REDUCTION OF N_2O BY BIOLOGICAL N_2-FIXING SYSTEMS

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Nitrous oxide, cyanide, azide, carbon monoxide and nitric oxide have been reported as inhibitors of nitrogen fixation[1]. We have now been able to show catalysis of the reduction of some of these inhibitors by the N_2-fixing system of Azotobacter vinelandii or Clostridium pasteurianum[2], e.g.,

$$N_2O \longrightarrow N_2 + H_2O \quad (1)$$
$$HCN \longrightarrow CH_4 + NH_3 \quad (2)[3]$$
$$N_3^- \longrightarrow NH_3 + N_2 \quad (3)[3].$$

Schöllhorn and Burris also have evidence for reaction (3) and have extended the list to include acetylene[4]. The characteristics of these reactions-requirement for ATP, reductant and an extract with N_2-fixing activity and inhibition by CO-parallel those of nitrogen fixation[5-7].

Previous studies have shown that N_2-fixing extracts of A.vinelandii and C.pasteurianum have a reductant-dependent ATPase[8-10] and an ATP-dependent H_2 evolution[10-12]. This ATPase was proposed to function in the activation of electrons that are used for N_2 reduction or H_2 evolution[8,10]. Nitrous oxide was found to inhibit ATP-dependent H_2 evolution[7] but not reductant-dependent ATPase of A.vinelandii[8]. This lack of inhibition by N_2O of reductant-dependent ATP utilization concomitant with the inhibition of ATP-dependent H_2 evolution suggested that some of the electrons activated by this ATPase were utilized for reduction of N_2O rather than evolution of H_2. This communication reports the reduction of N_2O to N_2 by N_2-fixing extracts of A.vinelandii and C.pasteurianum.

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Table 1

N_2O Reduction By The N_2-Fixing System Of A.vinelandii

Gas Phase	Product Formed	Incubation System			
		Complete	-C~P, -ATP	$-Na_2S_2O_4$	-Enzyme
		μmoles in 1 hr. at 30°			
N_2O	N_2	37	10	11	12
	H_2	22	1	2	0
	Creatine	210		82	
	NH_3	0.8			
N_2	NH_3	13			
He	N_2	0		0	
	H_2	55		2	
	Creatine	200		84	

System contained per 4 ml in μmoles: Tris·HCl, 200; creatine phosphate (C~P), 224; ATP, 20; and $Na_2S_2O_4$, 80 all at pH 7.0; and $MgCl_2$, and in mg protein: heated extract of N_2-grown A.vinelandii, 15; and creatine kinase, 0.8. Gas phase flushed repeatedly and filled with 0.55 atm of N_2O, N_2 or He.

METHODS

Enzyme Sources - Heated extracts of cells of A.vinelandii, ATCC 12518, were prepared as described for N_2 fixation[8,12]. Those from cells grown on air (N_2-grown) had N_2-fixing activity while those from cells grown on urea with a gas phase of 80% A:20% O_2 (urea-grown)[13] did not. Cell free extracts of dried cells of C.pasteurianum, ATCC 6013, were also prepared as described for N_2-fixation[14]. Those from cells grown on N_2 (N_2-grown) had N_2-fixing activity while those from cells grown on NH_3 with a gas phase of A (NH_3-grown) did not.

Determinations - Gases were analyzed with a Consolidated Mass Spectrometer and the initial gas phase of N_2O or He was used as an internal standard. The spectrum of N_2 was corrected daily for the N_2 fragment from N_2O. Reductant-dependent ATPase was determined as the increase in creatine released from creatine phosphate in the presence of dithionite[8,15]. The synthesis of NH_3 from either N_2 or N_2O was measured by titration after micro-diffusion[16]; He replaced N_2 or N_2O as a control. Protein was estimated with biuret reagent.

