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Energy-Linked Reduction of Nicotinamide–Adenine Dinucleotide in Membranes Derived from Normal and Various Respiratory-Deficient Mutant Strains of *Escherichia coli* K12

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1. Assay conditions are described for the ATP-dependent, uncoupler-sensitive, energy-linked reduction of NAD^+ by succinate, DL-α-glycerophosphate or D-lactate in membranes from aerobically grown *Escherichia coli*. 2. The reaction may be demonstrated in electron-transport particles (ET particles) from cells grown in glycerol, but not in depleted particles washed in low-ionic-strength buffer, or in ET particles from cells grown in glucose. 3. The latter two classes of particles have low specific activities of ATPase (adenosine triphosphatase), succinate dehydrogenase, DL-α-glycerophosphate dehydrogenase and D-lactate dehydrogenase relative to undepleted ET particles from cells grown in glycerol. 4. Reconstitution of energy-linked NAD^+ reduction in particles from cells grown in glucose was done by : (*a*) addition of the high-speed supernatant fraction from sonicates of the same cells; (*b*) addition of a protein fraction, precipitated by $(NH_4)_2SO_4$ from this supernatant, or (*c*) addition of an $(NH_4)_2SO_4$-precipitated fraction from the low-ionic-strength wash of particles from cells grown in glycerol. 5. The use of $(NH_4)_2SO_4$-precipitated fractions from ATPase- or succinate dehydrogenase-deficient mutants grown in glycerol in the above reconstitution indicated that failure to demonstrate the reaction in particles from cells grown in glucose was a result of inadequate activities of appropriate dehydrogenases, rather than of ATPase. 6. Energy-linked NAD^+ reduction could be demonstrated in particles from a ubiquinone-deficient mutant only after restoration of NADH oxidase activity by adding ubiquinone-1. 7. The measured rate of the energy-linked reaction in particles from a haem-deficient mutant, however, was not stimulated after the ATP- and haematin-dependent acquisition of functional cytochromes. 8. Results are interpreted as evidence of the ubiquinone-dependent, but cytochrome-independent, nature of the site I region of the respiratory chain in *E. coli*.

Energy-linked reduction of NAD^+ by succinate was first observed by Chance & Hollunger (1957, 1960) in rat liver mitochondria. The phenomenon has been extensively studied, and generally interpreted as a transfer of reducing equivalents from succinate via succinate dehydrogenase and a span of the respiratory chain to NAD^+ by an energy-requiring process involving a reversal of normal electron transport at site I (see review by Ernster & Lee, 1967). The reaction has been observed in a small particular fraction from *Escherichia coli* by Kashket & Brodie (1963*a*,*b*). Their studies were extended by Sweetman & Griffiths (1971) to an investigation of the specificity of the electron acceptor, and the effects of pH, inorganic ions, inhibitors of electron transport and uncouplers of oxidative phosphorylation on the reaction.

The object of the present study is to define the role of the various membrane-bound redox components and of the ATPase* (EC 3.6.1.3; West & Mitchell, 1974) in the ATP-dependent reduction of NAD^+ by reversed electron transport in *E. coli*, by exploiting various phenotypic and genotypic variants of the energy-conservation system.

* Abbreviations: ATPase, Mg^{2+}-dependent, proton-translocating adenosine triphosphatase activity; the definitions of F_0 and F_1 components are those of Mitchell (1973).

Materials and Methods

Bacterial strains and genetic markers

The five strains derived from *E. coli* K12 used are described in Table 1. Strain sdh-3 was kindly supplied by Dr. J. R. Guest (Department of Microbiology, University of Sheffield, Sheffield S10 2TN, U.K.) and strain AN59 by Professor F. Gibson (Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia).

Growth conditions

The basic medium for aerobic growth contained (per litre): K_2HPO_4 (4g), KH_2PO_4 (1g), NH_4Cl (1g), $CaCl_2$ (0.01g), $MgCl_2,6H_2O$ (0.2g), K_2SO_4 (2.6g) and the mineral-salts solution (10ml) of Pirt (1967).

Table 1. *Strains of E. coli K12 used*

The nomenclature of *E. coli* genes is that of Taylor & Trotter (1972).

