Tabachnick, M., & Sobotka, H. (1959) *J. Biol. Chem. 234*, 1726.
Thiers, R. T. (1957) *Methods Biochem. Anal. 5*, 273.
Vallee, B. L., Riordan, J. F., Bethune, J. L., Coombs, T. L., Auld, D. S., & Sokolovsky, M. (1968) *Biochemistry 7*, 3547.
Westerman, P. W., Botto, R. E., & Roberts, J. D. (1978) *J. Org. Chem. 43*, 2590.
White, W. I., & Legg, J. I. (1976) *Bioinorg. Chem. 6*, 163.
Witanowski, M., & Webb, G. A. (1973) *Nitrogen NMR*, pp 231-232, 302-306, Plenum Press, London and New York.

Spinach Siroheme Enzymes: Isolation and Characterization of Ferredoxin-Sulfite Reductase and Comparison of Properties with Ferredoxin-Nitrite Reductase†

Rick J. Krueger‡ and Lewis M. Siegel*

ABSTRACT: Sulfite reductase (SiR) has been purified to homogeneity from spinach leaves. Two forms of the enzyme were separated by hydroxylapatite chromatography. One, with subunit M_r 69 000, appears to be proteolytically cleaved to give rise to the other, with subunit M_r 63 000, during the purification procedure. The two species have identical catalytic activities (on a per heme basis) when reduced methylviologen (MV^+) or ferredoxin (Fd_r) is used as electron donor for sulfite reduction, and they exhibit nearly identical optical and EPR spectra. Both enzyme forms exist in 50 mM phosphate buffer (pH 7.7) primarily as dimers at 20 °C. Spinach SiR contains 1 mol of siroheme and one Fe_4S_4 center per subunit. The heme iron is in the high spin Fe^{3+} state in the enzyme as isolated. Near quantitative reduction of the Fe_4S_4 center by dithionite could be achieved if SiR was either converted to the CO complex or treated with 80% dimethyl sulfoxide. Spinach SiR and nitrite reductase (NiR) both catalyze Fd_r- or MV^+-dependent six-electron reductions of SO_3^{2-} and NO_2^-, as well as the two-electron reduction of NH_2OH. V_{max} values are highest with the nitrogenous substrates. However, the K_m of SiR for SO_3^{2-}, and of NiR for NO_2^-, is at least 2 orders of magnitude less than with either of the other substrates. Rates of reduction with Fd_r as electron donor are greater than with MV^+ as donor. No immunological cross-reaction could be detected between spinach SiR and *Escherichia coli* SiR or between spinach SiR and NiR.

A key step in the assimilation of sulfate by plants and microorganisms is the six-electron reduction of SO_3^{2-} to S^{2-}, catalyzed by sulfite reductase (SiR)[1] (Siegel, 1975). This enzyme has been highly purified from *Escherichia coli* (Siegel et al., 1973) and several other microorganisms (Yoshimoto & Sato, 1968; Siegel et al., 1971; Lee et al., 1973a,b). The *E. coli* SiR has been separated into two distinct subunits, each consisting of a single type of polypeptide (Siegel & Davis, 1974). The "flavoprotein" subunit contains FMN and FAD and catalyzes transfer of electrons from NADPH to the "hemoprotein" subunit, which in turn binds sulfite and catalyzes its reduction to sulfide (Rueger & Siegel, 1976). The hemoprotein subunit of *E. coli* SiR exists in solution as a monomeric polypeptide of M_r 56 000; it contains one molecule of siroheme and one Fe_4S_4 center per peptide (Siegel, 1978). Siroheme is an iron dimethylurotetrahydroporphyrin, with the methyl groups attached to adjacent pyrrole rings of the porphyrin nucleus (Murphy et al., 1973; Scott et al., 1978). EPR and Mossbauer studies have indicated that the siroheme and Fe_4S_4 centers exchange spins through a common bridging ligand in the *E. coli* SiR hemoprotein subunit (Christner et al., 1981).

A second enzyme, ferredoxin-nitrite reductase (NiR), which catalyzes the six-electron reduction of NO_2^- to NH_3, has been highly purified from higher plants, algae, and fungi (Hucklesby et al., 1972; Zumft, 1972; Vega et al., 1975; Vega & Kamin, 1977). After some initial evidence suggesting the presence of an Fe_2S_2 center in the enzyme (Vega & Kamin, 1977), recent data of Lancaster et al. (1979) strongly indicate that spinach NiR contains an array of prosthetic groups identical with those of the *E. coli* SiR hemoprotein, i.e., one siroheme and one Fe_4S_4 center on a monomeric polypeptide (in the case of NiR, M_r 61 000).

Asada et al. (1969) have reported isolation of an SiR from spinach which was stated to contain no more than 1 g-atom of Fe/mol of enzyme. The SiR preparation exhibited the absorption spectrum of a hemoprotein, and the sulfite-reducing activity was markedly inhibited by the heme ligands CO and CN^-. The dye MV^+, but not the physiological electron donor ferredoxin, could serve as reductant for stoichiometric reduction of SO_3^{2-} to S^{2-}. The possibility of obtaining an SiR enzyme which could transfer six electrons in a single reaction yet was structurally simpler than the siroheme-Fe_4S_4 protein from *E. coli* prompted our efforts to purify and characterize the spinach SiR. The present paper reports the isolation of SiR from spinach leaves, describes a number of physical, chemical, and catalytic properties of the enzyme, and compares those properties with those of *E. coli* SiR and spinach NiR. We find that spinach SiR is in fact a siroheme-Fe_4S_4 protein

† From the Departments of Biochemistry and Botany, Duke University, and the Veterans Administration Hospital, Durham, North Carolina 27705. *Received December 18, 1981.* This work was supported by Grant AM-13460 from the National Institutes of Health, Grant PCM-7924877 from the National Science Foundation, and Project Grant 7875-01 from the Veterans Administration.

‡ Present address: Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139. This work is part of a dissertation submitted by R.K. to Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] Abbreviations: BSA, bovine serum albumin; Fd_r, reduced spinach ferredoxin; Fe_2S_2, binuclear iron-sulfur center; Fe_4S_4, tetranuclear iron-sulfur center; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; MV^{2+}, oxidized methylviologen; MV^+, reduced methylviologen; NiR, nitrite reductase; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; SiR, sulfite reductase; 63K SiR, spinach SiR species with subunit molecular weight 63 000; 69K SiR, spinach SiR species with subunit molecular weight 69 000.

0006-2960/82/0421-2892$01.25/0

like the other two enzymes and that each of the enzymes can catalyze the six-electron reductions of SO_3^{2-} and NO_2^-. Spinach SiR is, however, immunologically distinct from both *E. coli* SiR and spinach NiR.

Experimental Procedures

Materials. MV^{2+} dichloride was purchased from K & K Laboratories, PMSF from Sigma, BSA from Miles Pentex, and Bio-Gel HT hydroxylapatite from Bio-Rad; dextran sulfate, dextran T-500, and phenyl-Sepharose CL-4B were from Pharmacia. High purity Ar, N_2, H_2, and CO gases were purchased from Matheson Coleman & Bell; all except CO were freed of residual O_2 prior to use by passage through columns of BASF R3-11 catalyst (Chemical Dynamics) maintained at 190 °C. CO gas was deoxygenated by passage through solutions of MV^+. 5′-Deazaflavin was a generous gift from Dr. D. Lambeth. Spinach ferredoxin was purified by the procedure of Borchert & Wessels (1970). Spinach NiR was purified by the method of Vega & Kamin (1977) as modified by Lancaster et al. (1979). *E. coli* SiR was purified by the method of Siegel et al. (1973), and its hemoprotein subunit was prepared according to Siegel & Davis (1974). All other chemicals were of reagent grade and were used without further purification.

Assay of MV^+–Sulfite, Nitrite, and Hydroxylamine Reductase Activities. MV^+ was prepared as follows: A few milligrams of 5% Pt asbestos were added to a solution of oxidized MV^{2+}, 2 mg/mL, in 0.05 M potassium phosphate buffer, pH 7.75, in a Becton-Dickinson Vacutainer tube. The Vacutainer stopper was applied and the solution deoxygenated by bubbling with O_2-free Ar for 15 min. The MV^{2+} was then reduced by bubbling the solution with O_2-free H_2 for 15 min, and the Pt asbestos was removed by centrifugation. We have found that MV^{2+} is approximately 50% reduced to the MV^+ form by this procedure. Solutions of MV^+ generated in this fashion remain stable for several weeks if protected from air.

MV^+-dependent reduction reactions were measured at 22 °C in anaerobic cuvettes, 1-cm path length, tightly fitted with Vacutainer stoppers. Reaction mixtures contained, in 1.5 mL total volume, 50 mM potassium phosphate buffer, pH 7.75, 0.14 mM MV^+, acceptor (1 mM Na_2SO_3 in the standard MV^+–SO_3^{2-} reductase assay), and an appropriate amount of enzyme. The reaction mixtures were prepared as follows: 1.35 mL of buffer was added to the cuvette, the Vacutainer stopper fitted in place, and the solution bubbled through a syringe needle with Ar for 15 min; 0.03 mL of enzyme and 0.04 mL of MV^+ solution were then added, and the rate of MV^+ oxidation followed spectrophotometrically at 600 nm for 3 min with a Cary Model 14 spectrophotometer. An anaerobic solution of acceptor (Na_2SO_3, $NaNO_2$, or NH_2OH) in buffer (pH adjusted to 7.75), 0.05 mL, was then added and the rate of MV^+ oxidation again followed. The difference in rates of MV^+ oxidation after and before acceptor addition was taken as the rate of the MV^+–acceptor reductase reaction.

Controls showed that the rate of MV^+ oxidation in the absence of enzyme was the same whether or not SO_3^{2-} or NH_2OH was present; this rate (which was identical with the MV^+ oxidation rate with enzyme but not acceptor added) was generally <0.003 ΔA_{600}/min. The amounts of enzyme added were adjusted so that the observed rates were at least 0.030 ΔA_{600}/min.

When $NaNO_2$ served as acceptor, 20 μM sodium diethyldithiocarbamate was also included in the reaction mixture. This concentration of diethyldithiocarbamate was found to totally inhibit a rapid nonenzymatic oxidation of MV^+ by 20 mM $NaNO_2$ otherwise observed under our assay conditions. Diethyldithiocarbamate was without effect on the rates of enzymatic MV^+ oxidation observed with $Na_2SO_3^{2-}$ or NH_2OH as acceptors.

The extinction coefficient used for MV^+ was 1.3×10^4 M^{-1} cm^{-1} at 600 nm (Thorneley, 1974); oxidized MV^{2+} does not absorb at this wavelength. One unit of MV^+–SO_3^{2-} reductase activity is defined as that amount of enzyme which catalyzes oxidation of 1 μmol of MV^+/min under the standard assay conditions with 1 mM SO_3^{2-} as electron acceptor.

Purification of Spinach SiR. Spinach, obtained from local supermarkets, was destemmed and rinsed with cold tap water. Excess water was drained off, and the leaves were stored in 1.5-kg batches in polyethylene bags at −15 °C.

The first three steps of the procedure described below were performed on 21-kg quantities of spinach leaves. The pooled eluates resulting from hydroxylapatite chromatography were stored at −15 °C until three 21-kg batches of leaves had been processed through step 3. The pooled material derived from 63 kg of spinach leaves was then subjected to steps 4 and 5. The entire procedure was carried out at 0–4 °C unless otherwise indicated.

Step 1: Extraction and $(NH_4)_2SO_4$ Fractionation. Each bag of frozen spinach was allowed to partially thaw at room temperature for 90 min. Each 1.5 kg of leaves was then homogenized with 1 L of 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA and 0.5 mM PMSF, for 2 min at low speed in a gallon volume Waring Blendor. (PMSF was added to buffers immediately before their use, by addition of a 0.1 M PMSF stock solution in 95% ethanol.) The homogenate was forced through eight layers of cheesecloth with a fruit press.

