AN ENERGY-DEPENDENT HYDROGEN-EVOLUTION FROM DITHIONITE IN NITROGEN-FIXING EXTRACTS OF CLOSTRIDIUM PASTEURIANUM[1]

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Nitrogen-fixation by extracts of Clostridium pasteurianum requires both a reductant and an ATP-generator (1,2,3). Subsequently, identical requirements were demonstrated for the $N_2 \rightarrow NH_3$ conversion by extracts of Azotobacter vinelandii and Rhodospirillum rubrum (4). Reductants for one or more of these systems include H_2, KBH_4, NADH, and $Na_2S_2O_4$; phosphagens include acetyl phosphate and creatine phosphate. The interdependence of reduction and phosphagen was indicated by an ATP requirement for H_2-evolution from dithionite by N_2-fixing extracts of A. vinelandii (4), and an H_2-stimulation of acetyl phosphate hydrolysis in extracts from N_2-grown but not NH_3-grown C. pasteurianum (5).

The present report: 1. demonstrates an ATP requirement for H_2-evolution from dithionite in N_2-fixing extracts of C. pasteurianum; 2. shows the presence and distribution of a reductant-dependent ATPase in these extracts; and 3. presents a mechanism for the function of ATP and reductant in N_2-fixing extracts from both aerobic and anaerobic organisms.

METHODS

Enzyme Sources - C. pasteurianum was grown either on N_2

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or NH_3 plus argon. Extracts were prepared as described earlier from this laboratory (6) and stored under H_2 at room temperature until used. These extracts were treated in four ways: 1. stirred

TABLE 1

ENERGY-DEPENDENT H_2-EVOLUTION

Preparation	Gas Phase	H_2-Evolution (mole % of atm) Complete	-C~P	-$Na_2S_2O_4$	-Enzyme
1. *C. pasteurianum*-N_2 ↓	A	8.6	7.8	1.3	1.4
2. "	CO	2.8	0.4	0.1	0.1
3. " DEAE-Treated	A	4.2	1.1	1.2	-
4. *C. pasteurianum*-NH_3	CO	0.8	0.5	0.2	-
5. *A. vinelandii*-N_2	CO	3.2	0.2	0.1	0.2

↓ Extracts from N_2-grown and from NH_3-grown cells indicated by N_2 or NH_3.

Complete system contained in μmoles per 4 ml: K cacodylate, 100; $Na_2S_2O_4$, 100; C~P, 160; and ATP, 10, all at pH 6.5 for *C. pasteurianu* and 7.0 for *A. vinelandii*; and $MgCl_2$, 2.0; and in mg per 4 ml : *C. pasteurianum* or *A. vinelandii* extract 40, and creatine kinase, 0.2. The gas phase was flushed repeatedly, and filled with 0.92 atm of CO or A prior to 1 hr incubation at 30°C. Hydrogen is expressed as mole % of atm/hr/incubation flask on the basis of A or CO as 100%. One percent of an atm equals 13 μmoles of gas. Corrections of mass 2 due to arsine were negligible.

at pH 6.5 with 2 mg semi-dry DEAE-cellulose/mg protein to remove ferredoxin (2); 2. stirred at pH 7.0 with 0.5 volumes of Dowex-1 per volume of extract to remove ADP; 3. stirred with phosphate gel to produce a partially purified nitrogenase (7); and 4. heated under H_2 for 15 min at 60° or 70°C to produce heat-treated preparations. *A. vinelandii* was grown and extracts prepared according to (4).

Determinations - Nitrogen-fixation was measured as net NH_3 synthesis (1), H_2-evolution as H_2% of atm in a Consolidated Mass Spectrometer, and ATPase as release of inorganic phosphate from ATP (8) or of creatine from creatine phosphate (9).

RESULTS

H_2-Evolution - The data on H_2-evolution are given in Table 1.

C. pasteurianum extracts catalyze a substantial H_2-evolution from dithionite solutions via ferredoxin and hydrogenase without

TABLE 2

REDUCTANT-DEPENDENCE OF ATPase

Preparation	Treatment	Gas Phase	ATPase		
			$+Na_2S_2O_4$	$-Na_2S_2O_4$	ΔReductant
			C~P hydrolyzed (mμmoles/min/mg protein)		
1. Extract-N_2↓	-	A	65	24	41
2. "	-	H_2	-	77	53
3. "	-	CO	41	18	23
4. "	DEAE-cellulose	A	46	19	27
5. "	"	H_2	-	22	3
6. "	Dowex-1	CO	40	15	25
7. " -ADP	"	CO	0	1	-1

↓ Extracts from N_2-grown C. pasteurianum.