Strain	Known relevant genetic loci	Other information
C-1	*nal* (prototroph)	Derived from strain EMG-2 [Haddock (1973) by the method of Hane & Wood (1969)]
A103c	*unc*A⁻-273,*met*E⁻	Schairer & Haddock (1972)
AN59	*ubi*B⁻, *leu*-6⁻, *thi* 1⁻ *thr*-1⁻	Cox *et al.* (1969)
A1004a	*hem*A⁻, *ilv*⁻, *met*E⁻,	Haddock (1973)
sdh-3	*sdh*⁻, *trpA*⁻	Spencer & Guest (1974)

Carbon sources, glycerol, glucose and, for strain sdh-3, glycerol plus sodium fumarate were each added to a final concentration of 0.5% (w/v). $MgCl_2,6H_2O$ (1M) and, where appropriate, glucose were autoclaved separately and added aseptically to the cooled, sterile medium. Strain C-1 was grown in this medium without further supplements, with either glucose or glycerol as carbon source. Growth of strains A103c (with glycerol) and A1004a (with glucose) was after supplementation with amino acids as described by Haddock (1973). Strain sdh-3 was grown on glycerol plus fumarate, supplemented with tryptophan (0.01%), and strain AN59 on glucose supplemented with threonine, leucine and methionine (all 0.01%) and thiamin (0.001%).

Growth of strains C-1 and sdh-3 was in 5–8 litre volumes of medium in a 10-litre fermentor vessel; vigorous stirring was employed and the air-flow rate was approx. 5 litres/min. Such cultures were harvested in the late exponential phase of growth when E_{420} (10mm light-path; 1/10 dilution) had reached 0.3–0.5. Strains AN59, A103c and A1004a were grown in 2-litre unbaffled conical flasks each containing 625ml of growth medium, agitated in a rotary shaker (L. H. Engineering Co. Ltd., Stoke Poges, Bucks., U.K.) operating at about 200 rev./min; these cultures were harvested in the early exponential phase of growth at E_{420} (10mm light-path) 0.5–0.7. The growth temperature was always 37°C.

Preparation of cell-free extracts and their fractionation

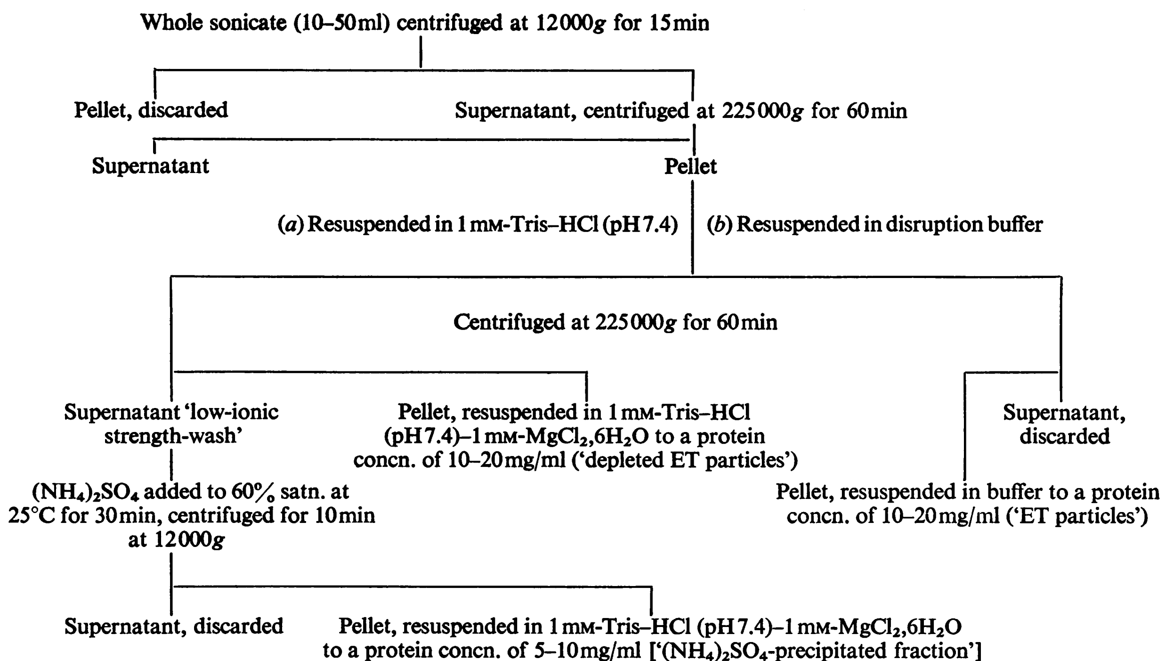
Cells were harvested, washed and broken by ultrasonic disruption as described previously (Schairer & Haddock, 1972) in a medium which contained 50mM-Tris–HCl, 2mM-$MgCl_2$ and 1mM-EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] (pH 7.4). The whole sonicate was further fractionated as shown in Scheme 1, to yield a supernatant fraction S and either ET particles (electron-transport particles) or depleted ET particles plus an $(NH_4)_2SO_4$-precipitated fraction.

