BIOCHEMICAL CHARACTERIZATION OF THE *unc*A PHENOTYPE OF *Escherichia coli*

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SUMMARY

The amounts of cross-reacting material (CRM) for antibody against ATPase (EF_1) in five *unc*A strains were determined. Considerable amounts were found in strains AN120, DF360 and DL54, but little in strains NR70 and NI44. The cross-reacting materials were solubilized by washing the membranes with EDTA and identified as inactive EF_1 containing non-covalently bound adenine nucleotides. All the washed membranes, except those of strain NI44, bound EF_1 (active ATPase) with restoration of the ^{32}Pi-ATP exchange reaction (RES). These results indicated that the *unc*A mutation is pleiotropic and that both CRM and RES, in addition to EF_1, are *biochemical* properties affected by the mutation. The subgrouping of the five strains based on these criteria is: AN120, DF360 and DL54#, [EF_1^-, CRM^+, RES^+]; NR70, [EF_1^-, CRM^+, RES^+]; NI44, [EF_1^-, CRM^+, RES^-].

INTRODUCTION --- Mutants of *Escherichia coli* with a phenotypic defect of *uncoupled* oxidative phosphorylation were first isolated by Butlin *et al.* (1) and classified into two subgroups, *unc*A ($ATPase^-$) and *unc*B ($ATPase^+$). This finding received much attention and a number of mutants showing the same phenotypic defects have since been isolated (2-15). As reviewed (16-18), these mutants, and particularly *unc*A strains, were useful in studies on energy transduction in oxidative phosphorylation, active transport and the transhydrogenation reaction.

It is known that the cells had no detectable ATPase activity (EF_1^-) but other biochemical defects of EF_1 molecules affected by the *unc*A mutation have not yet been established. Recently we developed an immunochemical procedure for determining

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Abbreviations: EF_1, adenosine triphosphatase (EC 3.6.1.3:coupling factor of oxidative phosphorylation) of *E.coli*; CRM, cross-reacting material for antibody against EF_1; RES, restoration of ^{32}Pi-ATP exchange activity; EDTA, ethylenediamine tetraacetate.

We have examined the capabilities of the four strains, AN120, DF360, NR70 and NI44 to reconstitute the ^{32}Pi-ATP exchange reaction. As another energy transducing reaction (ATP-driven transhydrogenase) could be restored in strain DL54 (27), we classified this strain as RES^+.

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the amount of EF_1 and found that one *unc*A mutant, strain AN120 has cross-reacting material containing bound adenine nucleotides (19).

This technique enabled us to analyze biochemical traits affected by the *unc*A mutation. This paper reports determinations of the contents of cross-reacting materials (CRM) in several *unc*A strains of *E.coli*. Restoration of the ^{32}Pi-ATP exchange reaction (RES) by incubating washed-membranes of these strains with purified EF_1 was studied and on the basis of quantitative analyses the five *unc*A mutants were classified into three subgroups.

MATERIALS AND METHODS

Bacterial Strains --- The strains of *E.coli* K12 used were: AN180 (wild type) and its derivatives, AN120 (*unc*A) (1) and AN382 (*unc*B) (7), (provided by F.Gibson); strains DF364 (*pgi*$^-$), DF360 (*pgi*$^-$, *unc*A) and DF362 (*pgi*$^-$, *unc*B) (transductants of AN180, AN120 and AN382, respectively, obtained from D.Fraenkel); strain 7 (wild type) and NR70 (*unc*A) (8) (from B.P.Rosen) and strain NI44 (*unc*A) (2) and *E.coli* ML308-225 and its derivative DL54 (*unc*A) (4) (obtained through L.A.Heppel).

