BBA 46178

OXIDATIVE PHOSPHORYLATION IN *AZOTOBACTER VINELANDII*

EFFECT OF INHIBITORS AND UNCOUPLERS ON P/O RATIO, TRYPSIN-INDUCED ATPase AND ADP-STIMULATED RESPIRATION

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(Received April 9th, 1971)

SUMMARY

1. The effect of various inhibitors and uncouplers of mitochondrial oxidative phosphorylation on phosphorylation by Azotobacter particles is reported.

2. The ATPase activity of Azotobacter particles and of the soluble factor involved in oxidative phosphorylation is stimulated 10–20-fold by incubation with trypsin. The trypsin-induced ATPase is Mg^{2+} or Ca^{2+} dependent, Mg^{2+} being the more effective cation. The ATPase is stimulated somewhat by 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole, desaspidin and carbonyl cyanide *m*-chlorophenylhydrazone, and inhibited by atebrin and Dio-9.

3. In previous work ADP was shown to stimulate the oxidation of NADH but not of the Site-II substrates malate, lactate or succinate. Stimulation by ADP of oxidation of malate has now been observed by inhibiting electron transport by 2-heptyl-4-hydroxyquinoline-*N*-oxide or by increasing the electron flow by adding lactate. The stimulation is, therefore, not confined to phosphorylation Site I.

INTRODUCTION

Membrane fragments from *Azotobacter vinelandii* can catalyse oxidative phosphorylation with a high rate of oxidation and phosphorylation[1–5]. The P/O ratios are usually low although recently values up to 1.1 have been reported[6,7]. There are at least two phosphorylation sites, one between NADH and ubiquinone and one between ubiquinone and oxygen (see, however, ref. 7).

The reactions associated with phosphorylation, *i.e.* ATPase, $^{32}P_i$–ATP exchange and respiratory control, have received little attention and therefore an investigation of the ATPase activity was started. This activity is usually very low (0.02–0.04 μmole/min per mg protein) compared with the rate of ATP synthesis (up to 3 μmoles/min per mg protein), even in the presence of Mg^{2+} or uncouplers[2,3]. A latent ATPase

Abbreviations: HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

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in chloroplasts and mitochondria can be unmasked by treatment with trypsin[8,9]; this is attributed to the removal of a protein factor shown to be a trypsin-sensitive ATPase inhibitor[10]. It seemed interesting, therefore, to examine the effect of trypsin on the ATPase activity of Azotobacter preparations.

As shown previously[4,5] the NADH oxidation in phosphorylating particles is stimulated by ADP; it was concluded that this stimulation is connected only with the first phosphorylation site. This effect has now been investigated especially in connection with Site-II phosphorylation.

Several authors have shown that Azotobacter particles, like other bacterial systems, are less affected by 2,4-dinitrophenol than mitochondria[2,11–14]; the same is true for carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), dicoumarol and pentachlorophenol[3], while Azotobacter particles and mitochondria are equally sensitive towards menaquinol-0 and chlorpromazine[3]. In this paper the effect of some additional uncouplers considered to be useful for the study of oxidative phosphorylation in Azotobacter is presented.

EFFECT OF INHIBITORS AND UNCOUPLERS

The effect of several substances on respiratory-chain phosphorylation in Azotobacter particles is shown in Table I. Higher concentrations of 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB)[15] and Dio-9 (ref. 16) are required to affect phosphorylation in Azotobacter than in mitochondria. Atebrin, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), desaspidin and arsenate, however, are active in both

TABLE I

INFLUENCE OF VARIOUS UNCOUPLERS ON THE P/2*e* RATIO

Reaction medium: 30 mM phosphate (pH 7.4) (1 mM in Expt. 5a, 5 mM in Expt. 5b), 5 mM $MgCl_2$, 1 mM EDTA, 10 mM glucose, 0.5–0.8 mM ADP, 0.5–0.6 mM NADH, 1–2 units of hexokinase and 0.15–0.25 mg of particle protein per ml. In Expts. 1, 2 and 5–8, the reaction was started by the addition of substrate after 3–5 min preincubation of the particles with the reaction mixture. In Expts. 3 and 4 the reaction was started by the addition of particles.

