The Energy-Linked Transhydrogenase Reaction in Respiratory Mutants of *Escherichia coli* K 12

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1. Energy-linked and non-energy-linked transhydrogenase activities were assayed in membrane preparations from normal *Escherichia coli* K 12 and from various mutant strains. 2. The energy-linked transhydrogenase, which uses ATP as energy source, was dependent for activity on the presence of a functional $Mg^{2+} + Ca^{2+}$ -stimulated adenosine triphosphatase. 3. Neither of the quinones formed by *E. coli*, namely ubiquinone-8 and menaquinone-8, was required for normal ATP-dependent energy-linked transhydrogenase activity. 4. The energy-linked transhydrogenase was inhibited by piericidin A at a site unrelated to the sites of inhibition of the electron-transport chain by piericidin A.

Kaplan, Colowick & Neufeld (1952) demonstrated an essentially irreversible reduction of NAD^+ by NADPH in cell extracts of *Pseudomonas fluorescens*. A similar activity was demonstrated in ox heart particles, except that the reaction was readily reversible (Kaplan, Colowick & Neufeld, 1953). Danielson & Ernster (1963), working with phosphorylating submitochondrial preparations from rat liver or ox heart, demonstrated an ATP-dependent reduction of $NADP^+$ by NADH in the presence of Mg^{2+} . They concluded that this reaction was different from the reaction described by Kaplan *et al.* (1953) because of the requirement for ATP. The inhibition of the ATP-dependent transhydrogenase by oligomycin, plus the observation that oxidation of a suitable substrate via the electron-transport chain could replace the requirement for ATP, led to the conclusion that a 'high-energy' intermediate of oxidative phosphorylation was involved in the reaction. Evidence has since accumulated that the transhydrogenase activity demonstrated by Kaplan *et al.* (1953) and the energy-linked transhydrogenase demonstrated by Danielson & Ernster (1963) may be performed by different forms of the same enzyme. Thus both activities are inhibited by tri-iodothyronine and thyroxine (Ball & Cooper, 1959; Stein, Kaplan & Ciotti, 1959; Hommes & Estabrook, 1963), and antibodies against the purified non-energy-linked transhydrogenase inhibited not only the non-energy-linked but also the energy-linked transhydrogenase (Kawasaki, Satoh & Kaplan, 1964). Studies with labelled adenine nucleotides indicated that both the energy-linked and non-energy-linked

transhydrogenases were A:B transhydrogenases involving direct hydrogen transfer (Griffiths & Robertson, 1966).

The energy-linked transhydrogenase was first demonstrated in bacterial preparations by Murthy & Brodie (1964), working with a particulate fraction of *Escherichia coli*. Thyroxine and tri-iodothyronine inhibited the reaction, but oligomycin had no effect. Asano, Imai & Sato (1967) demonstrated the reaction in *Micrococcus denitrificans*, but the activity in this organism was sensitive to oligomycin. The energy-linked transhydrogenase has also been demonstrated in the photosynthetic organisms *Rhodospirillum rubrum* (Keister & Yike, 1966) and *Rhodopseudomonas spheroides* (Orlando, Sabo & Curnyn, 1966). The transhydrogenases in *E. coli* have been studied more extensively by Sweetman & Griffiths (1971) who demonstrated that, whereas the energy-linked reaction required Mg^{2+} for maximum activity, the non-energy-linked reaction was inhibited by Mg^{2+} . In the energy-linked reaction, 1–2 mol of ATP was utilized/mol of NADPH formed, and the reaction was sensitive to 2,4-dinitrophenol, piericidin A, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide or thyroxine but insensitive to oligomycin.

Mutant strains of *E. coli* K 12 have been isolated with mutations in the following genes: the *uncA* gene affecting Mg, Ca -ATPase* (Butlin, Cox & Gibson, 1971); the *ubiB* or *ubiD* genes affecting ubiquinone biosynthesis (Cox, Young, McCann &

* Abbreviation: Mg, Ca -ATPase, $Mg^{2+} + Ca^{2+}$ -stimulated adenosine triphosphatase activity.

