THE ELECTRON TRANSPORT SYSTEM IN NITROGEN FIXATION BY AZOTOBACTER. IV. SOME OXIDATION-REDUCTION PROPERTIES OF AZOTOFLAVIN

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Summary: The oxidation-reduction potentials (E_1 and E_2) of azotoflavin determined by equilibration with indicator dyes were -464 and -270 mV, respectively, at pH 7.7. The reduction of azotoflavin to the semiquinone was greatly stimulated by catalytic concentrations of methyl viologen and was observed with three diverse reducing systems: dithionite, illuminated chloroplasts, and NADPH plus ferredoxin-NADP reductase. The rate of semiquinone oxidation was increased 10- to 15-fold by methyl viologen at 6.25×10^{-6} M. Biological activity of azotoflavin in the nitrogenase reaction was considerably stimulated by addition of low concentrations of azotobacter ferredoxin or methyl viologen. The joint requirement for azotoflavin and ferredoxin for maximal nitrogenase activity is discussed in reference to recent suggestions on renaming azotoflavin.

Azotoflavin, a flavoprotein electron transport carrier isolated from the aerobic nitrogen-fixing bacterium Azotobacter vinelandii, is active in the nitrogenase system of this organism (1). Evidence showing azotoflavin to be an endogenous reductant in the nitrogenase system of A. vinelandii came from experiments in which the reducing power generated by illuminated chloroplasts was coupled to the nitrogenase system. Illuminated chloroplasts donated electrons to nitrogenase only through intermediate electron carriers, azotoflavin (1) and azotobacter ferredoxins I and II (Fd I and Fd II) (2,3). The physiological electron donor system for A. vinelandii nitrogenase appears to be NADPH-linked, requiring, in addition to azotoflavin, azotobacter Fd I and ferredoxin-NADP reductase (4). A protein with the same chemical properties as those of azotoflavin (Shethna flavoprotein) was previously isolated by Shethna et al. (5) and by Hinkson and Bulen (6) from A. vinelandii; however, no biological activity was reported.

This paper reports the oxidation-reduction potentials of azotoflavin, the influence of methyl viologen on its oxidation and reduction, and the stimulatory effect of ferredoxin and methyl viologen on azotoflavin-linked nitrogenase activity.

METHODS

Azotoflavin was purified according to Benemann et al. (1) to near homogeneity (absorbance ratio $A_{274}:A_{452} = 4.8$). Azotobacter nitrogenase was prepared and assayed with chloroplasts as a reductant as previously described (1,4). Ferredoxin-NADP reductase was supplied by R. K. Chain. Azotobacter

Fd I and II were prepared as described by Yoch and Arnon (3); azotobacter iron-sulfur proteins I and II, as described by DerVartanian et al. (7).

Chloroplast preparation.--Chloroplasts were prepared by blending 20 g of fresh spinach leaves (freed of midribs) in a Waring blendor for 20 sec with 65 ml of preparative solution (0.4 M sucrose; 0.05 M Tris-HCl buffer, pH 7.8; and 0.01 M NaCl). The slurry, filtered through several layers of silk, was centrifuged at 2,500 x g for 1 min. The supernatant was discarded and the intact chloroplasts in the pellet were disrupted and washed free of ferredoxin by resuspension in 50 ml of preparative solution diluted 1:10. Chloroplast fragments were collected by centrifugation (10,000 x g, 5 min) and resuspended in a small volume of 5 mM Tris buffer, pH 7.8, and chlorophyll was estimated (8). The chloroplasts were heated at 55° for 5 min to destroy their oxygen-evolving capacity.

Measurement of oxidation-reduction potentials.--Oxidation-reduction potential measurements of azotoflavin were as previously described by Vetter and Knappe (9) for Escherichia coli flavodoxin. The oxidation-reduction potential (E_1)* for the couple azotoflavin semiquinone-fully reduced azotoflavin was determined from equilibrium reactions (system reduced with dithionite and reoxidized step-wise with air) with equimolar mixtures of azotoflavin and methyl viologen; E_2, the potential of the couple oxidized azotoflavin-azotoflavin semiquinone, was determined from equilibrium reactions with equal concentrations of benzyl viologen or safranine T. A catalytic concentration of methyl viologen (10^{-6} M) was required to facilitate the equilibrium between azotoflavin semiquinone and safranine T [because of stability of semiquinone to air oxidation (5,12)]. At equilibrium, the ratio of oxidized to reduced dye and azotoflavin was determined and the resulting midpoint potential (E_1 or E_2) of the azotoflavin redox couple was calculated from the Nernst equation (see ref. 9).

