ASSEMBLY OF FUNCTIONAL *b*-TYPE CYTOCHROMES IN MEMBRANES FROM A 5-AMINOLAEVULINIC ACID-REQUIRING MUTANT OF *ESCHERICHIA COLI*

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1. Introduction

The investigation of the properties of the respiratory chain components in *E. coli* has been greatly aided by the analysis of various mutants [1–3]. The formation of functional enzymes may be studied through investigation of the effects of genetic lesions on the synthesis of apoproteins and prosthetic groups, and their assembly. *E. coli* is capable of synthesizing a number of distinct cytochromes [4,5] but the isolation of mutants deficient in their ability to produce specific cytochromes has not been reported. However cytochrome biosynthesis has been investigated using mutants requiring 5-aminolaevulinic acid, a presursor of haem biosynthesis, for non-fermentative growth [6–8]. Functional cytochromes are synthesized by these mutants during growth in the presence of 5-aminolaevulinic acid, but when grown in the absence of this compound no cytochromes are spectrally detectable and oxidase activities are extremely low [7]. When membranes derived from cells grown aerobically in the absence of 5-aminolaevulinic acid are incubated with haematin and ATP, NADH oxidase activity can be reconstituted in vitro. This reconstitution was shown to be independent of protein synthesis [7] implying that apocytochromes were pre-assembled in the membrane. Since these membranes were incubated with haematin (haem *b*), only *b*-type cytochromes were reconstituted. The role of ATP in the reconstitution of NADH oxidase activity is unclear but it has been shown that hydrolysis of the ATP by an enzyme other than F_1-ATPase is a necessary step [8].

It is the aim of this study to investigate which cytochromes are reconstituted during incubation of haem-deficient membranes with haematin and ATP. The reconstituted cytochromes have been studied by potentiometric redox titrations and low-temperature spectrophotometry of membrane preparations, and compared with the cytochromes found in prototrophic *E. coli* [4]. The requirement for ATP during reconstitution has been further investigated by similar studies with haem-deficient membranes which had been incubated with haematin in the absence of ATP; cytochromes are apparently reconstituted but these membranes lack NADH oxidase activity.

2. Materials and methods

2.1. *Growth, harvesting and breakage of cells*

Escherichia coli strain A1004a (K12Ymel *ato fadR hemA ilv lacI metE rha*) was grown aerobically, as 500 ml batches in 2 l flasks, with vigorous shaking at 37°C, in the mineral salts medium of [9] 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). Cells were harvested, washed and broken by ultrasonic disruption [10], and membrane particles prepared by differential centrifugation [6].

2.2. *Reconstitution of oxidase activity*

Membrane particles (~10 mg protein/ml) were incubated with haematin (1 μM) and ATP (2 mM) for 30 min at 37°C. NADH-dependent O_2 uptake, detected with an oxygen electrode, was reconstituted in these particles as in [7]. Another batch of mem-

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branes was similarly incubated but in the absence of ATP, under which conditions no measurable NADH oxidase activity was reconstituted.

2.3. *Redox titrations and spectrophotometry*

Optical redox potentiometry was performed as in [4] except that the Soret band absorbance was monitored. The mediators used were: duroquinone, *N,N,N',N'*-tetramethylphenylenediamine, thionine, indigodisulphonate, dibromophenolindophenol and tetrachlorohydroquinone, each at 20–40 μM. Data were analysed using a computer program made available by J. Bowyer, University of Illinois. Data were fitted for 1, 2 or 3 components; fitting for greater numbers of components was impractical because of the running time of the program. Low-temperature spectrophotometry was performed as in [4].

