TRAPPING OF AN INTERMEDIATE IN THE OXIDATION–REDUCTION CYCLE OF CYTOCHROME *d* IN *ESCHERICHIA COLI*

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1. Introduction

Cytochrome *d* is a terminal oxidase in the respiratory chain of many bacteria [1]. In spite of the importance of this membrane-bound cytochrome relatively little is known about its reaction mechanism. It has not been purified although the structure of its unique chlorin prosthetic group has been determined [1]. Cytochrome *d* is characterized in reduced *minus* oxidized difference spectra as an absorption peak at 628 nm and a trough at 648 nm. The Soret absorption band has not been recorded since cytochrome b_1, which is generally present with cytochrome *d*, has a strong absorption band in this region [1]. The absorption bands at 628 and 648 nm and usually attributed to the reduced and oxidized forms of cytochrome *d*. However, there are some anomalies. Thus, the components responsible for these absorption bands are not *direct* oxidation–reduction products of one another but pass through an intermediate form, cytochrome *d**, which has no absorption band in the 600–700 nm region [2,3]. Potentiometric titration of the 628 nm component gives a midpoint oxidation-reduction potential of + 260 mV (n = 1) (M. R. Pudek and P. D. Bragg, submitted for publication). However, the 648 nm component does not follow normal redox behaviour in potentiometric titrations. Even at potentials as high as + 450 mV it is not formed unless oxygen is present. This suggests that the 648 nm component might be analogous to the 'oxygenated' cytochrome oxidase of mitochondria [4]. Further studies are required to determine the redox relationships between the 628 and 648 nm components and cytochrome *d**.

Although cytochrome *d** can be trapped as a compound with cyanide [3,5] its low steady state level relative to those of the 628 and 648 nm components makes further study of it very difficult [2,3]. In the present paper we show that the amount of cytochrome *d* as the intermediate species can be greatly increased in the aerobic steady state by reduction of membrane particles with ascorbate in the presence of PMS at temperatures below 0°C.

2. Experimental

Respiratory particles were prepared from *E. coli* NRC 482, grown on 1.4% disodium succinate to the stationary phase of growth, as previously described [6]. The respiratory particles were suspended in 0.05 M potassium phosphate buffer, pH 7.0, and 50% ethylene glycol to a concentration of 14 mg particle protein per ml. Ethylene glycol, which has been shown not to interfere with electron transport even at high concentrations, is present to prevent freezing of the samples at low temperatures [7].

A Perkin Elmer/Hitachi 356 spectrophotometer with the cryogenic attachment was employed for spectroscopic analysis at low temperatures. The particle suspension was aerated by mixing in air at room temperature and 1.0 ml was then placed in both the reference and the sample compartment of the cuvette holder. The path-length of the cuvette employed was 3 mm unless indicated otherwise. The cuvette holder was immersed in the Dewar flask of the cryogenic system which contained ethanol cooled with dry ice to give the desired temperature of 1°C to –38°C. The temperature of the sample

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was measured directly using a Yellow Springs Instrument Co. model 42SC tele-thermometer. After equilibration for 10–15 min at the desired temperature, 15 μl of 0.25 M ascorbate in 50% ethylene glycol and 10 μl of 1 mM phenazine methosulfate in 50% ethylene glycol were added to the sample cuvette. The difference spectra of the aerobic steady state were scanned against the oxidized reference compartment immediately and at 2–3 min intervals thereafter to ensure that a steady state had been reached. The temperature in the sample cuvette during the experiment was stable to within ± 1°C. At temperatures above 1°C the system became anaerobic too quickly so that the aerobic steady state difference spectrum could not be obtained.

To obtain absolute spectra of cytochrome *d* the oxidized sample was scanned against a sample reduced with dithionite and containing 20 mM KCN. The cyanocytochrome *d* so formed in the reference sample has no absorption bands in the 600–700 nm range [3,5].

When the inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) (11 mM in EtOH) was used it was added prior to cooling the samples. All spectra were normalized to the baseline.

3. Results and discussion

Only a small fraction of the cytochrome *d* exists as the intermediate species cytochrome *d** in the aerobic steady state at room temperature in particles oxidizing NADH [2,3]. However, from difference spectra of the aerobic steady state of particles oxidizing ascorbate in the presence of PMS measured at 77°K 60–70% of the cytochrome *d* appeared to be trapped as the intermediate species. This was not observed with particles oxidizing succinate or NADH. This behaviour was examined under more controlled conditions.