RESULTS AND DISCUSSION

Oxidation-reduction potentials.--Spectral analysis showed that methyl viologen equilibrates with only the couple azotoflavin semiquinone-fully reduced azotoflavin, while benzyl viologen and safranine T equilibrate with the couple oxidized azotoflavin-azotoflavin semiquinone. The oxidation-reduction potential (E_1) for the couple azotoflavin semiquinone-fully reduced azotoflavin was -464 mV, while E_2, the couple oxidized azotoflavin-azotoflavin semiquinone, determined with two different indicator dyes, was approximately -270 mV (Table I).

The potentials, E_1 and E_2, of azotoflavin, while similar to those of E. coli flavodoxin (9), are approximately 75 and 150 mV, respectively, more

*Notation recommended for flavoproteins (10) and flavodoxins (11).

Table I

OXIDATION-REDUCTION POTENTIALS OF AZOTOFLAVIN DETERMINED BY USE OF INDICATOR DYES

E_1			E_2					
Indicator dye: Methyl viologen[a]			Indicator dye: Benzyl viologen[b]			Indicator dye: Safranine T[c]		
$\frac{[\text{oxid.dye}]}{[\text{red. dye}]}$	$\frac{[\text{red. azotoflavin}]}{[\text{oxid.azotoflavin}]}$	$E_m^{7.7}$	$\frac{[\text{oxid.dye}]}{[\text{red. dye}]}$	$\frac{[\text{red. azotoflavin}]}{[\text{oxid.azotoflavin}]}$	$E_m^{7.7}$	$\frac{[\text{oxid.dye}]}{[\text{red. dye}]}$	$\frac{[\text{red. azotoflavin}]}{[\text{oxid.azotoflavin}]}$	$E_m^{7.7}$
		(mV)			(mV)			(mV)
0.165	2.700	-467	1.64	6.26	-298	4.47	3.50	-255
0.529	0.850	-466	3.70	4.06	-288	4.96	2.07	-269
1.122	0.568	-458	8.05	2.56	-244	7.70	0.88	-284
1.545	0.322	-464	15.50	0.99	-250	10.02	0.48	-286
3.570	0.178	-458						
9.323	0.039	-472						
	Mean	-464		Mean	-265		Mean	-273

[a] Methyl viologen $E_m^{7.7}$ = -446 mV (ref. 13), n = 1.

[b] Benzyl viologen $E_m^{7.7}$ = -359 mV (ref. 13), n = 1.

[c] Safranine T $E_m^{7.7}$ = -324 mV (ref. 14), n = 2.

Reaction mixtures contained (in 2.0 ml) the following (μmoles): phosphate buffer, pH 7.7, 200; azotoflavin, 0.080; and methyl viologen, benzyl viologen, or safranine T as indicated, 0.075. Stoppered cuvettes evacuated and flushed four times with argon. Ratio of oxidized to reduced azotoflavin and dye determined from composite spectrum obtained after reduction with 0.25 μmoles dithionite. Temperature, 20°.

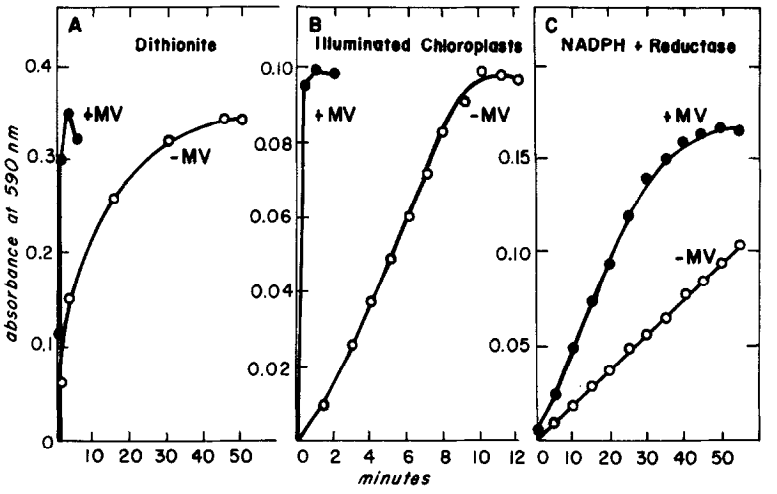

Fig. 1. Effect of methyl viologen on the rate of azotoflavin reduction with various reducing systems. Reaction mixtures (2.0 ml) contained phosphate buffer, pH 7.0, 200 μmoles; methyl viologen (MV), where indicated, 0.001 μmoles, plus the following: (A) azotoflavin, 0.130 μmoles, and dithionite, 1.2 μmoles; (B) azotoflavin, 0.045 μmoles, spinach chloroplasts, 100 μg chlorophyll, ascorbate, 10 μmoles, 2,6-dichlorophenol indophenol (DPIP), 0.05 μmoles; (C) azotoflavin, 0.062 μmoles, NADPH, 0.02 μmoles, and ferredoxin-NADP reductase, 0.01 mg. Cuvettes, stoppered, evacuated, and flushed (5 times) with argon. Anaerobic solutions of dithionite and NADPH added by syringe to start appropriate reactions. Chloroplast system illuminated at saturating light intensity.

