OXIDATION REDUCTION PROPERTIES OF NITROGENASE FROM *CLOSTRIDIUM PASTEURIANUM* W5

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SUMMARY

Dithionite reduced azoferredoxin and molybdoferredoxin from *Clostridium pasteurianum* W5 were oxidatively titrated with various electron acceptors. The AzoFd gave up 0.87 electrons per AzoFd monomer (27,500 mol. wt.). The oxidation reduction potential of AzoFd, determined by equilibrium with redox dyes, was -0.240 V. Dithionite reduced MoFd gave up 3.6 electrons per MoFd tetramer (220,000 mol. wt.). The oxidation reduction potential for MoFd was -0.070 V. Because the potential of *this* MoFd half cell is so positive, the electrons removed during this oxidation may not be those that reduce dinitrogen.

INTRODUCTION

Nitrogenase from *Clostridium pasteurianum* W5 has been resolved into two protein components, AzoFd and MoFd. Both components, plus ATP·Mg, are required for the transfer of electrons from electron donor to dinitrogen (1). Reduced ferredoxin or flavodoxin serve as physiological reductants for nitrogenase (2,3,4,5) but artificial electron donors such as sodium dithionite and reduced viologen dyes also were good electron donors (6,7).

Both AzoFd and MoFd have been shown to have spectrally distinct oxidized and reduced states, similar to the ferredoxins (8,9). The purpose of this study was to determine both the number of electrons accepted by and the oxidation-reduction potentials of both components of nitrogenase in order to determine the order of electron flow in the system.

MATERIALS AND METHODS

AzoFd and MoFd were prepared as described by Mortenson (10). The AzoFd used in this study had a specific activity of 2.3 μmoles of acetylene

Abbreviations: AzoFd, azoferredoxin; MoFd, molybdoferredoxin; Fd, ferredoxin.

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Table 1

Electrons Transferred by AzoFd

Electron donor	Electron acceptor	Electrons per monomer
Reduced AzoFd	Methylene blue	0.87
Reduced AzoFd	Potassium ferricyanide	0.82
Reduced AzoFd[a]	Methylene blue	0.71
Reduced AzoFd[a]	O_2 (as air-saturated buffer)	0.71, 0.71
Sodium dithionite	Air-oxidized AzoFd[b]	1.03

[a]AzoFd, after storage 8 months in liquid N_2

[b]AzoFd was only 30% oxidized before titration to avoid oxygen damage. The number of electrons to reduce fully oxidized AzoFd was calculated by extrapolation.

reduced per min per mg protein. The specific activity of the MoFd used was 1.7 μmole of acetylene reduced per min per mg. Acetylene reduction was assayed as described by Dalton et al. (9). The gas phase used was 0.75 atm Argon and 0.25 atm acetylene. The biuret method and the Folin method were used for protein determinations (11,12). Dried bovine serum albumin (Sigma) was used as a standard.

Spectrophotometric titrations were performed using matched 10 mm suprasil cells with teflon stoppers. The teflon stoppers were drilled and fitted with butyl rubber septa. These stoppers were secured with tape, and a positive pressure was maintained in the cuvettes. Gases and reagents were added by needle through the butyl rubber septa. Reagents were added by syringe and gases were added through a manifold equipped with a vacuum pump and fitted with syringe barrels and needles.

To remove O_2 the spectrophotometer cells were evacuated and filled 10 consecutive times with Argon that was passed over heated, reduced copper to remove traces of O_2. The buffer was deoxygenated separately and added by syringe.

Table 2

Oxidation-Reduction Potentials of AzoFd and MoFd

Indicator	Em 7.5 of Indicator (v)	Em 7.5 protein (v)
AzoFd		
Nile blue A	-.147	-.232 (± .018)
Methyl viologen*	-.446	-.252 (± .005)
Safranine O	-.268	-.242 (± .005)
MoFd		
Pyocyanine	-.060	-.070 (± .008)
Methylene blue	-.005	-.067 (± .014)

*The fact that oxidized methyl viologen has no absorbance at 665 nm and reduced methyl viologen does permits accurate determination of the reduced methyl viologen present, even under conditions where more than 99% of the methyl viologen is oxidized.

The equilibria between reduced AzoFd and MoFd and a number of dyes were observed.

The formula

$$Eh = E_m 7.5 \text{ (dye)} + \frac{RT}{nF} \ln \frac{\text{(oxidized dye)}}{\text{(reduced dye)}} = E_m 7.5 \text{ (protein)} + \frac{RT}{nF} \ln \frac{\text{(oxidized protein)}}{\text{(reduced protein)}}$$

was used to calculate the oxidation-reduction potential of the protein (13). A pH of 7.5 and temperature of 25° were used in these studies.

