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PROPERTIES OF AZOFERREDOXIN PURIFIED FROM NITROGEN-FIXING EXTRACTS OF *CLOSTRIDIUM PASTEURIANUM*

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SUMMARY

Azoferredoxin has been purified 34–35 fold from N_2-fixing extracts of *Clostridium pasteurianum* by three steps of protamine sulfate fractionation and Sephadex G-100 gel filtration. The protein contains two nonheme iron atoms and two acid-labile sulfide groups per molecular weight of 40000. It is decolorized by the sodium salt of *O*-((3-hydroxymercuri-2-methoxypropyl)carbamyl)phenoxyacetic acid (mersalyl) and the effect of mersalyl on its spectrum is similar to the effect of mersalyl on the spectrum of ferredoxin. Azoferredoxin is cold labile and the rate of cold inactivation is decreased in the presence of 10% ethanol or 10% acetone. Exposure of azoferredoxin to O_2 for 5 min causes complete inactivation. The effect of cold inactivation on azoferredoxin is different from that of oxidation as shown by distinct differences in spectral changes, measurable Fe^{2+} content and mercury titratable sulfhydryl and sulfide groups of the protein.

INTRODUCTION

Azoferredoxin is one of the two proteins necessary to catalyze nitrogen or acetylene reduction by *Clostridium pasteurianum*[1,2]. Crude extracts from N_2-fixing cells of *C. pasteurianum* stored at 0–5° lose their ability to catalyze N_2 fixation[3] and acetylene reduction[4] because of inactivation of azoferredoxin[4]. Azoferredoxin is also extremely O_2 sensitive[1]. In this communication a method for preparing azoferredoxin purified 34–35 fold over crude cell extracts is described. Some of the properties of this purified azoferredoxin are reported with particular emphasis on the changes which take place after inactivation by cold storage and by oxidation with O_2.

EXPERIMENTAL

Preparation of crude extracts

Cells of *C. pasteurianum* were grown in a nitrogen-free medium and cell-free extracts were prepared from dried cells[5]. Lysozyme (1 mg/10 ml extracting buffer) was added to the extraction mixture since it increased the yield of soluble protein released.

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Purification of azoferredoxin

All steps in the following purification procedure were performed under strictly anaerobic conditions. The crude extract (2800 ml) was subjected to protamine sulfate fractionation and the fraction obtained between 4 and 15 % protamine sulfate by weight of extract protein was collected and stirred for 90 min with 250 ml 0.05 M Tris–HCl buffer at pH 8.0 containing 18 g phosphocellulose. The mixture was centrifuged (30000 × *g* for 5 min) and the sediment was washed with 150 ml 0.05 M Tris–HCl buffer at pH 8.0. The washings and the supernatant solution were combined and refractionated with protamine sulfate. This time the fraction obtained between 2 and 5 % protamine sulfate by weight of the treated protein was collected, mixed with 60 ml 0.05 M Tris–HCl buffer at pH 8.0 containing 4 g phosphocellulose and stirred for 1 h. The mixture was centrifuged for 5 min at 30000 × *g* and the supernatant solution collected and introduced to an anaerobic column of Sephadex G-100 (5 cm × 48 cm) previously equilibrated with 0.05 M Tris–HCl buffer at pH 8.0. The fractions containing azoferredoxin were combined and refractionated with protamine sulfate. The fraction obtained between 3.5 and 6.5 % protamine sulfate by weight of treated protein was collected and this time the precipitate was stirred for 15 min with 10 ml of Tris–HCl buffer (0.05 M, pH 8.0) containing 0.45 g of phosphocellulose. The mixture again was centrifuged and the supernatant solution containing azoferredoxin was frozen as pellets in liquid N_2 (refs. 6, 7) and stored in liquid nitrogen. The protein during the last step of protamine sulfate fractionation was extremely O_2 sensitive and all manipulations were carried out under an atmosphere of H_2.

