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Subunit Structure of Azoferredoxin from *Clostridium pasteurianum* W5*

George Nakos and Leonard Mortenson†

ABSTRACT: Azoferredoxin from *Clostridium pasteurianum* W5 has a molecular weight of 55,000 ± 5000 as determined by gel filtration. Treatment of azoferredoxin with sodium dodecyl sulfate results in the dissociation of the "native" enzyme into two subunits of mol wt 27,500 ± 1350. The subunits are identical in size and they appear to be chemically similar. Each dimer contains 4 iron atoms and about 4 acid-labile sulfide groups.

Azoferredoxin is one of the two proteins required by *Clostridium pasteurianum* to catalyze nitrogen fixation (Mortenson *et al.*, 1967). Moustafa and Mortenson (1969) reported that azoferredoxin of 90–95% purity had an estimated molecular weight of 40,000 and 2 iron and 2 acid-labile sulfide atoms per molecule.

This paper presents evidence that azoferredoxin exists in solution as a dimer of two polypeptide chains of identical molecular weights and that the molecular weight of the dimer is 55,000 and contains 4 iron and 4 acid-labile sulfide atoms per molecule.

Materials and Methods

Chemicals. The chemicals used were obtained: from Eastman Organic Chemicals, acrylamide and *N,N'*-methylbisacrylamide; from Sargent Co., *N,N,N',N'*-tetramethylethylenediamide; from Fisher Scientific Co., sodium dodecyl sulfate and bromophenol blue; from Mann Research Laboratories, comassie brilliant blue R-250, from Pharmacia, Sephadex G-200 and Blue Dextran 2000. All other reagents were commercially obtained and of the best available grade.

* From the Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907. *Received September 14, 1970.* This research was supported by Grant AI 04865-08 of the National Institutes of Health.

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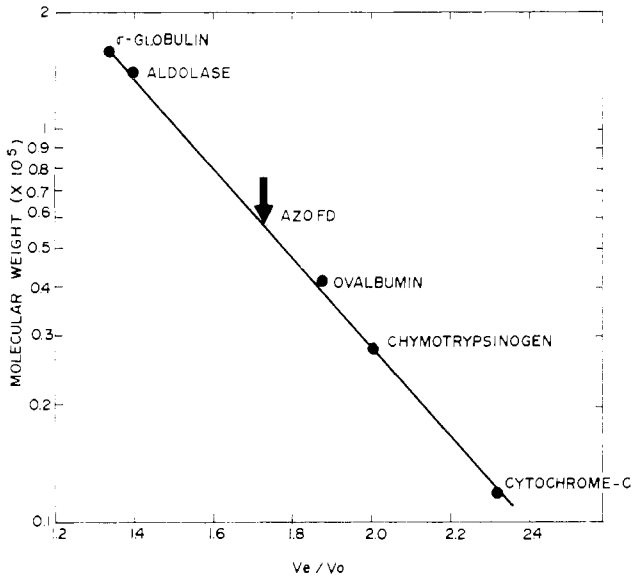

FIGURE 1: Molecular weight estimation of "native" azoferrodoxin by Sephadex G-200 filtration. The solid circles indicate the position of peak midpoints for proteins of known molecular weights. The arrow indicates the position of azoferrodoxin. Each protein peak was found in 5–7 tubes. The column flow rate was 3.2 ml/hr. Fractions of 1.6 ml were collected by use of a fraction collector.

TABLE I: Molecular Weights of the Native Protein Markers and Their Polypeptide Chains (from Weber and Osborn, 1969).

Proteins	Molecular Weights: Native Protein	Molecular Weights: Polypeptide Chain
Aldolase	140,000	40,000
Catalase	240,000	60,000
γ-Globulin	160,000	50,000[a]
		23,500[b]
Fumarase	220,000	49,000
Ovalbumin	43,000	43,000
Glyceraldehyde phosphate dehydrogenase	140,000	36,000
Chymotrypsinogen	25,700	25,700
Myoglobin	17,200	17,200
Cytochrome *c*	11,700	11,700

[a] H chain. [b] L chain.

Azoferredoxin Samples. Cells of *C. pasteurianum* were grown using N_2 as the sole nitrogen source and cell-free extracts were prepared from dried cells by the method of Mortenson (1964). Azoferredoxin purified according to Moustafa and Mortenson (1969) was kindly supplied by Dr. T. Devanathan of our laboratories. ^{35}S-Labeled azoferredoxin was prepared by the same purification method except that cells were grown with [^{35}S]Na_2SO_4 in the culture medium.