A 170-g sample of solid $(NH_4)_2SO_4$ was added slowly, with stirring, to each liter of filtered crude extract. After 45 min of additional stirring, the mixture was centrifuged for 45 min at 8000 rpm in a Sorvall RC-2B centrifuge (G-3 rotor). A 80-g sample of solid $(NH_4)_2SO_4$ was then added, with stirring, to each liter of supernatant fluid. After 30 min of further stirring, the mixture was centrifuged at 7000 rpm for 30 min. The resulting pellet was triturated with sufficient buffer A (10 mM potassium phosphate, pH 8.0, containing 0.5 mM EDTA and 0.5 mM PMSF) to give a suspension with a total volume equivalent to 50 mL/kg of leaves initially processed. This suspension was centrifuged at 45000*g* for 90 min. The resulting supernatant fluid was placed in an Amicon DC-2 hollow fiber apparatus equipped with an H1-P10 fiber cartridge; a total of 20 L of buffer A serves as dialysate. The protein solution was removed from the desalting apparatus when its conductivity (measured at 25 °C with a Radiometer CDM-2e conductivity meter) was less than 2.2 $M\Omega^{-1}$, and any precipitated protein was removed by centrifugation for 30 min at 23000*g*.

Step 2: DEAE-cellulose Chromatography. The enzyme solution was applied, at a flow rate of 22 mL cm^{-2} h^{-1}, to a 7.5 × 70 cm column of Whatman DE-52 equilibrated with buffer A. The column was washed, at the same flow rate, with 4 L of buffer A, and then a linear gradient composed of 6 L each of buffer A and buffer A containing 0.4 M KCl was applied at a flow rate of 10 mL cm^{-2} h^{-1}. Fractions were collected and analyzed for MV^+–sulfite reductase activity and protein content, and those fractions with specific activity >0.02 unit/mg of protein were pooled.

Step 3: Hydroxylapatite Chromatography. The enzyme solution was applied, at a flow rate of 15 mL cm^{-2} h^{-1}, to a 5 × 42 cm column of Bio-Gel HT hydroxylapatite equilibrated with buffer B (identical to buffer A but pH 7.7). The column

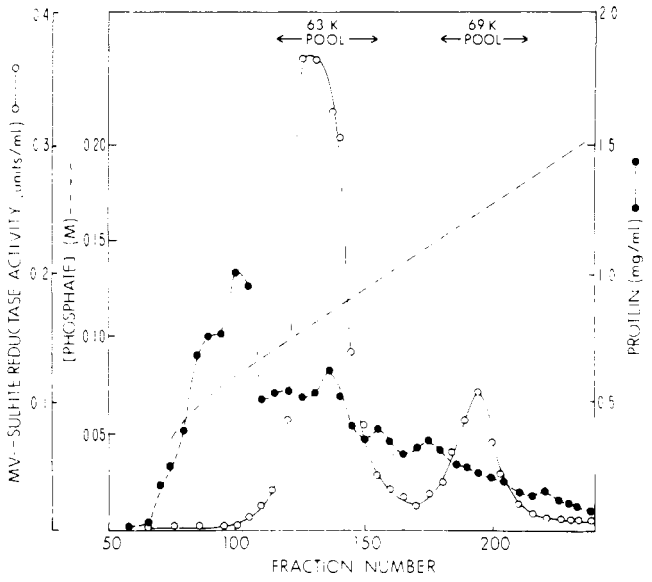

FIGURE 1: Chromatography of spinach sulfite reductase on Bio-Gel HT. 21 kg of spinach leaves were purified through the DEAE-cellulose chromatography step of the sulfite reductase purification procedure, and the resulting solution was applied to a column (5 × 42 cm) of Bio-Gel HT hydroxylapatite. For details of buffers used and the elution procedure, see the text. Fraction volumes were 20 mL. Assays were performed as described under Experimental Procedures. Protein concentration (●); MV^+–SO_3^{2-} reductase activity (○); phosphate concentration (measured as conductivity at 25 °C with a Radiometer Model CDM-2e meter and appropriate potassium phosphate standards) (···).

was washed, at a flow rate of 7.5 mL cm^{-2} h^{-1}, with 1.5 L of buffer B, and then a linear gradient composed of 2 L each of buffer B and 0.4 M potassium phosphate, pH 7.7, containing 0.5 mM EDTA and 0.5 mM PMSF, was applied at a flow rate of 5 mL cm^{-2} h^{-1}. Fractions were collected and analyzed for MV^+–sulfite reductase activity and protein content. As shown in Figure 1, two well-resolved peaks of enzyme activity were generally found. The peak of activity which eluted first from the column contained a species of SiR whose subunit molecular weight, determined after further purification, proved to be 63 000. The second peak of activity eluted contained an SiR species of subunit M_r 69 000. The two different enzyme forms are subsequently referred to as the "63K" and "69K" forms of SiR, respectively. Fractions from each peak with specific activity >0.1 unit/mg of protein were pooled. All subsequent procedures were performed separately and in identical fashion for the 63K and 63K enzyme pools.

Each pooled enzyme solution was precipitated by addition of 390 g of solid $(NH_4)_2SO_4$/L. After 20 min of stirring, the precipitated protein was collected by centrifugation at 10000*g* for 30 min, and the pellets were stored at −15 °C (for up to 2 weeks) until three 21-kg batches of spinach had been processed through the hydroxylapatite chromatography step. The combined pellets for each enzyme pool were dissolved with the aid of a TenBroek homogenizer in 200 mL of 10 mM potassium phosphate buffer, pH 7.7, containing 140 g of $(NH_4)_2SO_4$/L (buffer C). Any undissolved material was removed by centrifugation for 20 min at 40000*g*.

Step 4: *Phenyl-Sepharose Chromatography*. The enzyme solution from step 3 was applied, at a flow rate of 20 mL cm^{-2} h^{-1}, to a 2.5 × 39 cm column of phenyl-Sepharose CL-4B equilibrated with buffer C. Elution was performed, at a flow rate of 5 mL cm^{-2} h^{-1}, with a linear gradient composed of 550 mL each of buffer C and buffer B containing 50% (v/v) ethylene glycol. Fractions containing enzyme with a specific activity >2.5 units/mg of protein were pooled.

Step 5: *Sephadex G-150 Chromatography*. Enzyme from step 4 was diluted with an equal volume of 50 mM potassium phosphate, pH 7.75, and the solution was concentrated to a volume of 5–6 mL with an Amicon concentrator. The protein was then applied to a 2.5 × 97 cm column of Sephadex G-150 (superfine) equilibrated with 50 mM potassium phosphate, pH 7.75, and elution was performed with the same buffer. For the 63K SiR, fractions with a specific activity >10 units/mg of protein (A_{278}/A_{384} < 2.0) were pooled. For 69K SiR, fractions with specific activity >8.4 units/mg of protein (A_{278}/A_{384} < 2.2) were pooled. Pooled fractions were concentrated to 2–5 mg/mL in a Schleicher & Schuell ultrafiltration apparatus, and the resulting enzyme was stored at −70 °C prior to further use.

Assay of Fd_r–Sulfite and –Nitrite Reductase Activities. An anaerobic solution containing 1 mM EDTA, 1.2 μM 5′-deazaflavin, and spinach ferredoxin in a total volume of 0.48 mL of 0.05 M potassium phosphate buffer (pH 7.75) was added to an Ar-flushed anaerobic cuvette fitted with a Vacutainer stopper. An absorption spectrum of this solution was recorded and the cuvette then illuminated with a GE 200-W sealed beam lamp placed 30 cm from the cuvette. A Pyrex chromatography tank filled with an ice–water slurry was placed between the lamp and the cuvette to minimize heating of the ferredoxin solution. Absorption spectra were recorded at 2–3-min intervals to monitor the rate of ferredoxin reduction. Illumination was continued until the A_{422} of the ferredoxin solution was 49 ± 2% of its initial value. [The A_{422} of Fd_r is reported to be 49% of that for oxidized ferredoxin (Hosein, 1973). Assays were considered valid only if at least 90% of the initial oxidized ferredoxin absorbance was recovered on addition of O_2 or reaction with enzyme plus SO_3^{2-} or NO_2^- at the end of the assay period.] An anaerobic enzyme solution (0.01 mL) was then added to the Fd_r solution and the A_{422} measured. (There was usually a small rapid increase in A_{422}, probably due to O_2 added with the enzyme solution. The ΔA_{422} rapidly decreased to a rate of no more than 0.0002/min.) An anaerobic solution (0.01 mL) of electron acceptor in buffer was then added and the ΔA_{422} again measured as a function of time. (Addition of SO_3^{2-} or NO_2^- as acceptor also led to small rapid increases in A_{422}, presumably due to O_2 addition. In the absence of enzyme, the ΔA_{422} also rapidly decreased to a rate of no more than 0.0002/min.) The difference in rates of A_{422} increase before and after acceptor addition was taken as the acceptor-dependent rate of Fd_r oxidation. A typical assay is shown in Figure 2.

The extinction coefficient used for oxidized ferredoxin was $E_{422} = 9.7 \times 10^3$ M^{-1} cm^{-1} (Tagawa & Arnon, 1968), and the ΔE_{422} used for oxidized-minus-reduced ferredoxin was 5.0×10^3 M^{-1} cm^{-1} (Hosein, 1973).

Protein Determination. Unless otherwise indicated, protein concentrations were measured by the Zamenhof (1957) microadaptation of the biuret method, with the modification that absorbances of two control solutions, one containing $CuSO_4$ reagent but not protein and the other protein but not $CuSO_4$, were both subtracted from the absorbance at 310 nm of the complete assay mixture. For the first two steps of the SiR purification procedure, the biuret method of Layne (1957) was used, due to the presence of large amounts of nonprotein UV-absorbing material in the enzyme preparation. In all cases, bovine serum albumin, assayed spectrophotometrically with use of $E^{1\%}_{279} = 6.7$ cm^{-1} reported by Rideal & Roberts (1951), was used as protein standard.

$NaDodSO_4$ Gel Electrophoresis. Electrophoresis was performed on 100 × 150 × 0.75 nm gel slabs, 7.5% in acrylamide,

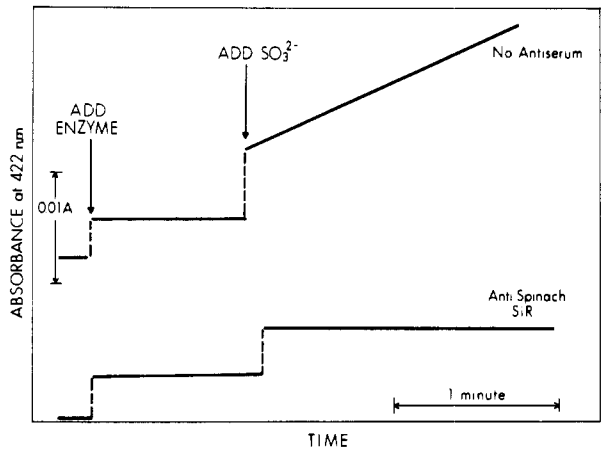

FIGURE 2: Sulfite-dependent oxidation of reduced ferredoxin catalyzed by spinach sulfite reductase. Assay conditions were as described under Experimental Procedures. Reduced ferredoxin concentration was 40 μM. Sulfite concentration was 1 mM. The assay contained 1.6 pmol of 63K spinach SiR (upper curve) or 1.6 pmol of 63K spinach SiR plus 0.005 mL of purified anti-63K SiR serum (lower curve). Enzyme and sulfite were added as indicated in the figure, and the absorbance at 422 nm was recorded as a function of time.

at 7 mA/slab for 8 h, according to the procedure of Laemmli (1970), except that $NaDodSO_4$ was omitted from the separating and stacking gels as suggested by Wyckoff et al. (1977). Protein samples were treated with sample buffer (62.5 mM Tris-HCl, pH. 6.8, containing 3% $NaDodSO_4$, 10% glycerol, and 5% 2-mercaptoethanol) according to Laemmli (1970), with the modification that the $NaDodSO_4$-containing protein solutions were incubated for 3 min in a boiling water bath prior to their application to the gel slabs. Coomassie Blue staining was used.

