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MUTANTS OF *ESCHERICHIA COLI* K12 UNABLE TO GROW ON NON-FERMENTABLE CARBON SUBSTRATES

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SUMMARY

Among a number of mutants unable to utilize non-fermentable carbon substrates, scoring for membrane ATPase and for ATP-driven transhydrogenase activity permitted to distinguish two phenotypes: (A) mutants lacking ATPase and ATP-driven transhydrogenase; (B) one mutant with an ATPase which behaved according to several criteria as released into solution instead of being membrane bound, a.o it exhibited no ATP-driven transhydrogenase activity. All A and B mutants exhibited a common nutritional pattern.

The ATPase-deficient group, when scored for ATPase-binding sites on its membrane particles revealed three different subgroups: (1) mutants having free ATPase-binding sites, (2) mutants with ATPase-binding sites made available by the procedure which releases ATPase from wild-type membrane, and (3) mutants with no detectable ATPase-binding sites.

Membranes of the mutant B with unbound ATPase also exhibited a deficiency in ATPase-binding sites, but its soluble ATPase was also found unable to bind to ATPase-binding sites of wild type membranes.

The double alteration, namely abnormal or inactive ATPase and absence of ATPase-binding sites on the membrane is compatible with a single mutational defect.

INTRODUCTION

Among the approaches to study oxidative phosphorylation the isolation and characterization of bacterial mutants deficient in this function have been rewarding. The complexity of the system in bacteria seems to be of the same order as in mitochondria of eucaryotes including a respiratory chain with presumably three sites of phosphorylation and on the ATP synthesizing side paths a terminal ATPase having a molecular weight and a subunit composition [1] similar to mitochondrial F1 and

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

Enzymes: ATPase, EC 3.6.1.3; alcohol dehydrogenase, EC 1.1.1.1.

For genetic loci designation, see: Taylor, A. L. et al. (1972) (Bacteriol. Rev. 36, 504–524).

chloroplast ATPase. Genetics of the system in yeast were the subject of much work but the additional difficulty of nuclear and mitochondrial genes involved in the formation of the system limited the impact of this research on the knowledge of the mechanism of oxidative phosphorylation.

Genetic studies in *Escherichia coli* have unraveled mutants deficient in the respiratory chain namely in porphyrin synthesis, and in ubiquinone synthesis [2, 3], and a presumably regulatory mutant with a high level of membrane ATPase [4]. More important in the context of the present article, two groups of mutants were found deficient in oxidative phosphorylation: *uncA*-type mutants deficient in the membrane ATPase that lack ATP-driven transhydrogenase at the same time [5–12] and *uncB*-type mutants deficient in the ATP-driven transhydrogenase that retain a normal ATPase activity [13–15].

They share the common property of being unable to grow on carbon sources which cannot generate ATP by pathways other than oxidative phosphorylation.

All mutations of these two groups were found to map in the same region of the chromosome (around 73.5 min) [5, 8, 9, 11–15].

The possibility to find a correlation between genes (cistrons) and ATPase subunits and between ATPase subunits and specific functions or functional details, and to detect gene products essential to oxidative phosphorylation other than ATPase subunits motivated the present research.

Using a selection technique based on the failure of non-fermentable carbon sources to support the growth a large number of mutants have been isolated and tested for membrane ATPase and ATP-dependent transhydrogenase, but also for their growth requirements and for the ability of their ATPase (when present) and of their membrane particles to reconstitute a *N,N'*-dicyclohexylcarbodiimide (DCCD)-sensitive ATPase and ATP-driven transhydrogenase with the wild type counterpart.

Besides mutants deficient in NaN_3-sensitive ATPase, one mutant was found with an ATPase which behaves in vivo as if released in solution.

The examination of ATPase-binding properties of the membrane particles derived from the various mutants provided a further differentiation of the ATPase-deficient mutants into three subgroups, one having free sites, another with occupied sites which can be cleared by the usual procedure, and a third subgroup in which no sites could be detected with present techniques.

MATERIAL AND METHODS

Strains

Strains used in this paper are described in Table I.

Isolation of mutants

After ultraviolet mutagenesis (giving 1–10 % survival) and penicillin enrichment on succinate (4 g/l) and D,L-lactate (4 g/l), bacteria were plated on minimal agar (with the required amino acids and bases) with 0.1 g/l glucose plus 4 g/l succinate and 4 g/l DL-lactate as carbon substrate. Small colonies appearing after 3 and 4 days were then picked and checked by replica plating for their inability to grow on non-fermentable substrates. Among the small colonies picked, around 6 % presented the right phenotype.

TABLE I

Strains	Relevant genetic loci	Other information
3300 (Hfr H)	*lac* i^-	
AB 3059 (Hfr 313)	*thi* 1, *leu* 6, *ilv* D132, *thy* A41, *thy* R23, *lac* Z4, *str* 8	Howard-Flanders [16]
CSH 57A (F^-)	*thi*, *leu*, *pro* C, *his*, *arg* G, *pur* E, *trp*, *ilv* *met* A or B, *lac* Y, *gal*, *mal* A, *ara*, *mtl*, *str* A	Miller [17]
ER E. Reich	*thi* 1, *asn* 31	Cedar and Schwartz [18]
KL 16 (Hfr)		Low [19]
MDA	derived from AB 3059 or CSH 57A	this paper
MDB	derived from AB 3059	this paper

Growth of bacteria

For cell fractionation and production of ATPase, membrane bound or soluble, bacteria were grown on medium 63 glucose as described previously [20], the required supplements being added at a concentration of 50 μg/ml. The same culture conditions were used for testing the utilization of various carbon sources in replacement for glucose at final concentrations of 4 g/l.