Measurement of energy-linked reduction of NAD^+

The assay system used was based on that of Sweetman & Griffiths (1971). The reaction mixture contained in a final volume of 3.0ml (10mm light-path cuvette) the following components (final concns.): 188mM-sucrose, 37.5mM-Tris–HCl (pH 8.0), 5mM-$MgCl_2,6H_2O$, bovine serum albumin (0.5mg), 0.17mM-dithiothreitol, 10mM-NaN_3, 1.5mM-NAD^+ and either 10mM-succinate, 3mM-D-lactate or 3mM-DL-α-glycerophosphate. Membrane preparation (1–2mg of protein) and, where appropriate, $(NH_4)_2SO_4$-precipitated fraction (0.5–1.0mg of protein) were added and the reaction was initiated by adding 1mM-ATP. Where indicated, carbonyl cyanide *m*-chlorophenylhydrazone or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (concentrated methanolic solutions) were added to a final concentration of 0.5–1μM. Reduction of NAD^+ was determined at 37°C by measuring the increase in E_{340} by using a Cecil CE 202 u.v. spectrophotometer (Cecil Instruments Ltd., Cambridge CB4 1TG, U.K.) with a Vitatron recorder type UR 40IL (Fisons Scientific Apparatus, Loughborough, Leics. LE11 0RG, U.K.), such that full-scale deflexion corresponded to an extinction change of 0.2. The observed change in E_{340} was shown to be the result of formation of NADH, by addition of alcohol dehydrogenase (EC 1.1.1.1) and acetaldehyde (Sweetman & Griffiths, 1971).

Other assay techniques

ATPase was assayed in a buffer containing 50mM-Tris–HCl (pH 7.6), 3mM-$MgCl_2,6H_2O$, 1mM-EGTA by using 0.05–0.5mg of bacterial protein in a total reaction volume of 1ml. The reaction was initiated by adding 6mM-ATP. After incubation for 30min at 35°C, the reaction was terminated by adding 1ml of 10% (w/v) trichloroacetic acid and, after centrifugation at 4°C to remove the acid-insoluble fraction, P_i was determined in the supernatants by the method of Fiske & SubbaRow (1925). Previous work with F_1-ATPase-deficient mutants of *E. coli* has shown that this assay is specific for the ATPase and less than 5% of the P_i released is due to the action of other ATP-hydrolysing enzymes in cell-free extracts (Schairer & Haddock, 1972). Succinate dehydrogenase (EC 1.3.99.1) and D-lactate dehydrogenase (EC 1.1.2.4) were assayed by a modification of the method of Arrigoni & Singer (1962) in the presence of 0.33mM-phenazine methosulphate and 33μM-2,6-dichlorophenol-indophenol. The method of assay of DL-α-glycerophosphate dehydrogenase (EC



Scheme 1. *Preparation of subcellular fractions from ultrasonically disrupted E. coli*

Unless otherwise specified, the buffer used was disruption buffer described in the text, and procedures were performed at 2–4°C. Resuspension of pellets was with a glass rod.

1.1.99.5) was that of Lin *et al.* (1962); reduction of 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide was measured at 550nm, an extinction coefficient of 62 litre·mmol^{-1}·cm^{-1} being used (Altman, 1969). All enzyme units are expressed as nmol of substrate transformed/min or as nmol of measured product formed/min.

Measurements of O_2 uptake by bacterial extracts in the presence of various added substrates were done at 35°C as described by Haddock (1973).

Protein was determined by the method of Lowry *et al.* (1951) with dry bovine serum albumin (fraction V; BDH Chemicals Ltd., Poole, Dorset, U.K.) as standard.

Reagents

Alcohol dehydrogenase (crystalline), ATP (disodium salt), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and NAD^+ (free acid) were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. D-Lactic acid (lithium salt, A grade) was obtained from Calbiochem, San Diego, Calif. 92112, U.S.A., and 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Carbonyl cyanide *m*-chlorophenylhydrazone, DL-α-glycerophosphate (disodium salt, grade X), L-methionine and phenazine methosulphate were all from Sigma (London) Chemical Co., London S.W.6, U.K. Ubiquinone-1 was a generous gift from F. Hoffman–La Roche and Co., CH-4002 Basel, Switzerland. All other reagents were from BDH Chemicals Ltd., and were of the highest available purity.

Results

Energy-linked reduction of NAD^+ in ET particles from glycerol-grown strain C-1

In the presence of 5mM-$MgCl_2$, 1mM-ATP gave maximal rates of NAD^+ reduction; this ATP concentration is somewhat lower than that reported by Sweetman & Griffiths (1971). ATP-dependent reduction of NAD^+ by substrates interacting with the respiratory chain at the level of cytochrome *b* requires inhibition of electron flow via terminal cytochrome oxidases. The reaction of cyanide with NAD^+ (or NADH) (Colowick *et al.*, 1951) renders

Table 2. *Activities of the energy-linked reduction of NAD^+ by various substrates and of oxidases in ET particles from glycerol-grown E. coli strain C-1*

ET particles were prepared from sonicated extracts of strain C-1. Assays of the energy-linked reduction of NAD^+ and of oxidase activities were performed as described in the Materials and Methods section by using 1.1 or 0.6mg of protein respectively. Concentrations of substrates used were 1mM (NADH), 3mM (DL-α-glycerophosphate and D-lactate) and 10mM (succinate). n.t., not tested; n.a., not applicable.

