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The interaction of ferredoxin-linked sulfite reductase with ferredoxin

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Spinach sulfite reductase has been shown to co-migrate during gel filtration chromatography at low ionic strength with spinach ferredoxin. No co-migration was observed at high ionic strength. These results indicate that the two proteins form a high-affinity, electrostatically stabilized complex, as had previously been demonstrated for three other ferredoxin-dependent, plant enzymes. Modification of 3–4 ferredoxin carboxyl groups had little detectable effect on the ferredoxin-sulfite reductase interaction.

Sulfite-reductase; Ferredoxin; (Spinach)

1. INTRODUCTION

A key step in sulfate assimilation by higher plants [1] is the six-electron reduction of sulfite to sulfide, catalyzed by the enzyme ferredoxin : sulfite oxidoreductase (EC 1.8.7.1, hereafter referred to as sulfite reductase). Sulfite reductase, located in the chloroplast [2], has been purified to homogeneity from spinach leaves [3–5]. The enzyme, which contains one siroheme and one [4Fe-4S] cluster as prosthetic groups [3–5], can utilize reduced methyl viologen as an electron donor [3,4] but the physiological electron donor is reduced ferredoxin [3,4].

Considerable evidence exists [6–11] that ferredoxin forms electrostatically stabilized complexes with a number of ferredoxin-dependent plant enzymes, including ferredoxin : $NADP^+$ oxidoreductase (EC 1.18.1.2, hereafter referred to as $NADP^+$ reductase), ferredoxin : nitrite oxidoreductase (EC 1.7.7.1, hereafter referred to as nitrite reductase) and ferredoxin-dependent glutamate synthase (EC 1.4.7.1). Chemical modification [12,13], cross-linking [10,13,14] and NMR studies [15] have implicated carboxyl groups on ferredoxin as supplying the negative charges involved in forming these complexes. As ferredoxin-dependent spinach sulfite reductase can be purified using a ferredoxin-Sepharose 4B affinity column [3], it appeared likely that sulfite reductase, like the other plant enzymes listed above, can form an electrostatic complex with ferredoxin. Evidence for such complex formation is presented below.

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2. MATERIALS AND METHODS

Spinach ferredoxin ($A_{422\,nm} : A_{277\,nm} = 0.45$) was prepared according to Tagawa and Arnon [16]. Ferredoxin was modified by treatment with glycine ethyl ester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) according to Vieira and Davis [12]. Sulfite reductase was purified by a modification of the procedure of Aketagawa and Tamura [3]. An isolation buffer consisting of 100 mM Tris-H_2SO_4 (pH 7.5), 200 mM NaCl, 2 mM Na_2SO_3, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride replaced that used previously. Acetone precipitation and anion-

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exchange chromatography were carried out as in [3] except that the 0–50% acetone precipitate, after re-dissolving in buffer, was concentrated by precipitation with 70% saturated ammonium sulfate and dialyzed prior to chromatography on a Whatman DE-52 DEAE cellulose column that had been equilibrated with 50 mM Tris-H_2SO_4 buffer (pH 7.5) containing 50 mM NaCl, 0.5 mM EDTA and 20% (v/v) glycerol. Active fractions were pooled, concentrated by precipitation with 70% saturated ammonium sulfate and, after dialyses, applied to an Ultrogel AcA 34 gel filtration column that had been equilibrated with 50 mM Tris-H_2SO_4 buffer (pH 7.5) containing 200 mM NaCl, 0.5 mM EDTA and 20% glycerol. Active fractions were pooled, concentrated by ammonium sulfate precipitation, dialyzed against 10 mM potassium phosphate buffer (pH 7.5) containing 140 g/l of ammonium sulfate, applied to a phenyl-Sepharose CL4B column equilibrated with the same buffer and eluted as described by Krueger and Siegel [4]. Fractions containing sulfite reductase with specific activity > 2.0 U/mg protein (see below) were pooled, concentrated by ammonium sulfate precipitation, dialyzed against 10 mM potassium phosphate buffer (pH 7.7) containing 10% glycerol and applied to a ferredoxin-Sepharose 4B affinity column [3] equilibrated with the same buffer. After washing the column with the equilibration buffer to remove undesired protein, sulfite reductase was eluted with 100 mM potassium phosphate buffer (pH 7.7). Fractions containing high specific activity were pooled and concentrated by membrane filtration using an Amicon PM-10 membrane. The final enzyme preparation had a ratio of $A_{385nm}:A_{280nm}$ of 0.20 and a specific activity of 5.3 U/mg protein with reduced ferredoxin as the electron donor.