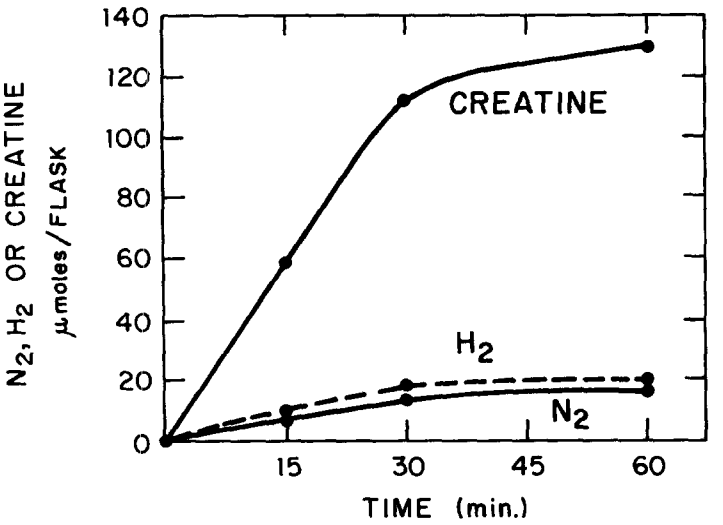

Fig. 1. Time course of N_2O reduction. System is described in Table 1; gas phase, 0.55 atm of N_2O. Values of N_2 were corrected with 0 time value of 14 μmoles/flask.

RESULTS

Requirements for N_2O Reduction - Extracts of N_2-grown A.vinelandii reduced N_2O to N_2 and traces of NH_3 (Table 1). The substrate requirements were the same as those for N_2 fixation, e.g., ATP and $Na_2S_2O_4$[7]. The complete system formed 26 μmoles of N_2/15 mg protein in 1 hr (corrected for average N_2 found in controls, e.g., -enzyme, -ATP source, and -$Na_2S_2O_4$) and 0.8 μmoles of NH_3 with 0.55 atm of N_2O as substrate while it formed 13 μmoles of NH_3 with 0.55 atm of N_2 as substrate. Thus, four times as much N_2O was reduced to N_2 (2 electrons required) as N_2 to NH_3 (6 electrons required). Evolution of H_2 was decreased when N_2O reduction occurred. This decrease in electrons evolved as H_2 corresponded to the electrons utilized for N_2 formation. Reductant-dependent ATPase activity was not affected by N_2O reduction.

Time Course of N_2O Reduction - The rates of the three related reactions, N_2O reduction to N_2, H_2 evolution and reductant-dependent ATPase are shown in Fig. 1. In this experiment the creatine phosphate was exhausted after about 30 min at which time all three reactions ceased. Evolved H_2 or even a gas phase of 30% of H_2 in place of $Na_2S_2O_4$ in the complete incubation mixture of Table 1 did not reduce N_2O to N_2.

Table 2

Dependency Of N_2O Reduction On Extracts Of N_2-Grown Cells

Extract	mg protein	Substrate Products	N_2O N_2	N_2O H_2	N_2 NH_3
			μmoles in 1 hr at 30°		
A.vinelandii					
N_2-grown cells	22		38	13	18
Urea-grown cells	27		4	1	0
C.pasteurianum					
N_2-grown cells	36		22	52	13
NH_3-grown cells	43		0	27	0

System for A.vinelandii, Table 1; for C.pasteurianum per 4 ml in μmoles: potassium cacodylate, 200; creatine phosphate, 224; ATP, 20; and $Na_2S_2O_4$, 80, all at pH 6.5; and $MgCl_2$, 20; and in mg: creatine kinase, 0.8. Gas phase, 0.55 atm. Product N_2 corrected with minus energy control.

Dependency on N_2-Fixing Extracts - The reduction of N_2O is dependent on the induced enzymes of N_2 fixation found in N_2-grown but not urea- or NH_3-grown cells. Extracts of N_2-grown A.vinelandii or C.pasteurianum both reduced N_2O to N_2 and fixed N_2 while those of urea-grown A.vinelandii or NH_3-grown C.pasteurianum had negligible (0-10%) N_2O-reducing activity and did not fix N_2 (Table 2).

Effect of CO - Reduction of N_2O to N_2 was inhibited 75-90% by 0.1 atm of CO (Table 3). Electrons not used for N_2O reduction were evolved as H_2. Thus the decrease by CO in N_2 synthesis from N_2O (31 μmoles) by extracts of A.vinelandii corresponded to the increase in ATP-dependent H_2 evolution (32 μmoles).

