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Cytochrome *d* induction in *Escherichia coli* growing under unfavorable conditions

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Growth of *E. coli* in the presence of the protonophorous uncoupler pentachlorophenol is shown to strongly enhance levels of cytochrome *d*, a putative Na^+-motive oxidase. This effect was found to be arrested by chloramphenicol and stimulated by high Na^+ concentration in the growth medium. The induction of cytochrome *d* takes place in a mutant deficient in the F_0F_1 ATP-synthase but does not occur in mutants deficient in either of two different components of the *Arc* system. Similar relationships were revealed when pentachlorophenol was replaced by ferricyanide and phenazine methosulfate, agents oxidizing the respiratory chain. Induction of cytochrome *d* is also shown to occur in riboflavin-deficient mutants growing in the presence of such low riboflavin concentrations as to be insufficient to maintain a high respiration rate. It is suggested (i) that it is $\Delta\bar{\mu}_{H^+}$ decrease rather than reduction of the respiratory chain that is the signal for the induction of cytochrome *d*, and (ii) the *Arc* system is involved in this type of metabolic regulation.

Cytochrome *d*; Na^+-motive oxidase; *Arc* system; *E. coli*

1. INTRODUCTION

Under aerobic conditions, the *E. coli* quinol oxidases of the *o* and *d* types are induced at the exponential and stationary growth phases, respectively [1]. Expression of these two oxidases is regulated by the *Arc* and *Fnr* systems [2,3]. The first system was shown to consist of a receptor protein, *Arc* B, and a regulatory protein, *Arc* A [4]. It was suggested that it is reduction of a respiratory chain component that serves as the signal for the *Arc* system-mediated increase in the cytochrome *d* level [2]. In apparent agreement with this suggestion, it was found that a decrease in the O_2 concentration [5] and the addition of cyanide [6] resulted in the induction of cytochrome *d*. Similar effects were produced by some respiratory chain mutations [2,7], changes in electron acceptors [8], and sulphur deficiency [9].

Puustinen et al. [10] showed that the *E. coli o*-type oxidase operates as an H^+ pump ($H^+/e^- = 2$), whereas the *d*-type oxidase cannot pump protons ($H^+/e^- = 1$), thus presumably forming $\Delta\bar{\mu}_{H^+}$ due to a transmembrane electron transfer.

In our group, it was shown that the *E. coli d*-type oxidase can operate as an Na^+ pump when *E. coli* is grown at low $\Delta\bar{\mu}_{H^+}$, i.e. in the presence of a protonophorous uncoupler (PCP) or at high pH [11]. For this reason, we propose that it is the $\Delta\bar{\mu}_{H^+}$ decrease that serves as the signal for induction of cytochrome *d* in *E. coli* [11]. In this paper, we report data of experiments planned within the framework of this concept.

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Abbreviations: $\Delta\bar{\mu}_{H^+}$; transmembrane difference in H^+ potentials; PMS, phenazinemethosulfate; PCP, pentachlorophenol.

2. MATERIALS AND METHODS

The following *E. coli* strains were studied: GR70N (F^- gal rpsL Str^R thi^-) from Prof. R.B. Gennis; B4089 (F^- thi^- hsdR hsdM), B4093 (F^- thi^- hsdR hsdM ribC::Tn5 Kan^R) and B4094 (F^- thi^- hsdR hsdM ribA::Tn5 Kan^R), all from the Russian collection of microorganisms [12]; LE392 (F^- supF supE hsdR galK trpB lacY tonA Δ(uncB–uncD) [13]; ECL547 [$sdh^+\phi$(sdh-lac); other characteristics as in ECL525], ECL590 [$sdh^+\phi$(sdh-lac)arcB1; other as in ECL525] and ECL968 [$sdh^+\phi$(sdh-lac)arcA131; other as in ECL525], all from Prof. E.C.C. Lin and Dr. S. Iuchi [14]. The ECL525 characteristics: F^- araD139 Δ(argF-lac) V169 rpsL150 relA1 fib5301 ptsF25 deoI1 Δfrd101 [14].

