ON THE STRUCTURE AND FUNCTION OF NITROGENASE FROM CLOSTRIDIUM PASTEURIANUM W5

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SUMMARY

Molybdoferredoxin from Clostridium pasteurianum W5 was fractionated into MoFd with two atoms of molybdenum per 220,000 daltons and a specific activity of 2.6 μmoles C_2H_2 reduced/min/mg protein and into a catalytically inactive species with an identical protein moiety but an incomplete active centre. Native MoFd is a tetramer composed of two 50,000 and two 60,000 dalton subunits. At low protein concentrations the tetramer is in equilibrium with a dimer. Under low ionic strength and at low pH further dissociation into monomers occurs. MoFd and azoferredoxin have distinct electron paramagnetic resonance spectra. The EPR spectrum of AzoFd and that of the combination of the two nitrogenase components undergoes characteristic changes upon addition of $MgATP^{2-}$.

INTRODUCTION

Reduction of dinitrogen to ammonia by N_2-fixing organisms requires the interaction of ATP with two iron-sulfur proteins. Unusual lability of both components has so far prevented a complete chemical and functional characterization. In this report we describe our recent findings which elucidate previous reported data and broaden our understanding of the nitrogenase complex of Clostridium pasteurianum. For a comprehensive discussion of N_2-fixation the reader is referred to a recently published review (1).

MATERIAL AND METHODS

AzoFd was purified as described elsewhere (2), omitting the final precipitation with protamine sulfate. Instead the material was passed through a 2.5 x 40 cm column of DE-52 cellulose (Whatman) by a linear NaCl-gradient (0.2 to 0.5 M NaCl in 0.05 Tris, pH 7.5, and 1 mM $Na_2S_2O_4$; total volume 600 ml). MoFd purified according to Dalton et al. (3) was chromatographed at least twice

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Abbreviations: AzoFd, azoferredoxin; EPR, electron paramagnetic resonance; MoFd, molybdoferredoxin; SDS, sodium dodecyl sulfate.

on a 2.5 x 40 to 60 cm DE-52 cellulose column. Elution was carried out at 30 ml/h by a linear KCl-gradient (0.15 to 0.3 M KCl in the above buffer; total volume 800 ml). The yellow fraction that eluted in front of the brown MoFd band was collected separately and three to four batches were rechromatographed on DEAE-cellulose like MoFd. Molybdenum was determined with the dithiol reagent (4). Sedimentation velocity was studied in a Beckman model E ultracentrifuge at 20°C with aluminum-filled Epon double-sector cells of 12 cm light path. EPR spectra were recorded in a Varian V 4500 spectrometer (5). Protein concentrations were estimated by the biuret method of Gornall et al. (6) or Goa (7). Activity of the proteins were assayed by acetylene reduction (2).

RESULTS AND DISCUSSION

MoFd when purified according to the previously described method (3) is pure with respect to its protein moiety. Disc gel electrophoretic analysis in the presence of SDS shows only two bands representing the 50,000 and 60,000 dalton subunits of MoFd (8). However, DEAE-cellulose chromatography removes a yellow protein from MoFd which is inactive in various nitrogenase reactions (Fig. 1). This protein in SDS polyacrylamide gel electrophoresis shows also the presence of the 50,000 and 60,000 molecular weight proteins in a ratio of approximately one. Sedimentation velocity studies of the yellow protein gave a $s_{20,w}$ of 8.7 which increased with increasing protein concentration. This behavior suggests the presence of a dimeric unit which associates unspecifically to give high molecular weight aggregates.

The immunoprecipitin test of this yellow protein is positive against a MoFd antiserum. Metal analysis shows a virtual absence of molybdenum and the presence of an average of two atoms of iron. The sulfide content is in the range of the iron content. From these results we conclude that previous MoFd preparations had a catalytic centre heterogeneity. The presence of this second species can be monitored by EPR spectroscopy. Catalytically active MoFd has in its reduced form resonances in the g = 4 range and at g = 2.01, but no 1.94 resonance, whereas the second MoFd species has a pronounced 1.94 type

