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Role of Molybdenum in Dinitrogen Fixation by *Clostridium pasteurianum*

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The role of Mo in the activity and synthesis of the nitrogenase components of *Clostridium pasteurianum* has been studied by observing the competition of Mo with its structural analogue W. Clostridial cells when fixing N_2 appeared strictly dependent upon the available Mo, showing maximal N_2-fixing activity at molybdate concentrations in the media of 10 μM. Cells grown in media with 3×10^{-6} μM Mo, although showing good growth, had only 15% as much N_2-fixing activity. In the presence of W the synthesis of both nitrogenase components, molybdoferredoxin and azoferredoxin, was affected. Attempts to produce nitrogenase in W-grown cells by addition of high molybdenum to the media in the presence of inhibitors of protein synthesis showed that Mo incorporation into a possible inactive preformed apoenzyme did not occur. Unlike other molybdoenzyme-containing cells, in which W either is incorporated in place of Mo to yield inactive protein or initiates the production of apoprotein, *C. pasteurianum* forms neither a tungsten substituted molybdoferredoxin nor an apoprotein. It is concluded that in *C. pasteurianum* molybdenum is an essential requirement for both the biosynthesis and activity of its nitrogenase.

It has been known for many years that N_2-fixing organisms require molybdenum for growth when fixing dinitrogen (3), this requirement being due to the presence of the metal in one of the two main proteins of the nitrogenase complex (6, 9, 12, 13, 19, 25).

Recently, attempts have been made to elucidate the function of molybdenum in the nitrogenase of *Azotobacter vinelandii* (2) and *Klebsiella pneumoniae* (5), and some conclusions have been drawn about the participation of this element in the induction and catalytic activities of the nitrogenase components.

In this paper we wish to clarify the role played by molybdenum in nitrogen fixation in *Clostridium pasteurianum* W5 cells.

MATERIALS AND METHODS

Growth of organism. *C. pasteurianum* W5 was grown as previously described (P. T. Bui, Diss. Abst. 29:909, 1968) except that molybdenum was omitted. When required, sodium molybdate and/or sodium tungstate were added at the indicated concentrations.

For enzyme purification, cells were grown under N_2 in a 160-liter steel fermentor maintained at pH 5.9 that was inoculated (2%) from a chemostat culture growing at an absorbance at 550 nm of 1.5. All chemostats used in this study were limited in sucrose. When an absorbance of 4.0 was reached the cells were harvested, washed and dried under vacuum, and stored until needed.

In the studies of tungsten incorporation the inocula were taken from a chemostat culture maintained under argon with 10^{-4} M Na_2WO_4 and 3×10^{-3} M $(NH_4)_2SO_4$, and the cells were grown under N_2 at the same concentration of W and ammonia. When the ammonia was exhausted (as indicated by the growth curve and by the absence of ammonia in the supernatant) the culture was derepressed under N_2 in the presence of W, and the cells were harvested and treated as described above.

In competition experiments, cells from a chemostat that contained 10 μM Mo were used as inocula, and subsequently the W:Mo ratio was adjusted as desired.

Cells for whole-cell experiments were grown under N_2 in 400-ml flasks in media buffered with 0.05 M succinate, pH 5.85. Cell growth was determined turbidimetrically during the log phase by absorbance readings at 550 nm in Baush and Lomb spectronic 20.

Preparation of extracts and specific activity determination. The crude extracts and purified nitrogenase proteins were prepared as previously described (33). When necessary, specific details are given in the text.

The nitrogen-fixing ability of the whole cells, crude extracts, and purified components was determined by measuring acetylene reduction activity, a known indicator of nitrogenase (11).

In whole cells acetylene reduction was followed at 30 C in 8.0-ml serum bottles filled with 0.20 atm of C_2H_2 and 0.80 atm of argon. The reaction mixture of 2 ml contained 1.9 ml of cell culture and 0.1 ml of 0.05 M succinate buffer at pH 5.85, 2% in sucrose.

Molybdoferredoxin and azoferredoxin activities were determined upon titration with the complementary purified component (33).

Specific activity of nitrogenase is defined as nanomoles of ethylene formed/minute per milligram of protein.

Adenosine 5'-triphosphate, creatine phosphokinase (EC 2.7.3.2), creatine phosphate, deoxyribonuclease I (EC 3.1.4.5), ribonuclease A (EC 2.7.7.16), rifamycin SV, chloramphenicol, tris(hydroxymethyl)aminomethane base, *N*-tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid, sodium molybdate, and sodium tungstate were purchased from Sigma (St. Louis, Mo.). Other chemicals used were of the highest purity available.