Sedimentation Studies. Experiments were conducted with a Beckman Model E ultracentrifuge equipped with electronic speed and temperature control units and photoelectric scanner with multiplexer accessories. Protein samples for all experiments were extensively dialyzed vs. 50 mM potassium phosphate–0.5 mM EDTA buffer, pH 7.75, and the dialysate was used as a reference solution.

Sedimentation equilibrium experiments were performed at 20 °C in An-J and and An-F rotors according to Chervenka (1969). Solution absorbance at equilibrium was measured at 384 nm and, where possible, also at 278 nm. Molecular weights (M_r) were calculated from the equation $M_r = 2RT(\mathrm{d} \ln A/\mathrm{d}r^2)/[w^2(1 - \bar{v}\rho)]$; $\bar{v}$ was determined from the amino acid composition by the method of Cohn & Edsall (1943), and ρ was determined pycnometrically. Values for $\mathrm{d} \ln A/\mathrm{d}r^2$ were obtained from linear regression analyses of the data. Measurements at 384 and 278 nm for a given experimental run in all cases yielded values for $\mathrm{d} \ln A/\mathrm{d}r^2$ which were not significantly different (±5%). Calculated molecular weights are reported as an average of the molecular weights determined for each wavelength scan.

Sedimentation velocity experiments were performed in the An-F rotor at 48 000 rpm, at 22 °C. The A_{384} of the protein solution as a function of r was recorded at intervals, and the position of the protein boundary was taken to be the position at which the absorbance was half-maximal.

For the determination of diffusion coefficients, the spreading of an initially sharp boundary between solvent and protein solution, formed with a synthetic boundary cell at 4000 rpm in the An-J rotor, at 22 °C, was followed by recording A_{384} as a function of r at intervals. The data were analyzed by the probability paper method of Markham (1967) in which the diffusion coefficient is obtained from the slope of a plot of σ^2 vs. $2t$. Both sedimentation and diffusion coefficients were corrected to the density and viscosity of water at 20 °C.

Amino Acid Composition. Amino acid analyses were performed according to Spackman et al. (1958) with a Beckman Model 120B automatic amino acid analyzer. A known amount of L-norleucine was added as a standard to each protein sample, which was then hydrolyzed in 6 N HCl in vacuo for 28, 48, and 72 h at 110 °C. Half-cystine was determined as cysteic acid after performic acid oxidation.

Standard Enzyme Assays. E. coli SiR was assayed by means of its NADPH–SO_3^{2-} reductase activity (Siegel et al., 1973), while concentrations of its hemoprotein subunit were determined spectrophotometrically, using $E_{587} = 1.8 \times 10^4$ M^{-1} cm^{-1} (Siegel & Davis, 1974). Concentrations of spinach NiR were determined spectrophotometrically, using $E_{386} = 7.6 \times 10^4$ (M heme)$^{-1}$ cm^{-1} (Lancaster et al., 1979).

Absorption Spectra. Spectra were measured in 1-cm path-length silica cells at 22 °C with an Aminco DW-2 spectrophotometer operating with a 2-nm band-pass.

Chemical Determinations. The total Fe content of enzyme samples was determined by the ferrozine method of Carter (1971); Fe wire dissolved in 20% HNO_3 and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ served as standards (both gave identical standard curves). Acid-labile S^{2-} was determined by the method of Siegel et al. (1973), except that assay volumes were scaled down to a total of 0.4 mL. Siroheme was measured in acetone–HCl extracts of SiR as described by Siegel et al. (1978).

For determinations of the stoichiometry of enzymatic SO_3^{2-} and NO_2^- reduction, concentrations of these substrates were determined spectrophotometrically by use of the known stoichiometry of the *E. coli* SiR catalyzed reductions with NADPH as electron donor (Lazzarini & Atkinson, 1961; Siegel et al., 1974). Such assays were performed under anaerobic conditions with limiting SO_3^{2-} or NO_2^-, and concentration of these substrates was determined as one-third the concentration of NADPH oxidized during the reaction.

S^{2-} concentration was determined colorimetrically (Siegel, 1965) or by radioactivity measurements (when $^{35}SO_3^{2-}$ served as substrate). Concentrations of standard S^{2-} solutions were determined by iodometric titration with $Na_2S_2O_3$ used as primary standard. S^{2-} determinations from radioactivity measurements utilized the volatility characteristics of this sulfur compound. Solutions containing [^{35}S]sulfide in phosphate buffer at pH 7.75 were bubbled for 20 min with O_2-free Ar. The volatilized radioactivity was taken as a measure of the [^{35}S]sulfide originally present in the solution. Controls showed that [^{35}S]sulfite is not volatilized under these conditions. Standards in which S^{2-} was determined by both the colorimetric and volatilization procedures gave identical results. For any $^{35}SO_3^{2-}$ solution used in stoichiometry experiments, the specific radioactivity was measured as the amount of radioactivity volatilized in the presence of *E. coli* SiR and excess NADPH divided by one-third the amount of NADPH consumed under anaerobic conditions. Radioactivity was measured with a Packard Model 3375 Tri-Carb liquid scintillation spectrometer.

EPR Measurements. EPR spectra were recorded on frozen samples with a Varian E9 spectrometer operating at 9.2 GHz with field modulation of 100 KHz. Temperature was maintained with use of an Air Products liquid helium cryostat. For quantitation of spins, all spectra were recorded under nonsaturating conditions, generally at 5 K for high-spin ferriheme and 20 K for reduced Fe_4S_4 centers. Ferriheme was quantitated by integration of the $g > 6$ peak according to the method of Aasa & Vangaard (1975) with *E. coli* SiR hemo-

Table I: Purification of Spinach Sulfite Reductase[a]

purification step	volume (mL)	protein (mg)	act. (units)	sp act. (units/mg)	yield (%)
(1) $(NH_4)_2SO_4$ fractionated extract	3390	77000	818	0.011[b]	(100)
(2) DE-52 chromatography	5115	14000	594	0.041	73
(3) Bio-Gel HT chromatography					
63K SiR pool	2365	1040	340	0.33	42
69K SiR pool	1060	244	117	0.48	14
(4) phenyl-Sepharose CL-4B chromatography					
63K SiR pool	135	63	239	3.8	29
69K SiR pool	140	34	96	2.8	12
(5) Sephadex G-150 chromatography					
63K SiR pool	9	18	190	10.3	23
69K SiR pool	3	7	60	8.9	7

[a] The purification procedure is described in the text. Assays for MV^+–SO_3^{2-} reductase activity and for protein were performed as described under Experimental Procedures. [b] The high rate of sulfite-independent oxidation of MV^+ in crude spinach leaf extracts has made accurate assays of MV^+–SO_3^{2-} reductase activity impractical in such extracts prior to $(NH_4)_2SO_4$ fractionation. Assays for activity and protein were performed after the $(NH_4)_2SO_4$ fractionated extract was desalted.

protein serving as standard. Studies of the temperature dependence of the magnitude of the integrals of the $g > 6$ features of spinach SiR and NiR and *E. coli* SiR hemoprotein showed that $D = 8 \pm 1$ cm^{-1} for all the ferriheme species (Christner et al., 1981), so that corrections for incomplete population of the lowest Kramers doublet at 5 K were negligible. Reduced Fe_4S_4 center EPR spectra were quantitated by double integration with CuEDTA as standard.

Preparation of Antisera. Antisera to spinach 63K SiR, spinach NiR, and *E. coli* SiR hemoprotein subunit were obtained by immunization of New Zealand white rabbits with 1 mg of purified enzyme in Freund's complete adjuvant, half injected into the toe pads and half injected subcutaneously in multiple sites. Sera were collected 2–4 weeks after immunization. Six weeks after the immunization, the rabbits were injected subcutaneously with another 0.5 mg of the same enzyme preparation, and sera were collected 2–3 weeks after this second injection.

Sera were partially purified by successive precipitations at 4 °C with 243 g of $(NH_4)_2SO_4$/L. Each precipitate was dissolved in a volume of 50 mM potassium phosphate buffer (pH 7.75) equal to the initial volume of serum. The resulting solution was dialyzed overnight vs. 100 volumes of the same buffer, and any precipitated protein was removed by centrifugation. Antisera were stored at −15 °C. Antisera were titered by reaction with a fixed amount of homologous enzyme; the ability to precipitate MV^+–SO_3^{2-} reductase (for SiR) or MV^+–NO_2^- reductase activity (for NiR) was used to determine equivalence ratios between each antiserum and its homologous antigen.

Results

Isolation of 63K and 69K Species of Sulfite Reductase. SiR was purified from spinach leaves as described under Experimental Procedures. Hydroxylapatite chromatography separated two species of MV^+–sulfite reductase activity (Figure 1), termed 63K and 69K SiR, respectively. The purification procedure, summarized in Table I, yielded 18 mg of 63K SiR (specific activity 10.3 units/mg of protein) and 7 mg of 69K SiR (8.9 units/mg of protein) from 63 kg of fresh weight of leaves. By the criterion of $NaDodSO_4$ gel electrophoresis, the 63K form appeared to be homogeneous, while the 69K form was at least 95% pure (Figure 3). The two enzyme forms were purified 800–1000-fold with respect to the material resulting from step 1 of the purification procedure, the total recovery of MV^+–sulfite reductase activity from that material being 30% in the sum of the two enzyme forms.

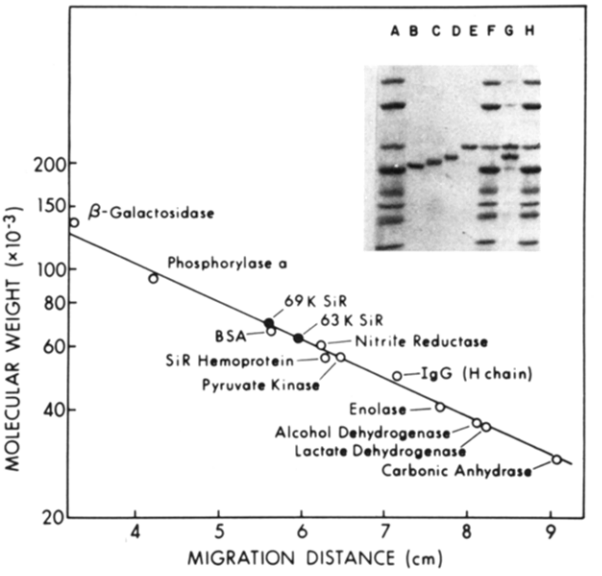

FIGURE 3: Electrophoresis of sulfite reductases in $NaDodSO_4$–polyacrylamide gels. Samples were prepared, and electrophoresis was performed on gel slabs as described under Experimental Procedures. Semilogarithmic plot of molecular weight vs. migration distance. (Inset) Photograph of gel pattern. Wells A, F, and H contained 0.5–1.0 μg each of the following standard proteins [subunit molecular weights (Weber & Osborn, 1969) in parentheses] in order of migration (top of gel to bottom): β-galactosidase (130000); phosphorylase *a* (94000); bovine serum albumin (BSA) (68000); pyruvate kinase (57000); IgG heavy chain (IgGH) (50000); enolase (41000); alcohol dehydrogenase (37000); lactate dehydrogenase (36000); carbonic anhydrase (29000); IgG light chain (23500). Well B contained 0.6 μg of *E. coli* sulfite reductase hemoprotein [molecular weight 56000 by $NaDodSO_4$ gel electrophoresis (Siegel & Davis, 1974)]. Well C contained 0.5 μg of spinach NiR [molecular weight 61000 (Vega & Kamin, 1977)]. Wells D and E contained 0.6 μg of 63K and 69K spinach SiR, respectively. Well G contained a mixture of 0.6 μg each of 63K and 69K spinach SiR and 0.05–0.1 μg of each of the standard proteins in well A.

