Biochem. J. (1973) **136**, 877–884
Printed in Great Britain

The Reconstitution of Oxidase Activity in Membranes Derived from a 5-Aminolaevulinic Acid-Requiring Mutant of *Escherichia coli*

By BRUCE A. HADDOCK
Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, U.K.

(*Received 5 June* 1973)

1. The reconstitution of oxidase activity in cell-free extracts of a mutant of *Escherichia coli* K12Ymel, that requires 5-aminolaevulinic acid for growth on non-fermentable carbon sources, is described. 2. The reconstitution is dependent on haematin or a haem extract from a prototrophic strain of *E. coli*, and the product of the reaction has been identified as NADH-reducible cytochrome *b*. 3. The requirement for haematin cannot be replaced by four other porphyrins. Coproporphyrin III does not inhibit the haematin-dependent reconstitution, mesoporphyrin IX and protoporphyrin IX apparently compete with haematin for a binding site on the cytochrome apoprotein(s) and deuteroporphyrin IX binds to cytochrome apoprotein(s) and cannot be subsequently replaced by haematin. 4. The properties of electron-transport particles from cell-free extracts of the mutant strain, grown aerobically in the presence or absence of 5-aminolaevulinic acid, are described. In the absence of 5-aminolaevulinic acid no detectable cytochromes are produced, and oxidase activities are lowered but there is no apparent effect on the activities of the NADH dehydrogenase and D-lactate dehydrogenase. 5. The reconstitution of oxidase activity by electron-transport particles from cells grown in the absence of 5-aminolaevulinic acid requires ATP and haematin, and the product of the reaction was identified as NADH-reducible cytochrome *b*. 6. It is concluded that the cytochrome apoproteins are synthesized and incorporated into the cytoplasmic membrane of *E. coli* in the absence of haem synthesis. The subsequent reconstitution of functional cytochrome(s) requires protohaem, but the nature of the side chain on the 2 and 4 positions of the porphyrin appears to be important.

Methods for the isolation of mutants auxotrophic for 5-aminolaevulinic acid (Wulff, 1967; Săsărman *et al.*, 1968*a*) and haematin (Beljanski & Beljanski, 1957; Săsărman *et al.*, 1968*b*) as well as porphyrin-accumulating mutants (Cox & Charles, 1973) have been reported for *Escherichia coli*. Such mutants may be used for the detailed analysis of the biochemistry, genetics and regulation of both porphyrin and cytochrome biosynthesis in this bacterium. We have demonstrated recently, in a mutant of *E. coli* requiring 5-aminolaevulinic acid for growth on non-fermentable carbon sources, that cytochrome apoproteins are synthesized in the absence of haem synthesis (Haddock & Schairer, 1973). The object of the present report is to characterize further the haematin-dependent reconstitution of functional cytochrome(s) in cell-free extracts of this mutant.

Materials and Methods

Organisms

Bacterial strains, genetic markers and nomenclature. *Escherichia coli*, strain A1004a (K12Ymel *ato*$^-$, *fad*R^c, *hem*A$^-$, *ilv*$^-$, *lac*I$^-$, metE$^-$, *rha*$^-$) used in the reconstitution experiments was derived from strain A201 (Haddock & Schairer, 1973) by P_1 transduction and was a generous gift from Dr. H. U. Schairer, Institut für Genetik der Universitat zu Köln, W. Germany. Mutations in the *hem*A allele presumably affect 5-aminolaevulinate synthetase (EC 6.3.–.–.) since strain A1004a, like strain A201, cannot grow on glycerol or intermediates of the citric acid cycle unless supplemented with 5-aminolaevulinic acid, though it will grow aerobically or anaerobically with glucose as carbon source. Strain EMG2 (K12Ymel prototroph) was from Dr. M. Peacey, University of Edinburgh, Edinburgh, U.K. The nomenclature of *E. coli* genes is that of Taylor & Trotter (1972).

Growth conditions. Strain A1004a was grown aerobically as described previously (Haddock & Schairer, 1973) in the mineral salts medium (CR medium) of Cohen & Rickenberg (1956) containing glucose (0.5%, w/v), vitamin-free casamino acids (0.1%, w/v), isoleucine (0.01%, w/v), valine (0.01%, w/v) and methionine (0.01%, w/v); 5-aminolaevulinic acid (30μM) was also added occasionally as indicated in the text. Strain EMG-2 was grown aerobically in

the same mineral salts medium supplemented with succinate (0.5%, w/v) and vitamin-free casamino acids (0.1%, w/v).

