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Involvement of a *d*-type oxidase in the Na^+-motive respiratory chain of *Escherichia coli* growing under low $\Delta\tilde{\mu}_{H^+}$ conditions

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An attempt has been made to find out which of the two terminal oxidases, the *d*-type or the *o*-type, operates as a Na^+ pump in *Escherichia coli* grown at low $\Delta\tilde{\mu}_{H^+}$ conditions. For this purpose, mutants lacking either *d* or *o* oxidase have been studied. It is shown that a d^-,o^+ mutant grows slowly or does not grow at all under low $\Delta\tilde{\mu}_{H^+}$ conditions (alkaline or protonophore-containing growth media were used). Inside-out subcellular vesicles from the d^-,o^+ mutant cannot oxidize ascorbate and TMPD, and cannot transport Na^+ when succinate is oxidized in the presence of a protonophore. The same vesicles are found to transport Na^+ when NADH is oxidized as if the Na^+-motive NADH-quinone oxidase were operative. On the other hand, a mutant lacking *o* oxidase (d^+,o^-) grows at low $\Delta\tilde{\mu}_{H^+}$ conditions as fast as the maternal *E. coli* strain containing both *d* and *o* oxidases. Corresponding vesicles oxidize ascorbate and TMPD as well as succinate, the oxidations being coupled to the protonophore-stimulated Na^+ transport. Growth in the presence of a protonophore is found to induce a strong increase in the *d* oxidase level in the maternal d^+,o^+ *E. coli* strain. It is concluded that oxidase of the *d*-type, rather than of the *o*-type, operates as a Na^+ pump in *E. coli* grown under conditions unfavorable for the H^+ cycle.

Low $\Delta\tilde{\mu}_{H^+}$; Na^+-motive terminal oxidase; *d*- and *o*-type oxidases; *Escherichia coli*

1. INTRODUCTION

As found in our group [1,2], growth of *Bacillus FTU* or *E. coli* under low $\Delta\tilde{\mu}_{H^+}$ conditions results in the appearance of a Na^+-motive terminal oxidase activity. To lower $\Delta\tilde{\mu}_{H^+}$, a protonophorous uncoupler was added to the growth medium, or the pH of the medium was increased to create an opposite ΔpH across the cytoplasmic membrane. In the case of *Bac. FTU*, the addition of cyanide at a low concentration specifically inhibiting the H^+-motive oxidase could also be used for the same purpose.

In *E. coli*, two terminal quinol oxidases, i.e. *d*- and *o*-type cytochrome complexes, were described (for reviews, see [3,4]). In the present paper, we have tried to elucidate a possible relation of these oxidases to the Na^+-motive one. Mutants lacking *d* oxidase or, alternatively, *o* oxidase were studied for this purpose. The results indicate that it is *d* oxidase, rather than *o* oxidase, that is competent in the Na^+ pumping.

Abbreviations: $\Delta\tilde{\mu}_{H^+}$, transmembrane difference in electrochemical H^+ potentials; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ETH 157, *N,N'*-dibenzyl-*N,N'*-diphenyl-1,2-phenylenediacetamide; PCP, pentachlorophenol; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene diamine.

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2. MATERIALS AND METHODS

E. coli K-12, strain GR 70N (F^-, gal, rpsL Str^R thi^-), GO 103 (F^-, Δcyd::kan gal rpsL Str^R Kan^R thi^-) and GO 104 (F^-, Δcyd::kan gal rpsL Str^R Kan^R thi^-) were a generous gift from Professor R.B. Gennis. In contrast to the maternal strain GR 70N, strains GO 103 and GO 104 were devoid of *o* and *d* oxidases, respectively [5]. The growth medium contained 22 mM potassium phosphate, 20 mM sodium phosphate, 10 mM NaCl, 10 mM $(NH_4)_2SO_4$, 50 mM glycyl-glycine, 0.05% yeast extract, 50 mM sodium succinate, 1 mM $MgSO_4$, streptomycin (0.1 mg × ml^{-1}), kanamycin (0.05 mg × ml^{-1}) and thiamine (10^{-6} g × ml^{-1}, pH 7.2, 7.5 or 8.6 (see figure captions).