Analytical methods. Molybdenum and tungsten were determined colorimetrically by the method of Cardenas and Mortensen (8). Ammonia was measured by nesslerization after diffuson and absorption of the gas in 5 mM H_2SO_4 in Conway dishes (10). Electron paramagnetic resonance (EPR) spectra were recorded at 20 K in a Varian V-4500 X-band spectrometer.

Protein was estimated by the biuret and Lowry methods (14, 24). In the whole-cell experiments the protein was measured in the following manner. Samples (10 ml) (absorbance at 550 nm between 1.0 to 1.5) were removed from the cultures and transferred to heavy-walled polyethylene centrifuge tubes containing 1.0 ml of 50% trichloroacetic acid. The contents were mixed thoroughly and centrifuged at 10,000 rpm for 5 min, and the supernatant was discarded. Five milliliters of 5% trichloroacetic acid was added to each tube, and the pellet was resuspended and recentrifuged under the same conditions as above. One milliliter of 2 N NaOH was added to each tube containing the washed cell pellet, and the tubes were incubated for 30 min in a 40 C bath and swirled gently until almost all cloudiness disappeared. Distilled water (9 ml) was then added to each tube, and 0.5-ml aliquots were assayed for protein content by the Lowry method (24).

RESULTS AND DISCUSSION

Effect of molybdenum on growth and nitrogenase activity. When the influence of added molybdate on clostridial cultures was studied, no significant difference in growth could be detected over the wide range of concentrations used (Fig. 1). At molybdate concentrations as low as 3×10^{-6} μM in the culture media (curve not presented) the cells showed "normal" growth rates and reached the stationary phase between 20 and 30 h. Molybdenum at a concentration of 10 mM inhibited the growth.

In contrast to the effect on growth, the activity levels of nitrogenase were affected markedly by the molybdate concentration in the media (Fig. 2). At a calculated concentration of 3×10^{-6} μM, when molybdenum was undetectable by any of the most sensitive analytical methods available, the cells retained 15% of their normal nitrogenase activity, whereas under the same conditions the cells grew normally. These results show that molybdenum is required for maximal nitrogenase activity. In addition they indicate that the

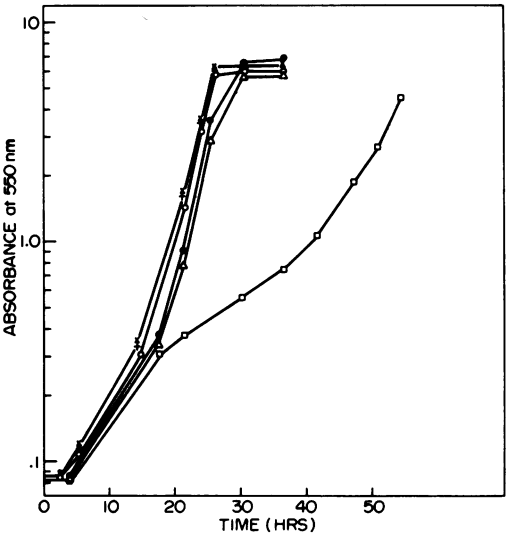

FIG. 1. *Effect of Mo addition on growth of C. pasteurianum at: ×, 0.25 μM; +, 1 μM; ○, 10 μM; ●, 100 μM; △, 1,000 μM; □, 10,000 μM. The cells were grown from 2% (vol/vol) inocula that contained 10 μM Mo in 400-ml flasks. Cultures with lower concentrations of molybdenum (curves not shown) were prepared by serial dilution from cells growing with molybdenum (0.25 μM) into media without molybdenum.*

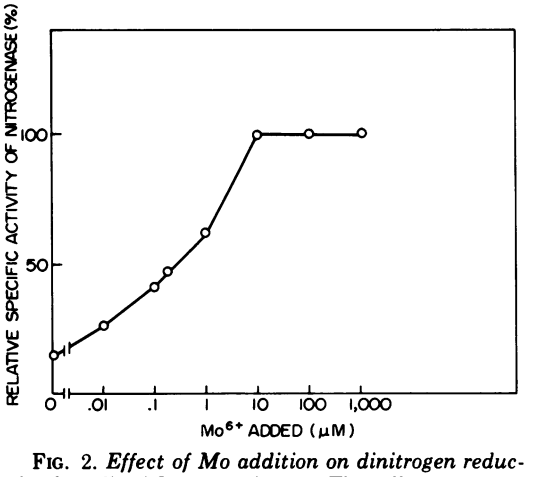

FIG. 2. *Effect of Mo addition on dinitrogen reduction by cells of C. pasteurianum. The cells were grown from 2% inocula into the conditions given in the legend of Fig. 1. Protein and activity were measured in mid-phase of growth. The Mo concentration of the lowest point was from a culture that has been serially transferred five times from cells growing with molybdenum (0.25 μM) into media without molybdenum. Relative specific activity of 100 corresponded to 186 nmol of C_2H_4 formed/min per mg of protein. The units of the abscissa are logarithmic.*