Expt.	*Addition*	*Concentration*	*P/2e ratio (expressed as percentage of a control without uncoupler)*			
			Substrate:			
			NADH	*Malate*	*Succinate*	*NADH → Q-*
1	Desaspidin	1.5 μM	25	—	—	—
2	TTFB	1.5 μM	45	—	—	—
3	Batho-phenanthroline	100 μM	75	—	—	—
4	Dio-9	25 μg/ml	80	—	—	—
5a	Arsenate	1.25 mM	80	—	—	—
		30 mM	15	—	—	—
5b	Arsenate	30 mM	40	—	—	—
5c	Arsenate	30 mM	80	—	—	—
6	Atebrin	0.25 μmole/mg protein	90	—	60	65
		0.5 μmole/mg protein	75	—	40	40
7	Atebrin	0.65 μmole/mg protein	—	60	35	—
8	HQNO	25 μM	90	—	—	10
		50 μM	70	—	—	7

systems in a similar concentration range (see refs. 15–22 for the data for mitochondria). As in mitochondria, the effect of arsenate is dependent on the phosphate concentration (Table I, Expt. 5). No lowering of the P/O ratio was observed with 40 μM 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol) or 30 μM 2-hydroxy-3-(3,7-dimethyloctyl)-1,4-naphthoquinone which uncouple phosphorylation completely in rat-liver mitochondria[23]. Of all the substances mentioned, only HQNO[5,24,25] and bathophenanthroline inhibited electron transport appreciably at the concentrations examined. One should interprete these data cautiously as the sensitivity of phosphorylation towards uncouplers is dependent on the nature of the substrate (Table I, Expts. 6–8). The P/2*e* ratios for malate and NADH oxidation are less affected by atebrin or HQNO than those observed with the oxidation of succinate or with the anaerobic reduction of ubiquinone-1 by NADH. A similar effect was found with 2,4-dinitrophenol (P. W. POSTMA, unpublished experiments). Variation of the effect of 2,4-dinitrophenol with the substrate used has also been observed by ROSE AND OCHOA[11] and HARTMAN *et al.*[12]. The explanation for this phenomenon may be found in the different rates of oxidation; the oxidation of NADH and malate in Azotobacter particles is about 10 times as fast as that of succinate. When the rate of oxidation was controlled by means of a malate-generating system (fumarate *plus* limiting amounts of fumarate hydratase, EC 4.2.1.2) the decrease of the P/O ratio by 0.7 μmole atebrin per mg protein was 50 % at an oxidation rate of 0.3 μatom O/min per mg protein but only 15 % at a rate of 3.0 μatoms O/min per mg protein. A similar dependence of uncoupling on the rate of oxidation in mitochondria has been reported by TSOU AND VAN DAM[26].

TRYPSIN-INDUCED ATPase

It was found that treatment of phosphorylating Azotobacter particles with trypsin stimulated the ATPase activity 10–20-fold. The measurements were carried out as described in EXPERIMENTAL. In the presence of phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40), the formation of inorganic phosphate and/or pyruvate was linear with time for 20 min. Without the ATP-regenerating system, the reaction was linear for 5–6 min. The induced ATPase was inhibited 70 % by 0.2 mM ADP whereas AMP (up to 1 mM) had no effect. The induced ATPase activity was dependent on the presence of Mg^{2+} with a maximal activity at a Mg/ATP ratio of about 1, which suggests that Mg–ATP^{2-} is the actual substrate.

Table II shows the effect of various inhibitors and uncouplers on the induced ATPase. While desaspidin, TTFB and CCCP stimulate the ATPase at concentrations that affect also the P/NADH ratio (*cf.* Table I and ref. 3), atebrin and Dio-9 are inhibitory. HQNO up to 200 μM had no effect. The effect of desaspidin and of atebrin on the ATPase was not expected in view of earlier experiments (*cf.* ref. 4) that led us to postulate that in Azotobacter particles desaspidin has an oligomycin-like effect, while atebrin acts as a true uncoupler. It seems now that these tentative conclusions are not justified.

Incubation of non-phosphorylating particles[3] or of the soluble factor[3] with trypsin also induces an ATPase (Table III), but the results are more variable than with phosphorylating particles. The correlation between induced ATPase activity and coupling activity of the soluble factor will be further investigated. It is not clear

whether the residual ATPase activity in non-phosphorylating particles is caused by incomplete extraction of the soluble factor or whether there is a particulate ATPase independent of this factor. The induced ATPase of the soluble factor and the particles can be stimulated by Ca^{2+} instead of Mg^{2+}, but the Ca^{2+}-ATPase is less active especially in the particles (Table III).

TABLE II

THE EFFECT OF INHIBITORS ON THE TRYPSIN-INDUCED ATPase OF PHOSPHORYLATING PARTICLES OF *A. vinelandii*

Experimental conditions as described in the text. Between 0.15 and 0.35 mg (based on particulate protein) of the trypsin-treated particles were used per incubation. The reaction was started by the addition of ATP after 4 min preincubation of the treated particles in the reaction medium containing the inhibitor.