Inside-out subcellular vesicles were obtained using a French press as described elsewhere [1]. To measure Na^+ uptake by the vesicles, the Penefsky's gel filtration–centrifugation procedure [6] and a flame photometer were employed (for details, see [1,7]). The incubation mixture contained 50 mM K_2SO_4, 30 mM $MgSO_4$, 5 mM Na_2SO_4, 5 mM potassium phosphate, 0.5 mM ethylenediamine tetraacetate, 100 mM Tricine, pH 7.75, 1.5×10^{-4} dicyclohexyl carbodiimide, and vesicles, ca. 5 mg protein × ml^{-1}.

Oxygen consumption was measured with a standard oxygen Clark-type electrode.

To measure cytochrome spectra of the vesicles, an Aminco DW-2000 spectrophotometer was used.

3. RESULTS AND DISCUSSION

The growth rates of three *E. coli* strains, i.e. d^+,o^+ (GR 70N), d^-,o^+ (GO 103) and d^+,o^- (GR 104) under different conditions are shown in Fig. 1. At neutral pH the maximal growth rate is observed in the maternal d^+,o^+ strain, whereas the *o* oxidase- or *d* oxidase-deficient mutants grow somewhat slower (Fig. 1A). At

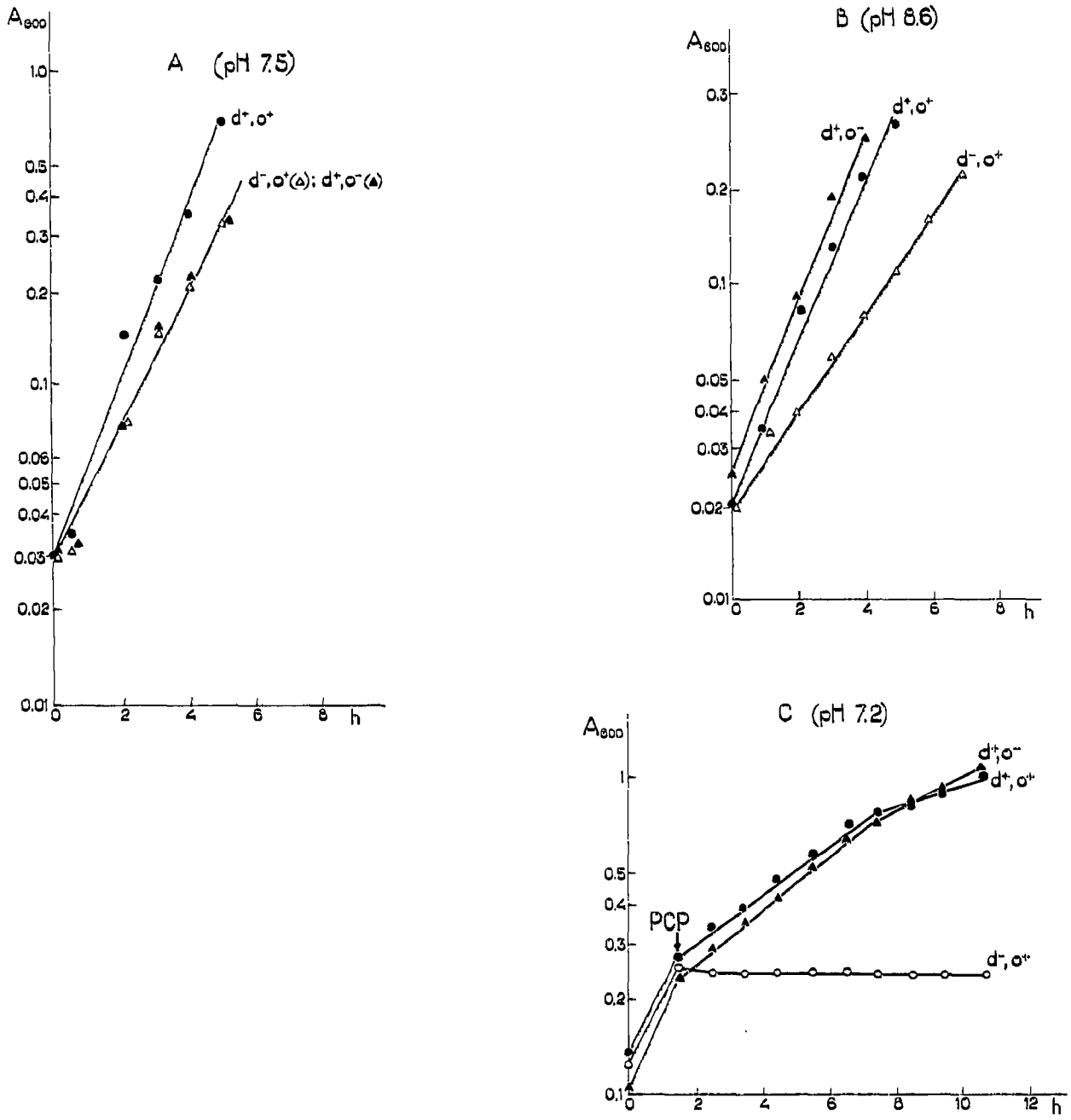

Fig. 1. *E. coli* strains grown at pH 7.5 (A), 8.6 (B) and 7.2 (C). In C, 8×10^{-5} M PCP was added as indicated.

alkaline pH, growth rates of the d^+,o^+ and d^+,o^- strains are similar, but the growth of the d^-,o^+ strain is much slower (Fig. 1B). The most dramatic difference in the growth rates between d^+,o^+ and d^+,o^-, on one hand, and d^-,o^+, on the other, was revealed with the addition of the protonophorous uncoupler PCP to the growth medium at neutral pH (Fig. 1C). Here the *d* oxidase-containing strains were still growing (the growth rate, though lower than without PCP, was quite measurable), whereas the d^-,o^+ mutant did not grow at all. As it was found in polarographic experiments, PCP stimulated respiration of both strains (not shown).

One may conclude therefore that it is *d* oxidase, rather *o* oxidase, that is essential for growth at low $\Delta\mu_{H^+}$ conditions.

Na^+ transport in the inside-out subcellular vesicles is shown in Fig. 2. Clearly, the addition of ascorbate and TMPD to the vesicles from the d^+,o^+ or d^+,o^- strains