Determination of azoferredoxin activity

Assay for azoferredoxin was performed by a previously published method[4]. Reaction mixture (a total volume of 2 ml) contained 115 μmoles potassium cacodylate buffer (pH 6.8), 10 μmoles $MgCl_2$, 4 μmoles ATP, 97 μmoles acetyl phosphate, 1 ml azoferredoxin-free extract (25–35 mg protein) and the appropriate amount of azoferredoxin fraction to be assayed for activity. The final pH was 6.8. Excess ATP: acetate phosphotransferase (EC 2.7.2.1) needed to generate ATP from acetyl phosphate, and H_2:ferredoxin oxidoreductase (EC 1.98.1.1) (hydrogenase) were present in the azoferredoxin-free extract used. The gas phase was 0.85 atm hydrogen and 0.15 atm acetylene. The rate of reaction was measured by estimating the amount of acetylene reduced to ethylene by gas chromatography[4,8] and expressed as nmoles ethylene produced per min per mg protein in the azoferredoxin fraction.

Determination of iron

The iron content of azoferredoxin was estimated from the atomic absorption at 372 nm measured with a Perkin–Elmer 303 spectrophotometer. All analyses at proper dilutions were made in the same buffer system since when different buffers were used different standards were needed for each buffer.

Determination of acid-labile sulfide

A modification of the method of FOGO AND POPOWSKY[9] as described by JENG AND MORTENSON[10] was used.

Determination of sulfhydryl plus sulfide content

The method developed by BOYER[11] was used. *p*-Hydroxymercuribenzoate (112 nmoles in 3 ml of 0.05 M phosphate buffer at pH 7.0) was rendered anaerobic in a Thunberg-type cuvette fitted with a rubber serum stopper. The *p*-hydroxymercuribenzoate was titrated with azoferredoxin which was added through the rubber stopper

from a gas tight syringe. Absorbance at 250 nm was read after each addition of azoferredoxin against a blank containing phosphate buffer alone to which the same amount of azoferredoxin was added.

Spectra

A Cary-14 recording spectrophotometer was used to measure all spectra.

RESULTS

Purity of azoferredoxin

Azoferredoxin prepared by the method described here is purified 34–35 fold over the crude extract (Table I). As the protein is extremely O_2 sensitive, it was difficult to determine the exact purity. From ultracentrifugation and disc-gel electrophoresis studies we found it contains one major protein component and a minor component; the purity was estimated to be 90–95 %.

TABLE I

PURIFICATION OF AZOFERREDOXIN FROM N_2-FIXING EXTRACTS OF *C. pasteurianum*

Determination of azoferredoxin activity was as described in the text.

Fraction	*Volume (ml)*	*Protein (mg/ml)*	*Total protein (g)*	*Spec. act. (nmoles ethylene per min per mg)*	*Purity*
Crude extract	2800	25.4	71.0	78.7	1
1st protamine sulfate	688	25.0	14.4	226.6	2.8
2nd protamine sulfate	34	65.0	2.2	846.1	10.7
Sephadex G-100	125	13.5	1.69	940.3	11.9
3rd protamine sulfate	9	60.0	0.54	2708.0	34.4

Molecular weight

The molecular weight of azoferredoxin was found to be in the order of 40000 (36000) as estimated by a Spinco Model-E ultracentrifuge fitted with absorption optics[12].

Iron and acid-labile sulfide

The number of iron atoms and sulfide groups per molecule of azoferredoxin (40000 molecular weight) are both 2 (Table II).

TABLE II

IRON AND ACID-LABILE SULFIDE IN AZOFERREDOXIN

*Protein (μmole/ml)**	*Iron (μmole/ml)*	*Acid-labile sulfide (μmole/ml)*
0.7	1.41	1.35
0.7	1.39	1.37

* Molecular weight of azoferredoxin = 40000 (assuming 95 % purity).