Preparation of cell-free extracts and electron-transport particles. Cells were harvested, washed and broken by ultrasonic disruption as described previously (Schairer & Haddock, 1972); this cell-free extract was used directly in some experiments. Electron-transport particles were prepared by the following procedure. The cell-free extract was centrifuged at 12000*g* for 15min, the supernatant was removed and then centrifuged at 225000*g* for 60min. The resultant supernatant was discarded and the pellet was washed by resuspension in CR medium lacking carbon source and amino acids and resedimentation at 225000*g* for 60min. The resultant pellet was suspended in CR medium to a final protein concentration of 10–20mg/ml.

Assay techniques

Protein was determined according to the method of Lowry *et al.* (1951), with dry bovine serum albumin fraction V (BDH Chemicals Ltd., Poole, Dorset, U.K.) used as standard. Measurements of oxygen uptake by bacterial extracts were performed in a vessel containing CR medium (3ml), air-saturated at either 30° or 37°C as indicated in the text, with an oxygen electrode (type YSI 4004, Yellow Springs Instrument Co., Ohio 45387, U.S.A.) calibrated as described by Chappell (1964); NADH (0.5mM) or D-lactate (5mM) were used as substrates.

The activities of NADH–ferricyanide reductase and of NADH– and of D-lactate–2,6-dichlorophenol-indophenol reductase were assayed at 30°C according to the procedure of Bragg & Hou (1967).

The concentration of NADH-reducible haem (cytochrome *b*) was determined from the difference between the spectra of cell extracts reduced with NADH (2mM) and oxidized with hydrogen peroxide (about 1mM) and catalase (EC 1.11.1.6, 50000units/ml) at the wavelength pair 560–575nm, a molar absorption coefficient of 17500 litre·mol^{-1}·cm^{-1} being used (Jones & Redfearn, 1966). These measurements, and those for the determination of porphyrin and hematin concentrations, were performed with a wavelength-scanning spectrophotometer (Haddock & Garland, 1971) with a spectral bandwidth of 1nm and optical cuvettes of 1cm light-path.

Preparation and assay of haems and porphyrins

A haem-extract from *E. coli* strain EMG-2 grown on succinate–CR medium was prepared according to the acetone–HCl extraction procedure of Basford *et al.* (1957). Haematin hydrochloride was purchased from BDH Chemicals Ltd., and a stock solution of haematin (0.25mM) was prepared as described previously (Haddock & Schairer, 1973). Deuteroporphyrin IX dimethyl ester and mesoporphyrin IX dimethyl ester were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and protoporphyrin IX and coproporphyrin III tetramethyl ester were from Calbiochem, Calif. 90054, U.S.A. Porphyrin methyl esters were hydrolysed to the free acid in methanolic 1% (w/v) KOH (Falk, 1964, p. 126) and stock solutions (0.1mM) were prepared in 100mM-Tris–HCl buffer, pH7.5. The haem concentrations of the solutions were calculated after preparation of an alkaline pyridine haemochrome and measurement of the reduced-minus-oxidized difference spectrum (Falk, 1964, p. 241). The porphyrin concentrations of the solution were calculated from the intensity of the Soret peak in aq. HCl solution (Falk, 1964, p. 236).

Other reagents

Hexokinase (EC 2.7.1.1), catalase, NADH (disodium salt), ATP (disodium salt), D-lactate (calcium salt), phosphoenolpyruvate (monosodium salt) and GTP (trisodium salt) were purchased from Boehringer Corp. (London) Ltd., London, W.5, U.K. Vitamin-free Casamino acids were from Difco (Detroit, Mich., U.S.A.), 2-heptyl-4-hydroxyquinoline *N*-oxide was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and 5-aminolaevulinic acid hydrochloride was from the Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.). All other reagents were from BDH Chemicals Ltd. and were of the highest available purity.

