Components of Cell-free Extracts of Clostridium pasteurianum W5 Required for Acetylene Reduction and N_2 Fixation

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Summary: The same two protein components, molybdoferredoxin and azoferredoxin, are required for acetylene reduction as were shown previously to be required for N_2 fixation. Neither protein component of the N_2-fixing system catalyzes either of these two reactions without the other. Both protein components are oxygen- and heat- (60^o) sensitive and one of them, azoferredoxin, is also cold- (0^o) labile. Neither molybdoferredoxin nor azoferredoxin is present in extracts of cells grown on NH_3. No indication of the need for a third component was observed.

The two protein components, molybdoferredoxin (MoFd) and azoferredoxin (AzoFd), of the nitrogenase system of Clostridium pasteurianum W5 have been partially purified from cells grown on N_2 (Mortenson et al., 1967). The same two protein components have been shown to be required for ATP-dependent H_2 evolution, electron-dependent ATP utilization (Mortenson, 1966), electron-independent ATP utilization (Kennedy & Mortenson, 1969; Jeng et al., 1969a) and acetylene reduction (Moustafa & Mortenson, 1967). Similar protein fractions have also been separated from the nitrogenase of Azotobacter vinelandii (Bulen & LeComte, 1966), Azotobacter chroococcum (Kelly, 1969) and soybean nodules (Klucas et al., 1968).

Recently, it was suggested that AzoFd of C. pasteurianum contains two fractions necessary for N_2 fixation and that one of these two fractions is present in cells grown on either N_2 or NH_3 (Taylor, 1969). In addition the latter author stated that MoFd (his C_1S_1 fraction) when added to an extract of NH_3-grown cells supported acetylene reduction activity but no N_2 fixation. In light of this report we have reevaluated our results and find that as previously reported 1) AzoFd and MoFd are single protein components that are required for both activities, 2) they are found only in cells grown on N_2,

3) the addition of MoFd to an extract of NH_3-grown cells does not give acetylene reduction activity and 4) there is no third component required.

Materials and Methods

A Cary 14 recording spectrophotometer was used to record the spectrum of AzoFd before and after treatment at 60°. Protein was measured by the Biuret method (Gornall et al., 1949). N_2 fixation was assayed manometrically (Mortenson, 1966). An extract of NH_3-grown cells was added as a source of the supporting enzymes, ATP : acetate phosphotransferase (for the ATP generating system), and H_2 : ferredoxin oxidoreductase (hydrogenase) and ferredoxin (for the electron donating system). In some experiments sodium dithionite (hydrosulfite) was used in place of the reduced Fd generating system (Burns & Bulen, 1965) and substrate levels of ATP (10mM) were used instead of an ATP generating system (Kennedy et al., 1968). Acetylene reduction was measured by the method of Koch & Evans (1966).

All treatments throughout the purification of MoFd and AzoFd were performed under anaerobic conditions. Cells and crude extracts were prepared as previously described (Mortenson et al., 1967). The two protein components of nitrogenase, MoFd and AzoFd, were separated as described by Mortenson et al., (1967). MoFd with a molecular weight of about 160,000 was further purified by the method of Mortenson et al., (1968) and Morris et al., (1969). It was about 95% pure based on a molybdenum content of 2 atoms per molecule and disc gel electrophoresis examination. AzoFd containing 2 iron and 2 acid-labile sulfide groups per molecule was isolated and purified (about 98% pure) and found to have a molecular weight of approximately 39,000 (Moustafa & Mortenson, 1969; Jeng, et al., 1969b).