Preparation of membranes

Membrane preparation has been described previously [20]. In brief, bacteria were harvested in the exponential growth phase, disrupted in a Ribi fractionator under 30 000 lb/inch^2 (approximately 1600 atm) pressure in 10 mM Tris/HCl buffer (pH 7.6) containing 1 mM $MgCl_2$ (Tris/HCl/$MgCl_2$ buffer). The crude homogenate was centrifuged at 66 000 × *g* for 30 min in a 30 rotor (Beckman Spinco L65). The pellet was discarded and the supernatant was centrifuged at 160 000 × *g* for 120 min (in a 50 Ti rotor). The 160 000 × *g* pellet (membrane) was washed once with cold Tris/HCl/$MgCl_2$ buffer and stored frozen at −20 °C in small fractions, in the same buffer (washed membranes) at a concentration of 50 mg/ml of proteins.

Release of ATPase from membranes

As described previously [21], the washed membranes were diluted in 20 vol. 1 mM Tris/HCl buffer (pH 7.6) containing no Mg^{2+} at room temperature, and centrifuged at 160 000 × *g* for 120 min. The pellet was resuspended in the original volume of 1 mM Tris/HCl (pH 7.6)/10 mM $MgCl_2$ (depleted membranes). The supernatant (depletion supernatant) contained the "solubilized ATPase" and was either used fresh or precipitated with $(NH_4)_2SO_4$ added solid to 80 % saturation.

ATPase assays

ATPase assays were performed as described previously [20] in 40 mM trieth-

anolamine/HCl buffer at pH 7.5 with 3.3 mM ATP and 1.66 mM Mg^{2+}. After 7 min of incubation at 30 °C, the inorganic phosphate was measured by the method of Martin and Doty [22].

For the screening of mutants an ATPase assay on toluenized bacteria was used. Bacteria, harvested during exponential growth on minimal medium, were filtered on Millipore (0.45-μm pore size), washed with 1 mM Tris/HCl pH 7.6 and 1 mM $MgCl_2$ at room temperature, and resuspended in the same buffer to a final density of about 250 μg/ml dry weight. Toluene was added at 1 % (v/v). The mixture was then vigorously stirred during 90 s. After centrifugation at 4 °C, the pellet was resuspended in the same volume of the above buffer and recentrifuged at 4 °C. The final pellet was resuspended in the buffer in order to give a density of about 1 mg/ml dry weight. ATPase assay was then immediately performed.

Azide at 10 mM inhibits membrane-bound as well as solubilized ATPase [5, 20, 23] and was therefore used to evaluate irrelevant phosphohydrolase activities.

ATP-driven transhydrogenase assays

The reaction mixture contained 100 mM Tris/HCl buffer (pH 7.5), 0.1 mM NAD, 30 mM ethanol, 300 μg/ml alcohol dehydrogenase, 0.5 mM NADP, 3 mM KCN, 4 mM $MgCl_2$, and washed membrane preparation (250–600 μg of protein). This mixture was incubated for 45 min at 25 °C in cuvettes of 1-cm light path. After a steady state was reached, 4 mM ATP was added and the increase of absorbance at 340 nm was recorded with a Gilford 240 spectrophotometer.

Reconstitution of membrane-bound ATPase and ATP driven transhydrogenase

Reconstitution was performed according to the technique of Roisin and Kepes [21]. Solubilized fraction containing ATPase activity was mixed with depleted membranes in 5 mM $MgCl_2$ and after 10 min incubation at room temperature, it was diluted in the reaction mixtures for ATPase and ATP driven transhydrogenase activity measurements (as above). Three criteria of reconstitution were applied routinely: an approximately 2-fold activation of ATPase activity at pH 7.5, appearance of DCCD sensitivity and of ATP-driven transhydrogenase activity. Occasionally a fourth criterion was used, namely the sedimentation of previously soluble ATPase activity upon binding to membranes. No discrepancies were observed between these criteria.

O_2 uptake in whole bacteria

Respiration of whole bacteria was measured with a Gilson Oxygraph with a Clark electrode. After growth overnight on minimal medium with the required supplements and with 4 g/l fructose and 4 g/l succinate as carbon substrates, bacteria were filtered on nitrocellulose membranes (0.45-μm pore size), washed with minimal medium and resuspended in the same medium at a density of approximately 0.28 mg dry weight per ml. The suspension was stirred for half an hour at 37 °C, and then the rate of respiration was measured with either fructose or succinate as carbon source.

Genetic mapping techniques

Mutations were mapped by transduction using P1 *vir*. The method of Castellazzi et al. [24] was followed. In particular, the phage was systematically ultraviolet treated with 900 erg · cm^{-2} before transduction.