3. Results

Membranes from *E. coli* strain A1004a grown in the absence of 5-aminolaevulinic acid were reconstituted with haematin and ATP; NADH oxidase activity was similar to that found in membranes from cells grown in the presence of 5-aminolaevulinic acid [7]. Since only *b*-type cytochromes are reconstituted by this procedure, the oxidase in these membranes must be cytochrome *o*. Cytochrome *o* has been shown to have a mid-point redox potential of +80 mV and an absorbance maximum at 556 nm at 77 K in reduced – oxidized difference spectra [4]. Redox titrations with reconstituted membranes were performed to determine the nature of the cytochromes present. The cytochrome content in the membranes was low (~0.07 nmol/mg protein) and the signal-to-noise ratio was not favourable. Best fits to the data were obtained using a computer program; single-electron transfer ($n = 1$) was assumed for the cytochromes and two components titrating at +80 mV and +262 mV were resolved (fig.1a), contributing 70% and 30%, respectively, to the total observed absorbance change.

When titrations of these membranes were performed in the presence of CO the total absorbance change over the titrated range of potentials decreased to ~60% of that observed in the absence of CO. The data were fitted by two components with mid-point potentials of +81 mV and +227 mV, contributing 23% and 77%, respectively, to the total observed absorbance change (fig.1b). It is of interest that the component titrating at +80 mV is observed in the presence of CO, albeit only a small fraction of the total amount of this component observed in the absence of CO. This may indi-

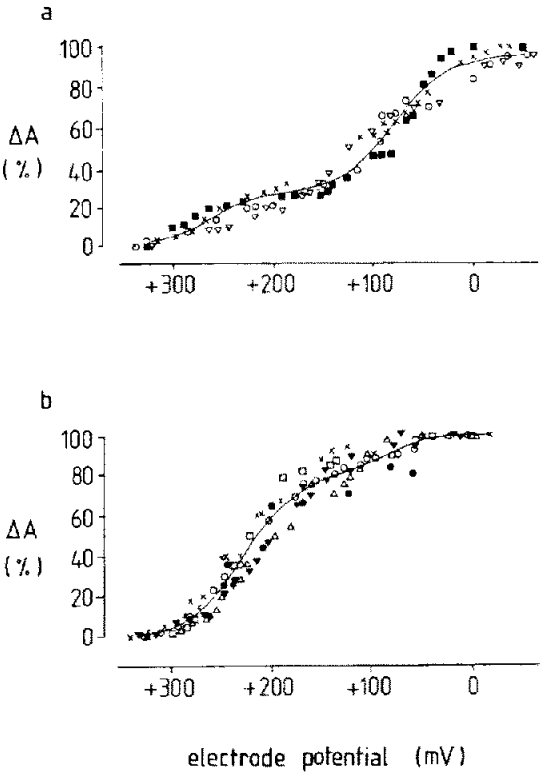

Fig.1. Redox titrations of reconstituted membranes from *E. coli* A1004a performed in the presence and absence of CO. Reconstituted A1004a membranes were suspended in 250 mM Hepes, (pH 7.1) and redox titrations were performed under an atmosphere of either argon (a) or CO (b). Data were normalized to give a range of absorbance change from fully oxidized to fully reduced of 100%. The data from several titrations were used to find the best fit using a computer program. The best fit is shown as a solid line, with data points from different titrations represented by different symbols. In (a) the best fit shown is for two components with mid-points at +80 mV and +262 mV; the data were not well-fitted by a single redox component but the sum of squared deviations for a three-component fit was similar to that for a two-component fit. However the three-component fit gave E_m-values of +90 mV and +230 mV for the major components and an additional component titrating at −90 mV contributing 11% to the total absorbance change. The two-component fit is preferred because there is no evidence for a component titrating at −90 mV either here in the presence of CO (b) or in a prototrophic strain [4], and because the E_m-values determined by the two-component fit correspond well with with those of *b*-type cytochromes in the prototroph. For redox titrations performed in the presence of CO a best fit is obtained for two components titrating at +81 mV and +227 mV (b).