concentration of nitrogenase in cells with excess Mo is much greater than needed to maintain growth. A similar requirement has been established for *Azotobacter* nitrogenase (2, 26) and for other molybdoenzymes (21, 29, 32) in which reduced molybdenum seems to play an essential catalytic role (1, 4, 23).

Effect of W on growth and activity. The role played by molybdenum in clostridial nitrogenase has been further established by studying the inhibitory effect of W on both growth and nitrogen-fixing ability.

Figure 3 shows the growth lag produced by addition of increasing amounts of sodium tungstate to the culture media. At ratios of W:Mo of 400 and higher the growth lag period was over 30 h. The maximum growth rate was restored when the W:Mo ratio was lowered by adding molybdenum.

The inhibitory effect of added tungstate on whole cell nitrogenase activity is presented in Fig. 4. At a W:Mo of 400 a decrease of 80% of the nitrogenase activity was obtained. The inhibition was reversed by adding molybdate (Table 1), which indicates that the W-Mo interaction is of a competitive nature. Similar effects on growth and activity have been reported for dinitrogen fixation in *A. vinelandii* (2, 3, 22, 31), *A. chroococcum* (15), and *K. pneumoniae* (5) and for other molybdoenzymes (17, 21, 27, 28, 32). All of these studies emphasize the essential role of molybdenum in the catalytic reductions mediated by molybdoenzymes.

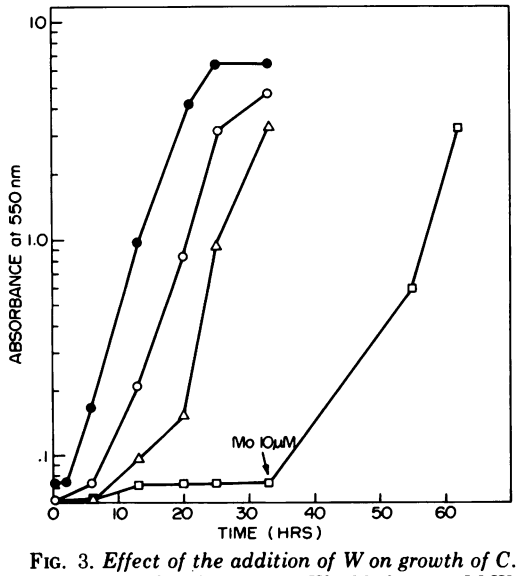

FIG. 3. *Effect of the addition of W on growth of C. pasteurianum. Symbols: ○, no W added; ●, 1 μM W; △, 10 μM W; □, 100 μM W. The starting Mo concentration of the cultures was 0.25 μM.*

Site of tungsten inhibition. The inhibition by tungsten of molybdenum-supported N_2 fixation was studied further. In Table 2 the decrease in nitrogenase activity in the presence of tungsten is compared to the external and internal concentration of both molybdenum and tungsten. The data strongly suggest that W inhibits molybdenum uptake by cells of *C. pasteurianum*.

When cells grown on media low in molybdenum were switched to a high tungsten concentration, nitrogenase production practically ceased, whereas growth still increased exhausting the excess molybdenum previously stored

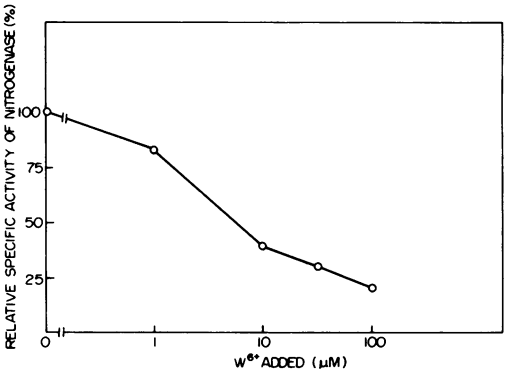

FIG. 4. *Effect of the addition of W on dinitrogen reduction by cells of C. pasteurianum. The culture media contained a constant concentration of Mo (0.25 μM). Relative specific activity of 100 corresponded to 105 nmol of C_2H_4 formed/min per mg of protein. The units of the abscissa are logarithmic.*