Results

Reconstitution of oxidase activity in cell-free extracts

It has previously been established that the reconstitution of oxidase activity, in cell-free extracts of a mutant strain of *E. coli* that requires 5-aminolaevulinic acid for growth on non-fermentable carbon sources, is dependent upon the addition of haematin and ATP (Haddock & Schairer, 1973). The dependence on haematin has been investigated further by using both a sample of commercially available haematin and a haem fraction derived by extraction from the prototrophic *E. coli* strain EMG-2. The data in Fig. 1(*b*) show that the concentration of haem required for the reconstitution of half-maximal rates of NADH oxidase activity in cell-free extracts from strain A1004a, grown in the absence of 5-aminolaevulinic acid, is about 0.5μM for the commercial sample of haematin, or about 1.5μM for the haem extract prepared from strain EMG-2. The difference in the amount of haem required with the two haematin preparations and in the specific activity of the reconstituted rates of NADH oxidation with higher concentration of haem

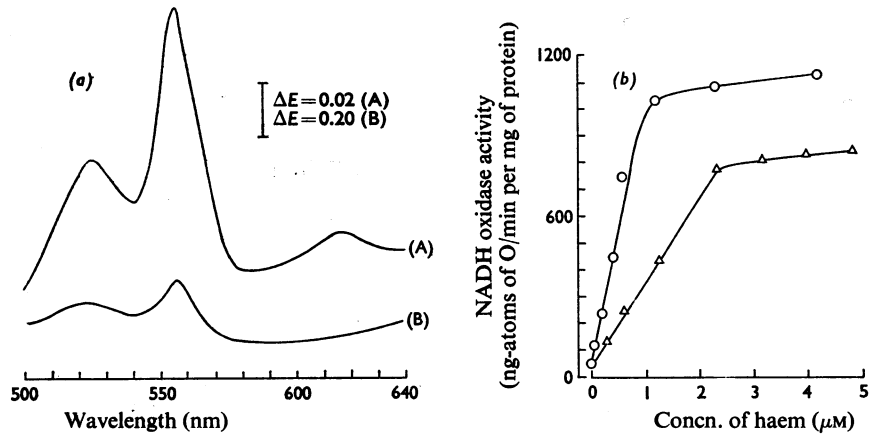

Fig. 1. *Absorption spectrum of pyridine haemochromes and the dependence on haem for the reconstitution of NADH oxidase activity in cell-free extracts of Escherichia coli strain A1004a*

The reduced-minus-oxidized difference spectra of alkaline pyridine solutions of a haem extract from *E. coli* EMG-2 (A) and haematin (B) are shown in (*a*) and were obtained as indicated in the Materials and Methods section. The comparative effectiveness of these two haem solutions for the reconstitution of oxidase activity in cell-free extracts of A1004a is shown in part B. Strain A1004a was grown aerobically in the absence of 5-aminolaevulinic acid, harvested, washed twice with CR medium and resuspended to a final protein concentration of 7.0mg/ml. Portions (1ml) of the suspension were broken by ultrasonic disintegration and ATP (10mM) was added. The suspension was mixed and 0.1ml portions were pipetted into small test tubes containing various concentrations of haematin (○) or the *E. coli* haem extract (△). The test tubes were transferred to a water bath maintained at 37°C and shaken for 60min. After this time the test tubes were removed and placed in ice and the NADH oxidase activities were determined polarographically at 37°C as described previously (Haddock & Schairer, 1973). In control experiments with haematin (4.1μM) or *E. coli* haem extract (4.9μM) present, but in the absence of ATP, the rate of NADH respiration was 50 or 55ng-atoms of O/min per mg of protein respectively.

(>4μM) is possibly due to the presence of haems from both *b*- and *d*-type cytochromes in the *E. coli* haem extract, as shown by the peaks at 557nm and 615nm respectively (Barrett, 1956) in the reduced-minus-oxidized difference spectrum of the alkaline pyridine haemochromes of this preparation (Fig. 1*a*).

The haematin required for the reconstitution of NADH oxidase activity is incorporated into NADH-reducible cytochrome *b* (Fig. 2). There is a linear relationship between the amount of added haematin and the amount of substrate-reducible cytochrome *b* up to a concentration of 1–1.5μM-haematin, after which the presence of excess of haematin has little effect on either the reconstitution of NADH oxidase activity or the amount of substrate-reducible cytochrome *b*. From the data in Fig. 2 the concentration of reconstituted, substrate-reducible cytochrome *b* required for the maximum rate of reconstituted NADH oxidase activity was 0.09nmol/mg of protein, a figure that is in close agreement with the concentration of substrate-reducible cytochrome *b* in cell-free extracts of strain A1004a grown aerobically in the presence of 5-aminolaevulinic acid (results not given) and of strain A201 (Haddock & Schairer, 1973).