The basic growth medium containing 22 mM potassium phosphate, 20 mM sodium phosphate, 34 mM NaCl, 10 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 5 mM glycyl glycine, 0.05% yeast extract and 0.4% glycerol, pH 7.5, was supplemented with (i) thiamine (1 $\mu g \times ml^{-1}$) and streptomycin (0.1 $mg \times ml^{-1}$) (GR70N), (ii) 0.1% tryptone and streptomycin (0.1 $mg \times ml^{-1}$) (ECL547, ECL590 and ECL968), (iii) 0.1% tryptone and thiamine (10 $\mu g \times ml^{-1}$) (B4089) and (iv) 0.1% tryptone, thiamine (10 $\mu g \times ml^{-1}$) and kanamycin (0.05 $mg \times ml^{-1}$) (B4093 and B4094). When the low Na^+ medium was used, all the Na^+ salts were replaced by the K^+ salts so that the final concentration of Na^+ was found to be about 1 mM. When indicated, the growth medium was supplemented with 2.5 mM ferricyanide and 1.5×10^{-5} M PMS at an intermediate stage of growth ($A_{600} = 0.5$). Then cells were allowed to continue to grow with these components for 2 h. In all cases, cells were grown up to the mid exponential phase at 37°C with shaking in the dark.

All the measurements of respiration and ferricyanide reduction were carried out on whole cells. The Clark-type oxygen electrode and Hitachi-557 spectrophotometer were used in the former and in the latter cases, respectively.

To measure cytochrome spectra, the membrane fraction was obtained. The cells were sedimented at 7.500 × *g* (10 min, 4°C) and washed twice with a medium containing 0.15 M NaCl, 5 mM $MgSO_4$ and 10 mM Tris-HCl, pH 7.5. The sediment was suspended in 0.1 M K_2SO_4, 5 mM $MgSO_4$, 0.5 mM EDTA and 10 mM Tricine-KOH, pH 7.75 and French-pressed (16,000 psi). After this treatment, the mixture was centrifuged at 50,000 × *g* (90 min, 2°C). The sediment was suspended in a solution of the same composition as that used previously to suspend the cells. To estimate the amount of the *d*-type and *b*-type cytochromes in the membranes, dithionite *minus* ferricyanide difference spectra were measured using the Hitachi-557 spectrophotometer. The following molar extinction coefficients were used: for *d*-type cytochrome, $E_{630-614} = 8.5$, and for *b*-type cytochromes, $E_{560-575} = 17.5$ [15].

3. RESULTS AND DISCUSSION

An example of the PCP-induced increase of *d*-type cytochrome levels in the *E. coli* membranes is given in Fig. 1. In agreement with our previous observation [11], growth in the presence of PCP and high Na^+ concentration resulted in a strong increase of the light absorption maximum at 630 nm which is known to be cytochrome *d*-specific [1]. Such an increase was completely arrested by chloramphenicol and was much less pronounced when the Na^+ concentration in the growth medium was decreased from 100 mM to 1 mM. This finding is consistent with the assumption that *E. coli* cytochrome *d* is induced by a $\Delta\bar{\mu}_{H^+}$ decrease in a Na^+-dependent fashion. Such relationships seem quite reasonable taking into account that cytochrome *d* operates as an Na^+ pump when *E. coli* is grown at low $\Delta\bar{\mu}_{H^+}$ conditions [11]. In this context, it should be mentioned that the same two conditions, i.e. $\Delta\bar{\mu}_{H^+}$ decrease and high Na^+ concentration, were shown to be required for the induction of Na^+-ATPase in *Streptococcus faecalis* [16–18].