TABLE 1. *Effect of W on nitrogenase activity in clostridial cells and the reversal of the effect by Mo*

Culture	Mo added (μM)	W present (μM)	Sp act (U/mg of protein)	Inhibition (%)	Recovery (%)
1	None	None	114		
2	None	25	45	60	
3	None	50	32	71	
4	—[a]	25	89		79
5	—[a]	50	95		83

[a] Starting molybdenum concentration of the control cultures was 0.25 μM. After 15 h acetylene reduction activity and protein were determined. Then the molybdenum concentrations of cultures 4 and 5 were increased to 25 and 50 μM, respectively, and acetylene reduction was measured 4 h later. The specific activity of culture 1 at the end of the experiment was 112 U/mg of protein. One unit is defined as the amount of protein that forms 1 nmol of C_2H_4 per min under the standard assay conditions as described.

TABLE 2. *Effect of W on the intracellular concentration of Mo in clostridial cells*[a]

External conc (μM)		Internal conc (nmol/g of packed cells)		Sp act (%)	W:Mo ratio	
Mo	W	Mo	W		Inner	Outer
0.25	0	70	0	100	0.0	0
0.25	1	45	8	68	0.2	4
0.25	10	8	21	32	2.7	40
0.25	100	Traces	150	19	Very high	400

[a] Relative specific activity of 100 corresponds to 110 nmol of C_2H_4 formed/min per mg of protein. The external concentration of W and Mo was determined in the supernatant of the cultures at the beginning of the experiment. Protein and internal concentration were determined after 20 h from pellets washed twice with succinate buffer (0.05 M, pH 5.85) as described.

(Table 3). In contrast, the control cells doubled their nitrogenase content during the same time period. These data corroborate the conclusion that W acts by inhibiting Mo permeation into the cell. In addition W may affect other aspects of Mo metabolism like Mo storage, Mo transport within the cell, or its participation in nitrogenase synthesis.

Antagonistic effects of tungstate on molybdate uptake have been reported for *A. vinelandii* (22) and recently have been confirmed in *C. pasteurianum* (B. Elliott and L. E. Mortenson, unpublished data) using ^{99}Mo.

It has been claimed that tungsten is incorporated into the Mo-Fe protein of *A. vinelandii* (2) and other molybdoenzymes (21, 27, 28, 32). If tungsten was incorporated in clostridial cells, it could provide an additional explanation of its inhibitory effect and a greater understanding of the role of molybdenum in nitrogenase catalysis.

To test this possibility the cells were grown at room temperature in a low concentration of molybdenum and 50 μM tungsten. At mid-exponential phase (after 15 to 20 h) the culture medium was made 100 μM with molybdenum both in the presence and absence of inhibitors of protein synthesis, rifamycin, and chloramphenicol (16, 18). After 4 h of treatment the cells were analyzed for nitrogenase activity and their content of protein, Mo, and W. In the presence of rifamycin the results shown in Fig. 5 were obtained. Similar results also were obtained with chloramphenicol. The data show that the nitrogenase remained constant in the cultures containing rifamycin, whereas in its absence specific activity doubled in the presence of molybdenum. The permeation of molybdenum in the presence of the inhibitor was not affected significantly since 80% of the Mo found in the control could be detected in the treated cells. The tungsten levels within the cells varied according to the outer molybdenum concentration. The obvious interpretation of these results is that an in vivo incorporation of molybdenum into a pre-existing nitrogenase-inactive apoenzyme did not take place, possibly either because W is unable to induce the formation of the apoenzyme or because Mo under the experimental conditions used is not capable of substituting for W firmly bound to the protein.

