REDUCTANTS AND ELECTRON TRANSPORT IN NITROGEN FIXATION

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Energy in the form of an ATP-generator and a suitable reductant, such as KBH_4, is required for nitrogen fixation by cell-free extracts of Clostridium pasteurianum (Hardy and D'Eustachio, 1964). Thus, the complex and labile pyruvate catabolic pathway need no longer be relied upon to study the N_2-fixation process. The nonbiological character of KBH_4 as a reductant raised the question of its mode of action and led to the assignment of H_2 evolution as the most probable route. The ability of KBH_4, H_2, NADH, or NADPH to act in combination with an ATP-generator to promote ammonia synthesis or N^{15}-enrichment, is presented in this paper. Ferredoxin is shown to be involved in electron transport from the active reductants to N_2.

Experimental methods and chemicals used are similar to those reported in the preceding paper (Hardy and D'Eustachio, 1964). Enzymatically reduced pyridine nucleotides were obtained from Sigma Chemical Co. and Calbiochem, Inc.

Studies of the rate of decomposition of KBH_4 in the cell-free extract showed that it was no longer available after 5 minutes of incubation, while NH_3 synthesis or N_2-fixation continued for 30-40 min. longer. Mass spectrometric analysis of the covering gas after incubation showed H_2 to be present. If the atmosphere in a reaction flask was removed after KBH_4 decomposition and replaced with a 1:1

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Abbreviations and New Terms:
For an explanation of "pre-addition" and "substrate" borohydride, see the preceding paper.
Fd = ferredoxin, MV = methyl viologen.

mixture of N_2 and H_2, ammonia synthesis continued. When the atmosphere was replaced with 100% N_2, no further ammonia synthesis occurred. An extract containing only "pre-addition" KBH_4 (insufficient to support N_2-fixation), acetyl phosphate and covered with a 1:1 mixture of N_2 and H_2 produced substantial fixation (3.8 mμmoles NH_3/mg protein/min.). Therefore, hydrogen is a primary "natural" reductant for nitrogen fixation.

Many reductants have been tested as supporters of N_2-fixation by our cell-free system. Typical results of net ammonia synthesis and atom % excess N^{15} produced by cell-free extracts of C. pasteurianum supplemented with acetyl phosphate (0.05 M) and active reductants are summarized in Table 1. Activity of hydrogen, "substrate" borohydride or enzymatically reduced pyridine nucleotides is compared to that of a system containing pyruvate. All systems were treated with "pre-addition" KBH_4. Acetyl phosphate in combination with 0.01 M "substrate" KBH_4 produced an ammonia level that was 58% of that produced by 0.11 M pyruvate. Both 0.4 atm. of H_2 and 0.01 M NADH served as active reductants producing ammonia synthesis that was 64% and 55% respectively, of that obtained with KBH_4. The activity of these three reductants was confirmed by N^{15}-enrichment; values of atom per cent excess N^{15} parallel those of ammonia. The maximum net ammonia syntheses so far shown with 0.01 M "substrate" KBH_4, 0.4 atm. H_2 and 0.01 M NADH in combination with acetyl phosphate are 7.9, 6.7 and 2.7 mμmoles NH_3 formed/mg protein/min. A number of other reductants, such as NADPH and ascorbic acid, have demonstrated slight activity, but of those examined so far, KBH_4, H_2 and NADH are the most active. Hydrogen and NADH are active in the absence of "pre-addition" KBH_4 but to a lesser degree, indicating that a pre-reduced enzyme system or removal of traces of oxygen may be required for maximum N_2-fixation.

We are reporting the first positive activity of H_2 and NADH as reductants for N_2-fixation by extracts of C. pasteurianum. The only previous report of activity of these substances was made by Arnon et al. (1961) who obtained less than 0.05 atom per cent excess N^{15} when cell-free extracts of Chromatium were supplied with 96 atom per cent excess N^{15}. Our extracts incorporated 0.06-0.08 atom per cent excess

TABLE 1

The Ability of Selected Reductants and ATP-Generators to Support N_2-Fixation

Reductant	Energy Source	Ammonia Formed	N^{15}
μmoles	μmoles	mμmoles/mg protein/min.	Atom % Excess
	Pyruvate 455	5.80	0.11
KBH_4 38.7	Acetyl Phosphate 200	3.34	0.09
H_2, 0.4 atm.	Acetyl Phosphate 200	1.84	0.06
NADH 40	Acetyl Phosphate 200	2.0	0.08
NADPH 40	Acetyl Phosphate 200	1.0	-
None	Acetyl Phosphate 200	0.0	0.03

The incubation mixture contained the following in a 4 ml volume: 100 μmoles KH_2PO_4, pH 6.5; 1 mg "pre-addition" KBH_4; 44 mg extract protein; dilithium acetyl phosphate, sodium pyruvate and various reductants as indicated; N_2^{15}, 7.60 atom % excess; incubation at 30°C for 45 min. with shaking.