Substrate	Energy-linked reduction of NAD^+ (nmol of NADH formed/min per mg of protein)	Oxidase (ng-atoms of O/min per mg of protein)
NADH	n.a.	406
DL-α-Glycerophosphate	5.6	212
D-Lactate	1.1	127
Succinate	4.6	67
Succinate+carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone	2.4	n.t.
Endogenous	0.9	7.5

this inhibitor unsuitable in the present system. It was shown that 2.5mM-Na_2S (Sweetman & Griffiths, 1971) inhibited oxidation by ET particles of NADH, succinate and DL-α-glycerophosphate by only 50%. More extensive inhibition (up to 70%) was obtained with 10mM-NaN_3; consequently, this inhibitor was used in all further experiments. Under the conditions described in the Materials and Methods section, the rate of NAD^+ reduction was dependent on the amount of bacterial membrane protein present, and was stimulated by the presence of bovine serum albumin (Asano *et al.*, 1967) and dithiothreitol (Schuurmans-Stekhoven *et al.*, 1971). The inhibitory effect of an uncoupling agent, carbonyl cyanide *m*-chlorophenylhydrazone (Table 2) and the dependence of the reaction on ATP are in agreement with the observations of Sweetman & Griffiths (1971) and demonstrate the coupling of ATP hydrolysis to the reversal of electron flow at site I. The nature of the uncoupler-insensitive proportion of the observed activity has not been further investigated.

Energy-linked reduction of NAD^+ in ET particles from glucose-grown strain C-1

Under the conditions described, the maximum rate of NAD^+ reduction by succinate observed in ET particles prepared from glucose-grown cells was less than 0.5nmol of NADH formed/min per mg of protein; this activity was ATP-independent and completely uncoupler-insensitive.

Fractionation by differential centrifugation of whole sonicates from glucose- and glycerol-grown strain C-1

Failure to demonstrate the energy-linked reduction of NAD^+ in ET particles from glucose-grown cells could be due to the repression or loss of appropriate dehydrogenases, the ATPase or some other membrane-bound component(s) required for oxidative phosphorylation at site I. A comparative study of the activities and distributions of these enzymes in extracts of both glucose- and glycerol-grown cells was therefore undertaken. The results presented in Table 3 establish the following points. (1) Specific activities of D-lactate dehydrogenase were similar in whole sonicates prepared from either glucose- or glycerol-grown cells, but the activities of succinate and DL-α-glycerophosphate dehydrogenases and ATPase were 3.5-, 10.5- and 2.1-fold higher respectively in glycerol-grown cells. (2) These increased specific activities were more apparent in ET particles from these cells. Thus activities of succinate- and DL-α-glycerophosphate dehydrogenases and of ATPase were 41-, 20.4- and 6.2-fold higher respectively in ET particles from glycerol-grown cells; that of D-lactate dehydrogenase was similar in both classes of particles. (3) Succinate dehydrogenase was undetectable in supernatant fraction (S) from glycerol-grown cells, but was present in ET particles at high specific activity. In contrast, almost 50% of the total succinate dehydrogenase in sonicates of glucose-grown cells failed to sediment under these conditions. The firm binding of succinate dehydrogenase to membrane fractions from *E. coli*, under conditions (in the presence of non-ionic detergents or repeated centrifugation) in which NADH, D-lactate and DL-α-glycerophosphate dehydrogenases become solubilized, has been described previously (Hendler & Burgess, 1972; Kung & Henning, 1972). (4) Dicyclohexylcarbodi-imide inhibited particulate ATPase activity in ET particles from glycerol-grown cells by 85%, but by only 53% in ET particles from glucose-grown cells. Corresponding degrees of inhibition of the soluble ATPase activities were 32% and 43% respectively. Solubilized ATPase (F_1-ATPase) of *E. coli* exhibits an increased resistance to this inhibitor (Roisin & Kepes, 1973); thus the varying degrees of inhibition and the relative distributions of ATPase in glucose- and glycerol-grown cells suggests that the enzyme is more readily solubilized in the former cell type.

Reconstitution of the energy-linked reduction of NAD^+ in particles from glucose-grown strain C-1

(*a*) Reconstitution of the activity in membranes from glucose-grown cells (which contain significantly decreased activities of at least two components, the appropriate dehydrogenase and ATPase, required

Table 3. *Subcellular fractionation by differential centrifugation of extracts of glucose- and glycerol-grown E. coli strain C-1*

Cell suspensions were sonicated to yield a whole sonicate which was fractionated by the procedure shown in Scheme 1, alternative (*b*). Enzyme and protein determinations were made on all recovered fractions, and recoveries thus calculated. The distributions of enzymes in ET particles, and supernatant fractions and in whole sonicates only are presented. Specific activities are expressed as enzyme units/mg of protein.