DISCUSSION

Nitrogen-fixing extracts of both A.vinelandii and C.pasteurianum are shown to reduce N_2O to N_2. The substrate requirements and extract dependency for this reduction are identical to those for N_2 fixation, reductant-dependent ATPase or ATP-dependent H_2 evolution[5-12]. Therefore, it is proposed that the reduction of N_2O is catalyzed, at least in part, by enzyme(s) of the N_2-fixing system. Electrons activated by the reductant-

Table 3

Effect Of CO On N_2O Reduction

Extract	Substrate Products	N_2O		N_2O:CO(0.8:0.2)	
		N_2	H_2	N_2	H_2
	mg protein	μmoles in 1 hr. at 30°			
A.vinelandii	15	34	21	3	53
C.pasteurianum	36	22	52	6	135

System for A.vinelandii, Table 1; C.pasteurianum, Table 2. Gas phase, 0.55 atm. Product N_2 corrected with minus energy control.

dependent ATPase of the N_2-fixing complex are utilized for N_2O reduction. This conclusion is supported by the following observations: 1) N_2O doesn't affect electron activation as measured by reductant-dependent ATPase activity; 2) N_2O decreases ATP-dependent H_2 evolution in an amount approximately equal to the increase in N_2 formation; and 3) inhibition by CO of N_2O reduction eliminates the decrease in ATP-dependent H_2 evolution. The inhibition by CO of N_2O reduction also demonstrates that an enzyme or at least site in addition to the reductant-dependent ATPase is required for N_2O chemisorption and reduction. The activity of N_2O as a competitive inhibitor of N_2 fixation, the reduction of other inhibitors of N_2 fixation by N_2-fixing extracts[2-4] and the inhibition of N_2 fixation as well as N_2O reduction by CO suggests that "nitrogenase", the site of N_2 chemisorption and reduction, rather than an N_2O reductase is the site of N_2O reduction. Thus N_2O-reducing activity may occur in all organisms and extracts that have N_2-fixing activity. This suggestion is supported by the previously observed reduction of N_2O to N_2 by soybean root nodules[17] and assimilation of N_2O by cells of A.vinelandii[18].

REFERENCES

1. Lockshin, A., and Burris, R. H., Biochim. Biophys. Acta, 111, 1 (1965).
2. Hardy, R. W. F. and Knight, E., Jr., Bacteriol. Proc., ___, (1966).
3. Hardy, R. W. F. and Knight, E., Jr., in preparation.
4. Schöllhorn, R. and Burris, R. H., Fed. Proc., 25, 710 (1966).
5. Hardy, R. W. F. and D'Eustachio, A. J., Biochem. Biophys. Res. Commun., 15, 314 (1964).
6. Mortenson, L. E., Proc. Natl. Acad. Sci. U.S., 52, 272 (1964).

7. Bulen, W. A., LeComte, J. R., Burns, R. C. and Hinkson, J., in "A Symposium on Non-Heme Iron Proteins: Role in Energy Conversion" (editor), A. San Pietro, The Antioch Press, Yellow Springs, Ohio, 1965, p. 261.
8. Hardy, R. W. F. and Knight, E., Jr., submitted to Biochim. Biophys. Acta.
9. Dilworth, M. J., Subramanian, D., Munson, T. O., and Burris, R. H., Biochim. Biophys. Acta., 99, 486 (1965).
10. Hardy, R. W. F., Knight, E., Jr., and D'Eustachio, A. J., Biochem. Biophys. Res. Commun., 20, 539 (1965).
11. Burns, R. C., in "A Symposium on Non-Heme Iron Proteins: Role in Energy Conversion" (editor), A. San Pietro, The Antioch Press, Yellow Springs, Ohio, 1965, p. 289.
12. Burns, R. C., and Bulen, W. A., Biochim. Biophys. Acta., 105, 437 (1965).
13. Mumford, F. E., Carnahan, J. E. and Castle, J. E., J. Bacteriol., 77, 86 (1959).
14. Carnahan, J. E., Mortenson, L. E., Mower, H. F. and Castle, J. E., Biochim. Biophys. Acta., 44, 520 (1960), and U.S. Patent 3,236,741 (1965).
15. Ennor, A. H. in "Methods in Enzymology" (editors), S. P. Colowick and N. O. Kaplan, Vol. 3, Academic Press, Inc., New York, 1957, p. 850.
16. Mortenson, L. E., Anal. Biochem., 2, 216 (1961).
17. Hoch, G. E., Schneider, K. C., and Burris, R. H., Biochim. Biophys. Acta., 37, 273 (1960).
18. Mozen, M. M., and Burris, R. H., Biochim. Biophys. Acta., 14, 547 (1954).