Chemicals

ATP, NADP and alcohol dehydrogenase (yeast) were purchased from Boehringer; NAD from Mann Research Laboratories; KCN from Merck, *N,N'*-dicyclohexylcarbodiimide (DCCD) from Calbiochem, Dio 9 from Mycofarma Delft Holland. All other chemicals were reagent grade.

RESULTS

ATPase and ATP driven transhydrogenase activities in mutants

About 40 independent mutants were isolated and characterized for their ATP-driven transhydrogenase activity in crude homogenates, and for their ATPase activity in homogenates and/or toluenized bacteria.

30 mutants were found to be deficient in ATPase activity. Residual activity was 5–30 % of wild type. In order to further characterize the ATPase activity due to the enzyme involved in oxidative phosphorylation and to discount irrelevant phosphohydrolase activities, the NaN_3-resistant activity was systematically substracted. With this precaution, all mutants of this class had less than 5 % of the wild-type activity.

Measurement of ATPase in toluenized bacteria gave results consistent with the measurement in bacterial extracts. All ATPase-negative mutants also lacked ATP-driven transhydrogenase activity. These mutants are designated in the following as MDA mutants (Table II).

One mutant has lost ATP-driven transhydrogenase activity but retained 60 % of the ATPase activity compared to wild type. This is designated as MDB (Table II).

TABLE II

ATPase AND TRANSHYDROGENASE ACTIVITIES OF MUTANTS

ATPase activities were measured by two methods as described, corrected for azide-resistant activity. Activities were related to bacterial dry weight, as calculated from $A_{600\ nm}$ measurements just prior to toluenization or disruption.

Strain	No. of strains tested	ATPase activity corrected for azide-resistant activity				ATP-driven transhydrogenase (nmoles NADPH/mg protein/min in crude homogenate)
		In toluenized bacteria		In crude homogenate		
		μmoles P_i/mg dry wt/min	% of wild type	μmoles P_i/mg dry wt/min	% of wild type	
Wild type	3	0.48	100	1.02	100	12
MDA	14*	0.02	4.5	0.05	5	<1
MDB	1	0.29	61	0.65	63	<1

* More mutants have been assayed only in crude homogenate.

Utilization of carbon sources

Since mutants have been selected as unable to grow on a mixture of succinate and D,L-lactate but able to grow on glucose as sole carbon source, with the assumption that ATP synthesis at the substrate level was their essential requirement, it was felt necessary to make a broader survey of carbon sources for their ability to support the growth of the mutants.

TABLE III

UTILIZATION OF CARBON SOURCES TO SUPPORT GROWTH OF THE MUTANTS

Sole carbon sources	Growth of strains (number tested)		
	Wild type	MDA (30)	MDB (1)
D-Glucose, D-fructose, D-mannose, D-mannitol**, D-gluconate, lactose*, L-arabinose**	+	+	+
Succinate, D,L-lactate, malate, oxaloacetate, pyruvate, α-ketoglutarate, fumarate, cis-aconitate, D-glucuronate, D-galacturonate, melibiose, glycerol, xylose, D-galactose**, maltose**	+	−	−
D-Glucose*** in anaerobiosis (liquid medium or agar plate)	+	−	−
nutrient agar*** in anaerobiosis	+	−	−

* Lactose was tested only in 4 transductants.

** Mannitol, arabinose, galactose and maltose were tested only in 25 mutants derived from AB 3059.

*** Growth in GasPak jars H_2+CO_2 (BBL Cockeysville, Md., U.S.A.).

As shown in Table III not only glucose but also fructose, mannose, lactose, arabinose, gluconate and mannitol, were efficient as carbon sources to support the growth of all mutants. Similarly not only succinate and lactate, but also pyruvate, malate, fumarate, oxaloacetate, α-ketoglutarate and *cis*-aconitate gave negative results, except in the parent strains in conformity with the working hypothesis. Besides this predictable spectrum of carbon-source utilization, D-glucuronate and D-galacturonate, xylose, galactose, maltose, melibiose and glycerol were found unable to support the growth of MDA and MDB mutants. Anaerobic conditions with either glucose or nutrient agar as the culture medium resulted in absence of growth.

The reasons for failures to utilize such carbon sources as e.g. galactose which are normally metabolized via the Embden-Meyerhof pathway might be a defective energization of the relevant uptake system or a drop in inducibility of the metabolic pathway. A few attempts made to grow MDA mutants on galactose in the presence of 5 mM 3′,5′-cyclic AMP did not give positive results.

Another discrepancy between presumed ATP synthesis and growth was the inability of the mutants to grow on glucose under anaerobic conditions.

Further correlation of carbon-source utilization and ATP production was sought by measuring the growth yield on glucose. As shown in Table IV, all MDA and MDB mutants had a strongly diminished growth yield, 15–35 % of the wild type. As toward carbon source utilization MDA and MDB again behaved similarly for growth yield.

Since aerobiosis was strictly required for growth of mutants MDA and MDB

TABLE IV

GROWTH YIELD ON MINIMAL MEDIUM CONTAINING 0.8 g/l GLUCOSE

Strain	No. of strains tested	mg dry weight per ml from $A_{600\ nm}$* after 24 and 48 h	
		Mean	Range
Wild type	2	0.460	
MDA	21	0.113	0.067–0.160
MDB	1	0.118	

* A culture of $A_{600\ nm} = 1$ contained 0.275 mg dry weight bacteria per ml.