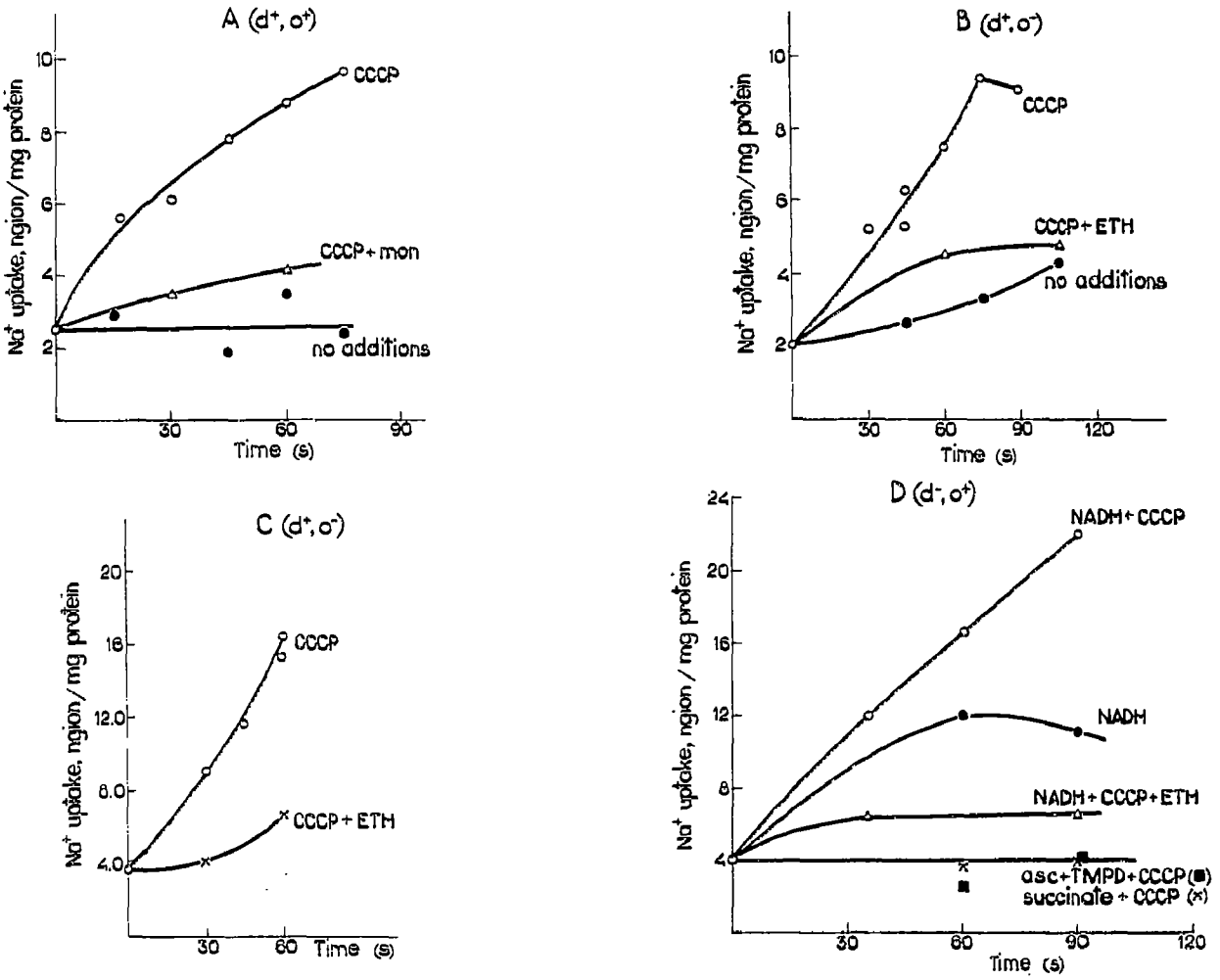

Fig. 2. Na^+ uptake by the inside-out subcellular vesicles of three *E. coli* strains. 0.5 mM TMPD and 50 mM ascorbate (A, B and D), 20 mM succinate (C, D) or 3 mM NADH (D) were added at zero time. Other additions: 1×10^{-5} M CCCP, 1×10^{-5} M monensin (mon), 2×10^{-4} M ETH 157.

resulted in some Na^+ uptake which was strongly inhibited by the Na^+/H^+ antiporter monensin or the Na^+ uniporter ETH 157 (Fig. 2A and 2B).

The same figures show that the protonophore CCCP has to be added for obtaining measurable Na^+ uptake. As found previously [1, 7–9], this effect results from the CCCP-mediated H^+ efflux which discharges the electric potential produced by the Na^+-motive oxidase. The stimulating action of CCCP does not make it possible to explain observed Na^+ uptake by co-operation of an H^+-motive oxidase and an endogenous Na^+/H^+ antiporter.

As shown in Fig. 2C, succinate was used as a respiration substrate for vesicles from the d^+,o^- strain. Oxidation of this compound was also coupled to the Na^+ uptake.

Fig. 2D deals with vesicles from the d^-,o^+ strain. They failed to transport Na^+ when ascorbate and TMPD or succinate were added. Polarographic experiments showed that the vesicles oxidized succinate and NADH but did not oxidize ascorbate and TMPD (not shown). NADH oxidation was coupled to the Na^+ transport, and CCCP stimulated this process (Fig. 2D). Thus vesicles from the d^-,o^+ strain were competent in the formation of a respiration-supported Na^+ gradient. Hence the absence of Na^+ uptake with the oxidation of succinate was most probably due to a lack of Na^+-motive terminal oxidase in the d^-,o^+ strain.

Fig. 3 shows cytochrome spectra of the d^+,o^+ strain growing with or without PCP. It is seen that PCP caused a strong increase in the absorption maximum at 630 nm which is known to correspond to cytochrome *d* [3,4].