	Glucose-grown cells						Glycerol-grown cells					
	Whole sonicate	ET particles		Supernatant			Whole sonicate	ET particles		Supernatant		
	Concn. (mg/ml)	Concn. (mg/ml)	Total (%)	Concn. (mg/ml)	Total (%)	Recovery (%)	Concn. (mg/ml)	Concn. (mg/ml)	Total (%)	Concn. (mg/ml)	Total (%)	Recovery (%)
Protein ...	7.0	4.2	8.5	7.3	68	118	8.3	3.9	7.9	6.8	77.5	106
	Specific activity	Specific activity	Units (% of total)	Specific activity	Units (% of total)		Specific activity	Specific activity	Units (% of total)	Specific activity	Units (% of total)	
D-Lactate dehydrogenase	28.6	58.0	17.3	16.3	28.6	118	22.4	44.4	23.0	7.4	36.2	68
Succinate dehydrogenase	16.7	1.3	9.4	8.1	45.7	87	58.6	53.3	27.3	<0.1	<1	35
α-Glycerophosphate dehydrogenase	8.8	3.8	12.1	1.8	26.4	46	92.0	77.3	10.5	44.2	32.5	134
ATPase	67	34	6.9	18	29.7	76	140	210	13.9	68	42.2	86.5
ATPase+dicyclohexyl-carbodi-imide	32	16	9.9	10	50.3	50	43	31	4.8	46	80.6	104

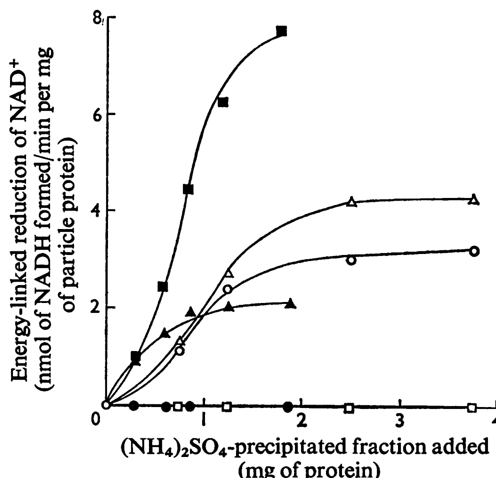


Fig. 1. *Effects of* $(NH_4)_2SO_4$*-precipitated fractions from both glucose- and glycerol-grown E. coli strain C-1 on the reconstitution of the energy-linked reduction of* NAD^+ *by various substrates in ET particles derived from glucose-grown cells*

Subcellular fractions were prepared from glucose-grown cells as described in the Methods and Materials section. The rate of energy-linked reduction of NAD^+ was measured before and after addition of an $(NH_4)_2SO_4$-precipitated fraction from either the supernatant (S) of glucose-grown cells (open symbols) or from a 'low-ionic-strength wash' of particles from glycerol-grown cells (closed symbols). Amount of ET particle protein used in all assays was 2.0mg. Substrates used were DL-α-glycerophosphate (□, ■), succinate (△, ▲) or D-lactate (○, ●). Observed rates of NAD^+ reduction were inhibited approx. 50% by 1 μM-carbonyl cyanide *m*-chlorophenylhydrazone.

for oxidative phosphorylation) was done by mixing such membranes with either (1) the supernatant fraction (S) from the same cells or (2) an $(NH_4)_2SO_4$-precipitated fraction from this supernatant. Results of the latter type of reconstitution experiment are shown in Fig. 1. With either D-lactate or succinate as substrate, maximal rates of NAD^+ reduction (3–4 nmol of NADH formed/min per mg of particle protein) were achieved on adding $(NH_4)_2SO_4$ fraction containing approx. 3 mg of protein. Reduction of NAD^+ by DL-α-glycerophosphate was never observed, owing to the low activities of the appropriate dehydrogenase in both particulate and soluble fractions. (*b*) Reconstitution was also achieved on adding an $(NH_4)_2SO_4$-precipitated fraction from a 'low-ionic-strength wash' of particles prepared from glycerol-grown cells as shown in Scheme 1. This procedure (Roisin & Kepes, 1973) resulted in significant losses of enzyme activity and protein from particles, the percentage (in parentheses) of each component lost being as follows: protein

(80%), D-lactate dehydrogenase (57%), succinate dehydrogenase (58%), DL-α-glycerophosphate dehydrogenase (61%) and F_1-ATPase (96%). The resulting membrane fraction (depleted ET particles) did not exhibit the energy-linked reduction of NAD^+, unless an $(NH_4)_2SO_4$-precipitated fraction from the 'low-ionic-strength wash' was added. The reconstitution of the energy-linked reaction in ET particles from glucose-grown cells by this $(NH_4)_2SO_4$ fraction is also shown in Fig. 1. In this case, rather lower concentrations of precipitated protein were required for reconstitution of the activity, and significant rates of NAD^+ reduction by DL-α-glycerophosphate were observed. Surprisingly, no reduction was observed with D-lactate as substrate.