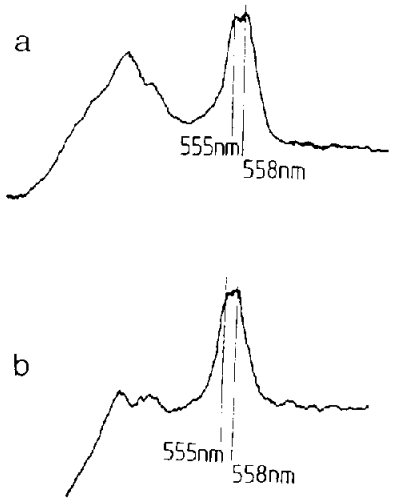

Fig.2. Low-temperature (77 K) difference spectra of reconstituted membranes from strain A1004a. Membranes from A1004a were incubated with haematin in the presence (a) or absence (b) of ATP as in section 2. Spectrum (a) is the difference between a sample poised at +180 mV and a fully oxidized sample. Spectrum (b) is the difference between a sample reduced with NADH (1 mM) and one oxidized with H_2O_2.

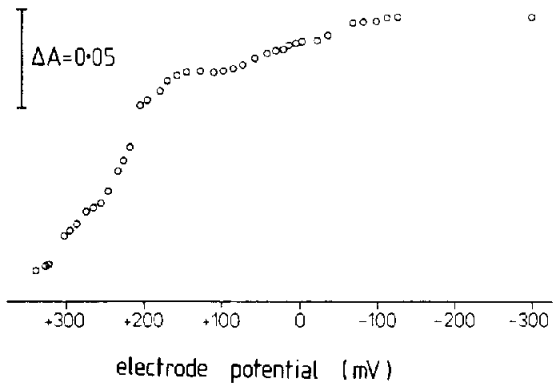

Fig.3. Redox titration of membranes from strain A1004a which had been incubated with haematin in the absence of ATP. Membranes from A1004a were incubated with haematin in the absence of ATP as in section 2. Membranes were resuspended to 17.1 mg protein/ml in 250 mM Hepes and a redox titration was performed. The absorbance change at 427 nm is plotted against electrode potential.

cate that a component other than cytochrome *o* also titrates at +80 mV in these reconstituted membranes, but no such species was observed in membranes from prototrophic cells [4]. The possible presence of a non-functional, non-CO-binding fraction of cytochrome *o* cannot be excluded. The decrease of the mid-point potential of the other *b*-type cytochrome from +262 mV to +227 mV in the presence of CO correlates with a similar drop from +260 mV to +200 mV observed in membranes from an aerobically-grown prototrophic strain [4]. However, difference spectra (fig.2a) show peaks at 556 and 558 nm in the high potential range, indicating the presence also of cytochrome b_{558} in these membranes. This cytochrome has a mid-point redox-potential of +250 mV in membranes from a prototrophic strain in the presence or absence of CO [4]. The component detected at +227 mV in the presence of CO in reconstituted membranes from A1004a may be a composite of cytochromes titrating at +200 mV and +250 mV; these were not resolved with the available data. In the absence of CO these cytochromes would be expected to titrate at +260 mV and +250 mV, too close to be resolved.

NADH oxidase activity was not reconstituted when membranes from A1004a grown aerobically in the absence of 5-aminolaevulinic acid were incubated with haematin in the absence of ATP. These membranes do, however, exhibit absorbance bands typical of cytochromes *b* (fig.2b), with maxima at ~556 nm and 558 nm in reduced – oxidized difference spectra at 77 K. Redox titrations of these membranes showed that most of the absorbance change was due to relatively high-potential components (fig.3) with no cyto-

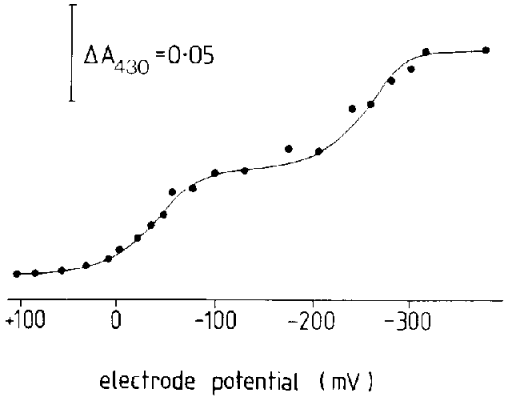

Fig.4. Redox titration of erythrocyte membranes which had been incubated with haematin. Human erythrocyte membranes were prepared and incubated with haematin as described; membranes were resuspended in 250 mM Hepes (pH 7.1). The solid line is drawn for two $n = 1$ components with mid-points at −35 mV and −260 mV. No significant absorbance change took place between +100 mV and +400 mV.

