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THE ANAEROBIC OXIDATION OF DIHYDROOROTATE BY *ESCHERICHIA COLI* K-12

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SUMMARY

The oxidation of dihydroorotate under anaerobic conditions has been examined using various mutant strains of *Escherichia coli* K-12. This oxidation in cells grown anaerobically in a glucose minimal medium is linked via menaquinone to the fumarate reductase enzyme coded for by the *frd* gene and is independent of the cytochromes. The same dihydroorotate dehydrogenase protein functions in both the anaerobic and aerobic oxidation of dihydroorotate. Ferricyanide can act as an artificial electron acceptor for dihydroorotate dehydrogenase and the dihydroorotate-menaquinone-ferricyanide reductase activity can be solubilised by 2 M guanidine · HCl with little loss of activity.

INTRODUCTION

One of the reactions in the biosynthesis of pyrimidines is the oxidation of dihydroorotate to orotate. In *Escherichia coli* this reaction is catalysed by the membrane-bound enzyme dihydroorotate dehydrogenase, and electrons from this biosynthetic oxidation are fed directly into either aerobic [1] or anaerobic electron transport sequences [2]. Under anaerobic conditions the oxidation of dihydroorotate in *E. coli* K-12 has been shown to require menaquinone, although in the absence of menaquinone, the other quinone formed by *E. coli*, ubiquinone, is partially functional [2]. Fumarate functions as the terminal electron acceptor when the cells are grown anaerobically on a glucose minimal medium [2].

In an attempt to further define this anaerobic electron transport system, dihydroorotate oxidation under anaerobic conditions has been studied in mutant strains lacking either cytochromes or the enzyme fumarate reductase. The stability of the system to solubilisation by the chaotropic agent, guanidine · HCl, is also reported.

MATERIALS AND METHODS

Organisms. All the bacterial strains used in this work were derived from *E. coli* K-12 and are shown in Table I.

TABLE I

STRAINS OF *E. COLI* K-12

Strain	Relevant genetic loci*	Other information
AN359	*entA403, hemA⁻, ilvC7, pyrE41*	
AN362	*entA403, ilvC7, pyrF40*	
R4*frd-1*	*metB1, frd-1*	Obtained from J. Guest
AN454	*entA403, frd-1, pyrF40*	Isolated following mating between R4*frd-1* and AN362
AB259	*thi⁻*	Obtained from J. Pittard

* Genetic nomenclature is that used by Bachmann et al. [3].

Genetic techniques. The technique for conjugation experiments was based on that described by Taylor and Thoman [4] and transduction experiments, in which the generalized transducing phage Plkc was used, were carried out as described by Pittard [5].

Media. The medium used was that described by Monod et. al. [6] as medium 56. To the sterilized mineral salts base were added the appropriate L-amino acids to give a final concentration of 0.2 mM; thiamine, 0.2 μM; 2,3-dihydroxybenzoate, 40 μM and uracil, 180 μM unless stated otherwise in the text. The carbon source, usually glucose, was added as a sterile solution at a final concentration of 30 mM.

Anaerobic growth. Flasks containing volumes of less than 1 l were placed in an anaerobic culture jar under hydrogen. For the anaerobic growth of 10-l cultures, 10-l bottles were fitted with rubber stoppers with two closeable outlets. After autoclaving, the medium was allowed to cool under a stream of sterile N_2. After the addition of glucose, appropriate growth factors and the inoculum, the bottle was flushed out with sterile N_2 and sealed.

Preparation of cell membranes. Membranes were prepared as described previously [7]. Briefly, washed cells were disintegrated by using a Sorvall Ribi Cell fractionator and the "membranes" were separated by ultracentrifugation and resuspended in a 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES) buffer system (pH 7.0) containing magnesium acetate, sucrose and ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA).

Protein concentrations were determined by using Folin's phenol reagent [8] with bovine serum albumin (fraction V; Sigma Chemical Co. St. Louis, Mo., U.S.A.) as standard.

Guanidine · HCl treatment of membranes. Membranes (about 30 mg/ml) were mixed with 0.75 M or 2 M guanidine hydrochloride for 15 min at 4 °C and then centrifuged at 160 000 $\times g$ for 60 min. The supernatant was dialyzed against 40 volumes of the 0.1 M TES buffer system at 4 °C for 12 h and is designated the "solubilised fraction". The pellet was resuspended in 0.1 M TES buffer system to half the original volume and is referred to as the "insoluble" fraction.

