 0.8 protons/enzyme molecule using an extinction coefficient of 24.2 $mM^{-1} \cdot cm^{-1}$ (redox, at 560–580 nm) for calculation of the cytochrome *bo* concentration [17]. Kinetic analysis shows that the time–course is well described by a single exponential with a rate constant of 8,000 s^{-1}.

In addition to the rapid uptake described above, we also observed uptake of more protons on a very slow time scale (seconds, not shown). The rate of this reaction is similar to the very slow phase observed previously [16] in the oxygen reaction of cytochrome *bo*. Although the rate is too low to be of catalytic significance, this suggests that the decay of the species present after the two rapid phases into the fully oxidized enzyme is accompanied by uptake of protons.

3.2. *pH dependence of the partial reduction of dioxygen*

The pH dependence of the absorbance changes coupled to the partial reduction of dioxygen by the three electron reduced (fully reduced) cytochrome *bo* was monitored in the Soret region at 430 nm. As found earlier [16], a biphasic course of the reaction with apparent rate constants of 2×10^4 and 4×10^3 s^{-1} was observed at an oxygen concentration of 1 mM. In the interval pH 6.0–9.5, no effect on the rate constants or the signal amplitudes was seen in the oxygen reduction kinetics.

3.3. *Solvent isotope effect on the partial reduction of dioxygen*

Fully reduced cytochrome *bo*, solubilized and carefully equilibrated in D_2O, was oxidized by molecular oxygen and the absorbance changes measured at 430 nm. None of the kinetic phases described above were affected by the exchange of H_2O to D_2O as solvent. The H_2O and D_2O experiments were performed at the same pH meter reading, pH* 7.5. This is, however, balanced by the equilibrium isotope effect on titrable groups in proteins, that commonly is of about the same magnitude, but of the opposite sign [18]. The ionisation state of the enzyme is therefore expected to be the same in the two experiments.

4. DISCUSSION

The aim of this work has been to characterise and compare the proton transfer reactions that are coupled to the reduction of dioxygen by fully reduced cytochrome *bo* from *E. coli*, and bovine cytochrome *c* oxidase. It should be noted that cytochrome *bo* lacks the low potential copper centre, Cu_A, and therefore cannot hold more than three electrons in its fully reduced state, whereas the bovine enzyme has four electrons. This makes the end points of the reactions different. The cytochrome *bo* reaction is expected to stop in a ferryl state whereas the bovine oxidase reduces dioxygen completely to water. From the suggested similarities in structure and function, however, the first three partial steps of oxygen reduction would be expected to be related. In the bovine enzyme, arrival of the first electron from the low potential site, i.e. cytochrome *a* oxidation

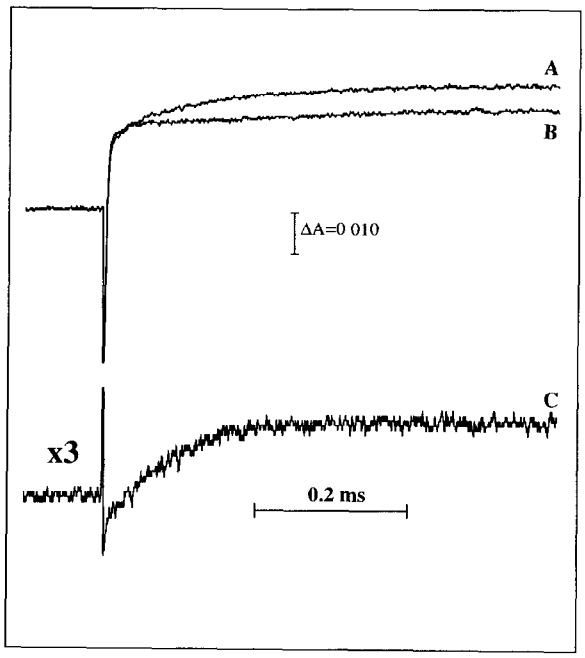

Fig. 1. Absorbance changes at 580 nm in the absence (A) and presence (B) of 0.1 M HEPES-KOH Concentrations are 30 μM Cresol red, 1 mM dioxygen and 5 μM fully reduced carbonmonoxy cytochrome *bo*. and the experiment is performed at pH 8.2 and at $T = 22$°C. Both traces are the averages of 20 transients. In C, the difference between the traces is shown, and it reflects the absorbance changes specifically due to the pH indicator dye.