Molecular Weight. (A) Subunit Molecular Weight. The molecular weights for the spinach SiR polypeptides were estimated by $NaDodSO_4$ gel electrophoresis with appropriate protein standards (Figure 3). Enzyme purified from the 63K SiR pool exhibited a single protein band with a mobility corresponding to M_r 63000. The purified 69K SiR preparation exhibited a predominant protein band at M_r 69000, with a small amount of contaminating protein (<5% of the intensity of the major component as determined densitometrically) at M_r 58000.

Table II: Sedimentation Equilibrium of Spinach Sulfite Reductases[a]

protein	rotor speed (rpm)	concn (mg/mL)	M_r
69K SiR	8 000	0.40	137 600
	8 000	0.20	134 300
	8 000	0.08	138 600
	8 000	0.04	154 400
	12 000	0.40	126 800
	12 000	0.20	137 300
	12 000	0.08	122 300
	12 000	0.04	120 100
			133 900 ± 11 000[b]
63K SiR	6 000	0.12	124 200
	8 000	0.40	119 700
	8 000	0.20	108 100
	8 000	0.04	103 400
	9 000	0.12	119 700
	12 000	0.40	109 800
	12 000	0.20	105 100
	12 000	0.04	109 000
			112 400 ± 7200[b]

[a] The methods used in making these measurements are described under Experimental Procedures. Protein solutions were in 0.05 M potassium phosphate buffer, pH 7.75, containing 0.5 mM EDTA. Centrifugation was performed at 20 °C. [b] Mean ± SD.

Table III: Molecular Parameters for 63K Spinach Sulfite Reductase[a]

parameter	concn (mg/mL)	exptl value
sedimentation coefficient ($s_{20,w}$)	0.2	5.8 S
diffusion coefficient ($D_{20,w}$)	0.2	4.8×10^{-7} cm^2 s^{-1}
Stokes' radius (gel filtration)	0.06[b]	4.7×10^{-7} cm
partial specific volume		0.736 cm^3 s^{-1}
molecular weight		
sed equilibrium	0.04–0.4	112 400 ± 7200
sed velocity–diffusion	0.2	114 000
sed velocity–Stokes' radius		119 000
subunit molecular weight (in $NaDodSO_4$)		63 000

[a] Methods are given in the text and under Experimental Procedures and in the legend to Figure 4. [b] Protein concentration at the peak of eluted MV^+–SO_3^{2-} reductase activity (Sephadex G-200 gel filtration).

(*B*) *Native Molecular Weight.* The molecular weights for 63K and 69K SiR were determined by sedimentation equilibrium analysis under nondenaturing conditions (50 mM potassium phosphate–0.5 mM EDTA, pH 7.75, 20 °C). Centrifugation was performed at several protein concentrations in the range 0.04–0.4 mg/mL and at several rotor speeds. In all cases, plots of ln A vs. r^2 appeared to be linear throughout the cell. The data for all experiments are presented in Table II. No consistent dependence of molecular weight on protein concentration was observed. If the $\bar{v}$ from amino acid analyses of SiR are used, average M_r values of 112 000 ± 7000 and 134 000 ± 11 000 (errors are standard deviations) were obtained for the 63K and 69K SiR, respectively. These results indicate that both forms of spinach SiR exist in solution, under the experimental conditions of Table II, primarily as dimeric proteins.[2] The molecular weight for 63K SiR was also determined by measurement of sedimentation and diffusion coefficients in the analytical ultracentrifuge. As shown in Table III, a 0.2 mg/mL solution of 63K SiR in 50 mM potassium phosphate–0.5 mM EDTA, pH 7.75, exhibited at 22 °C an $s_{20,w}$ = 5.8 S and a $D_{20,w} = 4.8 \times 10^7$ cm^2 s^{-1}. Only single boundaries were observed. Using $\bar{v}$ = 0.736 cm^3 g^{-1}, M_r = 114 000.

[2] It may be noted that molecular weights obtained at high rotor speeds (which should emphasize the low molecular weight components of a heterogenous mixture) were generally smaller than those obtained at lower rotor speeds. These results suggest the presence of a sedimenting species of lower molecular weight than those indicated in the enzyme solutions, possibly indicative of a rapid equilibrium between monomeric and dimeric enzyme species.

Table IV: Amino Acid Compositions of Spinach Sulfite and Nitrite Reductases[a]

residue	residues/subunit weight: 69K sulfite reductase	63K sulfite reductase	nitrite reductase[b]
Asx	66	62	64
Thr[c]	32	31	19
Ser[c]	31	31	28
Glx	70	65	70
Pro	31	32	34
Gly	52	52	47
Ala	36	31	39
Val	45	43	46
Met	14	13	14
Ile	31	29	27
Leu	59	53	49
Tyr	22	21	12
Phe	26	26	20
Lys	42	39	38
His	8	7	12
Arg	38	38	32
$^1/_2$-Cys	5[e]	5[e]	8[e]
Trp	*d*	*d*	5[f]

[a] Details of the hydrolyses are given under Experimental Procedures. Each value, except where noted, is the average of 24-, 48-, and 72-h hydrolyses, with L-norleucine serving as an internal standard for recovery of samples. Results are consistent to ±5%. [b] Data of Vega & Kamin (1977). [c] Calculated by extrapolation to zero time of hydrolysis. [d] Not determined. [e] Measured as cysteic acid after performic acid oxidation. [f] Determined spectrophotometrically.

Gel filtration of 63K SiR on a Sephadex G-200 column calibrated with a number of proteins of known Stokes' radius (bovine erythrocyte carbonic anhydrase, rabbit muscle enolase and pyruvate kinase, BSA, and yeast alcohol dehydrogenase) according to the method of Siegel & Monty (1966) yielded a Stokes radius of 4.7 nm. The enzyme was assayed by means of its MV^+–sulfite reductase activity; SiR concentration at the elution peak was 0.06 mg/mL. When this Stokes radius is combined with the $s_{20,w}$ obtained in the ultracentrifuge and the $\bar{v}$ from amino acid analysis, the calculated M_r = 119 000.

Amino Acid Composition. The results of amino acid analyses of the 63K and 69K forms of SiR are presented in Table IV. $\bar{v}$ = 0.736 and 0.737 cm^3 g^{-1}, respectively, were calculated for the two enzyme species. Compositions of the 63K and 69K forms are similar, with the 69K SiR containing significantly greater amounts of Leu, Ala, Glx, Asx, Val, and Lys than 63K SiR. Only in the case of Pro was any amino acid found to be present in greater amount in the 63K than in the 69K species, and the difference of 1 residue in 31 observed is well within the precision error of the amino acid analyses. The total recovered residue weight per siroheme (see below) for each enzyme was in good agreement with the subunit molecular weight as determined by $NaDodSO_4$ gel electrophoresis.

Spectral Properties. Absorption spectra of the 63K and 69K forms of SiR are shown in Figure 4. In both cases, maxima are found at 278, 384, 542, 587, and 712 nm. The absorbances at the wavelength maxima are found in the following ratio: 63K SiR, 1.97, 1.00, 0.21, 0.30, and 0.10; 69K SiR, 2.17, 1.00, 0.21, 0.30, and 0.10. The spectra of the 63K and 69K SiR

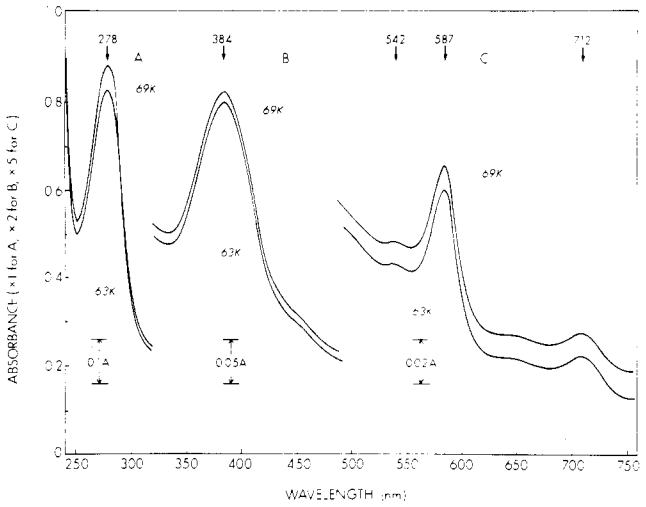

FIGURE 4: Absorption spectra of spinach sulfite reductases. Spectra of enzyme samples, 7.1 μM in enzyme subunits, in 0.05 M potassium phosphate, pH 7.75, were recorded vs. a blank containing buffer only. The spectrum of the 69K SiR has been displaced upward by 0.01A at 587 nm with respect to the 63K SiR. The spectra are in fact superimposable within the wavelength range 320–750 nm. The 69K enzyme exhibits greater absorbance per subunit than the 63K enzyme in the wavelength range 250–320 nm. For curves B and C, the absorbance scale has been expanded 2- and 5-fold, respectively, with respect to the absorbance scale for curve A.

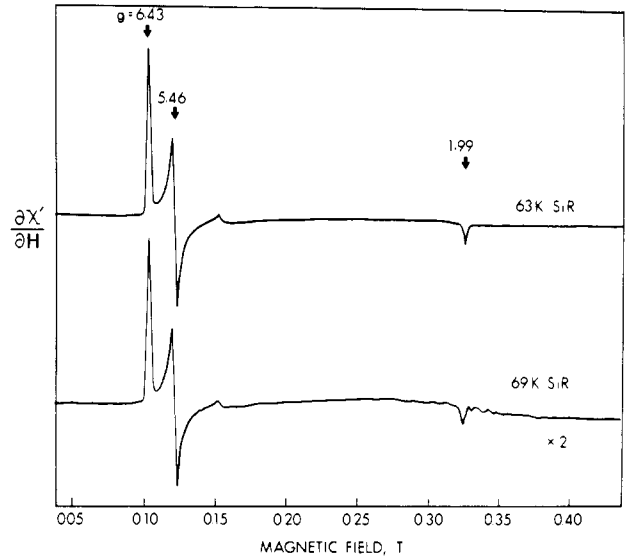

FIGURE 5: EPR spectra of spinach sulfite reductases. Solutions were in 0.05 M potassium phosphate buffer, pH 7.75, and contained 76 μM enzyme subunits for 63K SiR (upper spectrum) and 38 μM enzyme subunits for 69K SiR (lower spectrum). The instrument gain for the lower spectrum was set at twice that used to record the upper spectrum. Conditions of measurement were the following: temperature 20 K; microwave frequency 9.13 GHz; microwave power 50 mW; modulation amplitude, 1 mT.

are thus identical in the visible wavelength region but differ in the presence of more UV-absorbing material, indicative of a greater content of protein per prosthetic group in the 69K as compared to the 63K form. On the basis of protein content, values of $E^{1\%}_{587}$ = 2.83 and 2.55 cm^{-1} were found for 63K and 69K SiR, respectively; these values correspond to E_{587} = 1.78 × 10^4 and 1.76 × 10^4 (M subunit)$^{-1}$ cm^{-1} for the 63K and 69K enzymes, respectively.

EPR spectra of the 63K and 69K SiR are shown in Figure 5. Each spectrum exhibits signals at g = 6.43, 5.46, and 1.99, a pattern characteristic of high-spin ferriheme with rhombically distorted tetragonal symmetry (Peisach et al., 1971).

Table V: Analysis of Spinach Sulfite and Nitrite Reductases for Siroheme, Total Iron, and Acid-Labile Sulfide[a]

prosthetic group	mol/mol of subunit: 63K sulfite reductase	69K sulfite reductase	nitrite reductase[c]
siroheme			
chemical analysis	0.99	ND[b]	0.52 (1.00)
EPR ferriheme	0.96	0.98	0.48 (0.92)
total Fe	5.2	5.1	3.1 (5.9)
acid-labile sulfide	3.3	3.0	2.0 (3.8)
reducible iron–sulfur centers (EPR)[d]	0.90	ND[b]	0.46 (0.88)

[a] For details of analytical methods and EPR quantitation see Experimental Procedures and the text. All chemical and EPR determinations were performed at least in duplicate. Results differed in all cases by no more than ±10%. [b] ND = not determined. [c] Data of Lancaster et al. (1979). Mol/mol of siroheme in parentheses. [d] Determined with enzyme–CO complex. For details see legend to Figure 10 and Lancaster et al. (1979).