Gibson, 1969); the *menA* gene affecting menaquinone biosynthesis (Newton, Cox & Gibson, 1971). The effects of these mutations on the energy-linked and non-energy-linked transhydrogenase activities in *E. coli* are described in the present paper.

MATERIALS AND METHODS

Chemicals. Piericidin A was kindly provided by Professor S. Tamura, Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan. Ubiquinone-3 was kindly provided by Dr O. Isler of F. Hoffmann-La Roche and Co., Basle, Switzerland. Lactate dehydrogenase and glutathione reductase were supplied by Sigma Chemical Co., St Louis, Mo., U.S.A. Chemicals generally were of the highest purity available commercially and were not further purified.

Organisms. All the bacterial strains used in this work were derived from *E. coli* K 12 and have been described (Cox *et al.* 1969; Butlin *et al.* 1971; Newton *et al.* 1971).

Media and growth of organisms. The minimal medium used (medium 56) was that described by Monod, Cohen-Bazire & Cohn (1951). To the sterilized mineral salts base were added, where appropriate, L-leucine, L-threonine, L-methionine, L-isoleucine, L-valine and L-arginine, each at 0.2 mM, and thiamin at a final concentration of 0.02 μ M. Glucose was added as a sterile solution at a final concentration of 30 mM.

Cells were grown in 7-litre New Brunswick fermenters as described by Cox, Newton, Gibson, Snoswell & Hamilton (1970).

Preparation and fractionation of cell extracts. Cell extracts and membrane fractions were prepared as described by Cox *et al.* (1970). The protein content of the membrane preparations, determined with Folin's phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951), with bovine serum albumin (fraction V; Sigma Chemical Co., St Louis, Mo., U.S.A.) as standard, was about 25 mg/ml.

Measurement of oxygen uptake. Oxygen uptakes at 25°C were measured polarographically as described by Cox *et al.* (1970).

Measurement of the energy-linked transhydrogenase activity. Reduction of NADP⁺ by NADH was assayed by coupling the reaction to the NADPH-dependent glutathione reductase and measuring the decrease in E_{340} (Ernster & Lee, 1967). The reaction mixture in a 1 cm light-path cuvette contained, in a final volume of 2.9 ml, the following components (final concentrations): membrane fraction (1 mg of protein/ml), sodium cyanide (18 mM), tris-HCl buffer (80 mM, pH 7.5), Mg²⁺ (14 mM), GSSG (0.7 mM), NADH (0.7 mM) and an amount of glutathione reductase capable of oxidizing 1 μ mol of NADPH/min. The cuvette was then adjusted to 30°C in the temperature-controlled cuvette housing of a Cary 14 spectrophotometer. NADP⁺ (0.3 mM) and then ATP (3.4 mM) were added and the increase in the rate of decrease in E_{340} , after the addition of ATP, was taken as a measure of the energy-linked transhydrogenase activity. The reference cuvette contained sufficient membranes in tris-HCl buffer to compensate for turbidity. The assay system was checked as a routine to ensure that the transhydrogenase activity was proportional to the

amount of membrane protein added. The cyanide (200 μ l of 250 mM) and membranes (100 μ l) were pre-incubated at room temperature for 5 min before addition of the other components. Piericidin A, when used, was added as portions of a 40 mM ethanolic solution immediately before the cuvette was placed in the spectrophotometer.

Measurement of the non-energy-linked transhydrogenase activity. Reduction of NAD⁺ by NADPH was assayed by coupling the reaction to the NADH-dependent reduction of pyruvate by lactate dehydrogenase and measuring the decrease in E_{340} . The reaction mixture in a 1 cm light-path cuvette contained, in a final volume of 2.9 ml, the following components (final concentrations): membrane fraction (0.5 mg of protein/ml), sodium cyanide (9 mM), tris-HCl buffer (80 mM, pH 7.5), pyruvate (1.4 mM), NADPH (0.7 mM) and an amount of lactate dehydrogenase capable of reducing 1 μ mol of pyruvate/min. All other operations were as described for the measurement of the energy-linked reaction except that the reaction was initiated by the addition of NAD⁺ (final concentration 0.9 mM). The increase in the rate of decrease in E_{340} after the addition of NAD⁺ was taken as the measure of the non-energy-linked transhydrogenase activity.