In an attempt to identify more precisely the nature of the component(s), present in supernatant (S) and $(NH_4)_2SO_4$ fractions, essential for energy-linked NAD^+ reduction, $(NH_4)_2SO_4$-precipitated fractions were prepared from mutant strains of *E. coli* with low succinate dehydrogenase (sdh-3) and ATPase (A103c) activities respectively. A comparison of these activities and of other dehydrogenases in such precipitated fractions from strains A103c, sdh-3 and C-1 is shown in Table 4. Activities of D-lactate dehydrogenase and DL-α-glycerophosphate dehydrogenase were similar in all three strains tested, but the low activities of ATPase and succinate dehydrogenase reflect the presence of the appropriate mutant alleles in strains A103c and sdh-3 respectively.

Each of these $(NH_4)_2SO_4$-precipitated fractions was tested for its ability to function in the reconstitution of the energy-linked reaction in either ET particles or depleted ET particles derived from glucose-grown strain C-1 (Table 5). In the absence of $(NH_4)_2SO_4$ fractions, low rates of NAD^+ reduction were observed in both ET particles and depleted ET particles. The rate of NAD^+ reduction observed with DL-α-glycerophosphate as substrate was enhanced on addition of $(NH_4)_2SO_4$-precipitated fraction from any strain to give rapid rates of NAD^+ reduction (greater than 30nmol of NADH formed/min per mg of protein) in ET particles, but little stimulation was observed in depleted ET particles. Reduction of NAD^+ by succinate was enhanced in all cases, except when $(NH_4)_2SO_4$-precipitated fraction from mutant sdh-3 was used. Results with lactate were more variable; a significant stimulation of the rate of NAD^+ reduction was obtained consistently only when the $(NH_4)_2SO_4$ fraction from strain C-1 was added. In no case were significant rates of NAD^+ reduction observed in depleted ET particles (which

Table 4. *Specific activities of ATPase and dehydrogenases in $(NH_4)_2SO_4$-precipitated fractions from various strains of E. coli*

E. coli strains A103c, sdh-3 and C-1 were sonicated, and extracts fractionated by the procedure shown in Scheme 1. Specific activities are expressed as enzyme units/mg of protein.

	Enzyme specific activities in $(NH_4)_2SO_4$-precipitated fractions from strain:		
	A103c	sdh-3	C-1
D-Lactate dehydrogenase	57.0	92.0	71.5
Succinate dehydrogenase	19.1	1.22	52.5
α-Glycerophosphate dehydrogenase	98.0	109.0	144.0
ATPase	12	130	96

Table 5. *Effects of $(NH_4)_2SO_4$-precipitated fractions from various strains of glycerol-grown E. coli on the activity of energy-linked reduction of NAD^+ in ET particles and depleted ET particles derived from glucose-grown cells*

The preparation of ET particles and depleted ET particles from strain C-1 and of $(NH_4)_2SO_4$-precipitated fractions from all strains was performed as shown in Scheme 1. Energy-linked NAD^+ reduction was measured as described in the Materials and Methods section with 1.7mg of particle protein and, where indicated, $(NH_4)_2SO_4$ fractions from strains A103c (0.8mg of protein), sdh-3 (0.7mg of protein) or C-1 (0.7mg of protein). Observed rates of NAD^+ reduction were 50% inhibited by 1μM-carbonyl cyanide *m*-chlorophenylhydrazone. n.t., not tested.

		Energy-linked reduction of NAD^+ (nmol of NADH formed/min per mg of particle protein)			
		In absence of $(NH_4)_2SO_4$ fraction	In presence of $(NH_4)_2SO_4$ fraction from strain		
Membrane fraction	Substrate		A103c	sdh-3	C-1
ET particles	DL-α-Glycerophosphate	<0.05	28.1	62.2	45.2
	Succinate	<0.05	11.3	0.56	11.0
	D-Lactate	<0.05	<0.05	n.t.	8.4
Depleted ET particles	DL-α-Glycerophosphate	0.28	n.t.	<0.05	0.28
	Succinate	0.14	0.09	<0.05	2.69
	D-Lactate	0.26	<0.05	0.57	1.00

Table 6. *Activities of the energy-linked reduction of NAD^+ by* DL-*α-glycerophosphate and of oxidases in ET particles from glucose-grown respiration-deficient mutants of E. coli*

The preparation of ET particles from strains AN59 and A1004a, and assay methods are described in the Materials and Methods section. Energy-linked reduction of NAD^+ was measured in the presence of $(NH_4)_2SO_4$-precipitated fraction (0.7mg of protein) from strain C-1, with 1.7 (strain AN59) or 1.1 (strain A1004a) mg of particle protein. Corresponding oxidase rates were measured with 0.85 and 0.22mg of protein respectively. Where indicated, reconstitution in ET particles from strain AN59 was achieved on addition of 20μM-ubiquinone-1 (Cox *et al.*, 1970) and from strain A1004a by incubation with 5μM-haematin and 5mM-ATP at 37°C for 30min (Haddock, 1973).