Estimation of Molecular Weight of Azoferredoxin. Gel filtration was performed anaerobically on a 120 × 1.5 cm column of Sephadex G-200, according to Andrews (1965), and the proteins were eluted with 0.05 M Tris-HCl buffer, pH 8. Proteins of known molecular weights were purchased from Sigma Chemical Company: aldolase (rabbit muscle No. A-6253), α-chymotrypsinogen A (bovine pancreas), cytochrome *c* (horse heart, type VI), γ-globulins (bovine, Cohn fraction II, stock BG-II), and ovalbumin (grade V). The void volume (V_0) of the column was determined by measuring the volume required for Blue Dextran 2000 to pass through the column (peak tube).

Sodium Dodecyl Sulfate–Polyacrylamide Disc Electrophoresis. The method of Shapiro *et al.* (1967) was used. Additional protein markers were obtained: from Sigma Chemical Company, catalase (beef liver, stock C-100); from Calbiochem, glyceraldehyde phosphate dehydrogenase (rabbit muscle, lot 000440, A grade); from Boehringer Mannheim, fumarase (pig heart). The preparation of the protein solutions, the acrylamide gels, and the electrophoresis buffer, and the staining and destaining of the gels were performed as described by Weber and Osborn (1969). The glass columns used were 6.5 cm long with an internal diameter of 0.5 cm.

The radioactivity of ^{35}S-labeled azoferredoxin bands, after slicing the gels, was measured in a liquid scintillation counter (Beckman Model LS-150). For this purpose the gel slices were transferred into scintillation vials containing 7.0 ml of scintillation fluid (5.0 g of 2,5-diphenyloxazole and 50.0 g of naphthalene per l. of 1,4-dioxane). For a measure of the band density, gels were scanned in a microdensitometer (Joyce, Loeble & Co. Ltd, England).

Iron and Acid-Labile Sulfides. Iron was determined by the *o*-phenanthroline method by first heating the protein sample in 1.0% HCl for 10 min (Lovenberg *et al.*, 1963). Ferrous ammonium sulfate was used as the iron standard. The molar absorptivity of the ferroin complex was equal to 11,100. Measurement of iron by atomic absorption spectrophotometry (Perkin-Elmer Model 303) was used to follow losses of iron during dialysis of the various treatments of azoferredoxin.

Acid-labile sulfide was determined by a modification of the method of Fogo and Popowsky (1949) as described by Brumby *et al.* (1965). Ferredoxin was used as a standard. Its 390/280 ratio was 0.80 and it was assumed to have 7 acid-labile sulfide groups (Jeng and Mortenson, 1968).

Protein was determined by the method of Lowry *et al.* (1951). A factor of 0.70 was used to correct colorimetric protein determinations to dry weight values in the case of ferredoxin (Sobel and Lovenberg, 1966).

Results and Discussion

For the molecular weight estimation on Sephadex G-200 5–7 mg of each protein marker and of azoferredoxin was each dissolved in 0.5–1.0 ml of 0.05 M Tris-HCl (pH 8). The protein solutions were applied to the column one at a time with a time lapse between each protein to avoid protein–protein interactions. Such interactions were observed when all the protein markers and azoferredoxin were mixed and applied as a whole to the column. This leads to overlapping protein peaks with a resulting distortion of the elution volume (V_e) of each protein.

The molecular weights of the native proteins and their polypeptide chains used in this study are given in Table I. From several standard curves such as that of Figure 1 and from the elution volume of azoferredoxin the molecular

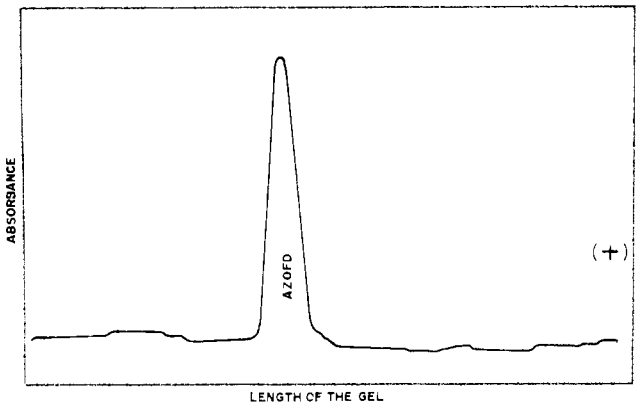

FIGURE 2: A microdensitometer trace of the results of sodium dodecyl sulfate–polyacrylamide electrophoresis of azoferredoxin treated either with sodium dodecyl sulfate or sodium dodecyl sulfate plus mercaptoethanol and dialyzed for varying periods of time.

weight was estimated at 55,000 ± 5000. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to analyze azoferredoxin for the present of subunits.