Sulfite reductase was assayed by monitoring the absorbance decrease at 604 nm due to the oxidation of reduced methyl viologen, using a modification of the procedure of Aketagawa and Tamura [3]. The reaction mixture (under an N_2 atmosphere in a Thunberg cuvette) contained, in a total volume of 2.5 ml, 100 μmol Tris-H_2SO_4 buffer (pH 8.0), 3 mg bovine serum albumin, 40 nmol ferredoxin, 5 μmol Na_2SO_3, 5 μmol Zn-reduced methyl viologen and enzyme. 1 unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol reduced methyl viologen per min. If ferredoxin was omitted from the reaction mixture, rates of sulfite and enzyme-dependent oxidation of reduced methyl viologen approx. 15% of those obtained with the complete reaction mixture were observed.

Absorbance spectra were obtained using an Aminco DW-2a spectrophotometer and CD spectra were obtained using a Jasco J-20 spectropolarimeter. Oxidation-reduction titrations were performed electrochemically using the optically transparent gold electrode/thin-layer cell system in [17]. Protein concentration was determined according to Bradford [18], using bovine serum albumin as a standard. Ferredoxin concentrations were determined using $\epsilon_{422nm} = 9.7 \times 10^3\ M^{-1} \cdot cm^{-1}$ [16]. Sulfite reductase concentration was calculated from protein concentration, assuming a molecular mass of 138 kDa [4].

3. RESULTS

Since the purification protocol used to obtain the spinach sulfite reductase for the experiments to be described below differed somewhat from those reported previously [3,4], it seemed appropriate to compare some of the preparation's kinetic properties with literature values [3,4]. The enzyme obeyed Michaelis-Menten kinetics with respect to the concentration of the electron donor. K_m values of 25 and 6 μM were obtained for reduced ferredoxin and reduced methyl viologen, respectively. When the enzyme was assayed (using reduced ferredoxin as the electron donor) at varying sulfite concentrations, a sigmoidal relationship between rate and sulfite concentration similar to that reported by Aketagawa and Tamura [3] was observed. Half-maximal velocity was observed at 120 μM sulfite and a Hill coefficient [19] of 2.1 (calculated by fitting the kinetic data using a linear least-squares regression program – GRAPH, Version 1 from Cricket Software – on a Macintosh Plus computer) was calculated. The visible absorbance spectrum (not shown) and apparent molecular mass, determined by gel filtration (see below), were essentially identical to those reported in [3,4].

The observation that spinach sulfite reductase can be purified using a ferredoxin-Sepharose 4B affinity column [3] strongly suggested that ferredoxin forms a complex with the enzyme. Additional evidence for complex formation comes from