Thus three independent pieces of evidence argue in favor of the conclusion that it is *d*, rather than *o* oxidase, that is involved in Na^+ pumping by the terminal span of the *E.coli* respiratory chain.

(i) *d* oxidase, not *o* oxidase, is essential for the growth at low $\Delta\tilde{\mu}_{H^+}$ conditions, such as an alkaline medium or a medium supplemented with a protonophore.

(ii) Growth in the presence of a protonophore induces

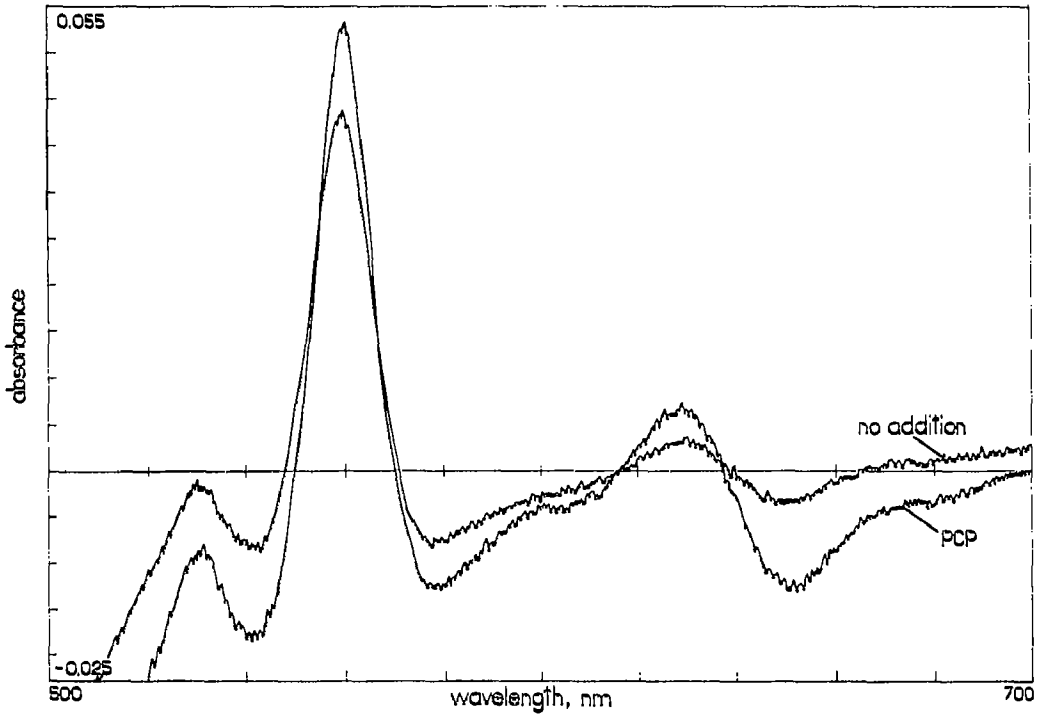

Fig. 3. Reduced (dithionite) minus oxidized (ferricyanide) difference spectra of subcellular vesicles from the d^+,o^+ *E. coli* strain grown at pH 7.2 with or without 9×10^{-5} M PCP.

a significant increase in the cytochrome *d* content in the d^+,o^+ strain.

(iii) Succinate oxidation by the *E. coli* subcellular vesicles is coupled to Na^+ uptake in the d^+,o^- strain but not in the d^-,o^+ strain. The former, not the latter, can oxidize ascorbate and TMPD, the oxidation being Na^+-motive.

The Na^+-motive terminal oxidase was discovered in our group when studying the alkalo- and halotolerant *Bac. FTU* [7,10]. Then this activity was described in *E. coli* [1,11] and *Vitreoscilla* [12,13]. In the latter case, the authors suggested that the oxidase must be of the *o* type. In *Bac. FTU* induction of the Na^+-motive oxidase was shown to correlate poorly with the cytochrome *d* level [8]. In this respect *E. coli* is clearly different than *Bac. FTU*. Here cytochrome *d* proved to be the best candidate to the Na^+-motive oxidase. Apparently in some other microorganisms the same function is performed by another enzyme species (for discussion, see [14]).

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