The genetic lesion in strain A1004a presumably results in the loss of 5-aminolaevulinate synthetase, so it was of interest to see if precursors of haem could substitute for haematin in the reconstitution of NADH oxidase activity. Neither the immediate porphyrin precursor of protohaem biosynthesis (protoporphyrin IX) nor coproporphyrin III, deuteroporphyrin IX and mesoporphyrin IX could replace haematin in the reconstitution; on the contrary, three of the porphyrins tested were inhibitors of the haematin-dependent reconstitution. If cell-free extracts of strain A1004a, grown in the absence of 5-aminolaevulinic acid, were preincubated with ATP and deuteroporphyrin IX, and then haematin was added and the incubation continued, the NADH oxidase activity of the sample decreased with increasing deuteroporphyrin IX concentration. A 90% inhibition of the reconstitution was achieved at a deuteroporphyrin IX concentration of about 1μM (Fig. 3*a*), a value very similar to the concentration of haematin required for the reconstitution of the maximum rate of NADH oxidase activity (Fig. 1*b*). This inhibition by deuteroporphyrin IX was not relieved by increasing concentrations of

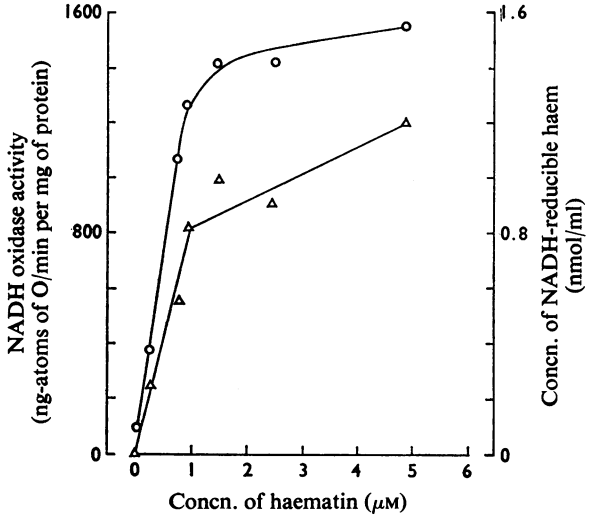

Fig. 2. *Appearance of NADH-reducible haem in the haematin-dependent reconstitution of NADH oxidase activity of cell-free extracts of Escherichia coli strain A1004a*

Strain A1004a was grown aerobically in the absence of 5-aminolaevulinic acid, harvested, washed twice with CR medium and resuspended to a protein concentration of 13.0mg/ml. Portions (2ml) of the suspension were broken by ultrasonic disintegration and ATP (8mM) was added together with various concentrations of haematin. The resultant suspension was incubated, with shaking, in 25ml conical flasks for 65min at 37°C. After this time the rate of NADH oxidation (○) was assayed polarographically at 37°C, as indicated in the legend to Fig. 1, and the concentration of NADH-reducible haem (△) was determined as indicated in the Materials and Methods section.

haematin (Fig. 3*b*). These results suggest that, in the presence of ATP, deuteroporphyrin IX can form a complex with the cytochrome apoproteins of the cell-free extract, which yields an inactive respiratory chain, and that there is no subsequent exchange of the deuteroporphyrin IX for haematin.

With mesoporphyrin IX and protoporphyrin IX, on the other hand, though both porphyrins inhibited the haematin-dependent reconstitution of NADH oxidase activity, the concentration of porphyrin required for such inhibition (4–6μM) was similar to the concentration of haematin in the assay. Also, in experiments of the type shown in Fig. 3(*b*), it was shown that the inhibition produced by a fixed concentration of either mesoporphyrin IX or protoporphyrin IX could be relieved by increasing the haematin concentration. From these results, it would appear that mesoporphyrin IX and protoporphyrin IX also form a complex with the cytochrome apoproteins of the cell-free extract, but that there is a subsequent exchange of mesoporphyrin IX or protoporphyrin IX for haematin.