Effect of sodium salt of O-((3-hydroxymercuri-2-methoxypropyl)carbamyl)phenoxyacetic acid (mersalyl)

Azoferredoxin in solution is yellow-brown. Mersalyl at a concentration greater than that required to combine with the free –SH groups and sulfide of azoferredoxin causes complete decolorization which results in the disappearance of light absorption at 375 nm and also a considerable decrease in light absorption in the ultraviolet region (260–280 nm) as shown in Fig. 1. The small absorption noted at 290–300 nm is probably due to the mersalyl bound to the protein. The addition of quantities of mersalyl smaller than that required to completely decolorize azoferredoxin caused a gradual decrease in light absorption at 375 nm and at 260–280 nm (Fig. 1). The effect of mersalyl on the spectrum of azoferredoxin as shown in Fig. 1 is similar to the effect of mersalyl on the spectrum of ferredoxin reported by LOVENBERG, BUCHANAN AND RABINOWITZ[13].

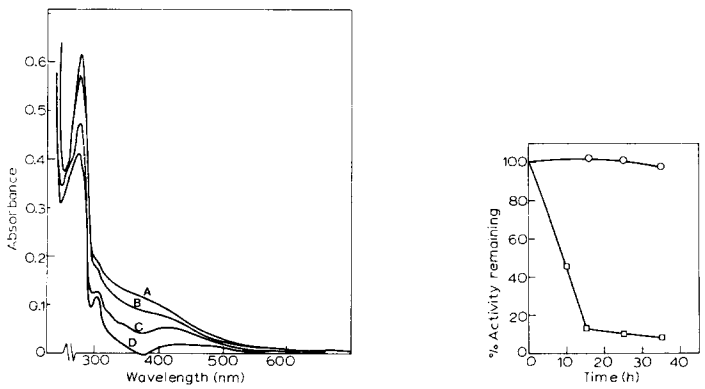

Fig. 1. Effect of mersalyl on the spectrum of azoferredoxin. 2.7 mg azoferredoxin in 0.05 ml was added anaerobically through a syringe to 2.95 ml 0.05 M Tris–HCl buffer (pH 8.0) which had previously been rendered anaerobic in a Thunberg-type cuvette fitted with a rubber serum stopper. The spectrum was read against a blank cuvette containing 3 ml of buffer alone. The spectrum was then read after each addition of mersalyl (0.01 ml containing 0.2 μmole mersalyl) to both blank and experimental cuvettes. A, spectrum without mersalyl; B, spectrum after adding 0.2 μmole mersalyl; C, spectrum after adding a total of 0.4 μmole mersalyl; D, spectrum after adding a total of 0.6 μmole mersalyl.

Fig. 2. Effect of storage temperature on the activity of azoferredoxin. Experimental conditions were the same as described in Table III. ○—○, stored at room temperature; □—□, stored at 0–1°.

Stability

Purified azoferredoxin does not lose activity when stored as pellets in liquid N_2 (refs. 6, 7) for up to 8 weeks. When it was stored under H_2 at room temperature in a container which in turn was placed in an anaerobic jar filled with H_2, it lost less than 5% of its activity over a period of 35 h (Fig. 2). In contrast, if azoferredoxin was stored anaerobically but at 0–1°, a rapid loss of activity resulted; after 15 h at 0–1°, 86% of the azoferredoxin activity was lost (Fig. 2). The rate of cold inactivation of azoferredoxin was decreased by adding 10% alcohol or acetone (Table III).

Effect of oxidation by O_2

When azoferredoxin at room temperature was exposed for 5 min to O_2 a complete and irreversible inactivation resulted. This was accompanied by a change in

TABLE III

EFFECT OF ETHANOL AND ACETONE ON STABILITY OF AZOFERREDOXIN DURING COLD STORAGE

In each of 12 tubes (10 ml capacity) 2 ml azoferredoxin (10 mg/ml) were stored anaerobically at the designated temperature in the presence or absence of 10% ethanol or acetone. Each of the tubes was used for one assay only to avoid any possibility of exposing azoferredoxin to O_2. When tested for azoferredoxin activity, 0.05 ml was transferred anaerobically by syringe to a reaction flask (see EXPERIMENTAL).