The extinction coefficients for the oxidized and reduced forms of the dyes were determined by titration against a standardized sodium dithionite solution. The amount of O_2 in air-saturated buffer was determined in the same manner.

RESULTS AND DISCUSSION

Spectra have been published showing the oxidized and reduced states

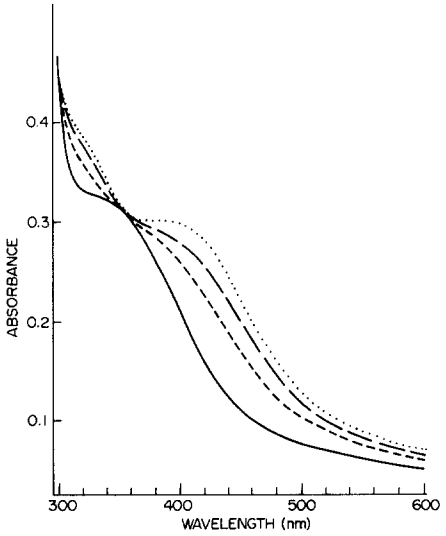


Fig. 1. Titration of reduced AzoFd with O_2 (added as air-saturated buffer). 2.3 mg AzoFd (43 nmoles AzoFd dimer) in 2.0 ml 0.05 M Tris-HCl buffer (pH 7.5) was titrated with O_2 as described in the text. The spectra were corrected for dilution. The amounts of O_2 were as follows: (——), none; (---), 16 nmoles; (— —), 24 nmoles; (·····), 35 nmoles.

of AzoFd and MoFd but the number of electrons transferred by the two proteins was not reported. This was partially because of the oxygen sensitivity of the nitrogenase components. Titrations of oxidized AzoFd with sodium dithionite, similar to the ferredoxins (14), would have been complicated by the breakdown of the iron-sulfur chromophore which occurs with time following oxidation (8). Oxidized AzoFd when reduced by sodium dithionite or reduced ferredoxin always had a spectrum which had less absorption than the original reduced spectrum. The greater the degree of oxidation, the greater was the "bleaching" which occurred. Another problem was the lack of highly purified protein. Preliminary studies using protein with a specific activity of 1 μmole acetylene reduced per min per mg protein suggested that one electron per dimer was transferred (Walker and Mortenson, abstract ASM, 1972).

The AzoFd used in this study had a specific activity of 2.3 μmoles acetylene reduced per min per mg protein. Titrations of AzoFd with various

oxidizing agents showed that one electron per monomer was transferred (Table 1). The oxidation of AzoFd was followed by measuring the increase in absorbance at 440 nm. Oxidation of AzoFd caused shoulders to appear at 320 nm and 420 nm (Fig. 1). There were isosbestic points at 295 nm and 360 nm. Reduced AzoFd was titrated with various oxidized, deoxygenated dyes, and the system was allowed to equilibrate between additions. The percentage of the protein oxidized was determined by following the absorption

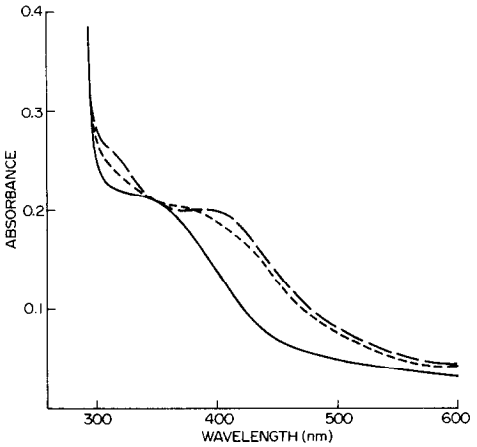
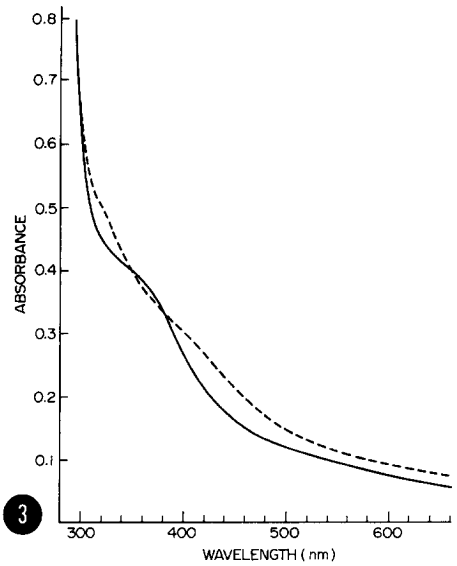