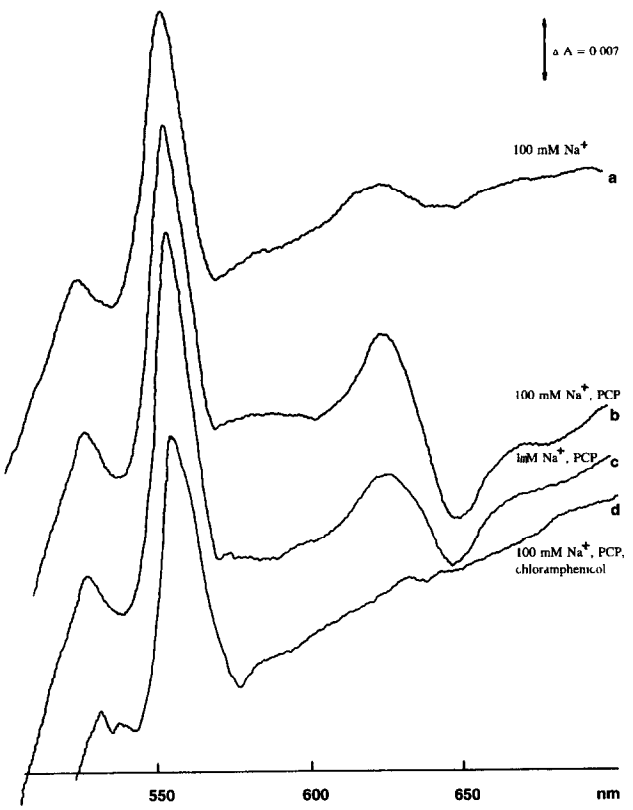

Fig. 1. Reduced *minus* oxidized difference spectra of *E. coli* GR70N membranes. The cells were grown with or without 8×10^{-5} M PCP and chloramphenicol, 0.1 mg × ml^{-1} in the presence of two different Na^+ concentrations. Protein concentrations were 3.1, 4.0, 4.5 and 4.1 mg protein × ml^{-1} in curves a, b, c and d, respectively.

An alternative explanation was not excluded, namely, that it is a change in reduction of respiratory chain components (rather than in the $\Delta\bar{\mu}_{H^+}$ level) that serves as the signal to induce cytochrome *d* [2]. As already mentioned, lowering of the O_2 concentration or addition of cyanide to the growth medium also results in a cytochrome *d* increase [5,6]. Therefore, it could be speculated that an increase in reduction of the respiratory chain is the signal [2].

To test this possibility, we measured the cytochrome *d* level when *E. coli* was grown for 2 h in the presence of an electron acceptor, ferricyanide, and a redox-mediator, PMS, i.e. under conditions favoring oxidation, rather than reduction, of the respiratory chain. If respiratory chain reduction were responsible for the cytochrome *d* induction, ferricyanide and PMS would be ineffective as inducers. On the other hand, if it is the $\Delta\bar{\mu}_{H^+}$ decrease that plays the role of the inducer, these compounds might be favorable for the induction since electrons from the respiratory substates are accepted by ferricyanide instead of being utilized by the respiratory chain to reduce O_2 in a $\Delta\bar{\mu}_{H^+}$-generating fashion. Measurement of the ferricyanide reduction rate on whole cells showed that, under the conditions used, it was as high as 0.7 μmol ferricyanide × mg $protein^{-1} \times min^{-1}$.

As can be seen from Table I, exp. 1, ferricyanide and PMS cause an increase in the cytochrome *d* level. This increase proved to be not as strong as that induced by PCP. This is not surprising since with PCP, the $\Delta\bar{\mu}_{H^+}$ decrease should be stronger than with these agents. In any case, the effect of ferricyanide and PMS does not support the idea that respiratory chain reduction is the signal for cytochrome *d* induction.

One extra piece of evidence against this idea was obtained in experiments with the *E. coli* mutants deficient in the system of riboflavin synthesis. Due to inhibition of the initial steps of respiration in these mutants, oxygen consumption was more than 5 times slower than that of the maternal strain (not shown). They fail to grow on succinate as the only energy source. As shown in Table I, exp. 2, these mutants had much higher cytochrome *d* levels when grown in the presence of minimal riboflavin concentrations. Addition of higher riboflavin concentrations lowered cytochrome *d* levels.

In the next series of experiments, we studied cytochrome *d* induction in a quite different *E. coli* strain, i.e. ECL, which, under the usual conditions, shows higher cytochrome *d* levels than the strains used in experiments

1 and 2. In this strain, two mutants deficient in *Arc* system components were described, namely, *arc*A$^-$ and *arc*B$^-$. As shown in Table I, exp. 3, PCP induces a significant cytochrome *d* increase in the maternal strain whereas in the mutants, PCP decreased cytochrome *d* levels or was without measurable effect. Ferricyanide and PMS decreased cytochrome *d* levels in both *arc*A$^-$ and *arc*B$^-$ mutants. Thus the *Arc* system is required for the induction of cytochrome *d* caused by the lowering of $\Delta\bar{\mu}_{H^+}$.