The other porphyrin tested, coproporphyrin III, did not inhibit the haematin-dependent reconstitution of NADH oxidase activity in cell-free extracts of strain A1004a grown in the absence of 5-aminolaevulinic acid.

Reconstitution of oxidase activity in electron-transport particles

From various experiments on the ATP- and haematin-dependent reconstitution of NADH oxidase activity in cell-free extracts of strain A1004a (B. A. Haddock, unpublished work) and strain A201 (Haddock & Schairer, 1973), it seemed likely that the cytochrome apoproteins were synthesized and incorporated into the cytoplasmic membrane in the absence of new synthesis of protein. To confirm this, the reconstitution of NADH oxidase activity in electron-transport particles, derived by differential centrifugation from cell-free extracts of strain A1004a grown in the absence of 5-aminolaevulinic acid, was studied.

Some properties of various preparations of electron-transport particles from strain A1004a are compared in Table 1. In the absence of cytochrome synthesis, the overall rates of both NADH and D-lactate oxidation are decreased 20- and 70-fold respectively but there is no effect on the specific activities of NADH–ferricyanide reductase and NADH– or D-lactate–dichlorophenol-indophenol reductase.

NADH oxidase activity could be reconstituted in electron-transport particles derived from strain A1004a, grown in the absence of 5-aminolaevulinic acid, provided that ATP and haematin were present, in about 30min at 37°C. The concentration of haematin required for the reconstitution of half-maximal rates of NADH oxidase activity was about 0.8μM (Fig. 4). It can be calculated that the concentration of reconstituted, NADH-reducible, cytochrome *b* required for the maximum rate of reconstituted NADH oxidase activity is about 0.11nmol/mg of protein, a value similar to that found in electron-transport particles derived from strain A1004a grown in the presence of 5-aminolaevulinic acid (Table 1).

The concentration of ATP required for the reconstitution of half-maximal rates of NADH oxidase activity was about 2mM, 1000-fold the required concentration of haematin. However, there will be other ATP-dependent enzymes in these electron-transport particles, e.g. Mg^{2+}-dependent adenosine triphosphatase (EC 3.6.1.3) activity, so little signi-

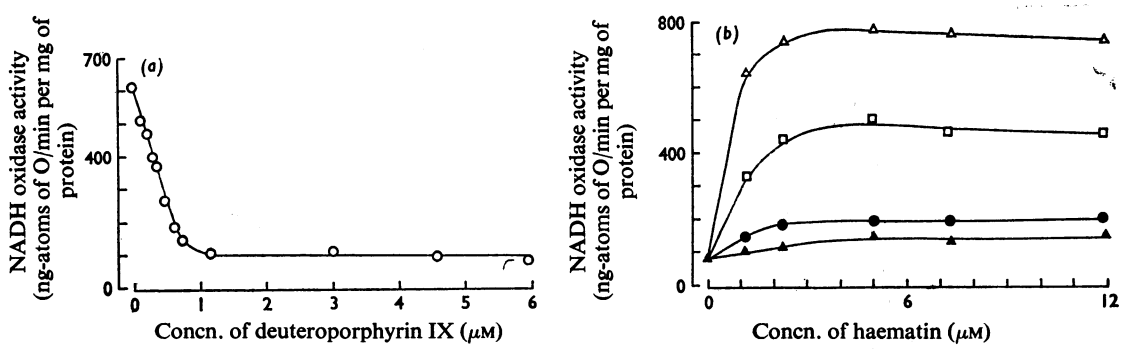

Fig. 3. *Effect of deuteroporphyrin IX on the haematin-dependent reconstitution of NADH oxidase activity of cell-free extracts of Escherichia coli strain A1004a*