	ET particles from			
	Strain AN59		Strain A1004a	
	Without ubiquinone	With ubiquinone	Without haematin and ATP	With haematin and ATP
	Energy-linked reduction of NAD^+ (nmol of NADH formed/min per mg of protein)			
Complete	<0.05	11.4	4.5	2.7
−ATP	<0.05	2.2	0.8	0.3
+1μM-carbonyl cyanide *m*-chlorophenylhydrazone	<0.05	6.0	2.4	1.5
	Oxidase rates (ng-atoms of O/min per mg of protein)			
NADH	30	430	50	800
D-Lactate	60	90	2	140

retain only 4% of the ATPase activity of ET particles) when $(NH_4)_2SO_4$ fractions from strain A103c (ATPase-deficient) were used. These data demonstrate the requirement for both an ATPase and the appropriate dehydrogenase for the energy-linked reduction of NAD^+, and further suggest that the failure to demonstrate significant rates of NAD^+ reduction in ET particles from glucose-grown cells, with either succinate or DL-α-glycerophosphate as substrate, results from the decreased activities of dehydrogenases, rather than ATPase, in these membranes.

Energy-linked reduction of NAD^+ in ET particles derived from glucose-grown respiration-deficient mutants

The ability to demonstrate the energy-linked reduction of NAD^+ in particles from glucose-grown cells enabled an investigation to be made of the role of membrane-bound redox components in oxidative phosphorylation at site I by the use of mutants defective in their ability to synthesize these components and thus unable to grow on glycerol.

The role of ubiquinone was investigated by using the ubiquinone-deficient mutant AN59. In the absence of exogenous ubiquinone, ET particles prepared from glucose-grown cells exhibited low NADH oxidase and lactate oxidase rates (Table 6). The energy-linked reduction of NAD^+ (by DL-α-glycerophosphate, D-lactate or succinate) could not be demonstrated, even on addition of an $(NH_4)_2SO_4$-precipitated fraction (approx. 1mg of protein) prepared from glycerol-grown strain C-1. Reconstitution of NADH oxidase activity (achieved on adding 20μM-ubiquinone-1; Cox *et al.*, 1970; Table 6) was accompanied by the ability to demonstrate the energy-linked reaction in the presence of the above $(NH_4)_2SO_4$ fraction. The observed activity was ATP-dependent and approx. 50% inhibited by 1μM-carbonyl cyanide *m*-chlorophenylhydrazone. The results clearly demonstrate a functional requirement for ubiquinone in electron transfer and oxidative phosphorylation in that span of the respiratory chain between NADH and the junction of the flavin-linked dehydrogenases.

The requirement for functional cytochromes in the site I region of the respiratory chain was investigated by a study of the energy-linked reduction of NAD^+ in the 5-aminolaevulinic acid-requiring mutant, A1004a. Cells of this strain, grown aerobically in the absence of 5-aminolaevulinic acid, synthesize cytochrome apoproteins in the absence of haem synthesis (Haddock, 1973). The NADH oxidase rates of ET particles from glucose-grown cells were low (Table 6). Nevertheless, in the presence of an $(NH_4)_2SO_4$-precipitated fraction prepared from glycerol-grown strain C-1, significant rates of ATP-dependent, uncoupler-sensitive reduction of NAD^+ by DL-α-glycerophosphate were observed. The acquisition of fully functional cytochromes, achieved by incubation of such particles with 5μM-haematin

and 5mM-ATP at 37°C for 30min (Haddock, 1973), was accompanied by a significant increase in the rate of NADH oxidation (Table 6). This reconstitution of oxidase activity was not concomitant with any increase in the activity of the energy-linked reaction, but rather a decrease, possibly owing to an alteration in the ATP/Mg^{2+} ratio (which is critical for maximal ATPase activity and hence reversal at site I) resulting from the high concentrations of ATP used in the reconstitution of functional cytochromes. These results are interpreted as evidence of the cytochrome-independent nature of the span of the respiratory chain associated with site I oxidative phosphorylation in *E. coli*. Similar conclusions as to the involvement of ubiquinone in this region of the respiratory chain have previously been made (Haddock *et al.*, 1974), on the basis of measurements of proton translocation coupled to ubiquinone reduction by endogenous substrates in intact cytochrome-deficient cells.