Aerobic steady state difference spectra of particles oxidizing ascorbate in the presence of PMS showed an increase in the size of the trough at 648 nm with decreasing temperature due to the disappearance of the absorption peak of this component in the sample cuvette without the concomittent appearance of the absorption peak of the 628 nm component. This indicated that the cytochrome *d* was being trapped in the cytochrome *d** form. The aerobic steady state level of reduction of cytochrome b_1 decreased slightly with the decrease in temperature. The upper curve shows the reduced *versus* oxidized difference spectrum of the respiratory particles employed in these experiments.

Fig.2 shows the relationship between the temperature and the amount of cytochrome *d* in the intermediate form in particles oxidizing ascorbate in the

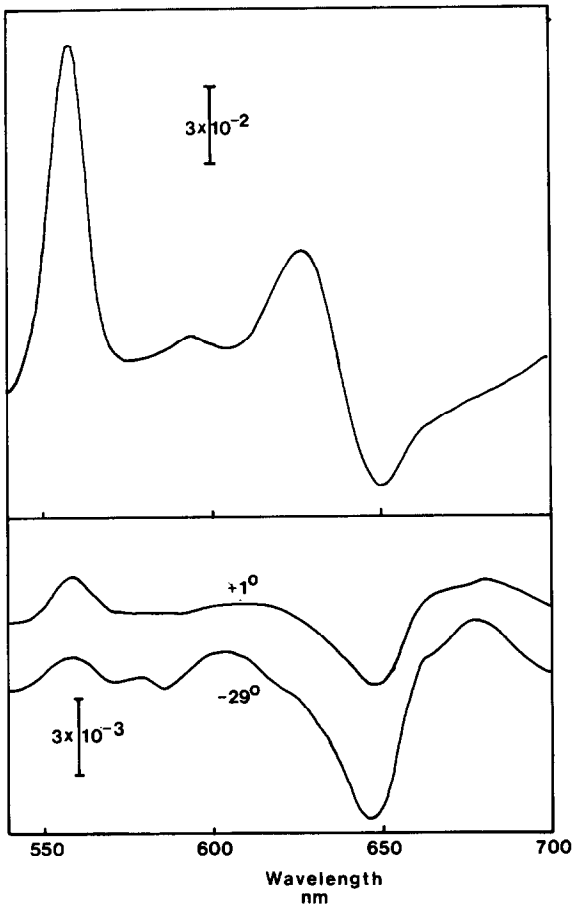

Fig.1. Difference spectra of respiratory particles of *E. coli*. The upper curve shows the dithionite-reduced *versus* air-oxidized difference spectrum of the respiratory particles employed in these experiments. The lower curves represent the difference spectra at 1°C and −29°C of the aerobic steady-state of respiratory particles oxidizing ascorbate (3.75 mM) in the presence of PMS (0.01 mM) *versus* the air-oxidized respiratory particles. The spectra were determined as described in Experimental except that for the upper curve a cuvette with a 10 mm light-path was used.

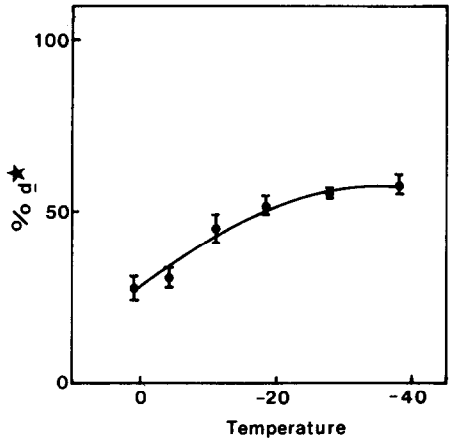

Fig.2. Effect of temperature (°C) on the amount of cytochrome *d* in the intermediate (*d**) form in the aerobic steady-state in particles oxidizing ascorbate with PMS. The experiment was carried out as in fig.1. The amount of cytochrome *d* in the *d** form was related to the peak height at 648 nm in the absolute spectrum shown in fig.3.

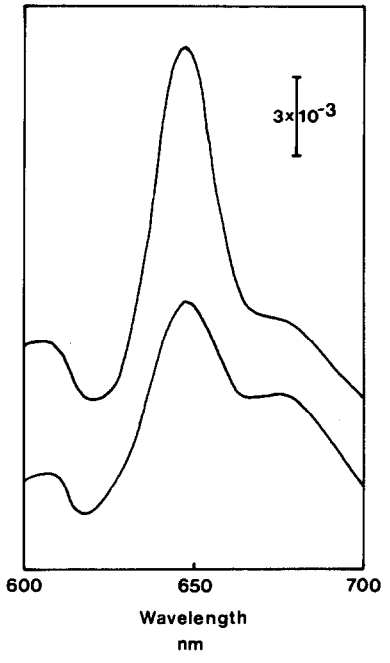

Fig.3. Absolute spectra of oxidized cytochrome *d* (upper curve) and cytochrome *d* of respiratory particles oxidizing ascorbate in the presence of PMS (lower curve). The spectra were obtained as described in Experimental at −18°C using the same particle preparation as in fig.1.

presence of PMS. The amount of cytochrome *d* in the intermediate form is expressed relative to the peak height of the 648 nm component in the absolute spectrum. The steady state level of cytochrome *d** increased from 28% at 1°C to a level of 57% at −38°C.