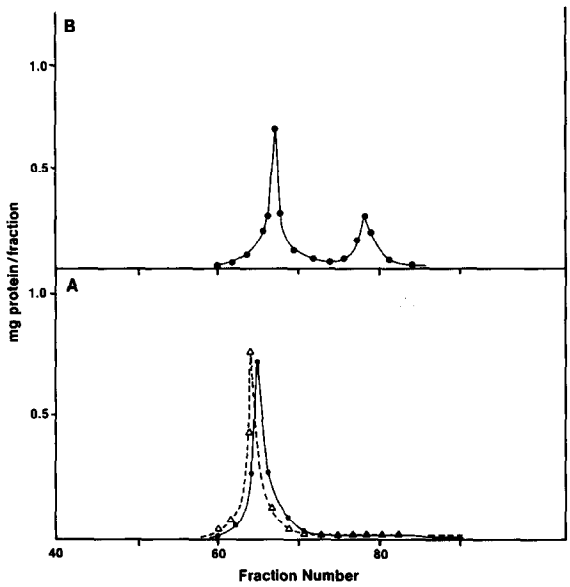

Fig.1. Gel filtration chromatography of ferredoxin plus sulfite reductase. Chromatography was performed on an Ultrogel AcA 34 column (1 × 30 cm). (A) Elution in 30 mM Tris-HCl buffer (pH 8.0). (Δ---Δ) Data for carboxyl-modified ferredoxin plus sulfite reductase; (●——●) data for unmodified, native ferredoxin plus sulfite reductase. (B) Elution in 30 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl. The data were obtained from an experiment with sulfite reductase plus unmodified, native ferredoxin.

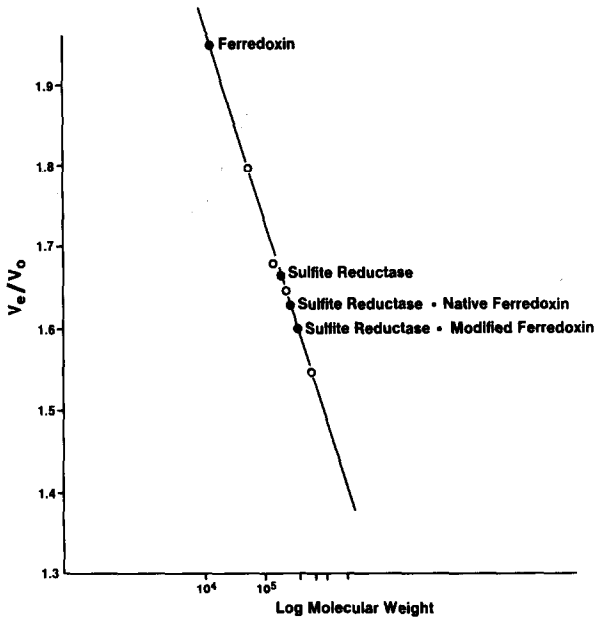

Fig.2. Apparent M_r values of ferredoxin-sulfite reductase complexes. Chromatography was performed as described in fig.1A. M_r standards used (○): bovine serum albumin (M_r = 66000); hexokinase (M_r = 110000); alcohol dehydrogenase (M_r = 150000) and bovine liver catalase (M_r = 240000).

the observation (not shown) when sulfite reductase was added to a ferredoxin-saturated DEAE-cellulose column, ferredoxin and sulfite reductase were co-eluted when 20 mM Tris-HCl buffer (pH 8.0) containing 80 mM NaCl was applied to the column. In the absence of sulfite reductase, ferredoxin remained bound to the column even when buffer containing 200 mM NaCl was used to elute the DEAE-cellulose. Similar results have been obtained previously in our laboratory [11] using $NADP^+$ reductase, nitrite reductase or glutamate synthase, three enzymes known to form complexes with ferredoxin.

More direct evidence for complex formation comes from the observation that ferredoxin and sulfite reductase co-migrate during gel filtration chromatography at low ionic strength. Fig.1A shows that when ferredoxin and sulfite reductase, in a 2:1 molar ratio, were applied to an Ultrogel AcA 34 gel filtration column in 30 mM Tris-HCl buffer (pH 8.0), the two proteins co-migrated. The apparent molecular mass for the ferredoxin-sulfite reductase complex (fig.2) was 160 ± 6 kDa (determined according to Andrews [20]). No protein was detected in fractions corresponding to molecular masses near 11 kDa, where ferredoxin was found during gel filtration in the absence of sulfite reductase (fig.2). However, at higher ferredoxin:sulfite reductase ratios, uncomplexed ferredoxin was observed in these fractions (not shown). Fig.1B shows that when the gel filtration chromatography was repeated under conditions of high ionic strength [30 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl] no co-migration was observed and sulfite reductase and ferredoxin eluted as separate components with elution volumes corresponding to molecular masses of 135 and 11 kDa, respectively. These values are in good agreement with those reported previously for sulfite reductase [4] and ferredoxin [21].