TABLE V

THE RESPIRATORY CHAIN IN MUTANTS

Bacteria were tested as described in Material and Methods.

Strain	No. tested	O_2 uptake per cent of wild type (range)	
		On 4 g/l fructose	On 4 g/l succinate
MDA	8	120 (60–160)	50 (25–60)
MDB	1	130	50

on glucose, it was rather obvious that oxidative processes were possible. Nevertheless, it was important to ascertain that the respiratory chain was not strongly impaired. Fructose and succinate were selected for in vivo respiration. Succinate is known to enter directly the respiratory chain, provided an inducible C4 dicarboxylic acid permease is present [25]. Growth on fructose plus succinate was utilized with the aim to circumvent the strong repressive effect of glucose on this induction process. With this precaution, respiratory rates ranging from 25 to 60 % of that of wild type were observed. Fructose, likely to feed reduced pyridine nucleotides into the respiratory chain, caused an O_2 uptake in all mutants virtually equal to that of wild type. These findings strongly support the assumption that a complete respiratory chain was present in all the mutants.

MDB has a DCCD-resistant unbound and uncoupled ATPase

As shown in Table II, MDB had about 60 % of the wild type ATPase activity, but negligible ATP-driven transhydrogenase activity. The ATPase was still azide sensitive, and showed a sedimentation coefficient on sucrose gradient similar to wild type. In contrast with wild type, the ATPase of MDB was DCCD resistant in the crude homogenate (or in toluenized cells, not shown) and Table VI shows that it was also resistant to the inhibition by Dio 9. The drug resistance was the same as that of the wild-type ATPase when released from the membrane. ATPase of MDB was largely non-sedimentable, and the small fraction which cosedimented with the membranes was removed by the first wash.

According to these findings it could be expected that the ATPase of MDB should be unable to function in ATP synthesis in vivo but would be active as a hydro-

TABLE VI

DISTRIBUTION DURING CELL FRACTIONATION AND DRUG RESISTANCE OF WILD-TYPE AND MDB MUTANT ATPases

Total activities, measured as described in Material and Methods are given as per cent of the total activity found in crude homogenates. DCCD was added to a final concentration of 10^{-4} M and Dio 9 to 100 μg/ml.

	Total ATPase activity					
	Wild type			Strain MDB		
		DCCD	Dio 9		DCCD	Dio 9
Crude homogenate	100	25	25	100	100	110
160 000 × *g* pellet	85	7	13	17	16	17
160 000 × *g* supernatant	10	—	—	83	83	99
Washed membranes	85	2	15	<5	—	—
Solubilized fraction	46	41	53			

lase. Hydrolase activity with an unbound ATPase could fail to perform a useful function such as contributing to establish a H^+ gradient and therefore could only be harmful for the economy of the cell. Conversely an inhibition of ATP hydrolysis can be beneficial.

NaN_3, long known as an uncoupler of oxidative phosphorylation, and as a proton-conducting agent, [26] also behaves as an inhibitor of ATPase [20]. This property is unchanged toward the MDB mutant. It was also shown that inhibition of ATPase by azide is obtained at lower concentrations, than the general uncoupling effect, as measured for example by the inhibition of active transport [27]. Since the phosphoenol pyruvate-dependent glucose phosphotransferase is insensitive to or even

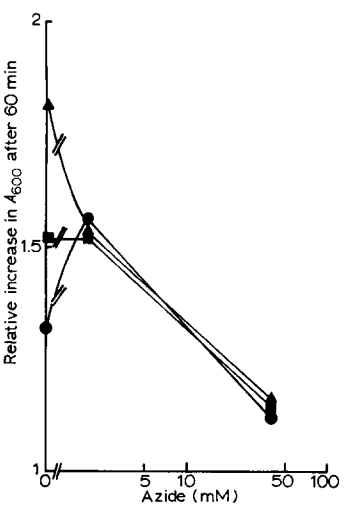

Fig. 1. Effect of azide on growth. NaN_3 was added at zero time to exponential cultures, and growth was measured by absorbance readings at 600 nm. ▲—▲, wild type; ■—■, strain MDA; ●—●, strain MDB. The relative increase in 60 min is plotted vs NaN_3 concentration.

stimulated by azide, the growth on glucose should not be affected at the transport level by azide. The effects of azide on the growth rate of wild type and mutant *E. coli* are represented on Fig. 1.

Azide at the concentration of 2 mM was slightly inhibitory for the growth of wild type, indifferent for the growth of MDA mutants but caused a significant stimulation of the growth of strain MDB. At higher concentrations inhibition became predominant on all strains.