Results

MoFd and AzoFd have been found only in extracts of cells that actively fix N_2. When either of these proteins was added to an extract of cells grown on NH_3, neither N_2 fixing nor acetylene reducing activity was observed (Fig. 1A & B). Similar results were obtained if sodium pyruvate was used as electron

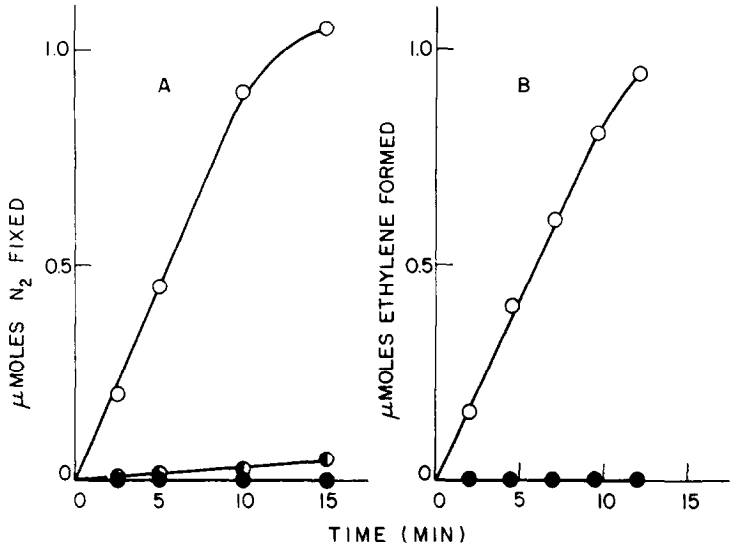

Fig. 1A Requirement of MoFd and AzoFd for N_2 fixation with H_2 as electron donor. The complete system in a total volume of 2.0 ml contained 100 μmoles of lithium cacodylate buffer at pH 6.75, 10 μmoles $MgCl_2$, 19.3 mg protein from extract of cells grown on NH_3, 5 mg MoFd, 1.15 mg AzoFd in the main compartment as well as 5 μmoles ATP and 50 μmoles acetyl phosphate in the sidearm. The center well contained 0.1 ml 20% KOH. The gas phase was 0.5 atm H_2 and 0.5 atm N_2 with a control of 0.5 atm A and 0.5 atm N_2. After 5 min equilibration at 30° under the specified gases, the reactions were started by tipping in the ATP and acetyl phosphate solution from the sidearm. O-O-O complete system under H_2 & N_2; ●-●-● complete system minus MoFd and AzoFd under H_2 & N_2; ●-●-● complete system under A & N_2.

Fig. 1B Requirement of MoFd and AzoFd for C_2H_2 reduction with H_2 as electron donor. The complete system in a total volume of 2.0 ml contained 100 μmoles lithium cacodylate buffer at pH 6.75, 10 μmoles $MgCl_2$, 8 mg protein from extract of cells grown on NH_3, 5 mg MoFd, 0.3 mg AzoFd, and 20 μmoles ATP. The gas phase was 0.85 atm H_2 and 0.15 atm C_2H_2. After 5 min equilibration at 30°under the specified gases, the reactions were started by injecting the ATP solution with a gas tight syringe into the reaction vials. To determine the amount of C_2H_2 reduced, a standard of ethylene was included. O-O-O complete system; ●-●-● complete system minus MoFd and AzoFd or either one of them.

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and energy source (Fig. 2). Therefore, both MoFd & AzoFd are required for both activities and neither is found in extracts of cells grown on NH_3.

Taylor (1969) claimed that AzoFd was heat stable at 60°, whereas MoFd was not. We find that when crude N_2-fixing extracts were treated at 60° under H_2 for 10 min., no N_2-fixing or acetylene reducing activity occurred unless MoFd was added; the total activity was always lower than that of the untreated