To rule out the latter alternative, attempts were made to incorporate tungsten into the molybdoprotein and to isolate this protein. In a first set of experiments clostridial cells growing for 2 weeks under argon in a chemostat culture (6 mM in ammonia and 10^{-4} M in Na_2WO_4) were used as an inoculum for a 160-liter culture which was grown under the same conditions but under an atmosphere of N_2. When ammonia was exhausted the cells were harvested and dried under vacuum overnight, and crude extracts and enzymes were obtained as indicated above. Additional cultures were prepared in the same manner except that the cells were allowed to remain under nitrogen, after ammonia exhaustion, for 1, 2, 3, 4, and 8 h to see whether derepression of nitrogenase complex took place. Neither molybdoferredoxin nor azoferredoxin could be detected in crude extracts of cells from any of the above cultures. In addition neither of the two nitrogenase components were isolated or detected by the usual means of purification, nor were EPR signals for molybdoferredoxin and azoferredoxin, which were clearly perceptible in parallel run controls, present in either crude extracts or purified fractions. Tungsten,

TABLE 3. *Time course of W inhibition of nitrogenase in clostridial cells*

Time[a] (h)	Total protein (mg)	Total act (U)	Sp act (units/mg of protein)	Remaining sp act (%)
0	136	16,600	122	100
1	193	18,000	93	76
2.30	237	19,000	80	66
3.30	281	19,400	69	57

[a] Cells were grown in the presence of 0.25 μM molybdate and when they were in the log phase of growth (15 h) the culture was made to 100 μM tungstate. Acetylene reduction activity and protein were measured at the indicated times as described. The total activity and protein of the control culture (0.25 μM in molybdate) at the end of the experiment were 35,460 U and 298 mg, respectively. Units are defined as in the footnote to Table 1.

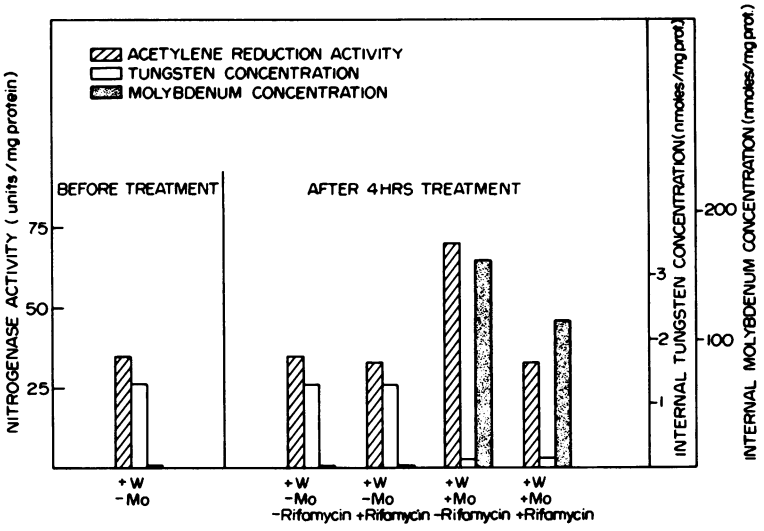

FIG. 5. *In vivo response to added molybdate of nitrogenase from clostridial cells grown on W in the presence and absence of rifamycin. +W = 50 μM; +Mo = 100 μM; R = rifamycin, 50 μg/ml of culture. The control cells were grown on 0.25 μM Mo and 50 μM W. In mid-phase of growth the cells were switched to 100 μM Mo with and without inhibitor. Two parallel controls of the original cells plus and minus rifamycin were run. After 4 h, activities, protein and metals were determined. Similar results were obtained with chloramphenicol (75 μg/ml of culture).*

nevertheless, entered the cells in considerable amount and concentrated to some extent in the early fractions where molybdoferredoxin would be present. The results of a typical experiment are presented in Table 4.

In another series of experiments clostridial cells grown normally under N_2 in a chemostat culture with 10 μM molybdenum were used as inocula for 160-liter cultures whose W:Mo ratios were adjusted to 100:1, 50:1, 25:1 and 10:1. The cells from these cultures were harvested at an absorbance between 2.0 and 4.0, dried, and treated as described. Similar cultures were prepared but supplied with limited amounts of ammonia. When the ammonia became exhausted, derepression of nitrogenase was allowed to occur for 4 h in the presence of the above W:Mo ratios.

During the purification of molybdoferredoxin from cells of all the above cultures, no tungsten was detected in any of the fractions after the first Sephadex G-100 purification step even though the W entered the cells together with molybdenum. The W:Mo ratio changed significantly in the early steps of purification and the W concentration in several early fractions resembled that found in the cells grown in the presence of tungsten alone where no molybdoferredoxin was formed. The results of one of these experiments are shown in Table 5.

It is concluded, therefore, that in *C. pasteurianum* tungsten is not incorporated into molybdoferredoxin in place of Mo. Its inhibition with respect of molybdenum is not exerted by producing an inactive molybdoferredoxin with W instead of Mo.