Ferredoxin, reacted with glycine ethyl ester in the presence of EDC, to modify 3–4 carboxyl side

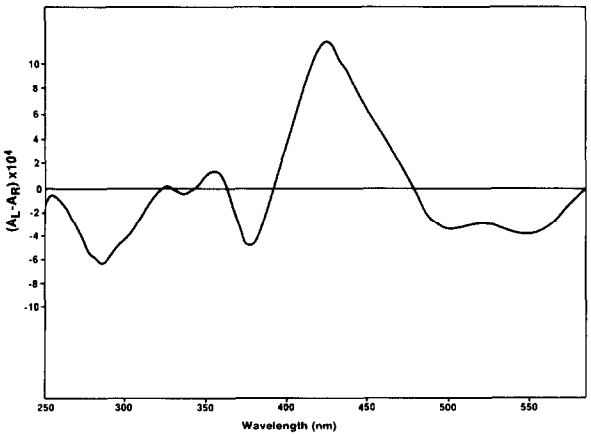

Fig.3. CD spectrum of carboxyl-modified ferredoxin. The cell (1 cm optical path length) contained 27 μM modified ferredoxin in 30 mM Tris-HCl buffer (pH 8.0).

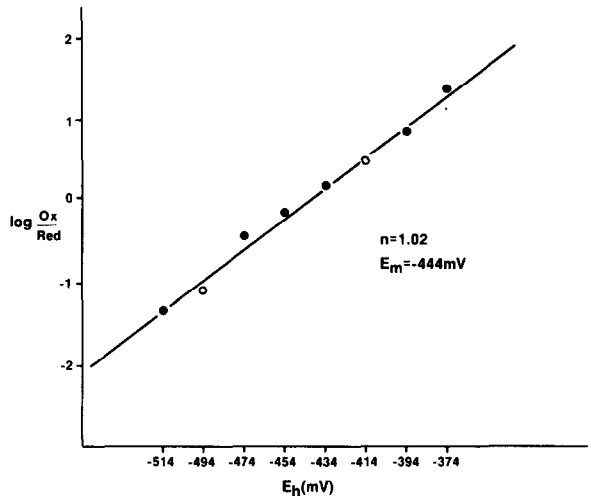

Fig.4. Oxidation-reduction titration of carboxyl-modified ferredoxin. The reaction mixture contained 200 μM modified ferredoxin in 30 mM Tris-HCl buffer (pH 8.0). 30 μM methyl viologen and 15 μM benzyl viologen were present to serve as redox mediators. The titration was conducted at 5°C under an N_2 atmosphere and the oxidation state of ferredoxin was monitored using $A_{420nm} - A_{390nm}$. Optical path length = 0.3 mm. Points taken (●) in the reductive direction and (○) oxidative direction.

chain groups [12,13], has been utilized to investigate the role of ferredoxin carboxyl groups in complex formation with a number of ferredoxin-dependent plant enzymes [11–13,22]. It was thus of interest to examine the effects of this carboxyl group modification on ferredoxin's ability to interact with sulfite reductase. Before doing so, it seemed appropriate to characterize further the modified ferredoxin to ensure that the carboxyl group modification affected neither the immediate environment around the protein's [2Fe-2S] prosthetic group nor the oxidation-reduction properties of the [2Fe-2S] cluster. Fig.3 shows the CD spectrum of the carboxyl-modified ferredoxin. An identical CD spectrum (not shown) was obtained for native ferredoxin. Both spectra are identical to those measured previously in our laboratory [17] and earlier by others [23] for unmodified, native ferredoxin. Fig.4 shows the results of an oxidation-reduction titration of the modified ferredoxin. A series of three titrations gave average values of -429 ± 15 mV for E_m and 0.98 ± 0.03 for n. All titrations were fully reversible. Titrations of unmodified, native ferredoxin (not shown) gave $E_m = -423 \pm 5$ mV ($n = 1.0$), in good agreement with literature values [17,24,25]. These observations, coupled with the previous observation that carboxyl group modification affects neither the visible absorbance spectrum of ferredoxin nor the protein's ability to be photo-reduced by chloroplast photosystem I [12,22], suggest that the modification has no effect on the redox properties or immediate environment of ferredoxin's [2Fe-2S] cluster. Fig.1A shows that modified ferredoxin, like native ferredoxin, co-migrates with sulfite reductase at low ionic strength. The apparent molecular mass of the complex was 180 ± 6 kDa, somewhat different from the 160 kDa value obtained for the enzyme complex with native ferredoxin. As was the case for native ferredoxin, no co-migration between modified ferredoxin and sulfite reductase was observed at high ionic strength. Modified ferredoxin, alone, migrated with an apparent molecular mass of 11 kDa, the same value obtained for native ferredoxin.