*Storage time** (*h*)	*Azoferredoxin activity (nmoles ethylene/min)*			
	Room temp. (20°)	0–1°	0–1° (+ 10% *acetone*)	0–1° (+10% *ethanol*)
0	760	763	758	765
8	740	560	740	620
24	600	20	540	540

* In a separate experiment azoferredoxin was frozen as pellets in liquid N_2 and stored in liquid N_2 and little activity was lost after 8 weeks.

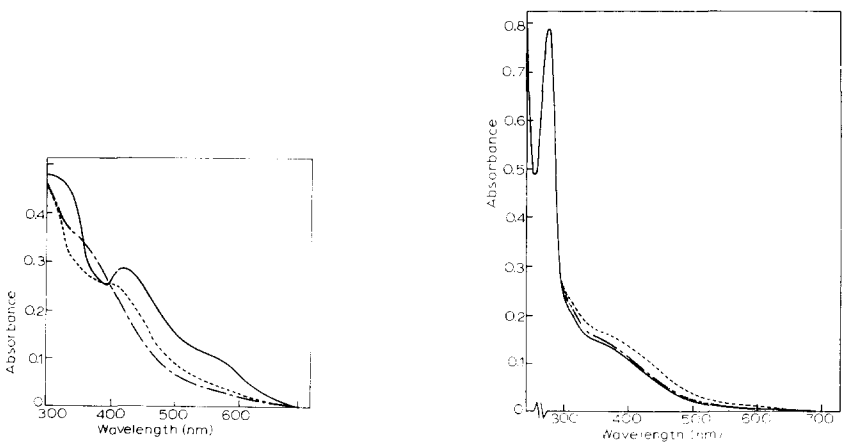

Fig. 3. Effect of oxidation on the spectrum of azoferredoxin. 5.4 mg in 0.1 ml azoferredoxin were added anaerobically through a syringe to 2.9 ml 0.05 M Tris–HCl buffer (pH 8.0) which was previously rendered anaerobic in a Thunberg-type cuvette fitted with a rubber serum stopper. The spectrum was then read against a blank containing 3 ml buffer alone. After oxidation for 5 min the spectrum of azoferredoxin was obtained before and after adding a hydrogenase system composed of H_2, 50 μg of H_2:ferredoxin oxidoreductase and 30 μg ferredoxin to both blank and experimental cuvettes. –·—·–, spectrum of active azoferredoxin (untreated); ———, spectrum of azoferredoxin after 5 min exposure to O_2; -------, spectrum of oxidized azoferredoxin after adding the hydrogenase system.

Fig. 4. Effect of $Na_2S_2O_4$ on the spectrum of azoferredoxin. 3.0 mg azoferredoxin were added to a cuvette under the same experimental conditions as described in Fig. 1. $Na_2S_2O_4$ (10 μg in 0.01 ml) was added to both the blank (buffer alone) and the experimental cuvette. -------, spectrum of active azoferredoxin (untreated); –·—·–, spectrum of azoferredoxin + $Na_2S_2O_4$ (10 μg); ———, spectrum of azoferredoxin + $Na_2S_2O_4$ (20 μg).