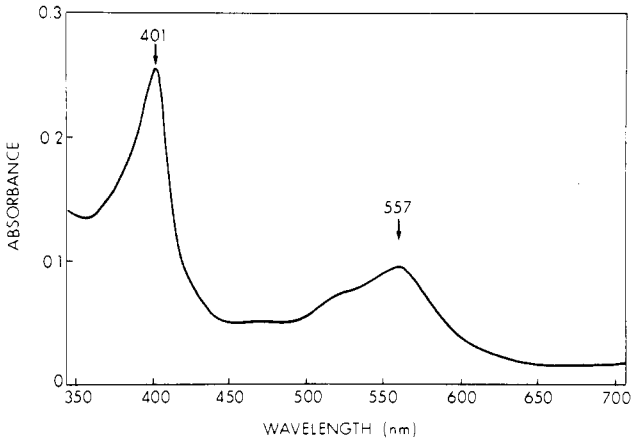

FIGURE 6: Absorption spectrum of spinach sulfite reductase heme in acetone–HCl containing 33% pyridine. To 0.06 mL of 63K spinach SiR in 0.1 M potassium phosphate (pH 7.7), A_{587} = 1.37 cm^{-1}, was added 0.54 mL of acetone–0.015 N HCl at 0 °C. After 5 min, the mixture was centrifuged for 2.5 min at 30000g at 0 °C, 0.30 mL of pyridine added to the supernatant, and the mixture again centrifuged. The absorption spectrum is shown. The $A_{557-700nm}$ of the extracted heme solution (0.9 mL) was 0.080 cm^{-1}, from which a siroheme concentration in the original enzyme solution of 76 μM can be calculated (Siegel et al., 1978).

(The preparations show a small variable signal at g = 4.3 which is probably due to a small amount of adventitious iron.) Quantitation of the ferriheme spectrum, as described under Experimental Procedures, yielded 0.97 ± 0.04 spin per subunit in both enzyme forms (Table V).

Prosthetic Groups. (*A*) *Siroheme.* The heme prosthetic group was extracted from a solution of 63K SiR (in 0.1 M potassium phosphate, pH 7.7) by treatment of enzyme with 9 volumes of acetone containing 0.015 N HCl at 0 °C. After centrifugation, the supernatant was made 33% (v/v) in pyridine and the absorption spectrum recorded. The spectrum (Figure 6), with maxima at 401 and 557 nm (A_{401}/A_{557} = 2.8) and a shoulder in the 520–530-nm region, is characteristic of siroheme in the pyridine/acetone/HCl solvent used (Siegel et al., 1978). By use of the reported $E_{557-700}$ = 1.57 × 10^4 M^{-1} cm^{-1} for siroheme in this solvent (Siegel et al., 1978), the 63K SiR was found to contain 0.99 mol of siroheme/mol of subunit (Table V). This result, taken together with the EPR quantitation data, indicates that each subunit of SiR contains 1 mol of siroheme and that substantially all of this heme is in the high-spin ferric state.

Further confirmation of the presence of siroheme in 63K SiR was achieved as follows: (a) An aliquot of the extracted

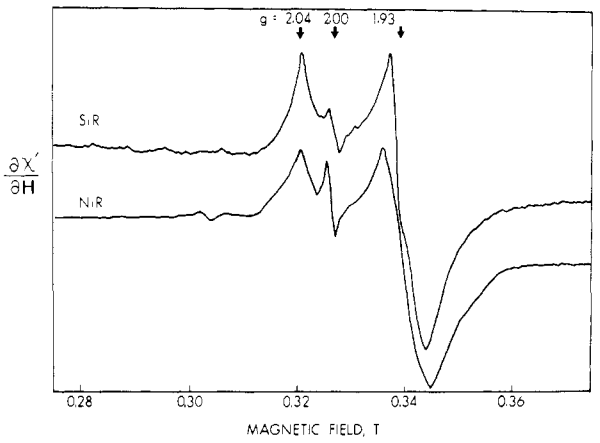

FIGURE 7: EPR spectra of spinach sulfite and nitrite reductase iron–sulfur centers in 80% dimethyl sulfoxide (Me_2SO). 0.16 mL of Me_2SO was added anaerobically to EPR tubes containing 0.04 mL of either 76 μM 63K spinach SiR (upper curve) or 370 μM spinach nitrite reductase (lower curve) in 0.05 M potassium phosphate, pH 7.75. The solutions were maintained just above the freezing point of Me_2SO with an ice bath, and the Me_2SO was added in four equal aliquots, with 15 s of cooling of the solution after each addition. 0.005 mL of 0.41 M $Na_2S_2O_4$ in the same buffer was added to each enzyme–Me_2SO solution, and the solutions were thoroughly mixed and subsequently frozen in liquid N_2. Conditions of measurement: temperature 20 K; microwave frequency 9.15 GHz; microwave power 10 mW; modulation amplitude 1 mT. The small radical signal at g = 2.00 was present in a control solution from which enzyme had been omitted.

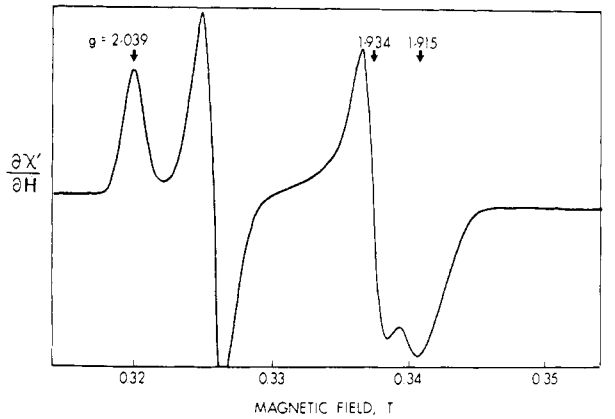

FIGURE 8: EPR spectrum of the iron–sulfur center of spinach sulfite reductase–CO complex. An anaerobic solution containing 20 μM 63K spinach SiR, 0.6 mM CO, and 0.1 mM MV^+, in 0.05 M potassium phosphate (pH 7.75), was incubated for 30 min at 23 °C. $Na_2S_2O_4$ was then added to a final concentration of 5 mM and the sample frozen in liquid N_2. Conditions of measurement: temperature 20 K; microwave frequency 9.13 GHz; microwave power 50 mW; modulation amplitude 1 mT. The large radical signal at g = 2.00 is due to MV^+.

heme solution of Figure 6 was taken to near dryness with a stream of N_2 and the resulting material taken up in a small volume of pyridine. The concentrated heme solution was added to a cuvette containing CO-saturated buffer to which $^1/_{6.5}$ volume of 0.1 N KOH and a few crystals of $Na_2S_2O_4$ had been added. The absorption spectrum of the resulting heme–CO complex exhibited a sharp α-band maximum at 602 nm and a well-defined β band at 558 nm, a spectrum identical with that previously reported for the CO complex of siroheme in the same solvent (Siegel et al., 1978). (b) An aliquot of the extracted heme solution of Figure 6 was taken to near dryness and the residue dissolved in glacial acetic acid. The heme was demetallated by the procedure of Siegel et al. (1978), and the resulting (highly fluorescent) porphyrin was taken up in 2 N HCl. The absorption spectrum of the porphyrin exhibited a triple Soret band at 374, 385, and 405 nm and visible absorption bands (in order of increasing intensity) at 499, 530, 573, and 612 nm. This spectrum is identical with that previously reported for the porphyrin moiety of siroheme, in 2 N HCl (Siegel et al., 1978).

(*B*) *Iron–Sulfur Center.* As shown in Table V, chemical analyses of the 63K and 69K SiR yielded 5.2 and 5.1 mol of total Fe per mol of subunits, respectively. Analyses for labile S^{2-} yielded 3.2 and 3.3 mol per mol of subunit, respectively. Taken together with the fact that only five half-cystines are present per SiR subunit (Table IV), these analyses suggest the presence of one $Fe_4S_4(Cys_4)$ center per subunit rather than, e.g., two $Fe_2S_2(Cys_4)$ centers per subunit.

Although it has proven difficult to elicit more than a small (<0.1 spin per heme) "g = 1.94" type of EPR signal characteristic of reduced iron–sulfur centers by treatment of native SiR with reducing agents such as $S_2O_4^{2-}$ or MV^+, such an EPR signal was readily observed when $S_2O_4^{2-}$ was added to (a) 63K SiR in 80% Me_2SO (g = 2.04, 1.93; Figure 7) or (b) the CO complex of 63K SiR (g = 2.039, 1.935, 1.915; Figure 8). In both cases, quantitation of the EPR spectra yielded 0.90 ± 0.02 spin per enzyme subunit, a result which indicates the presence of one iron–sulfur center as well as one siroheme per subunit. The saturation properties of the reduced enzyme EPR signal in 80% Me_2SO (no saturation of the signal was seen at 20K until microwave powers of greater than 20 mW were applied), as well as the fact that no signal could be detected at 77K, are consistent with the behavior expected for an Fe_4S_4 (but not an Fe_2S_2) center (Cammack, 1975).

Immunological Relationship between 63K and 69K SiR. Figure 9 shows that the 63K and 69K sulfite reductases appear to be immunologically identical when reacted with antisera prepared vs. the 63K enzyme. (No antisera were prepared vs. 69K SiR because of its more limited availability.) It seemed probable, in light of the close similarity between these two enzyme forms, that the 69K SiR might in fact be a precursor of the 63K form, the latter being generated by proteolysis during the purification procedure.[3]

So that this hypothesis could be tested, 50 g of spinach was processed through step 1 of the purification procedure for SiR (desalting omitted), and aliquots of the resulting crude enzyme solution were mixed with purified antisera prepared vs. either 63K spinach SiR or *E. coli* SiR hemoprotein subunit. The precipitates were taken up in the sample buffer of Laemmli (1970) and aliquots of the resulting solutions subjected to $NaDodSO_4$ gel electrophoresis, as described under Experimental Procedures, together with control samples of 69K spinach SiR (well A) and the reference proteins bovine serum albumin (M_r 68 000) and pyruvate kinase (M_r 58 000) (well E). The results are shown in Figure 10. The precipitate recovered from spinach extract which had been treated with anti-63K SiR (well B) exhibited a prominent protein band at M_r 69 000 as well as a much less intense protein band at M_r 63 000. In a control experiment, purified 63K SiR was treated with anti-63K SiR serum and the resulting precipitate processed in the same fashion as the spinach extract. As seen in Figure 10, well C, such a precipitate showed the expected protein band at M_r 63 000, but little or no protein could be detected at the position corresponding to M_r 69 000. The nonspecific protein precipitate recovered upon reaction of

[3] When PMSF was not included in buffers used for SiR purification, we found that SiR preparations, catalytically active in the MV^+–SO_3^{2-} reductase assay, were obtained which exhibited only polypeptide species on $NaDodSO_4$ gel electrophoresis of M_r 54 000–60 000. Thus proteolytic cleavage to species smaller than the 63K SiR is clearly possible in spinach extracts.