Preparation of French Press Vesicles --- Cells were grown in a semi-defined medium (20). Membrane fractions were prepared by the method of Futai *et al.* (21), except that 50 mM HEPES-NaOH buffer, pH 7.2, was used instead of Tris-HCl. Washed-membrane fractions were prepared by washing membranes twice with 0.5 mM EDTA containing 1 mM HEPES-NaOH, pH 7.2. Both membrane fractions were finally suspended in 50 mM HEPES-NaOH, pH 7.2, containing 10 mM $MgCl_2$ (approximately 50 mg protein per ml) and stored at -80° until use.

Analysis of Bound Nucleotides --- Cells were grown aerobically in a semi-defined, low phosphate medium (20 mg KH_2PO_4 per 200 ml) containing 0.5% casamino acids (Difco), 1 μg/ml of thiamine and 0.2% glucose as described previously (19). Carrier free ^{32}Pi (0.4 mCi) and ^3H-amino acid mixture (30 μCi) were added to 200 ml of the medium before cultivation. Fructose (0.3%) in place of glucose was used for growth of strains DF364, DF362 and DF360. Bound nucleotides were analysed after precipitating ATPase or cross-reacting materials with antiserum against EF_1 as described previously (19). The specific radioactivities of ^{32}P and ^3H-protein were determined by the published procedure (19).

Binding of EF_1 to Membranes --- The French press vesicles (2.4 mg protein) were incubated with ATPase (8.9 units) purified from strain ML308-225 in 50 mM HEPES-NaOH, pH 7.2, containing $MgCl_2$ as described previously (21,22). After centrifugation, the membranes were suspended in the above buffer and the activity of the ^{32}Pi-ATP exchange reaction was assayed by the method of Kanner *et al.* (23), except that the buffer contained 10 mM sodium phosphate and the protein concentration was 1.6 mg per ml.

Other Procedures and Materials --- ATPase activity (24) and protein (25) were measured by published methods. ^3H-L-Amino acid mixture (NET-250) was obtained from New England Nuclear Co. and ^{32}Pi from CEA, France. All adenine nucleotides were from Boehringer Mannheim. Other chemicals used were of the highest grade available commercially.

RESULTS AND DISCUSSION

Presence of Cross-reacting Materials in the Membranes of ATPase Mutants --- More

Table I. Effects of mutant membranes on the activity of antiserum against ATPase

Membranes incubated with serum	Amount of preincubated-serum causing 50% inhibition of ATPase
none	2.5 μl
AN120 membranes [a]	18.3
AN120 washed-membranes [a]	6.0
DF360 membranes [a]	21.0
DF360 washed-membranes [a]	8.4
NR70 membranes [b]	12.4
NR70 washed-membranes [b]	3.2
NI44 membranes [b]	7.0
NI44 washed-membranes [b]	2.6

Various amounts of antiserum against ATPase were mixed with membranes from mutant cells [(a), 160 μg or (b), 1.6 mg protein] in 50 μl of 50 mM HEPES-NaOH, pH 7.2, containing 10 mM $MgCl_2$. The mixture was incubated for 30 min at 25°, and then 2 μl of membranes of strain 7 (160 μg, 0.2 unit ATPase) was added and the mixture was incubated further for 60 min. ATPase activity was then assayed as described previously (20). The amount of serum causing 50% inhibition of ATPase of strain 7 was assessed from a plot of the amount of serum against the ATPase activity.

than 85% of EF_1 can be solubilized from the membranes of wild-type cells by washing them with buffer containing EDTA (21). However, it was not known whether inactive EF_1 in membranes of mutants could be solubilized in the same way. To examine this we tested for cross-reacting materials in the membranes of mutant cells before and after treatment with EDTA. We assayed for this materials by pretreating anti-EF_1 serum with the membranes of mutant cells and then testing its ability to inhibit ATPase activity, because antibody which had already reacted with cross-reacting material could not inhibit ATPase activity. As shown in Table I, several fold more serum pretreated with unwashed membranes was required for 50% inhibition of ATPase activity than of untreated serum or serum pretreated with washed-membranes. In this experiment washed-membranes of mutants were obtained by the same procedure as those of wild-type cells. These results suggest