N_2^{15} for NADH and H_2, when only 7.6 atom per cent N_2^{15} was supplied. Maximum incorporation is being determined with gas mixtures richer in N_2^{15} content.

The electron-transferring protein, ferredoxin, has been shown by Mortenson et al. (1963) to be a vital component of pyruvate metabolism in C. pasteurianum. While its role in transporting electrons from pyruvic dehydrogenase to hydrogenase for H_2 production could be readily shown, its direct involvement in N_2-fixation was not demonstrated. It is now possible to demonstrate a role in N_2-fixation by preferentially adsorbing ferredoxin from an active cell-free extract and thus remove ammonia synthesis ability. Activity can be restored by addition of ferredoxin. Figure 1 shows that 0.05 μmoles of purified ferredoxin (Buchanan et al., 1963) restored activity to the level of the extract prior to removal of ferredoxin, i.e., 5.75 mμmoles NH_3/mg protein/min. Figure 1 also

shows that the dye, methyl viologen, can substitute for ferredoxin as an electron carrier in ammonia synthesis. This resembles the situation reported by Mortenson et al. (1962) in which MV replaced ferredoxin in the production of H_2 from dithionite.

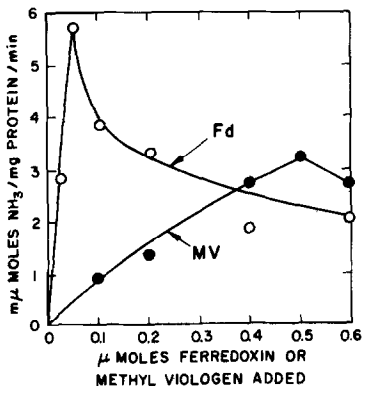

Figure 1. Catalysis of N_2-fixation by ferredoxin or methyl viologen in an extract devoid of ferredoxin. Reaction flasks contained in a 4 ml volume; 455 mμmoles pyruvate; 50 μmoles KH_2PO_4, pH 6.5; 200 μmoles cacodylate, pH 6.5; 44 mg extract protein; Fd or MV as indicated; incubation at 30°C for 1 hour with shaking. See Table 2 for a description of the method for removing ferredoxin.

The role of ferredoxin as the electron carrier for nitrogen fixation was further demonstrated by its requirement in the active reducing systems shown in Table 2. The data indicate that electron flow from reductant to N_2 must proceed via ferredoxin. Where methyl viologen has been tried as a substitute for ferredoxin, it has worked, namely with the pyruvate and the acetyl phosphate-KBH_4 systems. Both electron carriers have been reported to possess redox potentials in the vicinity of -400 mv, suggesting that the N_2-fixation system may operate at this low a potential.

The observations and interpretations reported here and in the preceding paper can be combined to formulate a current concept of the N_2-fixation pathway in C. pasteurianum.

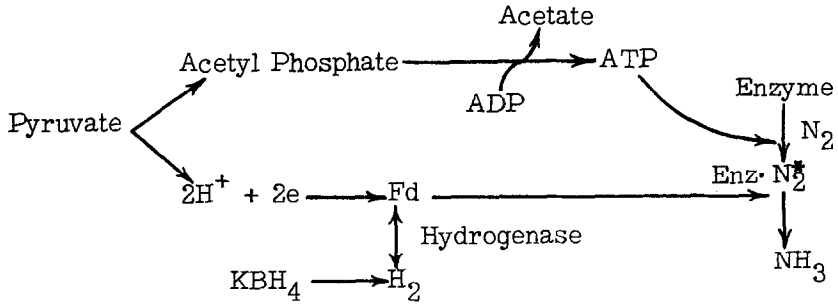

TABLE 2

Essential Role of Ferredoxin or Methyl Viologen in Electron Transport for N_2-Fixation

Reductant	Fd Added	MV Added	NH_3 Formed	
	μmoles	μmoles	mμmole/mg protein/min.	
			DEAE Treated	Untreated
Pyruvate	0	0	0.00	5.75
455 μmoles	0.05	0	5.75	-
	0	0.50	3.25	-
Hydrogen	0	0	0.03	3.66
0.5 atm.	0.05	0	2.62	-
	0.10	0	2.88	
KBH_4	0	0	0.0	3.18
38.7 μmoles	0.05	-	1.97	-
		0.50	1.97	-
	0.05		4.25[1]	-
NADH	0	0	0.01	2.62
40 μmoles	0.05	-	2.62	-

[1] 10 μmoles of ADP and 10 μmoles Mg^{++} added to reaction flask.

Ferredoxin was removed from the cell-free extract by adding 60 mg of DEAE-cellulose/ml of extract. After stirring at 1°C for 15 min. under a H_2 atmosphere, the adsorbed material was removed by centrifugation. The supernatant was maintained under H_2 until assayed as indicated. Experimental conditions are identical to those described in Table 1; all flasks contained 200 μmoles acetyl phosphate.

A more detailed report of the studies leading to this pathway will be presented subsequently.

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