Reconstitution of membrane bound ATPase and ATP-driven transhydrogenase with components derived from the mutants

It was shown previously in this laboratory [21] that solubilized ATPase can bind to homologous depleted membranes (strain 3300), with the result that ATPase activity at pH 7.5 was doubled and the typical sensitivity of membrane-bound ATPase to DCCD and Dio 9 was recovered. Moreover, titration of a fixed amount of depleted membranes with an increasing amount of solubilized ATPase could define an equivalence point that gave a measure of the number of binding sites for ATPase on membrane particles. Identical results were found with the parental strains here utilized. Furthermore, cross reconstitution between strain Hfr 3300 and the two parental strains used were successful. Subsequently depleted membranes and solubilized ATPase of strain 3300 were currently used as the wild type component in reconstitution experiments with mutant ATPase and mutant membranes respectively. The method used to determine equivalence between membrane sites and soluble ATPase is illustrated in Fig. 2. It is based on both stimulation at pH 7.5 and inhibition by DCCD giving concordant results.

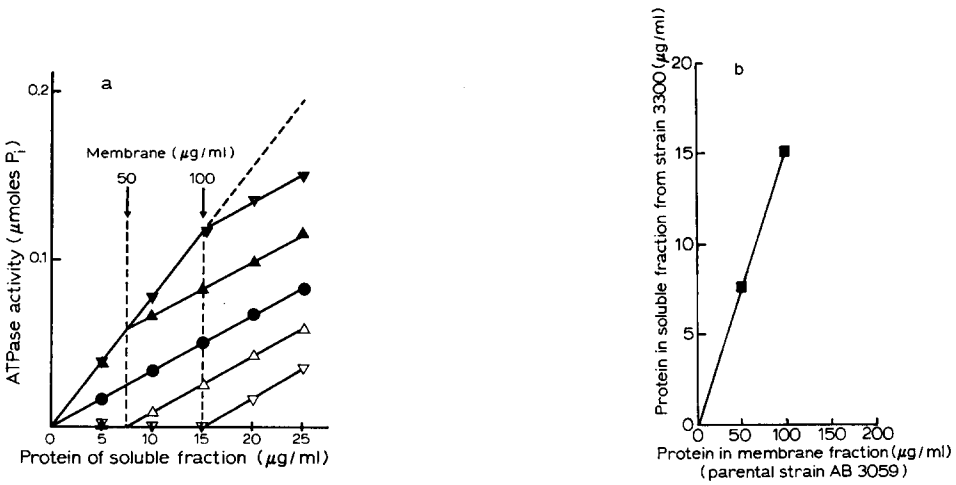

Fig. 2. Measurement of equivalence between solubilized ATPase and ATPase-binding sites in depleted membrane particles. Solubilized ATPase fraction from strain 3300 contained 0.5 mg protein per ml. Depleted membranes from parental strain AB 3059 contained 25 mg protein per ml. Incubation mixtures contained soluble fraction as indicated in abscissa and 0 μg, ●—●; 50 μg, ▲—▲; 100 μg, ▼—▼ of depleted membranes. Open symbols: DCCD, $5 \cdot 10^{-5}$ M was also added. The arrows directed toward the breaks in the curves indicate the soluble fraction equivalent to 50 μg and 100 μg membrane fraction respectively. These values were reported on Fig. b. The same representation was adopted in Fig. 3.