RESULTS

Transhydrogenase activities in the mutant strains lacking Mg,Ca-ATPase activity. The isolation of mutant strains of *E. coli* K 12 (AN120, AN183) carrying mutations in the *uncA* gene affecting the membrane-bound Mg,Ca-ATPase has been reported (Butlin *et al.* 1971). Strain AN180 is normal with respect to the Mg,Ca-ATPase activity and all three strains were derived, by transduction, from the same parental strain. Membrane fractions were prepared from the normal strain AN180 and from the two mutant strains AN183 and AN120 and the energy-linked and non-energy-linked transhydrogenase activities were examined (Table 1). The energy-linked transhydrogenase activity in strain AN180 was 64% of the non-energy-linked transhydrogenase activity, whereas in the two mutant strains this value decreased to only 3%. The NADH oxidase activities of the membrane fractions are also given in Table 1 and, as described previously (Butlin *et al.* 1971), a mutation affecting the Mg,Ca-ATPase activity results in an increase in the rate of electron transport. The oxidase activities are lower than those reported previously (Butlin *et al.* 1971) owing to the lower concentration of the mineral salts medium used for the growth of the cells in the present experiments.

Transhydrogenase activities in quinone-deficient mutants. A mutation in the *menA* gene affecting menaquinone biosynthesis has been characterized and a transductant (strain AN99) carrying the *menA*⁻ allele has been prepared from the normal strain AN98 (Newton *et al.* 1971). Ubiquinone-deficient mutants have also been genetically and

Table 1. *Comparison of transhydrogenase and NADH oxidase activities in a normal strain of E. coli and in mutant strains lacking Mg,Ca-ATPase activity*

Assays of enzymic activities are described in the Materials and Methods section.

Strain	Energy-linked transhydrogenase (a) (nmol of NADPH formed/ min per mg of protein)	Non-energy-linked transhydrogenase (b) (nmol of NADH formed/ min per mg of protein)	$100 \times (a/b)$	NADH oxidase activity (ng-atoms of O/min per mg of protein)
AN180	77	119	64	552
AN183 (<i>unc</i> ⁻)	4.2	142	3	696
AN120 (<i>unc</i> ⁻)	4.5	150	3	648

Table 2. *Comparison of transhydrogenase and NADH oxidase activities in a normal strain of E. coli and in quinone-deficient strains*

Assays of enzymic activities are described in the Materials and Methods section.

Strain	Energy-linked transhydrogenase (a) (nmol of NADPH formed/ min per mg of protein)	Non-energy-linked transhydrogenase (b) (nmol of NADH formed/ min per mg of protein)	$100 \times (a/b)$	NADH oxidase activity (ng-atoms of O/min per mg of protein)
AN98	58	87	67	384
AN99 (<i>menA</i> ⁻)	57	88	65	396
AN187 (<i>ubiD</i> ⁻), (<i>menA</i> ⁻)	78	107	73	162
AB2154	16	61	26	516
AN59 (<i>ubiB</i> ⁻)	13	31	42	73

biochemically characterized (Cox *et al.* 1969) and a transductant (strain AN187) has been prepared from strain AN99, which is deficient in both quinones (Newton *et al.* 1971). Strain AN187 therefore carries mutations in both the *menA* and the *ubiD* genes. The mutation in the *ubiD* gene causes only a partial metabolic block, allowing the formation of relatively small quantities of ubiquinone, but the aerobic growth and electron transport of any strain carrying this *ubiD*⁻ allele is severely affected (N. A. Newton, unpublished work). Strain AN59 was derived by transduction from strain AB2154 and carries a mutation in the *ubiB* gene. This transductant has been used in a detailed investigation of the function of ubiquinone in aerobic respiration (Cox *et al.* 1970). Membrane fractions were prepared from the parental strains AN98 and AB2154 and from the quinone-deficient strains AN99, AN187 and AN59, and the energy-linked and non-energy-linked transhydrogenase activities examined (Table 2). The ratios of energy-linked to non-energy-linked transhydrogenase activities of strains AN98, AN99 and AN187 were similar to that found above for strain AN180, indicating that neither menaquinone nor ubiquinone is involved in the energy-linked reaction. The parental strain AB2154 has less active non-energy-linked and energy-linked transhydrogenases

than the other strains and the energy-linked transhydrogenase activity is only 26% of the non-energy-linked one. In strain AN59, which completely lacks ubiquinone, the non-energy-linked transhydrogenase activity is only about half of that found in the parental strain AB2154, but the energy-linked activity remains about the same. Thus the ratio of energy-linked to non-energy-linked transhydrogenase activities is higher in the ubiquinone-deficient strain than in the parental strain.