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negative than those of clostridial flavodoxins (15,11,16). It is assumed that E_2, the couple oxidized azotoflavin-azotoflavin semiquinone, is too positive at -270 mV to be active in reducing nitrogenase and that therefore the semiquinone-fully reduced couple (-465 mV) of azotoflavin is active in nitrogen fixation.

Influence of methyl viologen on the oxidation-reduction properties of azotoflavin.--The observation of Benemann et al. (1) and Edmondson and Tollin (17) that a catalytic amount of methyl viologen greatly increases the rate of azotoflavin semiquinone formation when dithionite is used as reductant has been extended to show that the increased rate of azotoflavin semiquinone formation (indicated by an increase in absorbance at 590 nm) in the presence of methyl viologen is independent of the reducing system (Fig. 1). Both dithionite (Fig. 1A) and illuminated chloroplasts (Fig. 1B) rapidly reduced azotoflavin to the semiquinone in the presence of methyl viologen, whereas in its absence this transition was considerably slower. NADPH (plus ferredoxin-NADP reductase) also reduced azotoflavin to the semiquinone (Fig. 1C); although the rate of this reaction was much slower than with the stronger reductants, it was also stimulated by a catalytic concentration of methyl viologen.

The striking effect of methyl viologen on azotoflavin reduction may indicate that a protein electron carrier serves a similar function in the cell. A. vinelandii contains a number of low-potential iron-sulfur electron carrier proteins capable of one-electron transfers similar to those of methyl viologen. Azotobacter iron-sulfur protein II (5,7) and Fd I and II (2,3) were tested for their ability to stimulate azotoflavin reduction to the semiquinone with illuminated chloroplasts serving as the electron donor system. The results were inconclusive, as Fd I and II stimulated the rate of azotoflavin reduction on occasion but did not do so consistently, while azotobacter iron-sulfur protein II actually inhibited azotoflavin reduction.

Although the flavodoxins react slowly with O_2 (11), azotoflavin is unique among the flavoprotein electron carriers in that the half-time for its semiquinone oxidation is 2000-3000 min, a rate 20-25 times slower than that of flavodoxins from the obligate anaerobes Peptostreptococcus elsdenii and C. pasteurianum under the same conditions (17). This characteristic may reflect an adaptation by an obligate aerobe such as Azotobacter to generate under aerobic conditions the strong reductant required for nitrogen fixation.

Azotoflavin semiquinone oxidation by O_2 was greatly stimulated by the addition of catalytic concentrations of methyl viologen (Fig. 2), which had a concentration-dependent effect: the rate of oxidation increased with increasing methyl viologen concentration. The pseudo first-order rate constant of azotoflavin semiquinone oxidation was 1.05×10^{-4} min^{-1}; this rate was increased to 1.35×10^{-3} min^{-1} in the presence of 6.25×10^{-6} M methyl viologen. Other electron carriers that have been shown to stimulate semiquinone oxidation by O_2 are DPIP, benzyl viologen, ferricyanide, tetrazolium blue, and horse heart cytochrome c (6). Neither azotobacter iron-sulfur protein II nor Fd I or II would replace methyl viologen in stimulating azotoflavin semiquinone oxidation.

In studying the oxidation characteristics of azotoflavin, buffer and pH must be chosen with care, i.e., in phosphate buffer, pH 6.0, the half-time of the semiquinone oxidation is approximately 2000 min in an atmosphere of air at room temperature. Under the same conditions in MES [2-(N-morpholino)ethane sulfonic acid] buffer, pH 6.0, the half-time of the semiquinone oxidation is only 180 min (unpublished observations).

Biological activity of azotoflavin.--The marked stimulation by methyl viologen of the reduction of azotoflavin to the semiquinone (Fig. 1) and its reoxidation (Fig. 2) raised the question whether electron transfer in biological systems via azotoflavin may also be stimulated by one-electron carriers endogenous to azotobacter cells. The influence of three azotobacter non-heme iron electron carriers--iron-sulfur protein II (5,7), Fd I (2,3), and Fd II (3)--on