Strain A1004a was grown aerobically in the absence of 5-aminolaevulinic acid, harvested, washed twice with CR medium and resuspended to a protein concentration of 6.7mg/ml (*a*) or 10.0mg/ml (*b*). (*a*) A portion (2ml) of the suspension was broken by ultrasonic disintegration and ATP (10mM) was added. The suspension was mixed and samples (0.1ml) were pipetted into small test tubes containing various concentrations of deuteroporphyrin IX. The test tubes were transferred to a water bath maintained at 37°C and shaken for 30min. After this time haematin (4μM) was added to each tube and the incubation continued for a further 30min. The test tubes were removed and placed in ice and the NADH oxidase activities, at 37°C, were determined as indicated in the legend to Fig. 1(*b*). In control tubes containing deuteroporphyrin IX (at any concentration), but no haematin, the rate of NADH oxidation was 45ng-atoms of O/min per mg of protein. (*b*) Portions (1ml) of the suspension were broken by ultrasonic disintegration and transferred to 25ml conical flasks. ATP (10mM) and various concentrations of deuteroporphyrin IX (△, 0; □, 0.61μM; ●, 1.07μM; ▲, 5.15μM) were added and the flasks shaken for 30min at 37°C. After this time portions (0.1ml) were removed from the flasks and pipetted into test tubes containing various concentrations of haematin. The test tubes were then shaken for a further 30min at 37°C, and the NADH oxidation rates at 37°C were determined as indicated in the legend to Fig. 1(*b*).

Table 1. *Oxidase activities, dehydrogenase activities and substrate-reducible cytochrome content of various preparations of electron-transport particles from Escherichia coli strain A1004a*

Electron-transport particles were prepared from strain A1004a, grown aerobically in the presence or absence of 5-aminolaevulinic acid (30μM), as described in the Materials and Methods section. 'Reconstituted' electron-transport particles were prepared by incubating electron-transport particles (10mg/ml), from cells grown in the absence of 5-aminolaevulinic acid, with haematin (5μM) and ATP (5mM) for 60min at 37°C. All assays were performed at 30°C as described in the Materials and Methods section and are the average of at least two separate preparations. Units of specific activity are: for (1) and (2), ng-atoms of O/min per mg of protein; for (3)–(5), μmol of dye reduced/min per mg of protein; for (6), nmol/mg of protein. n.t., Not tested.

	Activity of electron-transport particles		
	From cells grown in the absence of 5-aminolaevulinic acid	From cells grown in the presence of 5-aminolaevulinic acid	'Reconstituted' particles
(1) NADH oxidase	50	1210	1600
(2) D-Lactate oxidase	2	145	140
(3) NADH–ferricyanide reductase	1.80	1.38	n.t.
(4) NADH–dichlorophenol-indophenol reductase	0.43	0.24	n.t.
(5) D-Lactate–dichlorophenol-indophenol reductase	0.19	0.05	n.t.
(6) NADH-reducible cytochrome *b*	not detectable (<0.01)	0.15	0.11

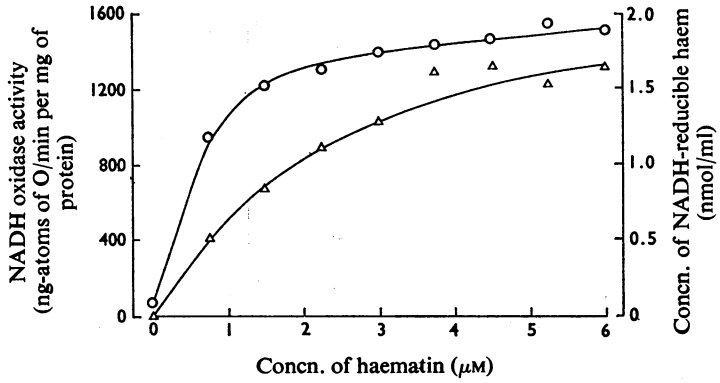

Fig. 4. *Appearance of NADH-reducible haem in the haematin-dependent reconstitution of NADH oxidase activity of electron-transport particles derived from Escherichia coli strain A1004a*

Assay conditions were as given in the legend to Fig. 2, except that electron-transport particles (15mg/ml) prepared from strain A1004a grown aerobically in the absence of 5-aminolaevulinic acid, as described in the Materials and Methods section, were used instead of the cell-free extract. ○, NADH oxidase activity; △, NADH-reducible cytochrome *b*. In a control experiment with haematin (6μM) present, but in the absence of ATP, the rate of NADH oxidation was 77ng-atoms of O/min per mg of protein.