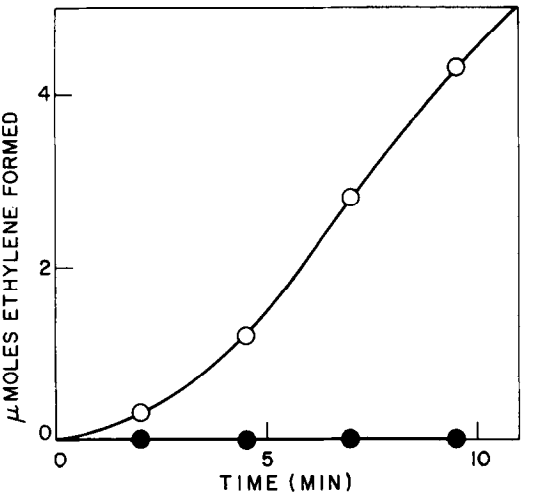

Fig. 2 Requirement of MoFd and AzoFd for C_2H_2 reduction with sodium pyruvate as energy and electron source. The complete system in a total volume of 2.0 ml contained 100 μmoles lithium cacodylate buffer at pH 6.75, 10 μmoles $MgCl_2$, 50 μmoles sodium phosphate at the same pH, 19.3 mg protein from extract of cells grown on NH_3, 5.0 mg MoFd, 1.15 mg AzoFd and 100 μmoles sodium pyruvate with 0.5 μmole of ATP. The gas phase was 0.85 atm A add 0.15 atm C_2H_2. After 5 min equilibration at 30° under the specified gases, the reactions were started by injecting the pyruvate and ATP solution with a gas tight syringe into the reaction vials. To determine the amount of C_2H_2 reduced, a standard of ethylene was included. O-O-O complete system; ●-●-● complete system minus MoFd and AzoFd or either one of them.

extract but the specific activity increased (Fig. 3A). However, when purified AzoFd was heated under similar conditions and the heated AzoFd then combined with MoFd, no activity resulted (Fig. 3B). Obviously certain components in the crude extract prevented AzoFd from being heat denatured. Even though no noticeable precipitation occurred when AzoFd was heated to 60°, AzoFd was indeed changed as evidenced by a large change in its spectrum (Fig. 4). Heat treated AzoFd did precipitate readily when added to the assay mixture; some sort of complexing with other components appeared to be necessary to observe denaturation visually. These results show that both MoFd and AzoFd are sensitive to heat (60°) treatment.

As a result of examining fractions of a crude AzoFd preparation collected from a Sephadex G-100 column, Taylor (1969) claimed there probably were two components present in our AzoFd preparation and that both were required for N_2

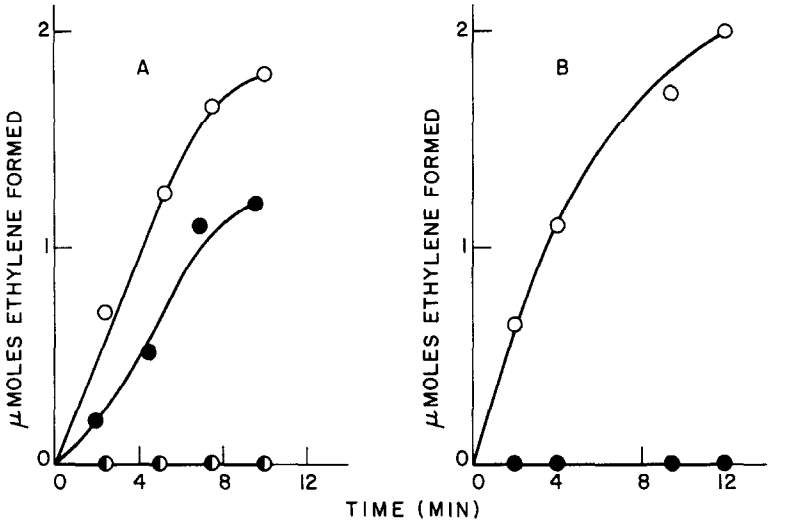

Fig. 3A Heat stability of AzoFd in an extract of cells grown on N_2. See text for the experimental conditions. The complete system in a total volume of 2.0 ml contained 100 μmoles lithium cacodylate buffer at pH 6.75, 10 μmoles $MgCl_2$, 0.5 ml of crude extract (21.4 mg/ml) or heat-treated crude extract (10.3 mg/ml after heat treatment) and substrate level of ATP (20 μmoles). Other conditions were the same as in Fig. 1B. O-O-O the original activity of the crude extract; ●-●-● the activity of the heat-treated crude extract plus 5.0 mg MoFd; ◐-◐-◐ the activity of heat-treated crude extract plus 2.0 mg AzoFd or alone.