Effect of piericidin A on the transhydrogenase activities. Studies on piericidin A inhibition of the NADH and D-lactate oxidase systems in *E. coli* K12 indicated that this inhibitor functioned as a ubiquinone analogue (Cox *et al.* 1970). It was therefore decided to examine the effect of piericidin A on both the energy-linked and non-energy-linked transhydrogenases in a normal strain (AN180). Piericidin A inhibits both transhydrogenase activities, but the energy-linked transhydrogenase is more sensitive to piericidin A than the non-energy-linked transhydrogenase (Fig. 1). After addition of ubiquinone-3 to the piericidin-treated system there was a slight initial increase in the rate of decrease in E_{340} , but the rate returned to the value observed before the addition. This effect is presumably caused by the decrease in E_{340} on reduction of the added ubiquinone-3, and therefore

Fig. 1. Inhibition of transhydrogenase activities in membranes from *E. coli* (AN180) by piericidin A. ●, Energy-linked transhydrogenase; ▲, non-energy-linked transhydrogenase.

piericidin A inhibition is not reversed by added ubiquinone-3.

DISCUSSION

The work described in the present paper established that Mg,Ca-ATPase activity is required for the energy-linked transhydrogenase activity when ATP is used as the energy source. Conversely, the non-energy-linked transhydrogenase activity is slightly derepressed in the Mg,Ca-ATPase-deficient mutants. Neither of the quinones formed by *E. coli*, namely ubiquinone and menaquinone, appear to be involved in the energy-linked transhydrogenase when ATP is used as the energy source. Sweetman & Griffiths (1970), who used a freeze-drying-pentane-extraction technique to deplete ox heart mitochondria of lipids, concluded that ubiquinone is involved in the energy-linked transhydrogenase. However, 67% of the energy-linked transhydrogenase activity was lost after freeze-drying, and the ability of ubiquinone-depleted lipid extract to re-activate the energy-linked transhydrogenase activity was not examined (see Albracht, van Heerikhuizen & Slater, 1971). The effect of lipid-depletion on the non-energy-linked transhydrogenase was not reported.

The energy-linked transhydrogenase activity in the parental strain AB2154 is only about 20% of that found in another parental strain (AN180). This result emphasizes the necessity of preparing a suitable set of strains by transduction when examining the effect of a mutation on a particular

enzymic activity. Thus if strain AN59 had been compared with strain AN180, the incorrect inference would have been drawn, that ubiquinone is concerned in the energy-linked-transhydrogenase activity.

Piericidin A appears to function as an inhibitor of a number of membrane-bound enzymic activities in *E. coli* at apparently quite different sites. Studies on the function of ubiquinone in *E. coli* indicated that piericidin A functioned as a ubiquinone analogue in inhibiting electron transport (Cox *et al.* 1970). In studies on the involvement of the Mg,Ca-ATPase in oxidative phosphorylation *in vitro* by membrane preparations of *E. coli*, piericidin A was shown to act as an uncoupler at a lower concentration than that required for the inhibition of electron transport (Butlin *et al.* 1971). This uncoupling activity of piericidin A may be reflected in the inhibition of the energy-linked transhydrogenase. Thus the inhibition of the energy-linked transhydrogenase by piericidin A is not reversed by the addition of ubiquinone, unlike the inhibition of electron transport, which is reversed by added ubiquinone. The energy-linked-transhydrogenase has been found (Sweetman & Griffiths, 1971) to be inhibited by higher concentrations of piericidin A than those described above, but the inhibition of the non-energy-linked transhydrogenase has not been reported before.

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