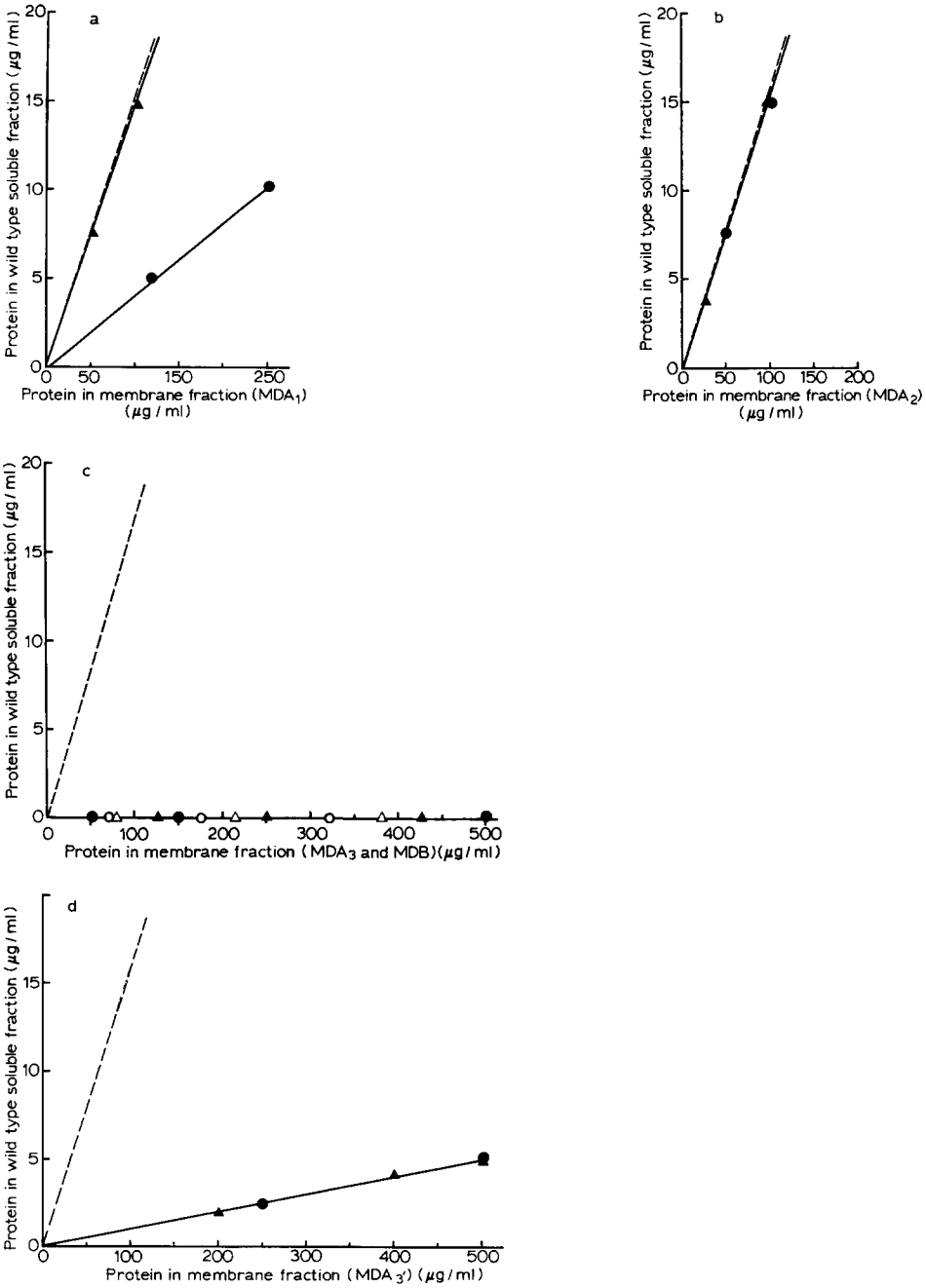

Fig. 3. Equivalence between ATPase binding sites in mutant membranes and wild-type solubilized ATPase fraction. ● — ●, washed membrane preparation; ▲ — ▲, membrane preparation submitted to the depletion process as described in Material and Methods. Dotted lines: equivalence of wild-type depleted membranes. a. Strain MDA_1. b. Strain MDA_2. c. Strain MDA_3 and MDB open symbols. d. Strain $MDA_{3'}$.

Eight MDA mutants were tested for the accepting capacity of their membrane particles. Results for four of them are given in Fig. 3. It can be seen that ATPase-deficient mutants could be further subdivided into 3 groups: (1) Mutants MDA_1 which exhibited a full complement of ATPase-binding sites after a depletion step of the membrane preparation according to ref. 20, whereas before the depletion procedure only a fraction of the sites were available. (2) Mutants MDA_2, membranes of which could accomodate wild-type ATPase in amounts comparable to wild-type depleted membranes whether submitted or not to the depletion procedure. (3) Mutants MDA_3 had membranes unable to bind wild-type ATPase before or after a depletion step.

Two of the strains tested gave a low level of binding (about 5 % of the wild-type binding sites) whether membranes were depleted or not. These might either be a leaky variant of MDA_3 or represent an independent group ($MDA_{3'}$).

ATP-driven transhydrogenase was measured with depleted membranes from one mutant of each group. When mixed with the solubilized fraction of the wild type, results were parallel to what was found for ATPase reconstitution using the same membranes. MDA_1 and MDA_2 membranes reconstituted ATP-driven transhydrogenase about as well as the parental type whereas MDA_3 did not reconstitute (Table VII).

MDB mutant membranes with the wild-type solubilized ATPase gave the same negative results as MDA_3 membranes, (Fig. 3c) but significantly, mixing depleted membranes of the wild type with the soluble ATPase of MDB did not reconstitute either DCCD-sensitive ATPase activity or ATP-driven transhydrogenase activity, (Table VIII, line 6). The failure of the reconstitutions according to all four criteria quoted in Methods with MDB cytoplasmic fraction was not due to the heavy contamination by cytoplasmic proteins, (Table VIII, line 7) or to the presence of some abnormal component having adverse effects on the reconstitution process altogether since MDB supernatant did not interfere with the reconstitution of wild type, (Table VIII, line 8).

TABLE VII

RECONSTITUTION OF ATP-DRIVEN TRANSHYDROGENASE

Soluble fraction	Depleted membranes from:	ATP-driven transhydrogenase (nmoles of NADPH per min per mg of membrane protein)
Released from wild-type membranes	wild type	15
	MDA_1	12.0
	MDA_2	11.9
	MDA_3	<0.1
	$MDA_{3'}$	0.8
	MDB	<0.1
MDB 160 000 × *g* supernatant	wild type	<0.1

TABLE VIII

RECONSTITUTION EXPERIMENTS WITH WILD TYPE AND MDB ATPase

Incubation mixture (abbreviation, protein content)	ATPase activity (nmoles P_i per min)			
	−DCCD		+DCCD (10^{-4} M)	
(1) Wild type depleted membranes (WT Mb, 130 μg)	8.5		0	
(2) Wild type solubilized ATPase (WT ATPase, 10 μg)	9.7		7.2	
(3) Wild type 160 000 × g supernatant (WT sup, 66 μg)	1.8		1	
(4) MDB 160 000 × g supernatant (MDB sup., 65 μg)	11.5		10	
(5) WT Mb + WT ATPase = (1) + (2)	32	(18.2)*	0	(7)*
(6) WT Mb + MDB sup. = (1) + (4)	18	(20)*	9	(10)*
(7) WT Mb + WT sup. + WT ATPase = (1) + (3) + (2)	34	(20)*	1	(8.2)*
(8) WT Mb + MDB sup. + WT ATPase = (1) + (4) + (2)	44	(29.7)*	7	(17.2)*

* Figures between parentheses represent the algebraic sum of activities of the components of the mixture.