Spectrophotometric assay of the conversion of dihydroorotate to orotate. Orotate formation was assayed according to the method of Newton et al. [2] by measuring increase of absorption at 280 nm.

Fluorimetric estimation of menaquinol. The formation and removal of menaqui-

nol was assayed by measuring the fluorescence (excitation, 340 nm; emission, 440 nm; uncorrected) as described by Newton et al. [2].

Estimation of quinones. The ubiquinone and menaquinone contents of cells were determined by continuously extracting 3 g wet weight of cells with acetone for 3 h as described by Newton et al. [2]. The acetone extract was evaporated and the residue extracted into light petroleum (b.p. 60–80 °C). The extract was then chromatographed on silica gel plates [9] and the yellow quinone bands eluted into absolute ethanol.

The concentration of ubiquinone was estimated by the difference in absorbance of the oxidized and the reduced quinol achieved by adding borohydride as described by Crane and Barr [10] ($E_{m,\,275} = 12\,700$). The menaquinone isolated by this procedure was a mixture of menaquinone and demethyl-menaquinone. Demethyl-menaquinone was estimated by the colorimetric assay of Baum and Dolin [11] ($E_{m,\,560} = 9100$). The total amount of menaquinone present was estimated by the method of Lester et al. [12]. From the change in absorbance at 245 nm on the reduction of the quinone the concentration of menaquinone was estimated using the molar extinction coefficients of 19 800 for demethyl-menaquinone and 25 800 for menaquinone.

Assay of cytochromes and flavins. Difference spectra were recorded at 77 K using an Aminco-Chance dual wavelength spectrophotometer, operating in the split-beam mode with a full scale deflection of 0.1 or 0.2 Å. Samples were frozen by the rapid injection technique [13] using 20 % glycerol (final concentration) for intensification. The spectral bandpass of the measuring light was 0.99 nm (0.18 mm slit width, 5.5 nm/mm reciprocal dispersion).

Membrane preparations were diluted to a concentration of 15–20 mg protein/ml and the differences between $Na_2S_2O_4$-reduced and oxygenated samples were recorded. The wavelength pairs employed [14] were: cytochrome *b*, $\Delta A_{558-575\ nm}$; cytochrome *d*, $\Delta A_{630-615\,nm}$; total flavin, $\Delta A_{510-465\,nm}$. The determination of flavoprotein by this method may be subject to error due to absorption by non-haem iron. Cytochrome *o* concentrations were determined from the $Na_2S_2O_4$-reduced+CO, minus $Na_2S_2O_4$-reduced difference spectra by using the wavelength pairs $\Delta A_{415-430\ nm}$.

RESULTS

Derepression of the enzyme catalysing the anaerobic oxidation of dihydroorotate

To derepress the dihydroorotate-fumarate reductase system, mutants affected in pyrimidine biosynthesis were cultured in the presence of a limiting concentration of uracil.

The rate of the fumarate-dependent anaerobic oxidation of dihydroorotate was fully derepressed in anaerobic cultures of *E. coli* strain AN362(*pyrF*$^-$) after about 6 h of uracil starvation (Table II). The specific activity of 339 nmol/min per mg protein for the dihydroorotate-fumarate reductase obtained in membrane preparations from fully derepressed cells represented about a 5-fold derepression during uracil starvation and about a 10-fold increase of this anaerobic enzymic activity over values obtained if the cells were grown aerobically under similar conditions of uracil starvation (Table II).

The anaerobic oxidation of dihydroorotate in the absence of cytochromes

Strain AN359 carrying mutations in the *pyrE* and *hemA* genes was used to

TABLE II

FUMARATE-DEPENDENT OROTATE FORMATION BY MEMBRANES FROM A URACIL AUXOTROPH

Membranes were prepared from cells of strain AN362 grown on limiting (45 μM) or excess (200 μM) uracil as indicated and assayed for fumarate-dependent orotate formation as described in Materials and Methods. The reaction mixture contained (final concentrations) in a final volume of 3 ml, 0.1 M Tris buffer, pH 8.0, approx. 1 mg of membrane protein, 10 mM D-lactate, and 7 mM fumarate. The reaction was initiated by stirring in 60 μl of 0.5 M L-dihydroorotic acid (final concentration 10 mM) and measuring the rate of increase of absorption at 280 nm.