the spectrum of the protein as shown in Fig. 3. The spectrum of the freshly isolated azoferredoxin had a narrow shoulder at 310 nm and a broad one at 325–375 nm. Exposure to O_2 for 5 min caused decrease in light absorption at 375 nm, an increase

in light absorption at 312 nm and an appearance of a peak at 410 nm. When azoferredoxin which had been exposed to O_2 was incubated under H_2 for 10 min with a H_2:ferredoxin oxidoreductase system (hydrogenase) composed of H_2, partially purified hydrogenase and ferredoxin from *C. pasteurianum*, no reactivation of azoferredoxin was observed but there was a change in the spectrum (Fig. 3). A decrease in light absorption at 310 nm and at 400–600 nm occurred but the loss in light absorption at 375 nm which resulted from oxidation by O_2 was not restored. This fact together with the fact that azoferredoxin activity was not restored when the hydrogenase system was added, suggested that the part of the structure of azoferredoxin responsible for the light absorption at 375 nm is at the active center of the protein. Since the absorption at 375 nm is also lost when the iron sulfide is lost or removed, it is probable that the part of the protein involved in binding of iron sulfide is also at the active site of azoferredoxin.

Reduction

The spectrum of active azoferredoxin (not exposed to O_2) did not change when a hydrogenase system composed of H_2, hydrogenase and ferredoxin was added. Since reduced ferredoxin acts as the electron donor in the N_2-fixing system[5,14], the absence of spectral change when the hydrogenase system was added to azoferredoxin suggested the following possibilities: (1) azoferredoxin is not the component that becomes reduced during N_2 fixation, (2) only when azoferredoxin and molybdoferredoxin are combined is the system reduced, (3) ATP involvement is necessary before reduction can occur, (4) azoferredoxin is already reduced or (5) reduction of the site on azoferredoxin involved in N_2 fixation does not change the spectrum.

Treatment of azoferredoxin with $Na_2S_2O_4$ did result in a spectral change (Fig. 4). The reduction of one molecule of azoferredoxin required one molecule of $Na_2S_2O_4$ suggesting that azoferredoxin is a 2-electron acceptor. However, since both reduced ferredoxin and $Na_2S_2O_4$ can serve as the electron donor in N_2 reduction, it is possible that the spectral changes observed in Fig. 4 might not reflect reduction of the part of azoferredoxin involved in N_2 fixation. It is also possible that azoferredoxin was not reduced by the hydrogenase system because of the low concentrations of hydrogenase and ferredoxin that could be used in the spectrophotometric analysis.

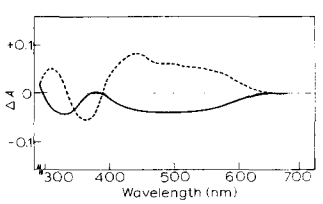

Fig. 5. Effect of cold storage and oxidation on the spectrum of azoferredoxin. 5.4 mg (in 0.1 ml) of either oxidized (5 min exposure to O_2) or cold inactivated azoferredoxin were added anaerobically through a syringe to 2.9 ml 0.05 M Tris–HCl buffer (pH 8.0) which had previously been rendered anaerobic in a Thunberg-type cuvette fitted with a rubber serum stopper. The difference spectrum of both oxidized and cold inactivated azoferredoxin *versus* untreated azoferredoxin was obtained by measurement against a cuvette containing 5.4 mg (in 0.1 ml) of active (untreated) azoferredoxin *plus* 2.9 ml buffer. -------, difference spectrum of oxidized azoferredoxin read against active azoferredoxin (untreated); ———, difference spectrum of cold inactivated azoferredoxin read against active (untreated) azoferredoxin.

Nature of cold lability

The changes that occur when azoferredoxin is subjected to low temperatures are distinct from those of oxidation by O_2. Fig. 5 shows the difference spectra of both cold inactivated and oxidized azoferredoxin obtained by reading each against a control containing untreated azoferredoxin. Whereas oxidation caused an increase in light absorption at 400–600 nm and at 310 nm, cold inactivation caused a decrease in light absorption in both these areas of the spectrum. The fact that cold treatment of azoferredoxin results in a different change from oxidation is also reflected in the ability of the iron in azoferredoxin to complex with α,α′-dipyridyl (Table IV). Oxidation decreased the total iron of azoferredoxin that could complex with α,α′-dipyridyl