Table VI: Stoichiometry of Sulfite Reduction Catalyzed by Spinach Sulfite and Nitrite Reductases[a]

enzyme	$[SO_3^{2-}]$ added	electron donor	[electrons] consumed	$[H_2S]$ produced	e^-/H_2S
63K SiR	10.2	MV^+	62.4	10.1	6.18
63K SiR	20.3	MV^+	118.3	20.0	5.92
63K SiR	50.9	MV^+	136.4	22.5	6.06
63K SiR	1017	MV^+	139.5	23.0	6.07
69K SiR	20.3	MV^+	113.8	19.1	5.96
69K SiR	40.7	MV^+	144.2	24.4	5.91
69K SiR	1017	MV^+	135.8	22.1	6.14
NiR	1017	MV^+	116.5	19.6	5.94
63K SiR	8.2	Fd_r	49.7	8.1	6.14
63K SiR	12.3	Fd_r	71.0	11.9	5.97
69K SiR	8.2	Fd_r	46.3	7.9	5.86
69K SiR	12.3	Fd_r	75.8	12.5	6.06

[a] Reaction mixtures were prepared in anaerobic cuvettes and were identical with those described under Experimental Procedures for the standard MV^+- or Fd_r-SO_3^{2-} reductase assays, with the exception that SO_3^{2-} concentrations were as stated in the table. MV^+ was 140 μM, Fd_r was 90 μM, SiR was 0.1 μM, and NiR was 2 μM. Reactions were initiated by addition of SO_3^{2-} and oxidation of MV^+ or Fd_r followed spectrophotometrically. Reactions either were allowed to go to completion or, when the colorimetric assay for sulfide was used, were stopped by addition of the colorimetric reagents for sulfide determination. The MV^+-dependent reactions utilized nonradioactive SO_3^{2-} in the reaction mixtures, and sulfide was determined colorimetrically. The Fd_r-dependent reactions utilized $^{35}SO_3^{2-}$ in the reaction mixtures (2.3×10^7 cpm/mol), and the volatility assay for sulfide was used. See Experimental Procedures for details of chemical determinations. All concentrations in the table are in μM.

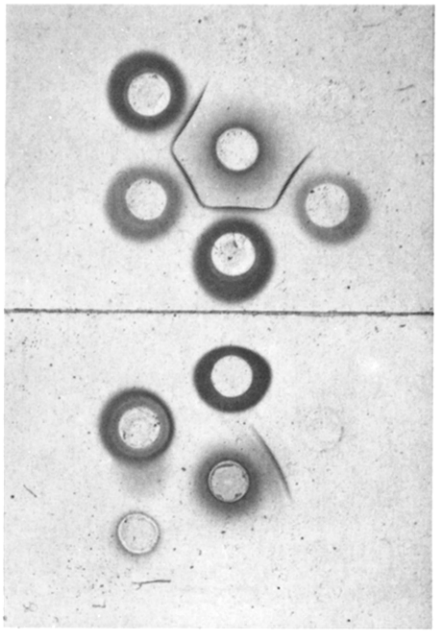

FIGURE 9: Immunodiffusion analysis of sulfite and nitrite reductases. The standard Ouchterlony immunodiffusion set LKB 6800 A-7 was used to carry out microdouble immunodiffusion tests as described under Experimental Procedures. (Upper pattern) The center well contained 0.01 mL of anti-63K spinach sulfite reductase. The outer wells contained (clockwise from top) 15 μg of (12 o'clock) spinach nitrite reductase, (2 o'clock) *E. coli* sulfite reductase hemoprotein, (4 and 8 o'clock) 63K spinach SiR, and (6 and 10 o'clock) 69K spinach SiR. (Lower pattern) The center well contained 0.01 mL of anti-nitrite reductase serum. The outer wells contained (clockwise from top) 15 μg of (12 o'clock) 69K spinach SiR, (2 o'clock) spinach nitrite reductase, (8 o'clock) *E. coli* sulfite reductase hemoprotein, and (10 o'clock) 63K spinach SiR. Wells at 4 and 6 o'clock contained buffer. The 69K SiR was observed to precipitate extensively in the region of the antigen well even in the absence of antiserum. Such precipitation was less marked with the 63K SiR and was not observed with nitrite reductase or *E. coli* sulfite reductase hemoprotein.

spinach extract with antiserum prepared vs. *E. coli* SiR hemoprotein subunit (this antiserum did not react with either 63K or 69K spinach SiR) exhibited no protein bands in the M_r 60 000–70 000 range upon $NaDodSO_4$ gel electrophoresis (well D).

The results of Figure 10 show that the subunits of spinach SiR exist during early stages of the purification procedure primarily in the form of 69 000 molecular weight polypeptides. By step 3 of the procedure, the enzyme is recovered primarily as a species composed of 63 000 molecular weight subunits (ratio of 69K:63K forms = 3 in Figure 1). By application of the antibody precipitation technique to enzyme at various stages of the purification procedure, we have determined that the conversion from the 69K to the 63K form occurs rapidly after the $(NH_4)_2SO_4$ precipitated enzyme solution is desalted in step 1 of the purification procedure.

Catalytic Properties of Spinach SiR. Tables VI–VIII show that the 63K and 69K forms of spinach SiR can catalyze the six-electron reductions of SO_3^{2-} (to S^{2-}) and NO_2^- (presumably to NH_3) as well as the two-electron reduction of NH_2OH. Both Fd_r and MV^+ could serve as electron donors for the enzyme. (The Fd_r–NH_2OH reduction reaction, though demonstrably catalyzed by the spinach SiRs, could not be studied quantitatively due to the high nonenzymatic rates of Fd_r oxidation by NH_2OH in our assay system.)

It is important to note that the six electrons per SO_3^{2-} or S^{2-} stoichiometry was maintained both under conditions where SO_3^{2-} was limiting and where reductant was limiting. This behavior is in marked contrast to that reported with "dissimilatory" sulfite reductases from *Desulfovibrio* and *Desulfotomaculum* species [see Siegel (1975) for review]

Table VII: Kinetic Parameters for MV^+- and Fd_r-Dependent Reduction Reactions Catalyzed by Spinach Sulfite and Nitrite Reductases[a]

		63K sulfite reductase			69K sulfite reductase			nitrite reductase		
electron donor	electron acceptor	K_A (μM)	K_D (μM)	V_{max} (e^- min^{-1} heme^{-1})	K_A (μM)	K_D (μM)	V_{max} (e^- min^{-1} heme^{-1})	K_A (μM)	K_D (μM)	V_{max} (e^- min^{-1} heme^{-1})
MV^+[b]	SO_3^{2-}	11		660	12		630	800		140
MV^+	NO_2^-	3 500		5 700	3 100		5 300	1.3		72 000
MV^+	NH_2OH	14 300		35 000	14 500		33 000	42 000		59 000
Fd_r[c]	SO_3^{2-}	21	21[d]	6 400	25	19[d]	6 000			
Fd_r	NO_2^-	3 800	25[e]	11 900	4 100	28[e]	11 200	17	33[f]	263 000

[a] Reaction conditions were as described under Experimental Procedures. Kinetic parameters were obtained from Lineweaver–Burk plots. $K_A = K_m$ value for electron acceptor. $K_D = K_m$ value for electron donor. V_{max} values were obtained at as close to saturation with both substrates as was experimentally feasible; these values are expressed as electrons transferred min^{-1} heme^{-1}. [b] At $[MV^+]$ = 140 μM. There was no variation in the MV^+–SO_3^{2-} reductase activity of SiR or of the MV^+–NO_2^- reductase activity of NiR (at saturating acceptor) when $[MV^+]$ was reduced from 140 to 30 μM. [c] When the concentration of acceptor was varied, $[Fd_r]$ = 90 μM. [d] At 2 mM SO_3^{2-}. [e] At 20 mM NO_2^-. [f] At 2 mM NO_2^-.

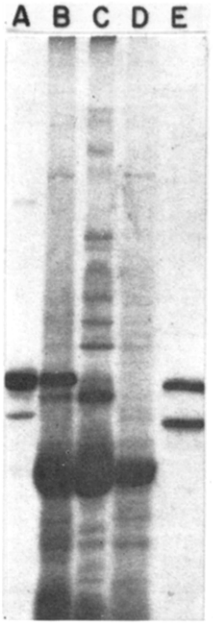

FIGURE 10: Protein species precipitated from $(NH_4)_2SO_4$ fractionated spinach leaf extract by anti-spinach SiR serum. 50 g of freshly harvested spinach (not frozen) was processed through the first step of the sulfite reductase purification procedure described in the text, except that stirring times were reduced to 15 min and centrifugations performed for 15 min at 40000*g*. The precipitated sulfite reductase fraction was suspended in buffer A to a volume of 8.5 mL, and the suspension was clarified by centrifugation for 15 min at 40000*g*. 0.25 mL of purified antiserum was then added to 2 mL of the supernatant fluid, and the mixture was centrifuged for 60 min at 2 °C in a tabletop centrifuge. Pellets were allowed to thoroughly drain of supernatant fluid. The pellets were then dissolved in $NaDodSO_4$-containing buffer and the solutions heated in a boiling water bath and subjected to $NaDodSO_4$ gel electrophoresis as described under Experimental Procedures. Well A contained 4 μg of purified 69K spinach SiR. Well B contained 5 μL of dissolved pellet obtained from reaction of anti-63K SiR serum with the spinach extract. Well C contained 5 μL of dissolved pellet obtained by reaction of anti-63K SiR serum with purified 63K spinach SiR. Well D contained 5 μL of dissolved pellet obtained from incubation of anti-*E. coli* SiR hemoprotein serum with the spinach extract. Well E contained 0.4 μg each of pyruvate kinase (subunit M_r 58 000) and bovine serum albumin (subunit M_r 68 000).

Table VIII: Stoichiometry of Nitrite Reduction Catalyzed by Spinach Sulfite and Nitrite Reductases[a]

enzyme	$[NO_2^-]$ added	electron donor	[electrons] consumed	e^-/NO_2^-
63K SiR	10.1	MV^+	61.3	6.07
63K SiR	20.2	MV^+	125.8	6.23
69K SiR	10.1	MV^+	60.8	6.02
69K SiR	20.2	MV^+	117.9	5.84
NiR	10.1	MV^+	58.6	5.80
NiR	20.2	MV^+	122.9	6.08
63K SiR	10.1	Fd_r	62.6	6.20
69K SiR	10.1	Fd_r	60.8	6.02
NiR	10.1	Fd_r	59.9	5.93

[a] Reaction mixtures were prepared in anaerobic cuvettes and were identical with those described under Experimental Procedures for the standard MV^+- or Fd_r-NO_2^- reductase assays, with the exception that NO_2^- concentrations were as stated in the table. MV^+ was 140 μM, Fd_r was 90 μM, SiR was 2 μM, and NiR was 0.01 μM. Reactions were initiated by addition of NO_2^- and were followed spectrophotometrically to completion. All concentrations in the table are in μM.

where more than six electrons were consumed for each SO_3^{2-} reduced, particularly at high ratios of $[MV^+]/[SO_3^{2-}]$. This high level of electron uptake was associated with release from the enzyme of the "byproducts" $S_3O_6^{2-}$ and $S_2O_4^{2-}$, which are presumed to be sulfite adducts of intermediate sulfur-containing compounds normally generated in the course of SO_3^{2-} reduction to S^{2-}. It is evident that such compounds are not released in any significant quantity by the spinach SiR and that this enzyme must therefore be considered as an assimilatory sulfite reductase.

Table IX: Inhibition of 63K Spinach Sulfite Reductase by Cyanide and CO[a]

expt	components in preincubation mixture	MV^+-SO_3^{2-} reductase act. (units/mL)	rel act.
A[b]	enzyme	18.3	100
	enzyme + CO	17.1	94
	enzyme + MV^+	17.4	95
	enzyme + MV^+ + CO	0.2	1
B[c]	enzyme	16.5	100
	enzyme + CN^-	15.2	92
	enzyme + MV^+	16.7	101
	enzyme + MV^+ + CN^-	0.1	<1

[a] Assays were performed as described under Experimental Procedures, except that, when indicated, 63K SiR plus buffer was preincubated in the anaerobic cuvette with 140 μM MV^+, 1 mM KCN, or 0.4 mM CO, as indicated, prior to addition of the remaining components necessary to initiate the MV^+-SO_3^{2-} reductase reaction. SO_3^{2-} concentration was 1 mM. [b] Preincubation time 43 min. [c] Preincubation time 5 min.