In the sodium dodecyl sulfate–polyacrylamide electrophoresis four different azoferredoxin treatments were employed with three dialysis periods for each treatment.

(a) Pretreatment of azoferredoxin (1 mg/ml) with 1.0% sodium dodecyl sulfate plus 1.0% mercaptoethanol for 2 hr followed by dialysis against 0.1% sodium dodecyl sulfate plus 0.1% mercaptoethanol for 2, 3, and 12 hr before electrophoresis.

(b) As in a but mercaptoethanol was left out in both steps.

(c) Direct dialysis against 0.1% sodium dodecyl sulfate plus 0.1% mercaptoethanol for 2, 4, and 16 hr.

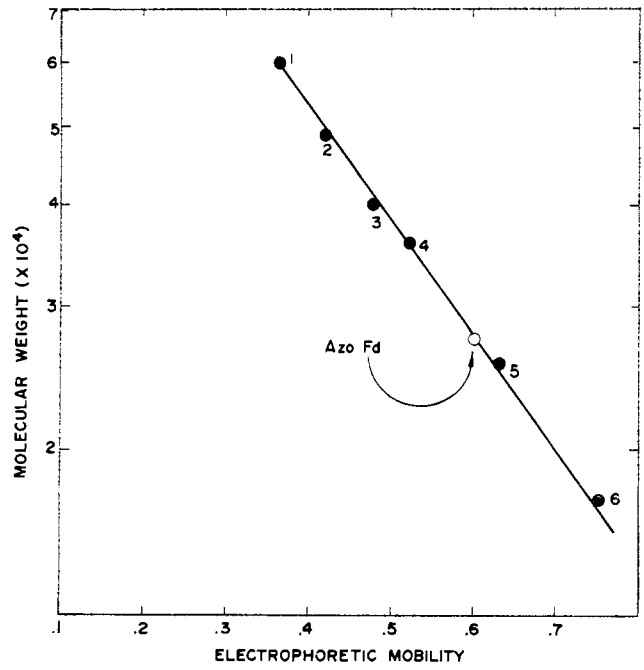

FIGURE 3: Molecular weight estimation of the polypeptide chain of azoferrodoxin from a set of 4 standard curves. The marker proteins were: 1, catalase; 2, fumarase; 3, aldolase; 4, glyceraldehyde phosphate dehydrogenase; 5, α-chymotrypsinogen A; and 6, myoglobin. (○) indicates position of azoferrodoxin.

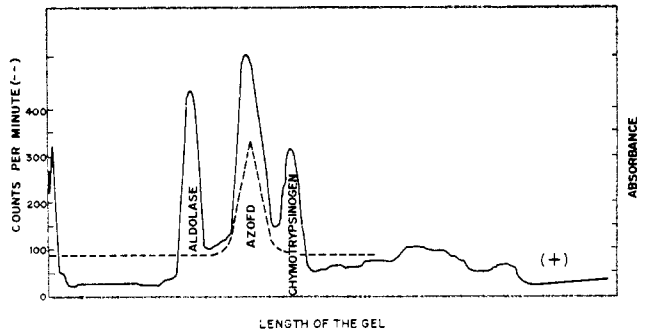

FIGURE 4: Relative position of an ^{35}S-labeled azoferrodoxin sample, from [^{35}S]Na_2SO_4 grown cells of *C. pasteurianum* with respect to aldolase (mol wt 40,000) and α-chymotrypsinogen A (mol wt 25,700) run on the same sodium dodecyl sulfate–polyacrylamide gel.

(d) As in c but mercaptoethanol was left out.

Regardless of treatment or dialysis time azoferredoxin samples gave only a single band when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2).

From the mobilities of about 40 azoferredoxin samples and with a standard curve (Figure 3) for each run of 24 gel columns, the azoferredoxin bands were found to have a molecular weight of 27,500 ± 1350 (99% confidence limit). This was confirmed (Figure 4) by running ^{35}S-labeled azoferredoxin on the same gel column as aldolase (subunit mol wt 40,000) and α-chymotrypsinogen mol wt 25,700). The results of this experiment excluded the possibility of azoferredoxin being a monomer of a molecular weight of 40,000 or a dimer with a subunit molecular weight of 20,000 and suggest that previous estimations were in error because the conditions in the ultracentrifugation run were such that a mixture of the monomer and dimer were present (possibly because of oxidation).