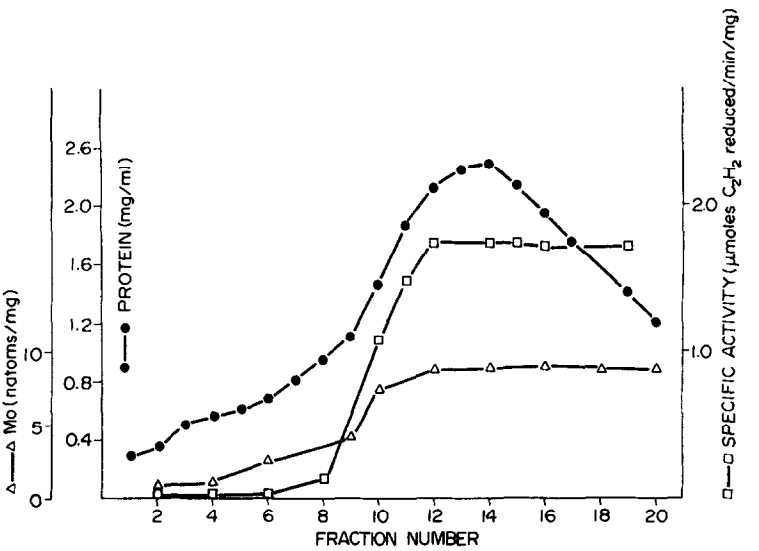

Fig. 1 Elution profile of molybdoferredoxin from a DEAE-cellulose column. MoFd was loaded on a 2.5 x 42 cm DE-52 column and was eluted as described under METHODS. Fractions of 9 ml were collected anaerobically and analyzed for protein, molybdenum and acetylene reduction (using in the latter case a 3-fold excess of AzoFd). Note the plateau in the specific activity which is matched by the molybdenum profile. The inactive MoFd species is eluted primarily in fractions 2 to 7 but tails out until fraction 10.

iron-sulfur signal with g-values of 2.05 and 1.92 and no resonance in the g = 4 region.

As a consequence of this further fractionation of MoFd the specific activity increased from 1,200 to 2,250 nmoles C_2H_2 reduced/min/mg protein. We have found a difference of approximately 15% between the biuret assay we usually employ (6) and the microbiuret assay (7) of other workers in the field, who have reported activities as high as 2,500 nmoles C_2H_2 reduced/min/mg protein (9). If we apply this correction, the specific activity of our best MoFd is 2,630 units. This value is achieved with a six-fold molar excess of AzoFd over MoFd. Lower ratios of AzoFd to MoFd (as low as 2) which are normally used for routine work yield lower specific activities, although AzoFd is assayed optimally at a ratio of 2. Moreover preliminary evidence indicates that AzoFd of a low specific activity, although homogeneous as a protein, is

inhibitory, presumably because the inactive species of the electron carrier forms an inactive nitrogenase complex.

As a further consequence of the removal of the inactive MoFd species, the molybdenum content of MoFd is higher than previously reported (3,10). Our data show the presence of 34.5 natoms of molybdenum per 20 nmoles of protein. On the basis of a minimal molecular weight of 118,000 derived from the amino acid composition (11) one atom of molybdenum would be present per minimal protein unit. Also the iron and sulfide content of active MoFd is higher. Current analyses indicate 11 to 12 atoms of iron and sulfide per minimal unit.

In solution MoFd is a complex with a molecular weight of 220,000 (8), composed of subunits of 50,000 and 60,000 daltons in a relative ratio of one (Fig. 2). The complex has a $s^{o}_{20,w}$ of 10.5. However, its sedimentation coefficient decreases when the protein concentration is decreased below 5 mg/ml and the Schlieren pattern at these lower concentrations shows a slightly skewed peak. We interpret this to mean that an equilibrium exists between a dimeric and a tetrameric form of the protein. This dissociation also would influence the sedimentation equilibrium behavior of the protein since very low protein concentrations are used and molecular weights derived from such studies should be considered with caution (3). It also should be pointed out, that this dissociation equilibrium might affect activity studies, depending on which species, dimer or tetramer, is the active one. The inhibition generally observed at high MoFd concentrations and activation at low protein concentrations could well be taken as an indication that the tetramer is inactive and only the dimer is active.

Under conditions of low ionic strength and low pH, MoFd can easily be separated into its dimeric and into its monomeric protein subunits. A Sephadex G-100 column (2.5 x 90 cm) equilibrated with 0.02 N HCl and 0.1% β-mercaptoethanol was found most suitable for isolation of the subunits (Fig. 3).