Carboxyl-modified ferredoxin was able to replace native ferredoxin as an electron donor in the sulfite reductase-catalyzed reduction of sulfite to sulfide with a K_m for ferredoxin (25 μM) and turnover number (2.3×10^4 s^{-1}) identical to those observed with unmodified, native ferredoxin (not shown).

4. DISCUSSION

The ability of sulfite reductase to elute ferredoxin from a DEAE-cellulose column and the co-migration of ferredoxin and sulfite reductase during gel filtration chromatography (fig.1A) provide convincing evidence for complex formation between the two proteins. The fact that co-migration does not occur at high ionic strengths is consistent with the ferredoxin-sulfite reductase complex being electrostatically stabilized. Sulfite reductase can thus be added to the list of ferredoxin-dependent, plant enzymes (along with $NADP^+$ reductase, nitrite reductase and glutamate synthase) that form electrostatic complexes with their physiological electron donor, ferredoxin. Previous investigations suggested that spinach sulfite reductase is a dimer of identical 69 kDa subunits [4]. The value of 135 ± 6 kDa for our preparation of sulfite reductase, estimated from gel filtration under non-denaturing conditions, agrees well with that of 134 ± 11 kDa estimated by Krueger and Siegel by sedimentation equilibrium. Our observation that no free ferredoxin is observed during co-chromatography at low ionic strength at ferredoxin:sulfite reductase ratios ≤ 2.0 is consistent with the presence of one ferredoxin-binding site on each of the identical subunits. However, a definitive determination of the binding stoichiometry will require additional quantitative measurements.

Surprisingly, modification of 3–4 carboxyl groups on ferredoxin to eliminate their negative charges had no effect on the kinetic parameters for the sulfite reductase-catalyzed, ferredoxin-dependent reduction of sulfite. The latter observation contrasts with that observed for other ferredoxin-dependent plant enzymes, such as glutamate synthase (where modification of ferredoxin carboxyl groups increased K_m 42-fold and decreased V_{max} 5-fold [11]), nitrite reductase (where modification decreased V_{max} 5-fold [11]) and $NADP^+$ reductase (where modification decreased activity 5-fold [12,22]). This result is similar, in one respect, to that obtained with spinach nitrite reductase (an enzyme that is closely related to sulfite reductase [4,5]) in that the K_m for ferredoxin was not affected by carboxyl group modification [11]. The results reported above raise the possibility that the binding site on ferredoxin for sulfite reductase may differ somewhat from that for other ferredoxin-dependent enzymes [11–13,22]. The difference in gel filtration migration patterns of the sulfite reductase complex with native and with modified ferredoxin (i.e. the differences in apparent molecular masses of the complexes; see fig.2) suggests the possibility that modification of ferredoxin carboxyl groups may have some effect on the protein's interaction with sulfite reductase so that either the stoichiometry or the geometry of the two complexes differs. To test the possibility of different interaction with native vs modified ferredoxin, quantitative measurements of the effect of ferredoxin carboxyl group modification on the protein's affinity for sulfite reductase must be conducted. Preliminary attempts to conduct such measurements, using spectral perturbation techniques [6–9,11], have not yet produced reproducible spectral changes on mixing the enzyme with ferredoxin. These experiments are continuing in our laboratory.

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