TABLE IV

REACTION OF AZOFERREDOXIN WITH α,α′-DIPYRIDYL

5.4 mg azoferredoxin in 0.1 ml were added anaerobically through a syringe to 2.9 ml of 0.05 M Tris–HCl buffer (pH 8.0) which has previously been rendered anaerobic in a Thunberg-type cuvette fitted with a serum rubber stopper. 0.01 ml α,α′-dipyridyl (2 mg) was added to the experimental cuvette only and after 2 min the *A* at 550 nm was read against a blank containing azoferredoxin but no α,α′-dipyridyl. Next 0.01 ml mersalyl (5 mg) was added to both the blank and experimental cuvettes and readings again were made. Where indicated 0.01 ml (10 μg) $Na_2S_2O_4$ was added to both the blank and experimental cuvettes. Under the conditions of the experiment the extinction coefficient for ferrous dipyridyl was 6980.

Treatment	*Absorbance at 550 nm*		*Measurable Fe** per azoferredoxin (μmole/μmole)*	
	− Mersalyl	*+ Mersalyl*	*− Mersalyl*	*+ Mersalyl*
None	0.16	0.56, 0.67*	0.5	1.8, 2.1*
Oxidized	0.19	0.28	0.6	0.9
Cold inactivated	0.26, 0.30**	0.52, 0.60*	0.8, 0.95**	1.6, 1.9*

* *A* or Fe^{2+} after addition of $S_2O_4^{2-}$.
** *A* or Fe^{2+} after 10 min in absence of mersalyl.

in the presence of excess mersalyl whereas the total iron reactive with α,α′-dipyridyl in the cold inactivated azoferredoxin was the same as that in the untreated azoferredoxin (Table IV). The rate at which iron complexed with α,α′-dipyridyl in the absence of mersalyl was greater in the cold inactivated azoferredoxin than that in the untreated azoferredoxin. This could mean that the change in the structure of azoferredoxin which caused its inactivation during cold storage rendered the 'ferrous' iron more available for complexing with α,α′-dipyridyl. The data (Table IV) also suggest that of the two iron atoms in azoferredoxin, one is more accessible to α,α′-dipyridyl than the other. In other words, the environment of the two iron atoms of azoferredoxin is not the same.

Of the two treatments, oxidation and cold inactivation, only oxidation affects the amount of mercury titratable sulfhydryl and sulfide groups in azoferredoxin. Fig. 5 shows the titration of a fixed amount of *p*-hydroxymercuribenzoate (112 nmoles) by azoferredoxin at pH 7.00. Untreated azoferredoxin contains 112 nmoles –SH (R–SH + S^{2-}) per 9 nmoles or 8 free –SH groups and 2 sulfide groups per molecule (mol. wt. 40000). When azoferredoxin was inactivated by cold storage, the combined R–SH and sulfide content remained the same as that in the untreated azoferredoxin

(Fig. 5), *i.e.*, 8 R–SH and two S^{2-} groups per molecule. However, when azoferredoxin was inactivated by O_2 treatment for 5 min, the combined R–SH and sulfide content decreased to 112 nmoles of –SH equivalent per 14 nmoles of azoferredoxin (Fig. 6). This meant that the azoferredoxin after exposure to O_2 for 5 min contained the equivalent of 8 –SH groups per 40000 molecular weight. Exposure to O_2 for longer

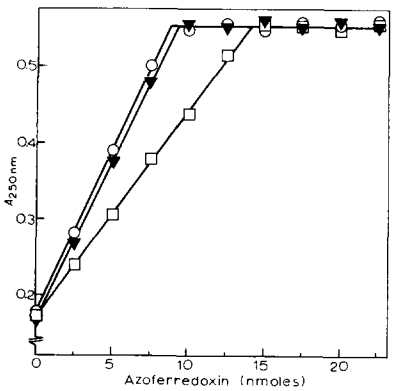

Fig. 6. Titration of *p*-hydroxymercuribenzoate with active, oxidized and cold inactivated azoferredoxin. 112 nmoles of *p*-hydroxymercuribenzoate in 3 ml of 0.05 M phosphate buffer (pH 7.0) were rendered anaerobic in a Thunberg-type cuvette fitted with a rubber serum stopper. The absorbance was read at 250 nm against a blank cuvette containing 3 ml of the same buffer and after each addition of 2.5 nmoles of the azoferredoxin sample to both the blank and experimental cuvettes. O—O, active azoferredoxin (untreated); ▲—▲, cold inactivated azoferredoxin; □—□, azoferredoxin oxidized for 5 min.