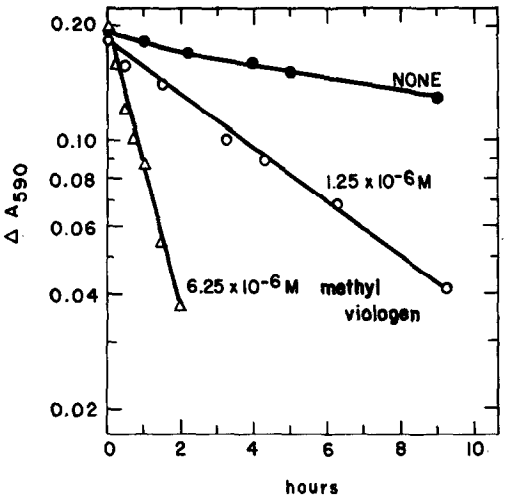

Fig. 2. Effect of methyl viologen on azotoflavin semiquinone oxidation by oxygen (first-order rate plot). Complete reaction mixture (2.0 ml) contained (μmoles): azotoflavin, 0.072; phosphate buffer, pH 7.7, 200; and methyl viologen as indicated. Azotoflavin initially reduced to the semiquinone with a slight excess of sodium dithionite.

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the effectiveness of azotoflavin as a link between the reducing power generated by spinach chloroplasts and azotobacter nitrogenase activity was measured, but only Fd I gave an appreciable stimulation of the azotoflavin-mediated nitrogenase activity (Table II, experiment A). The azotobacter nitrogenase preparation had previously been passed over a bed of DEAE-cellulose to remove all endogenous electron carriers (4). Nitrogenase activity with azotoflavin supplemented with Fd I was 1.5 to 2.0 times greater than the sum of the nitrogenase activity when each of the two carriers was added separately. Table II also shows that the addition of methyl viologen at a low concentration greatly stimulated azotoflavin-linked nitrogenase activity. Similarily, spinach ferredoxin, which at 2 μM gave little or no activity, gave some stimulation when combined with 10 μM azotoflavin. Benzyl viologen or other one-electron carriers of less negative potential did not substitute for methyl viologen.

When azotoflavin and azotobacter ferredoxin were found to link the reducing power generated by illuminated chloroplasts to azotobacter nitrogenase activity, their effectiveness as electron carriers appeared to be only additive (2). Subsequently, with NADPH as the source of reducing power, azotoflavin and azotobacter ferredoxin were both required as electron carriers for the azotobacter nitrogenase system (4)--consistent with the now-observed synergystic effect of these two electron carriers in linking the reducing power of illumi-

Table II

STIMULATION OF AZOTOFLAVIN-CATALYZED NITROGENASE ACTIVITY BY AZOTOBACTER FERREDOXIN OR METHYL VIOLOGEN

Experiment	Addition	Ethylene formed
		(nmoles/min)
A	Azotoflavin (10 μM)	11.7
	Azotobacter Fd I (1 μM)	25.2
	Azotoflavin (10 μM) + Azotobacter Fd I (1 μM)	60.2
B	None	0
	Azotoflavin (10 μM)	12.2
	Methyl viologen (0.67 μM)	1.8
	Azotoflavin (10 μM) + Methyl viologen (0.67 μM)	41.5

Reaction mixture (1.5 ml) contained DEAE-cellulose-treated azotobacter extract (5 mg), chloroplasts (300 μg chlorophyll), methyl viologen or azotobacter electron carriers as indicated; and the following (μmoles): HEPES buffer, pH 7.4, 50; ascorbate, 10; DPIP, 0.05; Mg^{++}, 5; creatine phosphate, 40; ATP, 4; and creatine phosphokinase, 0.05 mg. Light intensity, 9,000 ft-candles; gas phase, 73% argon + 27% acetylene; temperature, 30°.

nated chloroplasts to azotobacter nitrogenase activity (Table II). A joint requirement of azotoflavin and an iron sulfur-type protein (18) for nitrogenase activity has also recently been observed in cell-free extracts of nodule bacteroids (19).

When the biological activity of azotoflavin was initially characterized, it was noted that it differed from flavodoxin by its "total or virtual inability" (emphasis added) to replace ferredoxin in the photochemical reduction of NADP by illuminated chloroplasts (1). This low activity of azotoflavin as a substitute for ferredoxin in the photoreduction of NADP by chloroplasts was recently confirmed by van Lin and Bothe (20) who found that azotoflavin at a 20-fold higher concentration gave half of the rate of NADP reduction obtained with ferredoxin (Fig. 5 in ref. 20). However, van Lin and Bothe (20) use these data as a basis for classifying azotoflavin as a flavodoxin. Of all of the reactions which ferredoxins catalyze in bacteria (see review, 21), azotoflavin catalyzes only the nitrogenase reaction with an equimolar efficiency (approximately 30%) comparable to a ferredoxin. Since flavodoxin was introduced to describe a type of flavoprotein serving as a substitute for ferredoxin and formed by cells under conditions that do not favor the formation of ferredoxin (22), this classification of

azotoflavin (= Shethna flavoprotein) appears to be without merit. Both azotoflavin and ferredoxin are normal components of azotobacter cells and they appear to operate jointly in electron transfer to nitrogenase, characteristics not common to the flavodoxins.

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