Table II. Detection of materials cross-reacting with ATPase with bound nucleotides in crude ATPase fractions from various strains

Strain	ATPase activity (unit/mg protein)	Molar content ATP (moles/mole enzyme)	Molar content ADP (moles/mole enzyme)	Amount of cross-reacting material (Amount precipitated/ total protein)
AN180	2.4	1.7	0.8	4.4 %
AN382 (*unc*B)	1.9	2.0	1.0	3.1
AN120 (*unc*A)	0.03	1.5	0.5	3.1
DF364	2.6	2.3	1.0	4.9
DF362 (*unc*B)	1.0	2.1	1.3	1.5
DF360 (*unc*A)	0.03	2.1	1.3	3.9
ML308-225	1.9	1.5	0.8	2.7
DL54 (*unc*A)	< 0.03	1.1	0.8	1.2
7	2.5	2.5	0.8	4.7
NR70 (*unc*A)	< 0.03	1.6	1.1	0.5
NI44 (*unc*A)	< 0.03	1.5	0.5	0.6

The analytical procedures are described in MATERIALS AND METHODS.

that membranes of the mutant cells contained significant amounts of cross-reacting materials which could be solubilized by washing with EDTA solution.

It is noteworthy that about 10 times more membranes of strains NR70 and NI44 than those of AN120 and DF360 were required for titrations. This may be because these strains contained low concentrations of cross-reacting materials; the supernatant fractions obtained by centrifuging the cell extracts at 100,000 x g for 60 minutes did not contain cross-reacting materials, because the capacity of antiserum to inhibit ATPase did not change on preincubation with this fraction (0.3 mg protein) (data not shown).

The Amounts of Cross-reacting Materials in the Crude ATPase Fractions --- Crude ATPase fractions of the ATPase mutants contained different amounts of cross-reacting materials (Table II, right column); the amounts obtained from strains

Table III. Binding of ATPase and reconstitution of the ^{32}Pi-ATP exchange reaction in membranes of *unc*A strains

Source of membranes	Pre-treatment of membranes	ATPase [a]	^{32}Pi-ATP exchange reaction [b]
strain 7	unwashed	0.81	2.73
	washed	0.78	2.86
AN120	unwashed	0.24	0.22
	washed	0.77	0.53
NR70	unwashed	0.17	0.18
	washed	0.41	0.62
NI44	unwashed	0.20	< 0.02
	washed	0.44	< 0.02

Membranes were incubated with purified EF_1 (3.7 units/mg membrane protein) for 10 min at 37°. The mixture was centrifuged and the activities of ATPase and the ^{32}Pi-ATP exchange reaction of the membrane fractions were assayed as described in the text, and expressed as (a) unit/mg protein and (b) nmoles ^{32}P-ATP formed/min/mg protein, respectively. Membranes washed twice with 0.5 mM EDTA containing 1 mM HEPES-NaOH, pH 7.2 were used where indicated. The activities of ATPase and ^{32}Pi-ATP exchange reaction of strain 7 membranes before addition of EF_1 were 0.54 unit/mg protein and 1.47 nmoles/min/mg protein, respectively. Membranes of mutant strains (AN120, NR70 and NI44) before addition of EF_1 and washed-membranes of strain 7 had negligible activity of ATPase (0.03 unit/mg) or the exchange reaction (< 0.02 nmole/min/mg protein).

AN120 and DF360 were comparable to the amounts in the parent strains, while that obtained from DL54 was about 50% of that in the parent strain, confirming the results of Boonstra *et al.* (26). On the other hand, strains NR70 and NI44 contained less than 15% of the cross-reacting materials in the wild-type strains. It has been reported that no inactive EF_1 molecules could be detected in strain NR70 (8,26) or NI44 (23) by immunodiffusion. Thus our method is probably more sensitive than previous methods (8,23,26).