Fig. 2. Physiological oxidation of AzoFd. (——) 2.1 mg reduced AzoFd in 2.5 ml 0.05 M Tris-HCl buffer pH 7.5. (---) 2.1 mg physiologically oxidized AzoFd in 2.5 ml 0.05 M Tris-HCl buffer pH 7.5. The AzoFd was oxidized by incubation with 0.46 mg MoFd and 5 μmoles ATP·Mg, as described in the text. The spectra were corrected for absorption by reduced MoFd. (—— ——) 2.1 mg fully air-oxidized AzoFd in 2.5 ml 0.05 M Tris-HCl buffer pH 7.5.

at 440 nm. The reduction of Methyl viologen, Nile blue A, and Safranine O were observed at 604 nm, 640 nm, and 520 nm respectively. The 440 nm absorbance of AzoFd was corrected for the absorbance of the oxidized and reduced dyes. The correction was never more than 40% of the 440 nm absorbance. The oxidation reduction potential for AzoFd was found to be -0.240 V at pH 7.5 (Table 2).

Mortenson et al. (15) have shown in EPR studies that when AzoFd, MoFd, and ATP·Mg are incubated together and the reductant, sodium dithionite, becomes exhausted, AzoFd becomes oxidized while the MoFd returns to the



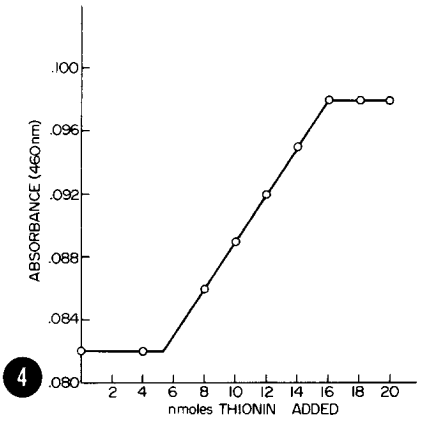


Fig. 3. Spectra of oxidized and reduced MoFd. (——) 2.3 mg reduced MoFd (10.5 nmoles) in 2.1 ml 0.05 M Tris-HCl buffer pH 7.5. (---) 2.3 mg oxidized MoFd in 2.1 ml 0.05 M Tris-HCl buffer pH 7.5. The sample had been exposed to air until no further change in absorbance at 440 nm occurred.

Fig. 4. Titration of MoFd with Thionin. 1.15 mg MoFd in 2.0 ml 0.05 M Tris-HCl buffer pH 7.5 was titrated with 10 μl increments of a 10^{-4} M Thionin solution. The values were not corrected for dilution. The initial 5 nmoles were required to remove dithionite present.

dithionite reduced state as isolated. This information was used to determine the spectrum of physiologically oxidized AzoFd (Fig. 2). The spectrum of physiologically oxidized AzoFd appears identical to that of artificially oxidized AzoFd.

When MoFd is oxidized it shows increased absorption between 300-340 nm and 280-700 nm (Fig. 3). There is a slight decrease in absorbance between 340-380 nm and isosbestic points are seen at 295 nm, 340 nm and 380 nm. The number of electrons given up by MoFd during this oxidation was determined by titrating dithionite reduced MoFd with deoxygenated, oxidized Thionin. The oxidation of MoFd was determined by the increase in absorbance at 460 nm and during oxidation MoFd gave up two electrons per dimer to Thionin (Fig. 4).

Since the activity of this MoFd was 1.7 μmole C_2H_2 reduced/min/mg

whereas activity as high as 2.3 units has been obtained (16) the number of electrons given up per MoFd dimer could be as high as three. However one of the major contaminants of MoFd is a second inactive species of MoFd (17) which can also give up electrons so that a value of two electrons per dimer seems more likely.

Reduced MoFd was titrated with oxidized, deoxygenated dyes as described for AzoFd (Table 2). The oxidation of MoFd was monitored at 460 nm. Oxidized pyocyanine and methylene blue were measured at 570 nm and 660 nm respectively. The midpoint potential of MoFd was found to be -0.070 V. Because the potential of these electrons is so positive with respect to the electron donor, reduced Fd, it seems unlikely that they are involved in dinitrogen reduction. EPR studies of this laboratory and others also suggest that the oxidized MoFd seen in these studies may not be formed during dinitrogen fixation. Instead, dithionite reduced MoFd may be further reduced in the presence of AzoFd and ATP·Mg, and this fully-reduced species may give up electrons to dinitrogen (15,18,19). Physiologically dithionite reduced MoFd may be the oxidized form of MoFd; chemically it would be the "semi-reduced" form.

ACKNOWLEDGMENT

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