Velocities of the MV^+-dependent reductions of SO_3^{2-}, NO_2^-, and NH_2OH and of the Fd_r-dependent reductions of SO_3^{2-} and NO_2^- were studied as a function of concentrations of electron donor and acceptor. Linear Lineweaver–Burk plots were obtained in all cases. Kinetic parameters derived from these studies are summarized in Table VII. These data show the following: (1) The 63K and 69K forms of SiR are functionally indistinguishable. We may conclude that the peptide cleaved during the 69K-to-63K conversion is not essential to catalysis with either MV^+ or Fd_r as reductant. (2) Higher V_{max} values can be obtained with either of the nitrogenous acceptors than with the presumed physiological substrate SO_3^{2-}. However, the K_m value for SO_3^{2-} is 2–3 orders of magnitude less than that for either NO_2^- or NH_2OH. (3) Considerably higher V_{max} values can be obtained with Fd_r than with MV^+ as electron donor.[4]

Inhibition by Cyanide and CO. CN^- and CO have been found to bind tightly to the siroheme prosthetic groups of *E. coli* SiR (Rueger & Siegel, 1976) and spinach NiR (Vega & Kamin, 1977). When bound, these heme ligands totally inhibited the ability of these enzymes to catalyze SO_3^{2-} and NO_2^- reduction, respectively. In the case of *E. coli* SiR, CN^- bound rapidly to the enzyme only when SiR was treated with a reductant, such as MV^+. NiR, in contrast, was found to bind CN^- rapidly when either oxidized or reduced (Lancaster et al., 1979). Both enzymes required addition of reductant to bind CO. This result is not surprising, since CO characteristically binds only to the ferrous state of hemoproteins.

Table IX shows that incubation of 63K spinach SiR with either CN^- or CO in the presence of the reducing agent MV^+

[4] The polyanion dextran sulfate was found to stimulate the MV^+-SO_3^{2-} and MV^+-NO_2^- reductase activities of spinach SiR and NiR, respectively, by 2.5–3-fold. Half-maximal stimulation was found with 2 μg/mL dextran sulfate in the standard assays. No stimulation was found with dextran T-500 or an appropriate mixture of dextran T-500 and Na_2SO_4. Dextran sulfate did not stimulate Fd_r-dependent reductase activities of either enzyme. The possibility must be entertained that at least part of the increased rate of electron transfer reactions involving Fd_r vs. MV^+ may be due to the polyanion nature of Fd_r itself.

results in nearly complete inhibition of MV^+–sulfite reductase activity. The MV^+–NO_2^- and NH_2OH reductase activities of the enzyme were also inhibited to the same degree as the MV^+–SO_3^{2-} reductase activity (data not shown). Although no systematic study of the dependence of CO inhibition of incubation time was performed, it was evident that CO inhibition of spinach SiR was a relatively slow process, with 20% of the MV^+–SO_3^{2-} reductase activity remaining after 10 min of reaction with 0.4 mM CO. In contrast, inhibition of 63K SiR by 1 mM KCN appeared to be complete within 1 min of reaction in the presence of MV^+.

Only slight inhibitions of MV^+–SO_3^{2-} reductase activity were observed if MV^+ was not included together with the potential inhibitor prior to addition of SO_3^{2-} to start the assay. As was previously observed with *E. coli* SiR (Siegel et al., 1973), SO_3^{2-} addition protects spinach SiR from further inhibition by either CO or CN^-. In contrast to the report of Asada et al. (1969), our preparation of spinach SiR was not inhibited upon incubation of the enzyme with MV^+ (Table IX).[5]

Nitrite Reductase Catalytic Properties. Because of the similarity in the nature of the enzyme active centers in spinach SiR and NiR, we have examined the specificities of the two enzymes for electron acceptor, with MV^+ serving as electron donor. Table VII shows that NiR, like SiR, can catalyze MV^+–SO_3^{2-}, NO_2^-, and NH_2OH reduction reactions. Tables VI and VIII show that the reductions of SO_3^{2-} and NO_2^- catalyzed by NiR are six-electron transfer processes. The ability of NiR to catalyze reduction of SO_3^{2-} has not been reported previously. The V_{max} and K_m values for each of the MV^+ oxidation reactions, obtained from Lineweaver–Burk plots (linear in all cases), are presented in Table VII. The K_m of NiR for its "physiological" substrate NO_2^- is nearly 3 orders of magnitude smaller than the K_m of the enzyme for SO_3^{2-} and over 4 orders of magnitude smaller than the K_m for NH_2OH. The V_{max} values for NO_2^- and NH_2OH are similar, while that for SO_3^{2-} is more than 2 orders of magnitude less than that for either nitrogenous substrate.

Table VII also shows kinetic parameters for the Fd_r–NO_2^- reductase reaction catalyzed by NiR. K_m values for both Fd_r and NO_2^- were in the micromolar range. The V_{max} value obtained for NO_2^- reduction with Fd_r as donor was more than 3 times the value found with MV^+ as reductant.

When the activities of SiR and NiR are compared (Table VII), the following patterns are evident: (1) Each enzyme can catalyze reduction of all three acceptors at significant rates. (2) Both enzymes exhibit considerably greater V_{max} values with the nitrogenous substrates than with SO_3^{2-}. (3) Each enzyme exhibits a much smaller K_m for its presumed physiological acceptor than for the other two substrates. (4) Higher V_{max} values can be obtained with Fd_r than with MV^+ as reductant.[4]

Table X: Effects of Anti-Sulfite Reductase and Anti-Nitrite Reductase Sera on Reduced Ferredoxin-Dependent Activities of Spinach Sulfite Reductase and Nitrite Reductase[a]

enzyme	antiserum added	vol of antiserum added (μL)	rate of Fd_r oxidation (ΔA_{422}/min)
SiR	none		0.0097
SiR	anti-SiR	2.5	0.0001
SiR	anti-SiR	5.0	<0.0001
SiR	anti-NiR	100	0.0100
NiR	none		0.0104
NiR	anti-NiR	10	0.0023
NiR	anti-NiR	30	0.0011
NiR	anti-NiR	100	0.0009
NiR	anti-SiR	10	0.0101

[a] Fd_r-dependent assays were performed as described under Experimental Procedures, except that, when indicated, antiserum was added to the ferredoxin-containing solution just prior to photoreduction. Antisera were partially purified as described under Experimental Procedures. Reduced ferredoxin concentration was 40 μM, and acceptor concentrations were 1 mM. Spinach nitrite reductase was assayed by means of its Fd_r–NO_2^- reductase activity; 0.2 pmol of enzyme heme was present in each assay. 63K spinach sulfite reductase was assayed by means of its Fd_r–SO_3^{2-} reductase activity; 2.0 pmol of enzyme heme was present in each assay.

Immunological Relationship between Spinach SiR and NiR. Antisera were prepared vs. purified NiR and 63K SiR in order to probe for the possible existence of a structural relationship between the spinach SiR and NiR proteins. Figure 9 shows that spinach NiR did not yield a precipitin line when reacted in Ouchterlony immunodiffusion analysis with anti-63K SiR serum and that 63K SiR did not yield a precipitin line when reacted with anti-NiR serum. (Neither antiserum reacted with *E. coli* SiR hemoprotein subunit, and neither spinach enzyme reacted with anti-*E. coli* SiR hemoprotein serum.)

As shown in Table X and Figure 2, the anti-spinach SiR serum totally inhibited the Fd_r–SO_3^{2-} reductase activity of spinach 63K SiR. The anti-SiR serum was without effect on the Fd_r–NO_2^- reductase activity of NiR, even when the antiserum was added in great excess of the amount required to inhibit an equivalent amount of SiR. Similarly, anti-NiR serum strongly inhibited the Fd_r–NO_2^- activity of NiR but did not inhibit the Fd_r–SO_3^{2-} reductase activity of spinach SiR. Thus there is no evidence for immunological cross-reaction between spinach SiR and NiR.[6]

When crude spinach extract was treated with anti-NiR serum and the precipitated protein subjected to $NaDodSO_4$ gel electrophoresis, the only protein band not present in the control (spinach extract reacted with anti-*E. coli* SiR hemo-

[5] A number of other substances were examined for effects on the MV^+–SO_3^{2-} reductase activity of 63K SiR. L-Methionine (1 mM) (a potential end product of the SO_4^{2-} assimilation pathway) and Na_2SO_4 (10 mM) had no effect on enzymatic activity. Glutathione-*S*-sulfonate (1 mM), a possible intermediate of the SO_4^{2-} assimilation pathway involving "bound intermediates" (Schiff & Hodson, 1973), neither affected the rate of MV^+–SO_3^{2-} reductase activity, under standard assay conditions, nor served as an electron acceptor for MV^+ oxidation catalyzed by the enzyme. When excess 2-mercaptoethanol was added to reaction mixtures containing 1 mM glutathione-*S*-sulfonate and MV^+, oxidation of MV^+ occurred in a reaction catalyzed by 63K SiR. Under these conditions, the sulfonate moiety of glutathione-*S*-sulfonate should be completely converted to free sulfite. NADPH (0.2 mM) neither served as an electron donor for SO_3^{2-} reduction catalyzed by 63K SiR nor affected the rate of the MV^+–SO_3^{2-} reductase reaction catalyzed by the spinach enzyme.

[6] It can be noted that the Fd_r-linked activities of spinach SiR and NiR were inhibited simply upon addition of homologous antiserum to the enzyme, without the necessity to removing the antigen–antibody complex from the solution prior to enzyme assay. In contrast, the MV^+–SO_3^{2-} reductase activity of spinach SiRs was not inhibited upon addition of anti-63K SiR serum unless the antigen–antibody complex was removed from solution by centrifugation and the supernatant fluid used for assays. This result indicates that soluble antibody–SiR complexes are active in catalyzing SO_3^{2-} reduction with the small molecule MV^+ as electron donor, but not with the much larger substrate Fd_r. When an amount of anti-SiR sufficient to inhibit 80% of the MV^+–SO_3^{2-} reductase activity of a fixed amount of spinach SiR (after centrifugation) was preincubated with excess NiR (10 times the amount of 63K SiR) prior to mixing with the SiR, no increase in MV^+–SO_3^{2-} reductase activity above that found with a control solution of antiserum not preincubated with NiR was detected.

protein serum) was one which migrated at M_r = 61 000, the position of purified NiR itself. Thus, there is no evidence for any multiplicity of NiR species in spinach extracts.

Discussion

Comparison of Plant Sulfite Reductases. The present preparation of spinach SiR, a dimer of 63 000–69 000-dalton subunits, differs in a number of significant respects from the SiR preparations previously described by Asada et al. (1969) for spinach, Tamura (1965) for garlic, and Saito & Tamura (1971) for *Porphyra*.

Molecular weights estimated (by gel filtration) for the spinach and *Porphyra* enzymes were both ca. 84 000. The garlic and *Porphyra* enzymes exhibited sedimentation coefficients of 4.5 and 4.7 S, respectively, in contrast to 5.8 S for the present 63K enzyme preparation. Although there is reason to believe that none of these early enzyme preparations were homogeneous, it is possible that the lower molecular sizes observed by other workers with plant SiRs represent (a) increased proteolytic degradation (proteolysis inhibitors were not included in any of the early preparations) and/or (b) a monomer–dimer equilibrium weighted more in the direction of the monomer under the previous conditions of measurement.

The most active of the previously described plant SiR preparations, that of Asada et al. (1969), exhibited a maximum MV^+–sulfite reductase activity of 2.3 units/mg of protein (compared to 10.3 for the present 63K SiR when tested under the assay conditions of Asada et al.). The absorption spectra of the garlic, *Porphyra*, and spinach SiR preparations previously reported are consistent with the presence of siroheme in those enzymes, since each of the spectra had peaks or shoulders in the 385- and 590-nm regions. However, the spectra suggest the presence of contaminating chromophores in each of the preparations. If one assumes that the Asada et al. (1969) preparation did contain siroheme, that the absorption peak at 589 nm in their reported spectra was due to this prosthetic group, and that the E_{587} found for the 63K SiR is applicable to their enzyme, one can calculate from Figure 8 of Asada et al. (1969) that their SiR preparation contained 300 000 g of protein/mol of siroheme. However, when calculated on a per heme basis, the catalytic activity of the Asada et al. enzyme, 700 MV^+ oxidized min^{-1} $heme^{-1}$, is comparable to that found with the present SiR preparation. Thus, we can conclude that the previous plant SiR preparations were substantially impure.