In the final series of experiments, the *E. coli unc*- mutant LE392 was studied. In this mutant, F_0F_1 ATP-synthase is absent [13] so that variations in $\Delta\bar{\mu}_{H^+}$ levels cannot affect the ATP concentration. It was found that in such a mutant, PCP is still quite effective in increasing the cytochrome *d* concentration. Thus it is a decrease in $\Delta\bar{\mu}_{H^+}$, rather than in the ATP level, that is responsible for the induction of cytochrome *d* synthesis.

This hypothesis may, in fact, explain all sets of observations concerning the induction of cytochrome *d* which can be caused by anaerobiosis (resulted, according to Kashket, in a strong $\Delta\bar{\mu}_{H^+}$ decrease [19]), cyanide, low sulfur growth medium, mutations resulting in deficiency of the respiratory chain components, etc.

Apparently, the bacterial cell monitors $\Delta\bar{\mu}_{H^+}$ levels to induce the Na^+-cycle enzymes when $\Delta\bar{\mu}_{H^+}$ drops [20]. Indications that a $\Delta\bar{\mu}_{H^+}$ receptor ('protometer') really exists at least in some bacteria, were recently obtained in our group [21]. In *E. coli*, the *arc*B protein seems to be a good candidate for the role of protometer.

Interestingly, an increase in the growth medium temperature was shown to induce accumulation of cytochrome *d*-specific mRNA in *E. coli*. This effect was totally absent from *arc*A or *arc*B mutants [22]. In this context it should be mentioned that, according to recent observation of the Konings' group [23], a shift to high temperature causes a much stronger increase in the H^+ conductance, than in the Na^+ conductance of the bacterial membrane. It was found that thermophilic *Clostridium fervidus* employs the Na^+ cycle to support the osmotic work of its membrane. For this purpose, Na^+-ATPase and Na^+ solute symporters are used [23]. One may speculate that also in *E. coli*, high temperature specifically increases the H^+ conductance, lowers $\Delta\bar{\mu}_{H^+}$and induces the Na^+-motive cytochrome *d*.

In conclusion, the above data indicate that low $\Delta\bar{\mu}_{H^+}$ conditions are favorable for the induction of *E. coli* cytochrome *d*, provided that the Na^+ concentration is high. This effect seems to be mediated by the *Arc* system.

Table I

The cytochrome *d* and *b* levels in some *E. coli* strains growing under different conditions (uncoupler, 8×10^{-5} M PCP)

Exp. No	*E. coli* strain	Additions to the growth medium	Cytochromes (nmol × mg protein^{-1})		*d*/*b*
			d	*b*	
1	GR70N	–	0.077	0.489	0.16
		uncoupler	0.244	0.428	0.57
		uncoupler [Na^+] is decreased down to 1 mM	0.129	0.405	0.32
		$K_3[Fe(CN)_6]$, PMS	0.187	0.501	0.37
2	B4089	–	0.076	0.310	0.24
	B4094 (*rib*A$^-$)	riboflavin, 5 mg × ml^{-1}	0.453	0.584	0.78
		riboflavin, 200 mg × ml^{-1}	0.153	0.332	0.46
	B4093 (*rib*C$^-$)	riboflavin, 20 mg × ml^{-1}	0.252	0.352	0.72
3	ECL547	–	0.179	0.403	0.44
		uncoupler	0.519	0.635	0.82
		$K_3[Fe(CN)_6]$, PMS	0.216	0.408	0.53
	ECL968 (*arc*A$^-$)	–	0.173	0.574	0.30
		uncoupler	0.098	0.522	0.19
		$K_3[Fe(CN)_6]$, PMS	0.016	0.322	0.05
	ECL590 (*arc*B$^-$)	–	0.194	0.501	0.39
		uncoupler	0.185	0.513	0.36
		$K_3[Fe(CN)_6]$, PMS	0.046	0.335	0.14
4	LE392 (*Δunc*)	–	0.277	0.540	0.51
		uncoupler	0.540	0.705	0.76

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