It is still possible that tungsten is incorpo-

TABLE 4. *Metal distribution during the purification of nitrogenase of clostridial cells derepressed under dinitrogen in the presence of tungsten*[a]

Fraction	Total protein (mg)	Total W (nmol)	/W/ (nmol/mg) of protein)
I. Centrifuged extract	25,500	187,000	7.3
II. 2.5% protamine sulfate supernatant	20,300	42,000	2
III. 12% protamine sulfate precipitate	1,170	13,500	11.5

[a] Cells were grown in a medium containing ammonia and 100 μM Na_2WO_4 and were allowed to "derepress" under N_2 for 4 h after ammonia was exhausted. Dried cells (215 g) were used for purification as described elsewhere (33). Neither molybdoferredoxin nor azoferredoxin activity could be detected during the course of purification. After filtration of fraction III through Sephadex G-100 only ferredoxin was identified, and the W was detected as free tungsten.

TABLE 5. *Metal distribution during purification of molybdoferredoxin from clostridial cells grown under dinitrogen in the presence of Mo and W*[a]

Fraction	Total protein (mg)	Total Mo (nmol)	Total W (nmol)	/Mo/ (nmol/mg of protein)	/W/ (nmol/mg of protein)
I. Centrifuged extract	23,500	11,850	28,000	0.5	1.2
II. 2.5% protamine sulfate supernatant	20,000	11,600	27,700	0.6	1.4
III. 12% protamine sulfate precipitate	2,600	2,400	485	1.0	0.2
IV. Sephadex G-100 filtrate	800	1,150	none	1.4	0.0
V. Eluted fraction from DE-52	172	620	none	3.6	0.0

[a] Cells were grown under the conditions described with a molar ratio W:Mo of 25:1. Dried cells (180 g) were used as starting material for the purification. Other conditions are decribed elsewhere (33).

rated into the molybdoenzyme but that its association with the protein is weak. However the above data indicated that the use of W to provide information on the role of molybdenum in the catalysis by nitrogenase of *C. pasteurianum* is clearly limited.

In contrast to these results, Benemann et al. (2) found in *A. vinelandii* an abnormally high tungsten uptake. In addition the W seemed to be incorporated into an inactive W-containing nitrogenase component. Similarly, W incorporation into nitrate reductase has been reported in *Chlorella* (28) and spinach (27), and very recently Johnson and co-workers succeeded in isolating a substituted W-sulfite oxidase from rat liver (20).

Role of molybdenum in the induction of the synthesis of nitrogenase components. The above results support the idea that molybdenum is an essential catalytic element for N_2 reduction in clostridial cells. However, it is also possible that it is required to induce molybdoferredoxin and azoferredoxin formation. Actually, *A. vinelandii* grown under derepression conditions in the presence of Na_2WO_4 with no added Mo synthesizes inactive molybdoferredoxin and normal levels of azoferredoxin (2, 26), whereas in *Klebsiella pneumoniae* neither nitrogenase component was synthesized in the absence of molybdenum (5).

The nitrogenase activities of clostridial cells grown at varying W:Mo ratios are given in Table 6. As observed, the activity of azoferredoxin always paralleled but was less concentrated than that of molybdoferredoxin. This suggests that in *C. pasteurianum* active molybdoferredoxin is required for azoferredoxin synthesis. An alternative explanation might be that molybdenum is an inducer of both nitrogenase components. However, the formation of azoferredoxin in *Azotobacter* in the absence of Mo (26) or in the presence of W (2) and the order of synthesis of both components in *C. pasteurianum* recently reported (30), strongly imply that in these cells molybdoferredoxin or a molybdenum cofactor is required to initiate the synthesis of their nitrogenase iron component.

TABLE 6. *Content of nitrogenase components at several W:Mo ratios in C. pasteurianum*

System	MoFd activity (U/mg of protein)[a]	AzoFd activity (U/mg of protein)[a]
Cells grown in the presence of W (100 μM) and no Mo added; derepressed under N_2 for 4 h	0	0
Cells grown in the presence of W:Mo ratio of 50[b]	40	16
Cells grown in the presence of Mo (10 μM)	104	89

[a] Molybdoferredoxin (MoFd) and azoferredoxin (AzoFd) activities were determined in crude extracts with the addition of the complementary purified component. One unit of MoFd or AzoFd corresponds to an amount of protein that together with the optimal amount of the other protein reduces 1 nmol of C_2H_2 per min under standard assay conditions (33).

[b] Cells grown in the presence of W:Mo ratios of 10, 25, and 100, like those at 50, exhibited lower AzoFd activities relative to their MoFd activity.

ACKNOWLEDGMENTS

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