Fig. 3B Heat stability of purified AzoFd. Purified AzoFd (22 mg/ml) was treated at 60° under a H_2 atmosphere for 10 min. The reaction mixture for acetylene reduction in a total volume of 2.0 ml contained 100 μmoles lithium cacodylate buffer at pH 6.75, 10 μmoles $MgCl_2$; 10 μmoles sodium dithionite as electron donor, 20 μmoles ATP, 5.0 mg MoFd and 1.1 mg AzoFd. Other conditions were the same as in Fig. 1B. O-O-O the activity in the presence of purified MoFd and AzoFd; ●-●-● the activities of MoFd or AzoFd alone and of purified MoFd plus heat-treated AzoFd.

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fixation but only one of them for acetylene reduction. To examine this point we subjected purified AzoFd to gel filtration on an anaerobic Sephadex G-100 column (43 x 2.5 cm) with a flow rate of 6.0 ml per hr. Fractions were collected and assayed for acetylene reduction and N_2 fixation. The two activities were not distinguishable, i.e. their ratio was the same in each fraction (Fig. 5). In addition, when fractions from each end of the activity peak were assayed alone and in combination, the activity for N_2 fixation was additive (Table I). There was no stimulation in activity as would be expected if two necessary components had been separated. These data indicate that the same single protein catalyzed both activities.

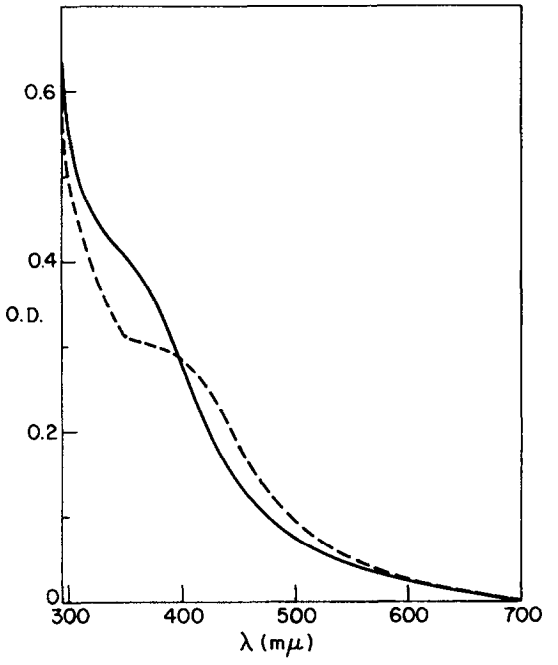

Fig. 4 Visible spectra of native (——) and heat-treated (-----) AzoFd under H_2 atmosphere at 25°. 6.5 mg AzoFd in total 3.0 ml was used.

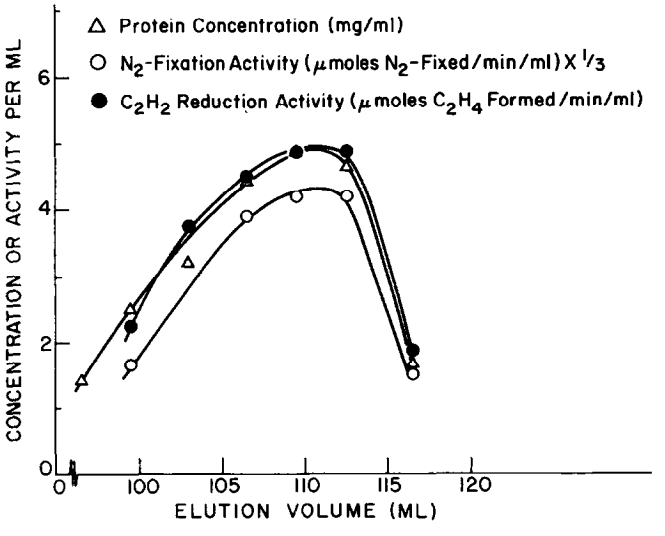

Fig. 5 Activity of fractions of purified AzoFd after chromatography on a Sephadex G-100 column. See text for the protein and activity measurements.