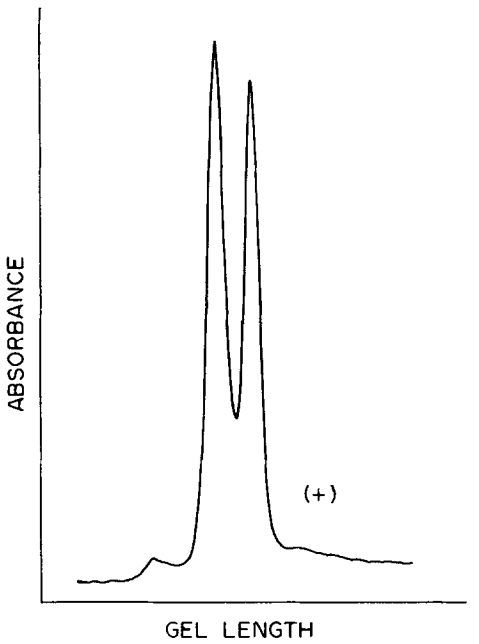

Fig. 2.

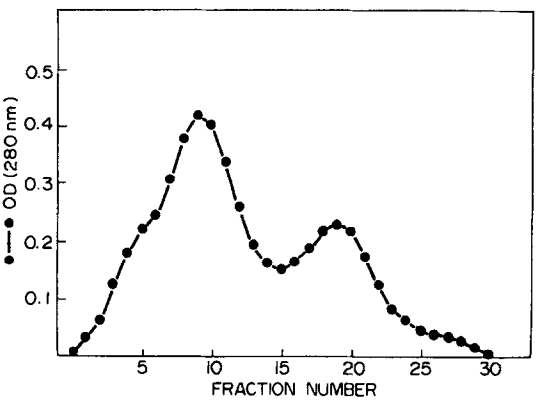

Fig. 3.

Fig. 2 Subunit pattern of molybdoferredoxin treated with SDS and mercaptoethanol when examined by disc gel electrophoresis. Ten μg of protein were separated and after staining with Coomassie blue the gel was scanned at 550 nm in a Gilford densitometer. The area of the 60,000 subunit is 8.7, that of the 50,000 subunit 7.3. When these relative areas are corrected for the molecular weight difference their ratio is one to one.

Fig. 3 Separation of the subunits of molybdoferredoxin by gel chromatography. 20 mg of protein were dialyzed for 24 h against H_2O and another 24 h against 0.02 N HCl, 0.1% with respect of β-mercaptoethanol. The treated protein was passed through a 2.5 x 90 cm Sephadex G-100 column, equilibrated with the dialysis solution and the effluent was monitored at 280 nm. The first peak is the 60,000 subunit, contaminated slightly in the front by some higher molecular weight protein (dimer), but clearly separated from the second peak containing the 50,000 subunit.

Electron paramagnetic resonance investigations have led to a characterization of both components of clostridial nitrogenase. AzoFd has in its reduced form a 1.94 type non-heme iron signal with g-values 2.06, 1.94 and 1.87. Reduced MoFd has a spectrum with nominal g-values of 2.01, 3.78 and 4.27 (5). As ATP is required for N_2ase activity, we have tested its effect on the EPR spectra of both separated and combined components. The EPR spectrum

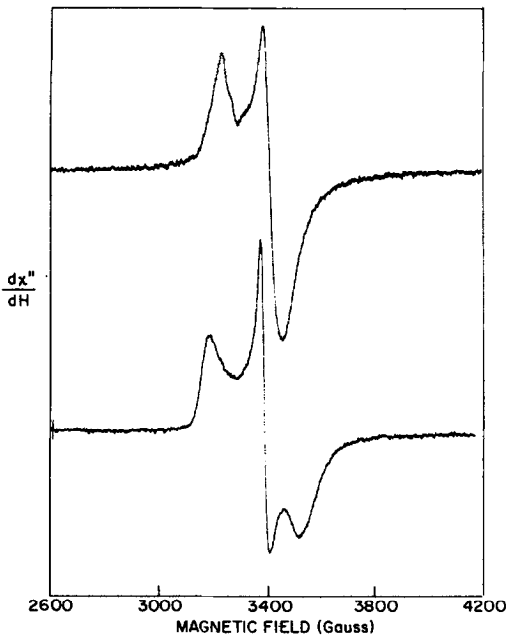

Fig. 4 Effect of $MgATP^{2-}$ on the EPR spectrum of reduced azoferredoxin. In the top spectrum 0.3 ml of AzoFd (20 mg/ml) and 7 μl of $MgATP^{2-}$ (0.1 M) were syringed into an EPR tube. The solution was mixed and frozen and the EPR spectrum recorded. Compare the top spectrum with the bottom spectrum, the results from an EPR tube that contained the same concentration of AzoFd but no $MgATP^{2-}$. The instrument settings were: gain, 100; sweep rate, 250 gauss/min; time constant, 0.3; frequency, 9.22 GHz; modulation amplitude, 4.5 gauss; temperature, 23 K.