Genetic mapping

Two MDA mutants were mapped by transduction using phage P1 *vir* grown on these strains, into strain ER E. Reich carrying an *asn* marker (asparagine synthetase). Cotransduction frequency of the two mutations with the *asn* gene was 0.49 and 0.52 respectively. A three-point test (mutation MDA *asn-ilva*) could not be achieved here presumably because of the proximity of the Hfr factor. The MDB mutation was cotransduced with the *asn* gene with a frequency of 0.51. This strain was found to be F^- and this allowed a 3-point test to be successfull (Table IX). Results favor the following order: *asn*-MDB-*ilva*.

TABLE IX

THREE-POINT TEST WITH MUTATIONS MDB, *asn*, *ilva*

Phage P1 *vir* grown on MDB was used to infect strain ER E. Reich (*asn*). Transductants were selected on medium 63 glucose without asparagine, supplemented with isoleucine (100 μg/ml) and valine (100 μg/ml). After purification on the same medium, colonies were tested for their ability to grow on 0.4 % succinate and for their dependence on added isoleucine-valine. The less frequent class corresponds to a double crossing over as shown on the following diagramme (Succ$^-$ = inability to grow on succinate).

Asn	ATPase	Ilv
+	−	−
−	+	+

Diagram of the genetic segment

(*asn*$^+$)	Number of colonies	Class frequency per cent of total
(*succ*$^-$) (*ilva*$^+$)	42	33.3
(*succ*$^-$) (*ilva*$^-$)	23	18.2
(*succ*$^+$) (*ilva*$^+$)	47	37.3
(*succ*$^+$) (*ilva*$^-$)	14	11.1
Total	126	100

DISCUSSION

The method of selection used here to isolate mutants of oxidative phosphorylation is similar in its principle to that used by others [5, 9, 10, 13] and does not rely on antibiotic resistence [8, 11, 12] the relevance of which to the actual deficiency is not obvious. Although only succinate and lactate were included in the selective medium, a single or double mutation specific of their two respective metabolic pathways did not bias the procedure, all mutants unable to grow on these two carbon sources were found also unable to grow on six other Krebs-cycle intermediates. In contrast, all mutants selected could grow on seven sugars including a disaccharide, two aldohexoses, one ketohexose, one hexitol, one hexonic acid and one pentose, three of which are known to have a constitutive metabolic pathway, the others being inducible.

It is thus striking that seven other sugars were unable to support the growth of all mutants, glucuronate, galacturonate, xylose, galactose, maltose, melibiose and also glycerol. All these nutrients are metabolized by inducible pathways.

All mutants of MDA therefore share a common nutritional pattern with MDB. Most of these features have not been reported by previous authors, but for two of them reports are conflicting. ATPase-deficient mutants of Kanner and Gutnik [8] grew on anaerobic glucose and those of Schairer [9] and of Shallenberger and Simoni [10] grew on glycerol. Growth on glycerol was utilized for the selection of ATPase negatives [9, 10].

The reasons for the non utilization of a number of sugars are poorly understood, but the most obvious hypothesis would be an impairment of the energization of the relevant transport systems, possibly amplified by the consequent low inducing potency of the transported substrate. The discrepancies between mutants isolated in different laboratories are unlikely to be due to differences in the selection procedure utilized, but might be due to minor differences in the genetic background concerning transport, leak, sensitivity to inducer, level of cyclic AMP, etc. These questions should find an answer after transfer of all the mutant genes to a common genetic background.

Mutants of class MDA can be compared to *uncA* mutants of Butlin et al. [5], since deficient in membrane ATPase. When reconstitution abilities are also taken into account, *uncA* as described [4] would be in our MDA_1 subclass and *unc* 405 [28] in our MDA_2 subclass.

MDB mutant has similarities with *uncB* mutations which have active ATPase, but lack ATP-dependent transhydrogenase activity [13, 14]. We have shown that unlike in *uncB* [13, 14] MDB ATPase was abnormal by not being membrane bound in the same sense as wild-type ATPase and unable to reconstitute a DCCD-sensitive ATPase activity or ATP-driven transhydrogenase activity with wild-type depleted membranes.

It still has a sedimentation constant on sucrose gradient similar to the wild-type ATPase, therefore includes probably the normal number of the larger peptides.

Evidence that this enzyme is working in vivo in the direction of hydrolysis rather than for ATP synthesis comes from the favorable effect of NaN_3 on the growth of this mutant whereas azide is inhibitory for the wild type, and has no obvious effect on MDA mutants at doses not exceeding 2 mM. The rarity of MDB-type mutations

might well be due to the harmful role of uncoupled ATPase.

The amount of ATPase activity found in the MDB mutant, about 60 % of the wild type, is compatible with a normal abundance of ATPase molecules taking into account the normal decrease in molar activity observed with the wild type upon dissociation of the ATPase from its binding site on the membrane (Table VI, last line). This remains to be confirmed with immunochemical techniques.