Table I. Demonstration by Sephadex G-100 chromatography of the purity of AzoFd required for N_2 fixation.*

Fraction #	Amount of Protein Added (mg)	N_2 Fixation Activity** ($m\mu$moles N_2 fixed/min)
#2 (corresponded to the fraction at 100 ml of the elution profile in Fig. 5)	0.26	70
#7 (corresponded to the fraction at 115 ml of the elution profile in Fig. 5)	0.14	50
#2 and #7	0.66***	200 (observed) 190 (calculated)

*The complete system in a total volume of 2.0 ml contained 100 μmoles lithium cacodylate buffer at pH 6.75, 10 μmoles $MgCl_2$, 4 μmoles ATP, 50 μmoles acetyl phosphate, 8.9 mg protein from the extract of cells grown on NH_3, 4 mg MoFd and indicated amount of AzoFd. Other conditions were the same as in Fig. 1A.

**Calculated from the maximum 5 min rate.

***0.52 mg of protein from fraction #2 and 0.14 mg of protein from fraction #7.

Discussion

Taylor (1969) has described the separation of the nitrogenase complex into at least three necessary protein components. All three fractions (C_1S_1, IV_n & IV_s) were required for N_2 fixation but only two of them (C_1S_1 & IV_s) were required for acetylene reduction. Fraction IV_s was also found in extracts of cells grown on NH_3. Both IV_n and IV_s were stable at 60^o under a H_2 atmosphere for 10 min. MoFd described by Mortenson et al., (1967), must have been a component of Taylor's C_1S_1 fraction. In contrast to the latter results with crude fractions we find that in addition to MoFd, AzoFd was the only other protein necessary for N_2 fixation and AzoFd is also required for acetylene reduction. An extract of cells grown on NH_3, which according to Taylor should have supported acetylene reduction if MoFd (C_1S_1) was added, was completely inactive when MoFd was added.

The conclusion that only AzoFd is required for the activities of N_2

fixation and acetylene reduction in the presence of purified MoFd, an energy source (ATP), Mg^{++} and an electron donor (dithionite) is inferred from the following considerations: 1) The purity of our AzoFd has been established from its behavior during ultracentrifugation and disc gel electrophoresis, by a consistent absence of tryptophan from several preparations and by the lack of further separation on Sephadex G-100 (Jeng et al., 1969b); 2) Neither MoFd nor AzoFd was found in extracts of cells grown on NH_3 even when N_2 was available; and 3) Only MoFd and AzoFd are required for the activity of N_2 fixation or acetylene reduction.

The history of the cells grown on NH_3 is important in the preparation of extracts for this work. Daesch & Mortenson (1968) demonstrated that the turnover rate of nitrogenase in whole cells is extremely low, i.e., it is decreased only by dilution during cell growth in the absence of further nitrogenase biosynthesis when a culture is switched from N_2-fixing conditions to an NH_3-supplemented medium. Therefore, in order to look for the presence or absence of components of nitrogenase, cells must be grown exponentially for at least 10 generations in the NH_3-supplemented medium before preparing the cell-free extract. This could account for Taylor's observations with extracts of NH_3-grown cells. Another possible explanation of his results could be that his C_1S_1 fraction was contaminated with AzoFd. The different requirements he finds when measuring acetylene reduction versus N_2 fixation could reflect procedural problems. For example, since the activities reported for acetylene reduction were very low, the measurement of N_2 fixation under such conditions would be very difficult.

Acknowledgments

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