Discussion

It has previously been shown that ET particles derived from *E. coli*, grown with succinate as sole carbon source, can catalyse both an energy-linked and non-energy-linked reduction of NAD^+ by succinate (Sweetman & Griffiths, 1971). By analogy with ET particles from mitochondria (Ernster & Lee, 1967), it might be expected that in *E. coli* energy for the energy-linked reduction of NAD^+ by succinate at site I could be obtained either by oxidation of succinate through the cytochrome-dependent region of the respiratory chain (Lawford & Haddock, 1973), or by ATP hydrolysis via the ATPase (West & Mitchell, 1974). Electron-transport-dependent site I reversal is experimentally difficult to achieve in *E. coli* ET particles since the rate of NADH oxidation is greater than that of other substrates. We have therefore restricted our investigation to the ATP-dependent uncoupler-sensitive site I reversal and have ignored the uncoupler-insensitive, presumably non-energy-linked component. It is particularly interesting that both azide and NH_4^+ ions, which are present in the reaction mixture and which are potential uncouplers of energy-linked reactions (both inhibit respiration-dependent atebrin quenching in ET particles, B. A. Haddock, unpublished work), do not inhibit the ATP-dependent site I reversal completely, i.e. there is still a carbonyl cyanide *m*-chlorophenylhydrazone-sensitive component.

When considering the rate of NAD^+ reduction via site I reversal, it should be remembered that the absolute rate may vary, not only between ET particles from different *E. coli* strains, but also in membranes prepared from the same strain grown under different conditions. Thus the absolute rate of site I reversal in any of the experiments described above are perhaps not comparable, and we have based our conclusions on the ability of a particular component to alter the rate of the reaction with a particular ET particle preparation from a single strain. In agreement with the previous conclusions of West & Mitchell (1974), the results suggest that sonication of *E. coli* results in the formation of some topologically closed inside-out vesicles rather than mere membrane fragments or closed vesicles that retain their outside-out orientation as *in vivo* [similar to the membrane-vesicle preparations of Kaback (1971)]. This conclusion is supported by the following experimental results obtained for site I reversal. (*a*) The reaction requires both ATP and NAD^+, though the cytoplasmic membrane is not readily permeable to either; (*b*) in certain cases it was necessary to add high-molecular-weight proteins, e.g. succinate dehydrogenase, to membranes in order to observe reversal, and such proteins are unlikely to be penetrants of the membrane, and (*c*) the uncoupler-sensitivity of at least part of the reaction, indicates the vesicular nature of the preparation. The proportion of inside-out closed vesicles in ET particle preparations is not known. The presence of membrane fragments, which would have a high NADH oxidase activity but exhibit no ATP-dependent site I reversal, would offer an explanation for the difference in the relative rates of NAD^+ reduction and NADH oxidation.

There are many examples in the literature of the general applicability of mutant-reconstitution methods in the study of the functional organization of membrane components in *E. coli*, including the reconstitution of D-lactate oxidase activity (Reeves *et al.*, 1973), nitrate reductase activity (Azoulay *et al.*, 1969; MacGregor & Schnaitman, 1973), and of respiration in haem- (Haddock, 1973), ubiquinone- (Cox *et al.*, 1970), and menaquinone- (Newton *et al.*, 1971) deficient mutants. Of particular interest are the reconstitutions of energy-linked reactions, e.g. of electron transport-dependent transhydrogenase activity in membranes lacking F_1-ATPase by addition of dicyclohexylcarbodi-imide, which decreases the proton permeability of the membrane by binding to F_0-ATPase (Bragg & Hou, 1973; Mitchell, 1973) and of ATP-dependent transhydrogenase activity in F_1-ATPase deficient mutants with F_1-ATPase from various sources (for review, see Cox & Gibson, 1974). Cox *et al.* (1971) have previously demonstrated with mutants that the energy-linked transhydrogenase reaction in *E. coli* membranes does not require ubiquinone or menaquinone.

In studies on the interaction between peripherally associated membrane proteins and their membranes, there are still unresolved problems, e.g. (*a*) the definition of a soluble or membrane-bound protein [the work of MacGregor & Schnaitman (1973) provides an excellent illustration], (*b*) the possibility of alterations to the membrane structure and com-

position after sonication (Hughes *et al.*, 1971) and (*c*) the possibility of competition for binding sites as suggested by Kung & Henning (1972) for several inducible dehydrogenases. Even with these limitations, the use of purified protein components from competent strains of *E. coli* to reconstitute functional enzyme activity in known membrane-protein-deficient preparations from mutant strains offers an alternative and complementary approach to the solubilization and reassembly method used by Racker and co-workers in their studies on the mitochondrial respiratory chain (see, e.g., Racker, 1972; Ragan & Racker, 1973).

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