chrome *b* titrating at +80 mV. The results of titrations performed in the presence and absence of CO were essentially identical; the total absorbance change was independent of CO. These data indicate that cytochrome *o* was not reconstituted in the absence of ATP.

It was considered likely that some haematin would bind non-specifically to the membranes during reconstitution. In order to investigate the properties of non-specifically bound haem a control experiment was performed using erythrocyte membranes which are devoid of endogenous haem. Human erythrocytes were washed thrice with isotonic NaCl then twice with isotonic sodium phosphate at pH 7.4. The cells were haemolysed in 7 mM sodium phosphate (pH 7.4) and washed 5 times with this low-ionic-strength buffer to remove haemoglobin. The membranes were incubated with haematin (10 μM) at 37°C for 30 min and centrifuged at 35 000 × *g* for 20 min at 4°C to remove unbound haematin. The membranes were resuspended in 250 mM Hepes (pH 7.1) and redox titrations were performed. The haem in this system titrated only at low redox potentials (fig.4) and thus free haem in the A1004a membranes would not be expected to contribute significantly to the absorbance changes at higher potentials attributed to cytochromes, assuming that the free haem would behave similarly in both membrane systems.

4. Discussion

The above results indicate that multiple components are reconstituted when membranes from *E. coli* A1004a grown aerobically in the absence of 5-aminolaevulinic acid are incubated with haematin and ATP. These membranes have a functional respiratory chain [7] containing cytochromes only of type *b*. The oxidase, cytochrome *o*, is reconstituted under these conditions in addition to components titrating at higher potentials; these other *b*-type cytochromes appear to correspond to cytochrome b_{558} titrating at +250 mV and the high potential (+260 mV) cytochrome *b* seen in membranes from aerobically grown cells of a prototrophic strain [4]. It has been noted that free haem exhibits an absorbance maximum at 558 nm in reduced – oxidized difference spectra at 77 K [6], but this absorbance band is broad. It appears unlikely that the absorbance at 558 nm observed at high potentials in the A1004a reconstituted membranes used here was due to free haem since:

(i) The absorbance band was relatively sharp (compare fig.2a with [6]);
(ii) The membrane-bound 'free' haem titrates at low redox potentials in erythrocyte membranes and presumably also in *E. coli* membranes.

No evidence was found for the presence in reconstituted A1004a membranes of a cytochrome *b* titrating at +140 mV where cytochrome b_{555}, associated with cytochrome *d* [4,11], would be expected to titrate. The cytochrome *d*-containing oxidase complex is therefore partly assembled in these reconstituted membranes since cytochrome b_{558} is present whereas cytochrome b_{555} apparently cannot be reconstituted and cytochrome *d* certainly cannot since the prosthetic group (haem *d*) is not present during the incubation.

That *b*-type cytochromes may be reconstituted in haem-deficient A1004a membranes implies that the cytochrome apoproteins are synthesized and inserted into the membranes in the absence of the prosthetic group. Similar haem-independent synthesis of cytochrome apoproteins appears also to occur in other bacterial systems [12,13]. However it has been shown that in some systems the synthesis of the prosthetic group controls the synthesis of the apoprotein [14,15].

The role of ATP in the reconstitution of a functional respiratory chain in A1004a membranes is poorly understood. The results shown here indicate that cytochrome *o* is not reconstituted in the absence of ATP and so it appears that ATP hydrolysis is directly linked to the association of the haem prosthetic group with apocytochrome *o*.

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