periods than 5 min (up to 15 min) caused no further increase in mercury titratable groups. The difference of 4 nmoles of –SH per azoferredoxin could account for either the complete oxidation of its two sulfide groups, the oxidation of one sulfide group and two –SH groups or the oxidation of four –SH groups. When the acid-labile sulfide content of untreated azoferredoxin, cold inactivated azoferredoxin and oxidized azoferredoxin, was determined, the results (Table V) showed that only half the sulfide content of azoferredoxin was lost as a result of 5 min exposure to O_2. These results together with those of Fig. 5 suggest that one sulfide group and two –SH groups of azoferredoxin are oxidized during the 5 min exposure to O_2. Since all activity of azoferredoxin is lost during contact with O_2, the data suggest that the oxidized groups are either at the active center of azoferredoxin or influence the active center possibly by causing a change in the structure of the protein.

TABLE V

EFFECT OF OXIDATION AND COLD STORAGE ON ACID-LABILE SULFIDE OF AZOFERREDOXIN

Treatment	*Protein* (*μmole/ml*)	*Acid-labile sulfide* (*μmole/ml*)	*Sulfide/azoferredoxin* (*μmole/μmole*)
None	0.69	1.35	2
Oxidized for 5 min	0.69	0.67	1
Cold inactivated	0.69	1.29	2

DISCUSSION

The properties of azoferredoxin, (1) its iron sulfide content which is lost on acid treatment, (2) its decolorization by mersalyl and (3) its loss of absorption between 300 and 600 nm on reduction with $S_2O_4^{2-}$, suggest that it is a ferredoxin-type protein.

One property which may reveal information on its structure and function, is its inactivation at low temperatures. The rate of inactivation increased as the storage temperature decreased below 10°. This cold inactivation occurred when azoferredoxin was either in the purified state or in the presence of other components of the N_2-fixing system[4] and the rate of inactivation is decreased in the presence of 10% ethanol or acetone. Other proteins have been found to be inactivated by exposure to low temperatures[16–20] and the low temperature inactivation of several of these proteins has been counteracted or prevented by the addition of alcohols. Our protein, azoferredoxin, behaves like chymotrypsin in that it becomes increasingly less stable when the temperature of storage is lowered from 10 to 0° and the low temperature instability is "counteracted" by the addition of 10% ethanol[21]. A precise chemical or physical explanation for these effects is not available. One could explain the cold lability by saying that hydrophobic bonding and consequently the structural stability of azoferredoxin decreased with decreasing temperatures and that azoferredoxin with this changed structure then aggregated irreversibly (under our conditions) so that inactive azoferredoxin with a changed spectrum resulted. One could then postulate that ethanol prevented the aggregation possibly by enhancing hydrogen-bonding or by preventing hydrophobic bonding between molecules of azoferredoxin that were structurally changed by the low temperature. Since exposed non-polar groups form clathrate structures at low temperatures, it is also possible that the aggregation or change in structure of azoferredoxin (denaturation) depends on clathrate formation and that alcohol prevents inactivation by preventing its formation.

Cold inactivation of azoferredoxin is distinct from oxidation of azoferredoxin as shown by differences in spectrum, in the measurable state of iron in the protein and in the mercury titratable sulfhydryl and sulfide content of azoferredoxin under the two conditions. In addition the rate of cold inactivation is decreased in the presence of ethanol or acetone whereas these solvents have no effect on oxidation.

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