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of MoFd does not change upon addition of ATP and magnesium. In contrast, AzoFd responds substantially to the addition of $MgATP^{2-}$ (Fig. 4). The integrated intensity is the same compared with the untreated protein, but the line shape changes to a spectrum with g-values of 2.04 and 1.93. The complete change of reduced AzoFd to this new signal requires the addition of approximately two moles of $MgATP^{2-}$ for each mole of AzoFd (mol. wt. 55,000). The observation that 5 M urea induces the same signal change of AzoFd as $MgATP^{2-}$, indicates that a conformational change of the protein leads to a rearrangement of the iron-sulfur centre(s) which is detectable by its altered EPR spectrum.

When both components of N_2ase are combined, an EPR spectrum of nitrogenase is obtained (Fig. 5). Addition of $MgATP^{2-}$ now causes changes not only

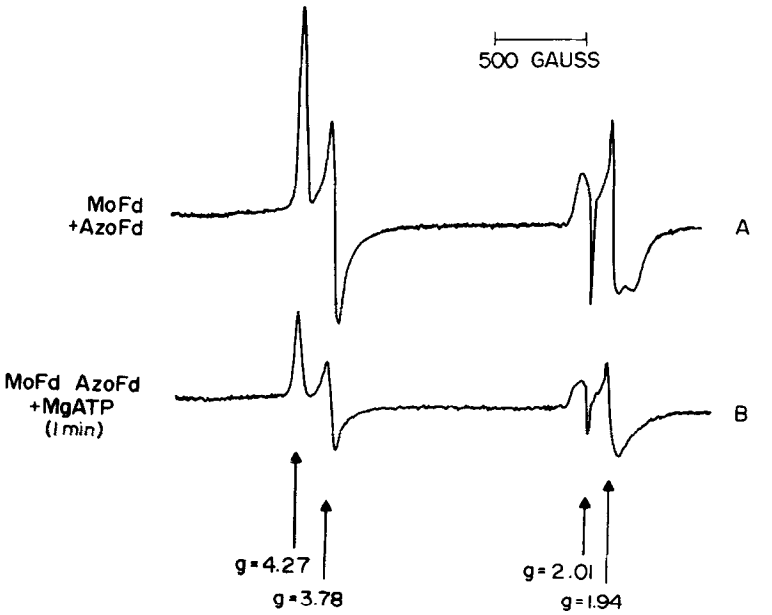

Fig. 5 Effect of $MgATP^{2-}$ on the EPR spectrum of nitrogenase. The nitrogenase solution in this experiments consists of 0.3 ml of reduced MoFd (36 mg/ml) and 0.15 ml of reduced AzoFd (36 mg/ml). The EPR spectrum of this nitrogenase was recorded (A). To this mixture 30 μl of 0.1 M $MgATP^{2-}$ were added and recorded in (B). The instrument settings were: gain, 50; sweep rate, 1000 gauss/min; modulation amplitude 8.9 gauss; otherwise like Fig. 4. Abscissa and ordinate are linear functions of dX"/dH and magnetic field respectively.

in the signal of AzoFd but of MoFd as well. Rapid freeze experiments (10 to 1000 msec) revealed that the signal of AzoFd changed first followed by the slower changing MoFd signal. Under the conditions employed (nitrogenase with a composition of a two-fold molar excess of AzoFd over MoFd and no ATP-generating system) 40% of the MoFd remained reduced. When an ATP-generating system was used and the dithionite but not the ATP was consumed, the AzoFd signal disappeared completely (complete oxidation), but MoFd was still 25% reduced. This natural oxidation of nitrogenase is similar to that obtained with certain dyes and it suggests that electrons flow from reduced AzoFd to MoFd and then to substrate reduction. The oxidation of reduced MoFd is dependent on ATP and AzoFd.

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