When cross-reacting materials of strains AN120 and DF360 were examined by gel electrophoresis in the presence of sodium dodecyl sulfate (19), 85% of the radioactivities were found in the positions of polypeptides identical to the

five subunits of purified EF_1 ($\alpha,\beta,\gamma,\delta,\varepsilon$). Essentially similar results were obtained with active ATPase from *unc*B mutants such as strains AN382 and DF362. The (^3H) radioactivities of the cross-reacting materials obtained from strains DL54, NR70 and NI44 were found in the positions of the three large subunits (α,β and γ). In this case no radioactivity could be detected in the positions of the small subunits (δ and ε) because of the low contents of the materials in these strains. These results suggest that the cross-reacting materials found in these strains were inactive EF_1 molecules bound to membranes.

Binding of EF_1 to Washed-membranes of the Mutants --- The presence of cross-reacting materials in the *unc*A strains was confirmed by demonstrating increase in the binding of purified EF_1 to mutant membranes after washing with EDTA: as shown in Table III, three times more EF_1 could bind to washed-membranes of strains AN120 and NR70 than to the corresponding unwashed membranes. Titration of the binding showed clear saturation kinetics and the specific activity of EF_1 bound to the membranes was essentially the same as that of wild-type membranes. The EF_1 bound to the membranes became capable of energy transduction, since ^{32}Pi-ATP exchange reactions were reconstituted. These results indicate that the binding sites for EF_1 in mutant membranes were increased after washing. As the binding sites for EF_1 in the membranes are specific for the whole ATPase complex (21), these results suggest that cross-reacting materials were present in these specific binding sites.

With increase in the amount of EF_1, the amount of EF_1 bound to the membranes of NI44 increased linearly. However, the bound enzyme did not reconstitute the ^{32}Pi-ATP exchange reaction. This agrees with the report of Bragg and Hou (27) that ATP-driven transhydrogenase was not reconstituted after incubation of membranes of this strain with EF_1. These results suggest that this strain has some defect of the membranes together with defect of EF_1 molecule.

Presence of Non-covalently Bound Nucleotides in Cross-reacting Materials --- Table II shows the molar contents of bound nucleotides found in cross-reacting materials of all the strains tested. In every strain 1 mole of inactive or active EF_1 contained 2 moles of ATP and 1 mole of ADP. These values were calculated

taking the molecular weight of inactive EF_1 as 300,000 (21). The results suggest that inactive EF_1 molecules contained essentially the same amount of non-covalently bound nucleotides as active molecules obtained from wild-type cells.

*unc*A Phenotype --- The results obtained indicate that the *unc*A phenotype is pleiotropic, although genetic evidence suggested that the defects in strains DL54 and NI44 were due to a single mutation (2,4). The content of cross-reacting material in the membranes and their ability to reconstitute Pi-ATP exchange with EF_1 are additional biochemical traits involved in the defect caused by the *unc*A mutation. It should be mentioned that the cross-reacting materials in all the five strains isolated in different laboratories (1, 2, 4, 8) were inactive EF_1, containing the same molar contents of bound adenine nucleotides as EF_1. We recently concluded that the bound adenosine triphosphate in EF_1 that can be measured by the immunochemical procedure is metabolically stable (unpublished observation). These results suggest that the nucleotides are essential, at least for the molecular organization of EF_1. It is therefore interesting to isolate and study strains that have cross-reacting materials without containing adenine nucleotides. The cross-reacting materials studied in this work seem to have polypeptides (at least those of strain AN120 and DF360) identical to the subunits of EF_1. Thus it is valuable to obtain mutants without one of the five subunits of EF_1. For some unknown reason we found that strain NI44 did not show restored ^{32}Pi-ATP exchange activity with added EF_1 [RES^-]. From our findings, an *unc*A phenotype of [EF_1^-, CRM^+, RES^-] seems possible but no mutant of this type has yet been isolated.

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