The present SiR preparation also differs in a number of catalytic properties from previously described plant SiRs. The most striking, perhaps, is the ability of the present SiR preparation to utilize NO_2^- as an electron acceptor and Fd_r in preference to MV^+ as an electron donor (Tamura, 1965; Asada et al., 1969; Hennies, 1975). The SiR K_m values determined for SO_3^{2-} in the present work are considerably smaller than the values of 600–700 μM obtained under similar assay conditions with the previous spinach, garlic, and *Pophyra* preparations. The present preparation, unlike that of Asada et al. (1969), is not inhibited by MV^+, does not require the presence of –SH-containing compounds for activity (no effect of 1 mM cysteine, 2-mercaptoethanol, dithiothreitol, or GSH or of 1 mg/mL BSA could be detected on the MV^+–SO_3^{2-} reductase activity), nor is it stimulated by ATP (1 mM tested in MV^+–SO_3^{2-} reductase assay).

Comparison of Spinach and E. coli SiR. Spinach SiR, as purified in the present work, is similar in many of its properties to the *E. coli* SiR hemoprotein subunit. The catalytic behavior of the two enzymes with respect to relative V_{max} and K_m values for SO_3^{2-}, NO_2^-, and NH_2OH as substrates is nearly identical. Each enzyme contains one siroheme and one Fe_4S_4 center per subunit. The *E. coli* hemoprotein is a monomeric 56 000-dalton peptide in solution at pH 7.7 even at high protein concentration (Siegel & Davis, 1974), whereas the spinach SiR is primarily a dimer of 63 000–69 000-dalton peptides under the same conditions. The absorption spectra of the two enzymes are nearly identical, each showing the same pattern and relative intensity of bands, with the *E. coli* enzyme visible wavelength maxima shifted 2–4 nm to longer wavelengths than that of the spinach SiR. Each enzyme has an E of 1.8×10^4 M^{-1} cm^{-1} at its α-band wavelength maximum. EPR spectra of the two enzymes show that all or nearly all of the siroheme is in the high-spin Fe^{3+} state in the enzyme as isolated, although the heme iron is in a somewhat more rhombically distorted tetragonal environment in the *E. coli* than in the spinach SiR (Christner et al., 1981). In both enzymes reduction of the Fe_4S_4 center is greatly facilitated by the presence of the heme ligand CO (Siegel, 1978). In *E. coli* SiR, this phenomenon appears to be related to the tight spin coupling which exists between the heme and Fe_4S_4 centers of the native enzyme (Janick & Siegel, 1979; Christner et al., 1981). This result suggests that such a coupling may exist between the prosthetic groups of spinach SiR as well.

As might be expected for enzymes derived from organisms as widely divergent as spinach and *E. coli*, there is no evidence for immunological cross-reaction between SiR enzymes derived from two species. Similarly, the amino acid compositions of the two enzymes are quite different (Siegel & Davis, 1974), although it may be noted that both enzymes contain only five cysteine residues per subunit. The natural electron donors for the two enzymes are, of course, quite different: the spinach SiR utilizes a small reduced Fe_2S_2 protein, ferredoxin, as its donor, while the *E. coli* SiR hemoprotein subunit is designed to form a tight complex with a 450 000 molecular weight flavoprotein moiety which catalyzes electron transfer from NADPH to the hemoprotein (Siegel et al., 1974; Faeder et al., 1974).

Comparison of Spinach SiR and NiR. The data reported in this work indicate that spinach SiR, like NiR, contains one siroheme and one Fe_4S_4 center per catalytically active polypeptide (Lancaster et al., 1979). Unlike SiR, NiR exists in solution primarily as a monomer (Vega & Kamin, 1977), and there is no evidence that there is any degradation of this 61 000-dalton NiR polypeptide during the course of enzyme isolation.

EPR spectra of SiR and NiR are similar (the heme containing rhombically distorted high-spin Fe^{3+} in both enzymes as isolated), and reduction of the Fe_4S_4 center of both SiR and NiR is facilitated by the presence of heme ligands (Aparicio et al., 1975; Lancaster et al., 1979). Both enzymes thus appear to have the same type of active center. The absorption spectra of SiR and NiR are distinct (Vega & Kamin, 1977), however, a result which may indicate the presence of a different heme ligand environment in the two enzymes. That the protein environment can exert pronounced effects on the properties of the siroheme prosthetic group is shown by the fact that the E_m values reported for the hemes of *E. coli* SiR (−345 mV; Siegel, 1978) and spinach NiR (−50 mV; Stoller et al., 1977) differ by ca. 300 mV. [Cammack et al. (1978) have reported an E_m of −120 mV for the siroheme moiety of *Cucurbita pepo* NiR.]

The present work demonstrates that both SiR and NiR possess the same range of catalytic activities. The ability of NiR to reduce SO_3^{2-} at a V_{max} the same order of magnitude as that of SiR is remarkable, particularly in light of the rel-

atively negative average E'_m for the SO_3^{2-} to H_2S reaction (−120 mV) when compared to that of the NO_2^- to NH_4^+ reaction (+330 mV) (Siegel, 1978). It is clear that both types of multielectron reduction reactions are characteristic of the siroheme–Fe_4S_4 active center. Each enzyme, when saturated with substrate, catalyzes reduction of its physiological substrate at higher V_{max} than does the other enzyme. In addition, the K_m of each enzyme for its physiological substrate is much smaller than the K_m of the other enzyme for that substrate. It is these factors which may cause SiR to properly be termed a "sulfite reductase" and NiR a "nitrite reductase".

Despite the marked similarities between spinach SiR and NiR with respect to the general nature of their catalytic centers, it is clear that they represent quite different proteins. Thus the amino acid compositions of SiR and NiR are distinct (Table IV), and we have been unable to detect any immunological cross-reaction between the two enzymes. There is therefore no reason to believe that SiR and NiR are modified forms of a common protein in spinach, although the possibility that the two enzymes may share a common evolutionary origin cannot be excluded.

Acknowledgments

We are grateful to Patricia Davis and James Wilkerson for preparation of spinach NiR and for their assistance in some of the experiments reported in this paper. We thank Dr. Harvey Sage for his guidance in preparation of the antisera.

References

Aasa, R., & Vangaard, T. (1975) *J. Magn. Reson. 19*, 308–315.

Aparicio, P. J., Knaff, D. B., & Malkin, R. (1975) *Arch. Biochem. Biophys. 169*, 102–107.

Asada, K., Tamura, G., & Bandurski, R. S. (1969) *J. Biol. Chem. 244*, 4904–4915.

Borchert, M. T., & Wessels, J. S. C. (1970) *Biochim. Biophys. Acta 197*, 78–83.

Cammack, R. (1975) *Biochem. Soc. Trans. 3*, 482–489.

Cammack, R., Hucklesby, D. P., & Hewitt, E. J. (1978) *Biochem. J. 171*, 519–526.

Carter, P. (1971) *Anal. Biochem 40*, 450–458.

Chervenka, C. H. (1969) *Manual of Methods for the Analytical Ultracentrifuge*, Beckman Instruments, Palo Alto, CA.

Christner, J. A., Munck, E., Janick, P. A., & Siegel, L. M. (1981) *J. Biol. Chem. 256*, 2098–2101.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids, and Peptides*, p 374, Reinhold, New York.

Faeder, E. J., Davis, P. S., & Siegel, L. M. (1974) *J. Biol. Chem. 249*, 1599–1609.

Hennies, H. H. (1975) *Z. Naturforsch., C: Biosci. 30C*, 359–362.

Hosein, B. (1973) Ph.D. Thesis, University of Michigan.

Hucklesby, D. P., Dalling, M. J., & Hagemann, R. H. (1972) *Planta 104*, 220–223.

Janick, P. A., & Siegel, L. M. (1979) Abstracts of the XIth International Congress of Biochemistry, Toronto, Canada p 427.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Lancaster, J. R., Vega, J. M., Kamin, H., Orme-Johnson, N. R., Orme-Johnson, W. H., Krueger, R. J., & Siegel, L. M. (1979) *J. Biol. Chem. 254*, 1268–1272.

Layne, E. (1957) *Methods Enzymol. 3*, 450.

Lazzarini, R. A., & Atkinson, D. E. (1961) *J. Biol. Chem. 236*, 330.

Lee, J. P., LeGall, J., & Peck, H. D., Jr. (1973a) *J. Bacteriol. 115*, 529.

Lee, J. P., Yi, C. S., LeGall, J., & Peck, H. D., Jr. (1973b) *J. Bacteriol. 115*, 453.

Markham, R. (1967) *Methods Virol. 2*, 275–302.

Murphy, M. J., Siegel, L. M., Kamin, H., & Rosenthal, D. (1973) *J. Biol. Chem. 248*, 2801–2814.

Peisach, J., Blumberg, W. E., Ogawa, S., Rachmilewitz, E. A., & Oltzick, R. (1971) *J. Biol. Chem. 246*, 3342–3355.

Rideal, E. K., & Roberts, R. (1951) *Proc. R. Soc. London, Ser. A 205*, 391.

Rueger, D. C., & Siegel, L. M. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 610–620, Elsevier, Amsterdam.

Satio, E., & Tamura, G. (1971) *Agric. Biol. Chem. 35*, 491–500.

Schiff, J. A., & Hodson, R. C. (1973) *Annu. Rev. Plant Physiol. 24*, 381–414.

Scott, A. I., Irwin, A. J., Siegel, L. M., & Shoolery, J. N. (1978) *J. Am. Chem. Soc. 100*, 7987–7994.

Siegel, L. M. (1965) *Anal. Biochem. 11*, 126–132.

Siegel, L. M. (1975) in *Metabolism of Sulfur Compounds* (Greenberg, D. M., Ed.) pp 217–286, Academic Press, New York.

Siegel, L. M. (1978) in *Mechanisms of Oxidizing Enzymes* (Singer, T. P., & Ondarza, R. N., Eds.) pp 201–214, Elsevier/North-Holland, New York.

Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.

Siegel, L. M., & Davis, P. S. (1974) *J. Biol. Chem. 249*, 1587–1598.

Siegel, L. M., Kamin, H., Rueger, D. C., Presswood, R. P., & Gibson, Q. H. (1971) in *Flavins and Flavoproteins* (Kamin, H., Ed.) pp 523–554, University Park Press, Baltimore, MD.

Siegel, L. M., Murphy, M. J., & Kamin, H. (1973) *J. Biol. Chem. 248*, 251–264.

Siegel, L. M., Davis, P. S., & Kamin, H. (1974) *J. Biol. Chem. 249*, 1587–1598.

Siegel, L. M., Murphy, M. J., & Kamin, H. (1978) *Methods Enzymol. 52*, 436–447.

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190.

Stoller, M. L., Malkin, R., & Knaff, D. B. (1977) *FEBS Lett. 81*, 271–274.

Tagawa, K., & Arnon, D. I. (1968) *Biochim. Biophys. Acta 153*, 602.

Tamura, G. (1965) *J. Biochem. (Tokyo) 57*, 207–214.

Thorneley, R. N. F. (1974) *Biochim. Biophys. Acta 333*, 487–496.

Vega, J. M., & Kamin, H. (1977) *J. Biol. Chem. 252*, 896–909.

Vega, J. M., Garrett, R. H., & Siegel, L. M. (1975) *J. Biol. Chem. 250*, 7980–7989.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Wyckoff, M. D., Rodbard, D., & Chrambach, A. (1977) *Anal. Biochem. 78*, 459–482.

Yoshimoto, A., & Sato, R. (1968) *Biochim. Biophys. Acta 153*, 576.

Zamenhof, S. (1957) *Methods Enzymol. 3*, 702.

Zumft, W. (1972) *Biochim. Biophys. Acta 276*, 363–375.