Expt.	*Addition*	*Concentration*	*ATPase activity (μmoles/min per mg protein)*
1	None	—	0.84
	TTFB	5 μM	0.90
		10 μM	1.05
2	None	—	0.41
	Desaspidin	1 μM	0.58
		3 μM	0.68
3	None	—	0.67
	CCCP	5 μM	0.78
		25 μM	0.83
4	None	—	0.82
	Atebrin	50 μM	0.51
		200 μM	0.15
5	None	—	0.94
	Dio-9	5 μg/ml	0.78
		25 μg/ml	0.57

TABLE III

ATPase ACTIVITIES OF VARIOUS AZOTOBACTER PREPARATIONS

The preparations were incubated with trypsin and the ATPase activity was measured as described in the text. Where mentioned, $MgCl_2$ was replaced by $CaCl_2$ or omitted. The pH of the reaction was 8.0. Pyruvate and pyruvate kinase were omitted.

Expt.	*Fraction*	*ATPase activity (μmoles phosphate formed/min per mg protein)*		
		Added cation:		
		Mg^{2+} (3 mM)	*Ca^{2+} (3 mM)*	*None*
1	Phosphorylating particles	0.40	0.05	0.00
	Non-phosphorylating particles	0.18	0.02	0.00
2	Soluble factor	0.51	0.37	0.00

Bathophenanthroline inhibits the ATPase activity of trypsin-treated particles but not that of the factor (Fig. 1). The concentrations used were about 60 times lower than those that inhibit the dinitrophenol-induced ATPase in rat-liver mitochondria[27]. An involvement of non-haem iron proteins in oxidative phosphorylation has been suggested by BUTOW AND RACKER[28]. It is possible that in Azotobacter preparations a non-haem iron protein is involved in the particulate but not in the soluble ATPase.

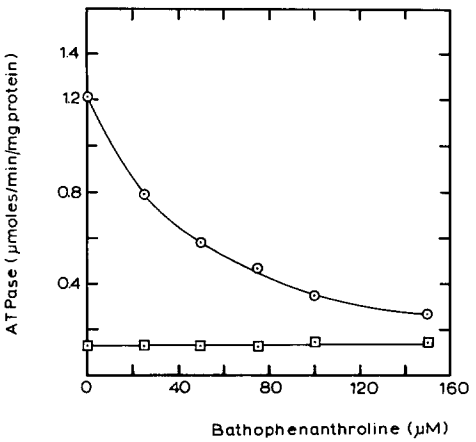

Fig. 1. The effect of bathophenanthroline on the trypsin-induced ATPase of phosphorylating particles and of the soluble factor of *A. vinelandii*. Experimental conditions as described in the text. ⊙—⊙, phosphorylating particles (0.21 mg/ml); ⊡—⊡, soluble factor (0.23 mg/ml).

ADP-STIMULATED RESPIRATION

It was shown in previous papers[4,5] that the oxidation of NADH can be stimulated by ADP even when the oxidase activity is inhibited 50 % by HQNO. With malate a slight stimulation by ADP was noticed with some preparations, whereas there was no effect of ADP on either succinate or lactate oxidation. From this it was concluded that the observed respiratory control was connected with Site I only (the oxidation of malate or lactate in Azotobacter particles is not NAD linked[24]). We also suggested[4,29] the existence of a phosphorylating and a non-phosphorylating pathway for electron transport in Azotobacter particles both sensitive towards inhibition by HQNO, with a transfer between both pathways at the ubiquinone level. If this were true, one would expect that stimulation by ADP of the oxidation of Site-II substrates would be dependent on the nature of the rate-limiting step in electron transport as also has been suggested recently by JONES *et al.*[30]. Table IV shows that, in agreement with this hypothesis, stimulation of oxidation by ADP with non-NAD-linked substrates is possible when the electron transport connected with malate oxidation is inhibited by HQNO and when the dehydrogenase capacity was raised by simultaneous oxidation of malate and lactate.

Another method of controlling the influx of electrons is to use a malate-generating system as mentioned above. Fig. 2 shows that ADP stimulates at the highest rates of oxidation. When less fumarate hydratase is added, there is little or no stimulation by ADP. In the presence of HQNO, stimulation by ADP is increased with

TABLE IV

STIMULATION OF MALATE AND LACTATE OXIDATION BY ADP; INFLUENCE OF HQNO

Oxidase activities were measured with a Clark oxygen electrode at 25°, in a reaction medium containing 30 mM phosphate buffer (pH 7.6), 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ADP (where mentioned), 10 mM L(−)-malate and/or D(−)-lactate (where mentioned), 30 or 60 μg particle protein per ml for malate and lactate oxidation, respectively. (a) No HQNO present; (b) 6.7 μM HQNO present.