with the rate 3×10^4 s^{-1} [19,20], triggers the proton uptake from the bulk water [1]. In this state the binuclear centre has three electrons, probably as a peroxy/a_3 compound with Cu_B reduced. Proton uptake then occurs with a maximal rate of 1×10^4 s^{-1}, rather than 3×10^4 s^{-1}, and titrates with a pK of about 8. The next step in the oxygen chemistry is expected to be a cleavage of the dioxygen bond followed by the formation of the ferryl intermediate. Indeed, this species is identified both optically in steady-state experiments involving reversed electron transfer in intact mitochondria [21] and kinetically with time-resolved resonance Raman [22,23]. In our earlier flow–flash experiments, collecting data in the visible and the near infrared regions, this event is reflected as a phase that exhibits a rate and a titration behaviour that is similar to that of the 1×10^4 s^{-1} proton uptake. In addition, the described reactions with cytochrome *c* oxidase also showed a deuterium solvent isotope effect [1]. We therefore concluded that proton transfers to the oxygen chemistry from a group situated in a proton link between the water bulk and the oxygen binding site could be rate limiting for the redox reactions on this time scale. The difference in rate between the reaction preceding proton uptake (2×10^4 s^{-1}) and the rate of the actual uptake of protons (8×10^3 s^{-1}) in cytochrome *bo* is similar to that observed in the bovine enzyme. In the latter system, however, the reaction preceding proton uptake (3×10^4 s^{-1}) is subject to a small, but significant, deuterium isotope effect, and the subsequent redox reaction (1×10^4 s^{-1}) was found to be both pH-dependent and D_2O-sensitive [1]. In the present study, we have observed neither an isotope effect, nor any pH dependence in any of the two phases resolved in the reaction between cytochrome *bo* and oxygen. These dissimilarities argue for a different role of protons in oxygen reduction and/or for differences in the mechanism for delivery of protons from the medium into the binuclear site in between the two enzymes. This assumes, however, that oxygen reduction follows a similar course in the two enzymes. At the present level of understanding of the oxygen reaction in cytochrome *bo*, differences also in this respect cannot be excluded.

Thus, our results suggest that the detailed catalytic mechanism is not the same in the bovine and *E. coli* oxidases. In this context it is worth noting that different K_M values for oxygen have been reported for the two oxidases, with a 3-fold higher K_M (O_2) for cytochrome *bo* [24,25]. Recently, dissimilarities in CO binding to the binuclear centres of the aa_3-type oxidases compared to cytochrome *bo* [26] have also been reported. These are other indications of differences between the two enzymes with respect to the chemistry of the binuclear site. We hope that future studies of these differences between the terminal oxidases will prove useful for understanding their mechanism.

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REFERENCES

[1] Hallén, S. and Nilsson, T. (1992) Biochemistry 31, 11853–11859.
[2] Wikström, M.K.F. (1977) Nature 266, 271–273.
[3] Matsushita, K., Patel, L. and Kaback, H.R. (1984) Biochemistry 23, 4703–4714.
[4] Puustinen, A., Finel,M., Virkki, M. and Wikström, M. (1989) FEBS Lett. 249, 163–167.
[5] Puustinen, A. and Wikström, M. (1991) Proc. Natl. Acad. Sci. USA 88, 6122–6126.
[6] Hosler, J.P., Ferguson-Miller, S., Calhoun, M.W., Thomas, J.W., Hill, J., Lemieux, L.J., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M.M.J., Babcock, G.T. and Gennis, R.B. (1993) J. Bioenerg. Biomembr. 25, 121–136.
[7] Lemieux, L.J., Calhoun, M.W., Thomas, J W., Ingledew, W.J. and Gennis, R.B. (1992) J. Biol. Chem. 267, 2105–2113.
[8] Minagawa, J., Mogi, T., Gennis, R.B and Anraku, Y. (1992) J. Biol. Chem. 267, 2096–2104.
[9] Chepuri, V., Lemieux, L., Au, D.C.T. and Gennis, R.B. (1990) J. Biol. Chem. 265, 11185–11192.
[10] Lanyi, J.K. (1992) J. Bioenerg. Biomembr. 24, 169–179.
[11] Okamura, M.Y. and Feher, G. (1992) Annu. Rev. Biochem. 61, 861–869.
[12] Gibson, Q. and Greenwood, C. (1963) Biochem. J. 86, 541–554.
[13] Oliveberg, M. and Malmström, B.G. (1992) Biochemistry 31, 3560–3563.

[14] Nilsson, T., Hallén, S and Oliveberg, M. (1990) FEBS Lett 260, 45–47.
[15] Minghetti, K.C., Chepuri-Goswitz, V., Gabriel, N.E., Hill, J.J., Barassi, C A., Georgiou, C.D., Chan, S.I. and Gennis, R.B. (1992) Biochemistry 31, 6917–6924.
[16] Svensson, M. and Nilsson, T. (1993) Biochemistry, in press.
[17] Puustinen, A., Finel, M., Haltia, T., Gennis, R.B., and Wikström, M. (1991) Biochemistry 30, 3936–3942.
[18] Schowen, K.B.J. (1978) in: Transition States of Biochemical Processes (Gandour, R.D., and Schowen, R L. eds), 225–283, Plenum Press, New York.
[19] Han, S., Ching, Y and Rousseau, D.L (1990) Nature 348, 89–90.
[20] Varotsis, C. and Babcock, G.T (1990) Biochemistry 29, 7357–7362
[21] Wikström, M. and Morgan, J E. (1992) J Biol. Chem. 267, 10266–10273.
[22] Babcock, G T. and Varotsis, C. (1993) J. Bioenerg. Biomembr 25, 71–80.
[23] Varotsis, C., Zhang, Y., Appelman, E.H. and Babcock, G.T. (1993), Proc. Natl Acad. Sci USA 90, 237–241.
[24] Anraku, Y. and Gennis, R B., (1987) Trends Biochem Sci. 12, 262–266.
[25] Petersen, L.C., (1977) Biochem. Biophys. Acta 460, 299–307.
[26] Woodruff, W.H. (1993) J. Bioenerg. Biomembr. 25, 177–188.