Table 2. *Requirement for ATP in the haematin-dependent reconstitution of NADH oxidase activity of electron-transport particles derived from Escherichia coli strain A1004a*

Electron-transport particles were prepared from strain A1004a grown aerobically in the absence of 5-aminolaevulinic acid as indicated in the Materials and Methods section and resuspended to a final protein concentration of 6.0mg/ml. Portions (1ml) were pipetted into 25ml conical flasks containing either (A) haematin (4μM) alone or (B) haematin (4μM), glucose (20mM) and hexokinase (0.1mg, 14units). The flasks were transferred to a water bath at 37°C, allowed to equilibrate for 10min with shaking, and then ATP (5mM), GTP (5mM), phosphoenolpyruvate (12.5mM) or acetyl phosphate (5mM) was added. After a further 20min incubation, the rate of NADH oxidation by small portions (10–50μl) of the electron-transport particles was measured as described in the legend to Fig. 1(*b*).

	Rate of NADH oxidation (ng-atoms of O/min per mg of protein)	
	(A)	(B)
Control, no addition	80	75
ATP	1260	615
GTP	995	580
Phosphoenolpyruvate	380	110
Acetylphosphate	920	155

ficance can be placed on the value for the required concentration of ATP. GTP, acetyl phosphate and phosphoenolpyruvate also stimulated the reconstitution of NADH oxidase activity, but none was as effective as ATP (Table 2). If glucose and hexokinase were included in the reaction mixture as an 'ATP trap', the rate of the reconstitution of NADH oxidase activity was inhibited, suggesting that GTP, acetyl phosphate and phosphoenolpyruvate, with decreasing efficiency, were not used directly but phosphorylated endogenous ADP present in the electron-transport particles.

Further experiments showed that it was possible to reconstitute both NADH and D-lactate oxidase activities, by using electron-transport particles from cells grown in the absence of 5-aminolaevulinic acid, to the values found with electron-transport particles grown in the presence of 5-aminolaevulinic acid (Table 1). It was also demonstrated that the NADH oxidase activity of reconstituted electron-transport particles (0.1mg of protein/ml) was sensitive to the inhibitors 2-heptyl-4-hydroxyquinoline *N*-oxide (75% inhibition with 2.3μg of inhibitor/ml) and potassium cyanide (100% inhibition with 1mM-inhibitor after 1min incubation).

Discussion

It has been shown here that, in the reconstitution of NADH oxidation, haematin is converted into NADH-reducible cytochrome *b* and that this

requirement for haematin can be replaced with a haem extract from a prototrophic strain of *E. coli*. This strain was grown aerobically with succinate as carbon source, and, under these conditions, the cells produce cytochromes b_{556} and *o* during the exponential growth phase with cytochrome *d* synthesis increasing in the later stages of the growth cycle (Haddock & Schairer, 1973). The haem extract contains haems derived from both *b*- and *d*-type cytochromes (Fig. 1*a*), so the data in Fig. 1(*b*) indicate that haem *d* (chlorin) is an inhibitor of the reconstitution, competing with protohaematin for the *b*-type cytochrome apoproteins.

The inability of various porphyrins to replace haematin in the reconstitution of NADH oxidase activity has been noted by other investigators in studies on the reconstitution of catalase activity in a haematin-requiring mutant of *E. coli* (Beljanski & Beljanski, 1957). However, it may prove possible to demonstrate a protoporphyrin IX-dependent reconstitution of oxidase activity by using assay conditions more appropriate to the conditions required for maximum ferrochelatase (protohaem ferrolyase; EC 4.99.1.1) activity (Porra & Jones, 1963; Porra *et al.*, 1972). The different inhibitory effects of the various porphyrins tested on the haematin-dependent reconstitution suggest that the nature of the substituent groups on the 2 and 4 positions of the porphyrin ring play an important role in haem–apocytochrome(s) interaction(s). However, in reconstitution experiments of apocytochrome *c* peroxidase (cytochrome *c*–H_2O_2 oxidoreductase; EC 1.11.1.5) with modified haem derivatives, the nature of the side chain at the 2 and 4 positions of the porphyrin ring was not considered to be important for enzymic activity (Asakura *et al.*, 1971).