To determine if this plateau level was due to only partial conversion of the 648 nm component to cytochrome *d**, or if it was due to the formation of a species with a new absorption peak overlapping at 648 nm, the absolute spectra of cytochrome *d* in the oxidized form and in the presence of ascorbate with PMS were measured at −18°C (fig.3). Substrate decreased the size of the peak at 648 nm without formation of a new peak or displacement of the absorption maximum. Thus, there is only partial conversion of cytochrome *d* to *d** at low temperatures.

The steady state level of cytochrome *d** at −20°C was decreased from 51% to 40% and 30% in the presence of 55 and 165 μM HOQNO, respectively (fig.4). The steady state level of

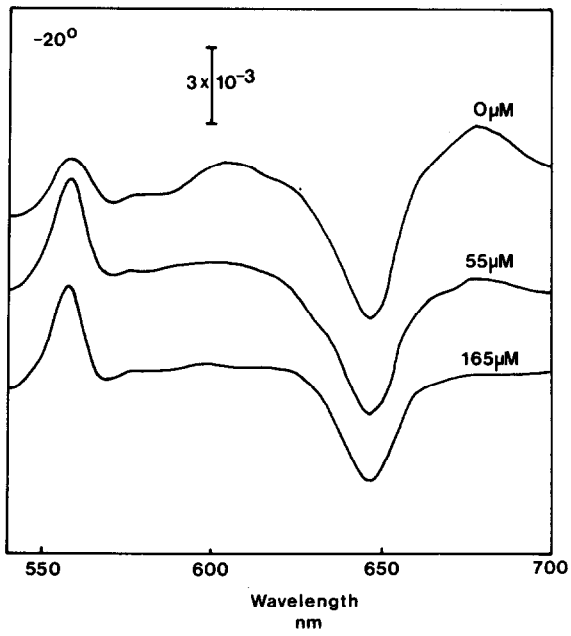

Fig.4. Effect of HOQNO on the aerobic steady state level of reduction of cytochromes in particles oxidizing ascorbate with PMS. The reduced *minus* oxidized difference spectra were measured at −20°C as in fig.1. HOQNO (55 μM and 165 μM) was added prior to cooling.

reduction of cytochrome b_1 increased from 4.7% to 9.7% and 9.1%, respectively, in the presence of HOQNO under these conditions.

We conclude that there is a temperature-sensitive reaction in the cytochrome *d* oxidation–reduction cycle which results in an increase in the steady state level of cytochrome *d**. This can only be observed in particles oxidizing ascorbate in the presence of PMS because PMS introduces electrons beyond the temperature-sensitive dehydrogenases. The studies with the inhibitor HOQNO suggest that electrons are fed into the respiratory chain before or after cytochrome b_1 but prior to the site of inhibition by HOQNO.

Because it is possible to trap a significant portion of the terminal oxidase in the intermediate form, cytochrome *d**, this method should allow further examination of the nature of this species to gain insight into the reaction mechanism of cytochrome *d*.

Acknowledgements

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References

[1] Lernberg, R. and Barrett, J. (1973) Cytochromes. Academic Press, London and New York.
[2] Kauffman, H. F. and Van Gelder, B. F. (1973) Biochim. Biophys. Acta 305, 260–267.
[3] Pudek, M. R. and Bragg, P. D. (1974) Arch. Biochem. Biophys. 164, 682–693.
[4] Okunuki, K. (1966) in: Comprehensive Biochemistry (M. Florkin and E. Stotz, eds.) Vol. 14, pp. 232–308, Elsevier, Amsterdam.
[5] Kauffman, H. F. and Van Gelder, B. F. (1974) Biochim. Biophys. Acta 333, 218–227.
[6] Bragg, P. D., Davies, P. L. and Hou, C. (1972) Biochem. Biophys. Res. Commun. 47, 1248–1255.
[7] Erecinska, M. and Chance, B. (1972) Arch. Biochem. Biophys. 151, 304–315.