Uracil concentration (μM) in growth medium	Condition of incubation	Time of uracil starvation (h)	Orotate formed (nmol/min per mg protein)
200	anaerobic	–	65
45	anaerobic	2	231
45	anaerobic	4	322
45	anaerobic	6	339
45	aerobic	6	32

determine whether or not cytochromes were required for the fumarate-dependent oxidation of dihydroorotate. Strains carrying mutations in the *hemA* gene form cytochromes when grown in media supplemented with 5-aminolevulinate. Under anaerobic conditions in the absence of 5-aminolevulinate strain AN359 grew at about one-third the rate at which it grew in the presence of excess (30 μM) 5-aminolevulinate. Membranes prepared from strain AN359 grown either in the presence or in the absence of 5-aminolevulinate, and starved for uracil as described above, gave similar activities for the fumarate-dependent anaerobic oxidation of dihydroorotate (Table III). Cytochromes could not be detected spectroscopically in the membranes from those cells grown in the absence of 5-aminolevulinate although the levels of the quinones were normal (Table III).

The requirement for fumarate reductase in the oxidation of dihydroorotate

A mutant strain carrying the *frd-1* allele has been shown by Spencer and Guest [15] to cause loss of fumarate reductase activity and to prevent growth under anaerobic conditions on a glycerol-fumarate medium. The fumarate reductase activity in membrane preparations from strain AN454(frd^-) was less than 1 % of that in membranes from strain AN362(frd^+) (Andrews, S., unpublished observations). The fumarate-dependent anaerobic oxidation of dihydroorotate in strain AN454 (16 nmol/min per mg protein) is less than 5 % of that in strain AN362 (339 nmol/min per mg protein).

The dihydroorotate-fumarate reductase system has been shown to be dependent on menaquinone [2] and the menaquinol formed during the oxidation of dihydroorotate is oxidized with fumarate as hydrogen acceptor. The oxidation of menaquinol by fumarate does not occur with membranes from strain AN454(frd^-) although the rate of dihydroorotate-dependent reduction of menaquinone is normal (cf. Figs. 1a and 1b).

Ferricyanide is also able to function as an electron acceptor in the anaerobic oxidation of dihydroorotate (see Table IV). Since the addition of ferricyanide causes

TABLE III

MEMBRANE COMPONENTS AND FUMARATE-DEPENDENT OROTATE FORMATION

Flavins and cytochromes were determined by direct spectrophotometric examination of suspensions of membranes at 77 K. Quinones were first extracted and partially purified before spectrophotometric determination. Orotate formation was measured in a basal system containing Tris · HCl buffer, membrane suspension and D-lactate (see Materials and Methods).

Membrane component or enzymic activity	Units	Concentration* of component in membranes from strain AN359 grown in	
		Absence of 5-aminolevulinate	Presence of 30 μM 5-aminolevulinate
Total flavin	ΔA/cm per mg protein	0.04	0.05
Cytochrome *b*	ΔA/cm per mg protein	absent	0.05
Cytochrome *d*	ΔA/cm per mg protein	absent	0.02
Cytochrome *o*	ΔA/cm per mg protein	absent	0.03
Ubiquinone	nmol/g wet weight cells	73	78
Menaquinone	nmol/g wet weight cells	33	31
Demethyl-menaquinone	nmol/g wet weight cells	50	52
Fumarate-dependent orotate formation	nmol of orotate formed/ min per mg protein	116	123

* The amount of cytochromes are expressed as the difference in absorption at the appropriate wavelength pairs (see Materials and Methods) rather than in molar concentration as the spectra were measured at 77 K.