It may be concluded from the results presented here, and from experiments on the reconstitution of catalase activity (Beljanski & Beljanski, 1957), that during the biosynthesis of haem-containing proteins in *E. coli* apoproteins are synthesized and incorporated into the cytoplasmic membrane, in the case of the *b*-type cytochromes, in the absence of haem synthesis. This conclusion is supported by work with other systems, e.g. the reconstitution of cytochrome–nitrate oxidoreductase (EC 1.9.6.1) activity in cell-free extracts of a haematin-requiring strain of *Staphylococcus aureus* (Chang & Lascelles, 1963), and studies on the synthesis and assembly of periplasmic cytochrome *c* in *Spirillum itersonii* (Garrard, 1972). However, in other studies, it has been concluded that the synthesis of the prosthetic group controls the synthesis of the apoprotein, e.g. in bacteriochlorophyll synthesis in *Rhodopseudomonas spheroides* (Takemoto & Lascelles, 1973) and in haemoglobin synthesis in rabbit reticulocytes (e.g. Gross & Rabinovitz, 1972). Indeed, with rabbit reticulocytes the control mechanism is more complex since deprivation of haematin results in an inhibition of the synthesis by lysates of not only globin but also membrane proteins and all other cytoplasmic proteins (Lodish, 1973).

The ability to reconstitute NADH oxidase activity and substrate reducible cytochromes in electron-transport particles from the mutant described here should prove useful in the investigation of the factors affecting the biosynthesis of cytochrome apoproteins and the role of ATP in the assembly of functional cytochromes from haem and apoproteins.

This work was generously supported by the Medical Research Council through Grant G970/772/B. The wavelength-scanning spectrophotometer was constructed with a grant (no. 318) from the North Atlantic Treaty Organization. I thank Professor P. B. Garland for critical discussions and advice, and Mr. A. Tucker for technical assistance.

References

Asakura, T., Drott, H. R. & Yonetani, T. (1971) in *Probes of Structure and Function of Macromolecules and Membranes* (Chance, B., Yonetani, T. & Mildvan, A. S., eds.), vol. 2, pp. 523–532, Academic Press, London and New York

Barrett, J. (1956) *Biochem. J.* **64**, 626–639

Basford, R. E., Tisdale, H. D., Glenn, J. L. & Green, D. E. (1957) *Biochim. Biophys. Acta* **24**, 107–115

Beljanski, M. & Beljanski, M. (1957) *Ann. Inst. Pasteur (Paris)* **92**, 396–412

Bragg, P. D. & Hou, C. (1967) *Biochim. Biophys. Acta* **119**, 194–201

Chang, J. P. & Lascelles, J. (1963) *Biochem. J.* **89**, 503–510

Chappell, J. B. (1964) *Biochem. J.* **90**, 225–237

Cohen, G. N. & Rickenberg, H. W. (1956) *Ann. Inst. Pasteur (Paris)* **91**, 693–720

Cox, R. & Charles, H. P. (1973) *J. Bacteriol.* **113**, 122–132

Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, Elsevier Publishing Co., Amsterdam, London and New York

Garrard, W. T. (1972) *J. Biol. Chem.* **247**, 5935–5943

Gross, M. & Rabinovitz, M. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 1565–1568

Haddock, B. A. & Garland, P. B. (1971) *Biochem. J.* **124**, 155–170

Haddock, B. A. & Schairer, H. U. (1973) *Eur. J. Biochem.* **35**, 34–45

Jones, C. W. & Redfearn, E. R. (1966) *Biochim. Biophys. Acta* **113**, 467–481

Lodish, H. F. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 1526–1530

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Porra, R. J. & Jones, O. T. G. (1963) *Biochem. J.* **87**, 181–185

Porra, R. J., Langman, L., Young, I. G. & Gibson, F. (1972) *Arch. Biochem. Biophys.* **153**, 74–78

Săsărman, A., Surdeanu, M. & Horodriceanu, T. (1968*a*) *J. Bacteriol.* **96**, 1882–1884

Săsărman, A., Surdeanu, M., Szegli, G., Horodriceanu, T., Greceanu, V. & Dumitrescu, A. (1968*b*) *J. Bacteriol.* **96**, 570–572
Schairer, H. U. & Haddock, B. A. (1972) *Biochem. Biophys. Res. Commun.* **48**, 544–551
Takemoto, J. & Lascelles, J. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 799–803
Taylor, A. L. & Trotter, C. D. (1972) *Bacteriol. Rev.* **36**, 504–524
Wulff, D. L. (1967) *J. Bacteriol.* **93**, 1473–1474