Substrate	*Oxidase activity (μatoms O/min per mg protein)*		*Stimulation* (%)
	−ADP	*+ADP*	
(a) Malate	6.1	6.0	0
Lactate	1.7	1.7	0
Malate + lactate	7.2	7.9	10
(b) Malate	4.4	5.3	20
Malate + lactate	3.7	4.8	30

all but the lowest concentration of fumarate hydratase; the degree of stimulation is still higher at the higher rates of oxidation. From this experiment it is clear that if electron transport is decreased by inhibition of the respiratory chain, stimulation by ADP is induced, but if it is decreased by lowering the influx of electrons, the stimulation by ADP disappears.

The experiments in Table IV and Fig. 2 show that, in agreement with the experiments of JONES *et al.*[30], there is indeed a relationship between the nature of the rate-limiting step and the presence or absence of stimulation by ADP.

These experiments are reminiscent of those described by LEE *et al.*[31], who found that respiratory control in sub-mitochondrial fragments was not influenced by the rate of oxidation when the respiratory chain was inhibited, whereas disappearance of

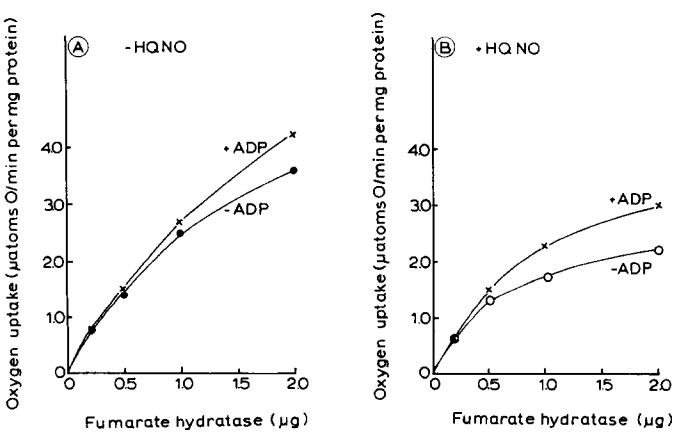

Fig. 2. The relation between the rate-limiting step of electron transport and stimulation of malate oxidation by ADP. Experimental conditions as described in Table IV. Substrate, 10 mM fumarate. 80 μg phosphorylating particles were added per ml. The reaction was started with the addition of fumarate hydratase. A. No HQNO present; ●—●, without ADP; ×—×, 1 mM ADP added. B. 3.3 μM HQNO present; ○—○, without ADP; ×—×, 1 mM ADP added.

respiratory control was observed in submitochondrial particles oxidizing alcohol, when the concentration of alcohol dehydrogenase was decreased.

EXPERIMENTAL

The preparation of phosphorylating and non-phosphorylating particles and of the soluble phosphorylation factor are described in ref. 3. Determination of P/O ratios and respiratory control are described in refs. 3 and 5. To measure the ATPase induced by trypsin, the particles (2–2.5 mg/ml) or the soluble factor (0.8–1.2 mg/ml) were incubated with 0.4–0.6 and 1.4–1.8 mg trypsin/mg protein, respectively, at 22–24°. After 4 min an equivalent amount of trypsin inhibitor was added. With these preparations the ATPase activity was measured in a medium containing 25 mM Tris–HCl buffer (pH 7.5 or 8.0), 3 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 3 mM ATP and 0.1 mg pyruvate kinase (EC 2.7.1.40) per ml. The reaction temperature was 22–25°. After 6–10 min the reaction was stopped by the addition of $HClO_4$ to a concentration of 4%. After neutralization with KOH and removal of the perchlorate, pyruvate and/or inorganic phosphate were determined as described in refs. 32 and 33.

Protein was determined in the particles by the biuret method[34] and in the soluble factor by measuring the absorption at 260 and 280 nm[35].

ACKNOWLEDGEMENTS

The authors thank Dr. L. Runeberg for a gift of desaspidin, Dr. V. Sprio for a gift of atractyloside, the N.V. Dutch Gist- en Spiritusfabriek for Dio-9, Dr. P. G. Heytler for CCCP, Dr. R. H. Büchel for TTFB and Dr. O. Isler for a gift of ubiquinone-1. We wish to thank Prof. E. C. Slater for his interest and advice and Mr. R. Th. P. de Vries and Mr. B. van Swol for technical assistance. Thanks are also due to Prof. E. F. Racker for his insistence that the effect of trypsin treatment on the ATPase of Azotobacter preparations should be investigated. This work was supported in part by a grant from the Life Insurance Medical Research Fund.

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