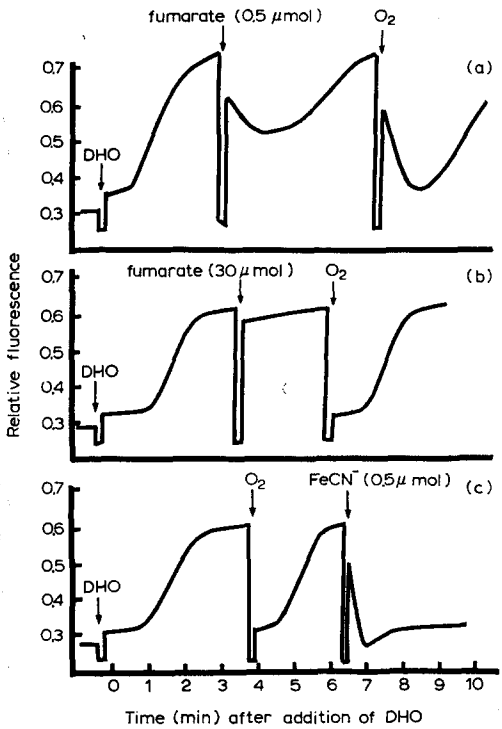

Fig. 1. Fluorimetric measurement of the reduction of menaquinone by dihydroorotate (DHO) and subsequent oxidation of the menaquinol formed with different hydrogen acceptors using membranes from a normal strain or from a mutant lacking fumarate reductase activity. The membranes used were from (a) strain AN362 (*frd*$^+$) or (b) and (c) strain AN454 (*frd*$^-$). The final volume of reaction mixture was 1 ml and contained 2–3 mg protein and 5 mM orotate. The cuvettes were aerated, where indicated, by gentle shaking.

TABLE IV

THE SOLUBILISATION OF THE DIHYDROOROTATE-FUMARATE REDUCTASE SYSTEM BY GUANIDINE · HCl

Orotate formation was measured in basal system containing Tris · HCl buffer, membranes from strain AN362, D-lactate and either fumarate (5 mM) or ferricyanide (0.8 mM) as described in Materials and Methods. The preparation was solubilised with 2 M guanidine · HCl as described in Materials and Methods.

Terminal oxidant	Orotate formation (nmol/min per mg protein) by	
	Original membrane preparation	Solubilised preparation
Fumarate	320	22
Ferricyanide	670	470

the re-oxidation of menaquinol in membranes from strain AN454 (Fig. 1c) such oxidation by ferricyanide is independent of the fumarate reductase activity.

Solubilisation of the dihydroorotate-ferricyanide reductase activity using guanidine · HCl

Membrane preparations from strain AN362(*pyrF*$^-$) grown under anaerobic conditions were mixed with final concentrations of 0.75 or 2 M guanidine · HCl and the solubilised components separated from the membrane residue by ultracentrifugation. The fumarate-dependent anaerobic oxidation of dihydroorotate activity remained in the insoluble fraction in the presence of 0.75 M guanidine and was almost completely inactivated by the 2 M guanidine treatment (Table IV). The dihydroorotate-ferricyanide reductase activity, however, was stable to treatment with 2 M guanidine and about 70 % of the activity was found in the "solubilised" fraction (Table IV). The solubilised preparation also contained menaquinone which was reducible by dihydroorotate and the menaquinol formed was reoxidized following the addition of ferricyanide.

DISCUSSION

The results presented above clearly indicate that in *E. coli* K-12 the anaerobic oxidation of dihydroorotate, in cells grown anaerobically in a glucose minimal medium, is linked via menaquinone to fumarate reductase and is independent of cytochromes. Mutations in the *pyrD* gene, the structural gene for dihydroorotate dehydrogenase, causes a uracil requirement under both anaerobic and aerobic growth conditions [16] indicating that the same dehydrogenase enzyme is linked to the aerobic as well as the anaerobic electron transport systems. The role of meanquinone in the fumarate-dependent anaerobic oxidation of dihydroorotate was established by Newton et al. [2] when the system was studied in a mutant strain lacking both menaquinone and ubiquinone.

The NADH- and α-glycerophosphate-fumarate systems studied by Singh and Bragg [17], and Haddock and Kendall-Tobias [18] are clearly analogous to the dihydroorotate-menaquinone-fumarate system. Furthermore, the results with the membranes from the strain carrying the *frd-1* allele show that the enzyme coded for by the *frd* gene is important under anaerobic conditions, not only for growth on α-glycerophosphate, but also for pyrimidine biosynthesis. The level of activity of the dihydroorotate-fumarate reductase, even in fully repressed cells, is higher than that reported for the NADH- or α-glycerophosphate-fumarate reductase activities. In the fully derepressed strain the dihydroorotate oxidation rate appears to be some 10-fold higher than that reported for NADH or α-glycerophosphate [17]. Under aerobic conditions the situation is reversed in that the rate of oxidation of dihydroorotate, even in membranes from fully derepressed cells, is only about 40 % of the NADH oxidase rate [16].

The stability of the dihydroorotate-menaquinone-ferricyanide reductase activity to solubilisation with 2 M guanidine · HCl should facilitate work on the purification of this part of the anaerobic electron transport sequence.

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