Complete system contained in μmoles per 2 ml: $Na_2S_2O_4$, 50; C~P 110; Tris.Cl, 100; and ADP, 2, all at pH 7.0; and $MgCl_2$, 5; and in mg per 2 ml: creatine kinase, 0.2 and C. pasteurianum extract, 20. Flasks were flushed and covered with 0.92 atm of indicated gas and incubated for 45 min at 30°C.

dependence on energy (line 1). This energy-independent evolution is inhibited 95% by CO (line 2) and 85% by the removal of ferredoxin with DEAE-cellulose (line 3). Addition of a phosphagen then permits the demonstration of an energy-dependent H_2-evolution. Creatine phosphate (C~P) (40 mM) increases H_2-evolution of DEAE-treated or CO-covered extracts by 3.4 to 7.0 fold (lines 2 and 3). Other phosphagens, e.g., 15 mM ATP or 50 mM acetyl phosphate, are also active. This energy-dependent evolution occurs in extracts of N_2-grown but not NH_3-grown C. pasteurianum (lines 2 and 4). It is quantitatively similar in extracts of C. pasteurianum and A. vinelandii (lines 2 and 5).

ATPase - Reductant-dependence of ATPase in N_2-fixing extracts of C. pasteurianum is shown in Table 2. Either dithionite or H_2

TABLE 3

DISTRIBUTION OF REDUCTANT-DEPENDENT ATPase

Preparation	Treatment	ATPase		
		+$Na_2S_2O_4$	-$Na_2S_2O_4$	ΔReductant
		C~P hydrolyzed (mMoles/min/mg protein)		
1. Extract-N_2 ↓	-	67	25	42
2. "	60°C 15'	36	24	12
3. "	70°C 15'	12	27	-15
4. "	Phosphate gel	2	2	0
5. Extract-NH_3	-	35	53	-18
6. "	60°C 15'	34	46	-12
7. 2 + 4 (1:1)		52	15	37
8. 3 + 4 (1:4)		26	8	18
9. 5 + 4 (1:1)		53	36	17

↓ Extracts from N_2-grown and NH_3-grown C. pasteurianum indicated by N_2 or NH_3.

Assay conditions as in Table 2; gas phase 0.92 atm CO.

stimulates the hydrolysis of C~P in the presence of its kinase and ADP (lines 1 and 2). Only dithionite is an effective stimulant under CO or after removal of ferredoxin with DEAE-cellulose (lines 3, 4, and 5). The reductant effect occurs at the site of ATP hydrolysis and not that of creatine phosphate since removal of ADP by Dowex-1 abolishes creatine release unless ADP is added (lines 6 and 7). Furthermore, the hydrolysis of 15 mM ATP is stimulated by either reductant.

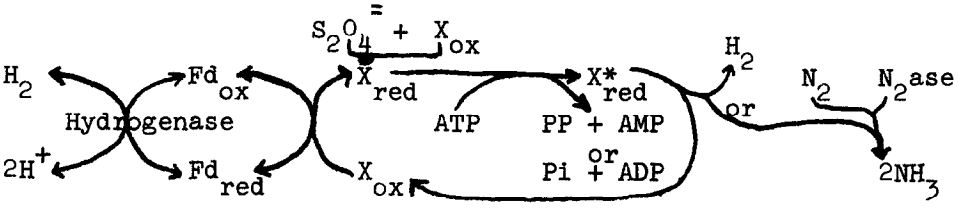
The distribution of the reductant-dependent ATPase in extracts of C. pasteurianum is shown in Table 3. It occurs only in extracts from N_2-grown cells (lines 1 and 5). It is decreased by heating at 60°C (line 2), destroyed by heating at 70°C (line 3) and removed by treatment with phosphate gel (line 4). As expected, the extract of NH_3-grown cells is still inactive after heating at 60°C (line 6). The reductant-

stimulated ATPase contains at least two enzymatic components as shown by recombination of partially or completely inactivated preparations (lines 7, 8 and 9).

N_2-Fixation - Dithionite is an active reductant for N_2-fixation by untreated or DEAE-cellulose treated extracts of C. pasteurianum in a system identical to that for H_2-evolution (Table 1). The untreated extract fixed 2.8 mμ moles of N_2/min/mg protein with dithionite as a reductant. Removal of ferredoxin with DEAE-cellulose did not reduce N_2-fixation by dithionite but reduced fixation by pyruvate to 3% of that of the untreated extracts. Addition of ferredoxin restores fixation by pyruvate

DISCUSSION

The following mechanism is compatible with the recent observations on N_2-fixation, energy-dependent H_2-evolution from dithionite, and reductant-dependent ATPase.



It is proposed that H_2 via hydrogenase and ferredoxin (Fd) reduces an electron acceptor X to X_{red} or, alternatively, dithionite, independently of hydrogenase and Fd, reduces X. The reduced acceptor X_{red} is activated to X^*_{red} by ATP through a coupling enzyme. The X^*_{red} is reoxidized by evolution of H_2 or by reduction of N_2 chemisorbed to nitrogenase (N_2 ase). All six of the electrons required for the reduction of one molecule of N_2 may not require activation by ATP.

The energy-dependent H_2-evolution and the reductant-dependent ATPase appear to be part of the N_2-fixation system since they are present in extracts of N_2-grown but not NH_3-grown cells. Their insensitivity to CO compared with N_2-fixation's sensitivity (7) indicates that the site of N_2-chemisorption is not involved

in either phenomenon.

The characteristics of the energy-dependent evolution of H_2 from dithionite by N_2-fixing extracts of A. vinelandii (4, 10) parallel those described here for C. pasteurianum. Thus, the mechanism with the exception of hydrogenase and Fd may be common to N_2-fixation by both aerobic and anaerobic organisms.

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