That the absence of coupling between this ATPase and the respiratory chain, the presumed absence of its proton-translocating properties and the lack of physical link between the ATPase and the membrane are not due exclusively to the defect of the membrane side but also to an abnormality of the soluble ATPase molecule, is strongly suggested by the inability of this released ATPase to bind to wild-type membranes.

One of the most striking observations is the double alteration in MDA_2, MDA_3 and MDB-type mutants. The ATPase and the ATPase-binding sites in the membrane are both modified: ATPase is inactive (MDA_2, MDA_3), or unable to bind to wild-type membranes (MDB), while ATPase-binding sites are not available on the mutant membrane (MDA_3, MDB), or on the contrary unoccupied, (MDA_2). This last combination is very easy to understand if ATPase is sufficiently altered to have no more affinity for its membrane site. But MDA_3 and MDB cannot be explained on this basis. It is unlikely that they are due to double mutations because ultraviolet mutagenesis was used. The most obvious explanation for MDB is the synthesis of one abnormal subunit out of the 5 different subunits generally recognized [1], which during the dissociation of the ATPase from the membrane site goes with the wrong partner or causes a genetically normal subunit to join the wrong side, entailing a functional alteration by its presence in one, and by its absence in the other component of the system. The same model could hold for MDA except that having no activity, the non dissociation of the mutant ATPase molecule cannot be ruled out.

It would be attractive to think that mutants MDA_3 and MDB have their genetic defects in a subunit located in the stalk region of ATPase [29]. In MDA_1 mutants an alteration of the head subunit would abolish enzyme activity, while the occupancy of binding sites and the conditions of dissociation, attributes of stalk subunits would remain normal.

Presently the genetic mapping cannot distinguish between the different mutants MDA and MDB. It is likely that several cistrons coding for subunits involved in ATPase activity are closely clustered around 73.5 min. This situation makes the adoption of a final genetic nomenclature for the designation of the different mutants premature.

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REFERENCES

1 Bragg, P. D. and Hou, C. (1972) FEBS Lett. 28, 309–312
2 Haddock, B. A. and Schairer, H. U. (1973) Eur. J. Biochem. 35, 34–45
3 Cox, G. B., Gibson, F. and Pittard, J. (1968) J. Bacteriol. 95, 1591–1598
4 Cox, G. B. Gibson, F., McCann, L. M., Butlin, J. D. and Crane, F. L. (1973) Biochem. J. 132, 689–695
5 Butlin, J. D., Cox, G. B. and Gibson, F. (1971) Biochem. J. 124, 75–81
6 Cox, G. B., Newton, N. A., Butlin, J. D. and Gibson, F. (1971) Biochem. J. 125, 489–493
7 Kanner, B. J. and Gutnik, D. L. (1972) FEBS Lett. 22, 197–199
8 Kanner, B. J. and Gutnik, D. L. (1972) J. Bacteriol. 111, 287–289
9 Schairer, H. U. and Haddock, B. A. (1972) Biochem. Biophys. Res. Commun. 48, 544–551
10 Simoni, R. D. and Shallenberger, M. K. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2663–2667
11 Yamamoto, T. H., Mevel-Ninio, M. and Valentine, R. L. (1973) Biochim. Biophys. Acta 314, 267–275
12 Rosen, B. L. (1973) J. Bacteriol. 116, 1124–1129
13 Butlin, J. D., Cox, G. B. and Gibson, F. (1973) Biochim. Biophys. Acta 292, 366–375
14 Gutnik, D. L., Kanner, B. I. and Postma, P. W. (1972) Biochim. Biophys. Acta 283, 217–222
15 Schairer, H. U. and Gruber, D. (1973) Eur. J. Biochem. 37, 282–286
16 Howard-Flanders, (1968) Adv. Biol. Med. Phys. 12, 299–317
17 Miller, J. H. (1972) in Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor
18 Cedar, H. and Schwartz, H. J. (1969) J. Biol. Chem. 244, 4112–4121
19 Low, B. (1965) Genet. Res. 6, 469–473
20 Roisin, M. P. and Kepes, A. (1972) Biochim. Biophys. Acta 275, 333–346
21 Roisin, M. P. and Kepes, A. (1973) Biochim. Biophys. Acta 305, 249–259
22 Martin, J. B. and Doty, D. M. (1949) Anal. Chem. 21, 965–967
23 Evans, D. J. (1969) J. Bacteriol. 100, 914–922
24 Castellazzi, M., George, J. and Buttin, G. (1972) Mol. Gen. Genet. 119, 139–152
25 Kay, W. W. and Kornberg, H. L. (1971) Eur. J. Biochem. 18, 274–281
26 Mitchell, P. (1970) Membranes and Ion Transport, (Bittar, E. E., ed.), Vol. 1, p. 192, Wiley Interscience
27 Cohen, G. N. and Rickenberg, H. V. (1956) Ann. Inst. Pasteur 91, 693–720
28 Cox, G. B., Gibson, F. and McCann, L. (1974) Biochem. J. 138, 211–215
29 Senior, A. E. (1973) Biochim. Biophys. Acta 301, 249–277