Correlation of the sodium dodecyl sulfate–polyacrylamide findings with the results from gel filtration lead us to the conclusion that azoferredoxin is a dimer with two monomers each with a molecular weight of 27,500 ± 1350. Preliminary studies to determine the amino acid sequence of azoferredoxin showed that the subunits have identical C- and N-terminal

TABLE II: Amount of Iron Lost During Dialysis of Azoferredoxin against 0.1% Sodium Dodecyl Sulfate or 0.1% Sodium Dodecyl Sulfate Plus 0.1% Mercaptoethanol.

Dialysis Time (hr)	Treatment	
	Sodium Dodecyl Sulfate (mμmoles of Fe/ml)	Sodium Dodecyl Sulfate + Mercaptoethanol (mμmoles of Fe/ml)
0	0	0
2	200	220
4	320	250
6	325[a]	270[b]

[a] Iron concentration at zero dialysis time equal to 350 mμmoles/ml (4.5 mg of azoferrodoxin/ml). [b] Iron concentration at zero dialysis time equal to 270 mμmoles/ml (3.5 mg of azoferrodoxin/ml).

TABLE III: Molecular Weight, Number of Polypeptide Chains, Iron and Acid-Labile Sulfides of Azoferredoxin.

Molecular weight	55,000
Polypeptide chains	2 (27,500 each)
Fe atoms/molecule (dimer)	4.05[a]
S^{2-} atoms/molecule (dimer)	4.0[b]

[a] A sample of azoferredoxin containing 1.5 mg/ml of protein (27.8 mμmoles/ml, mol wt 55,000) was found to have 112.5 mμmoles of iron per ml. [b] A sample of azoferredoxin (850 mμmoles/ml) contained 3400 mμmoles/ml of acid-labile sulfides when compared to ferredoxin (7 sulfide groups per molecule) as a standard.

amino acids (K. M. Dus and L. E. Mortenson, unpublished) and cyanogen bromide peptides (J. Chen and L. E. Mortenson, unpublished). Therefore, it is likely that the monomers are also chemically identical.

The iron and sulfide of azoferredoxin are removed by sodium dodecyl sulfate treatment followed by dialysis (Table II). Whether in sodium dodecyl sulfate azoferredoxin dissociates into monomers because of the removal of the iron and sulfide or whether the iron and sulfide are released for the same reason the monomers dissociate is not yet known. We are presently trying to establish whether azoferredoxin can dissociate into monomers that still contain iron and sulfide.

The number of iron atoms and sulfide groups per azoferredoxin dimer (mol wt 55,000) is four each (Table III). If the subunits are chemically identical each subunit would contain two iron atoms and two sulfide groups.

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Effect of Alcohols on the Rate of Autoxidation of Ferrocytochrome *c**

Laurence S. Kaminsky, Rodney L. Wright, and Allan J. Davison†

ABSTRACT: The effects of a homologous series of alcohols on the rate of autoxidation of ferrocytochrome *c* was studied in aqueous solution at pH 5 with the following results. The reaction was invariably first order with respect to ferrocytochrome *c*. Low concentrations of alcohol decreased the first-order rate constant, while higher concentrations produced a marked rate enhancement. Increasing the hydrocarbon chain length of the alcohols increased their effectiveness in enhancing autoxidation, while branching in the chain decreased their effectiveness. The order of effectiveness of the various alcohols in enhancing autoxidation was the same as their order of effectiveness in producing a conformational change exposing the heme of ferricytochrome *c*, suggesting that it is their ability to produce a similar conformational change in ferrocytochrome *c* which produces the enhanced reactivity toward oxygen. Greater concentrations of alcohol are required to produce an effect on ferrocytochrome *c*, no doubt because its native conformation is more stable. At higher temperatures, autoxidation was initiated by lower alcohol concentrations. The Arrhenius activation energy was calculated for autoxidation at high and at low alcohol concentrations and was found to be increased from 15 kcal/mole to 48 kcal/mole by increasing solute concentration sufficiently to induce rapid autoxidation. Evidently the autoxidation becomes permissible because the entropy of activation of some rate-determining process becomes more favorable to the extent of over 100 eu. This may reflect the fact that in alcohol mixtures exposure of hydrophobic groups in the heme crevice is less opposed by entropy considerations than in pure aqueous solution.

Knowledge of the various factors contributing to the stability of proteins is important for the determination of their structures in solution. In earlier papers we described the investigation of the effects of a series of organic solvents on ferricytochrome *c* (Kaminsky and Davison, 1969a,b). These studies provided qualitative evidence for a conforma-

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