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Activated Conformers of *Escherichia coli* Sulfite Reductase Heme Protein Subunit†

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ABSTRACT: The heme protein subunit of *Escherichia coli* sulfite reductase shows enhanced reactivity with its substrate and a number of other ligands after a cycle of reduction and reoxidation at alkaline pH. At pH 9.5 this variant of the enzyme possesses at least four EPR-detectable, chloride-sensitive high-spin conformers, in contrast to the single chloride-insensitive species observed in the oxidized, resting enzyme at pH 7.7. Quantitative reversal of the spectral and ligand-binding properties of the "activated" enzyme to those of the resting enzyme is observed on reacidification to pH 7.7. At intermediate pH values, there occurs an acid-catalyzed relaxation of the activated enzyme to the resting form. This reaction is distinct from the one responsible for the accelerated ligand binding and production of multiple EPR conformers, which appears to be regulated by a process with a pK of 8.5.

Many metalloproteins are activated for catalysis and/or ligand binding by reduction of their metal-containing prosthetic groups. In the case of hemoglobin and myoglobin, it is the ferrous state of the iron, per se, that is the factor of prime importance. The mechanism of activation for heart cytochrome *c* oxidase is more complex and appears to involve the sustained rupture of the bond between cytochrome a_3 and a bridging, sulfur-containing amino acid that occurs after reduction and turnover of O_2 (Antonini et al., 1977). A spin-state change and increased ligand affinity of the substrate-binding heme are coupled to the reduction of the metal centers in *Pseudomonas putida* cytochrome P-450 (Sligar, 1976) and *Pseudomonas aeruginosa* nitrite reductase (Walsh et al., 1979). Reoxidation reverses the activation in all of these proteins, but with cytochrome *c* oxidase the reversion to the starting species is slow enough (minutes) to be followed by using room temperature optical spectroscopy.

† Supported by Project Grant 7875-01 from the Veterans Administration and Grant GM 32210 from the National Institutes of Health.

0006-2960/88/0427-4991$01.50/0

Sulfite reductase heme protein subunit (SiRHP),[1] a siroheme 4Fe-4S protein, is also reductively activated in that reduction increases the k_{obsd} for sulfite binding by 5 orders of magnitude over that of the resting[2] form (Janick et al., 1983). We report in this paper that reduction and reoxidation of SiRHP at alkaline pH produces a ferric siroheme species which possesses an enhanced reactivity with substrate and other ligands. The kinetic profiles for ligation of sulfite and cyanide to the activated enzyme showed multiple phases, which could be modulated with respect to apparent rate constant and phase amplitude by non-heme-binding anions.

The optical and EPR spectra of this "activated" enzyme (in a pH range from 8.1 to 9.9) are very different from those of the pH 7.7 resting form. Multiple, rhombic high-spin EPR conformers are observed instead of the single high-spin species of the resting enzyme. In recent years it has become increasingly apparent that conformational heterogeneity in the active site of heme proteins is more the rule than the exception. Such heterogeneity can often be overlooked if only a single probe is used, and thus it is proper to employ a multipronged approach to the problem: optical and EPR spectroscopies to demonstrate conformers of resting cytochrome *c* oxidase (Brudvig et al., 1981); optical/infrared spectroscopy to delineate the four substrates of CO-complexed hemoglobin (Choc & Caughey, 1981), myoglobin (Shimada & Caughey, 1982), and cytochrome *c* oxidase (Yoshikawa & Caughey, 1982; Young et al., 1984); optical, MCD, and EPR spectroscopies to show multiple forms of oxidized *P. aeruginosa* cytochrome *c* peroxidase (Foote et al., 1985); and low-temperature optical kinetics to study the recombination of photodissociated CO to hemoglobin and myoglobin (Fraunfelder & Wolynes, 1985).

The conformer distribution in activated SiRHP was found to be sensitive to the composition of the supporting buffer and the presence of non-heme-binding anions such as chloride, sulfate, and nitrate. EPR spectra of samples taken during the course of ligand-binding experiments indicated that the most rhombic conformers reacted with added ligands first. Of further interest was the fact that significant amounts (up to 0.9 spin/heme) of EPR-silent heme were found in the adducts of the activated enzyme with sulfite, nitrite, cyanide, and azide. The changes in ligand reactivity and the EPR spectrum appeared to be regulated by a process having a pK of approximately 8.5, but an acid-catalyzed relaxation of the activated enzyme back to the resting form was also present.

Our findings of active-site conformational heterogeneity, activation by alkaline reduction/reoxidation, acid-catalyzed relaxation of the activated enzyme, and EPR silence of certain ferriheme species of SiRHP will be discussed with respect to structural aspects of the mechanism of substrate reduction catalyzed by the assimilatory nitrite and sulfite reductases.

Materials and Methods

NADPH–sulfite oxidoreductase holoenzyme and its heme protein subunit were isolated from *E. coli* B by using a minor modification of earlier procedures (Siegel et al., 1973; Siegel & Davis, 1974), as outlined below.

Nucleic acids were extracted with 10% poly(ethyleneimine) instead of protamine sulfate. There were no further changes in the holoenzyme protocol, and after the second ammonium sulfate fractionation, the yield and activity of holoenzyme were identical with those previously reported, although the A_{280}/A_{387} ratio of this product was significantly and reproducibly higher (8 ± 2 compared to 5 ± 1) than that reported by Siegel et al. (1973). Gel exclusion chromatography using Bio-Gel A 1.5-m resin removed contaminating protein and gave a purified sulfite reductase holoenzyme like that found by using the original methodology. If the heme protein subunit was to be studied, the product of the second ammonium sulfate fractionation was treated with either 4 M urea for 8 h at 4 °C or 3 M urea for 30 min at 23 °C followed by chromatography on DE-52 cellulose in 4 M urea. The A_{280}/A_{387} ratio of the effluent from this column was 4 ± 2, as compared to the literature value of 1.67 (Siegel & Davis, 1974). The latter ratio could be obtained if the protein was further chromatographed on benzyl-DEAE-cellulose at room temperature by using a gradient of potassium phosphate, pH 7.7, from 0.01 to 0.3 M. The spectral form, enzyme activity, and SDS–PAGE mobility of the final product were as described previously.

E. coli cells were grown in 80–90-kg batches on the minimal medium of Siegel et al. (1973) by Grain Processing Corp. of Muscatine, IA, and stored as 2.5-kg cakes at 77 K until used. Following isolation, the heme protein was concentrated to 100 μM in heme and kept at −20 °C in 0.1 M potassium phosphate plus 0.1 mM EDTA, pH 7.7 (standard buffer). SiRHP concentrations were measured from the absorption of the oxidized enzyme at 590 nm by using an extinction coefficient of 18.1 mM^{-1} cm^{-1}. Methylviologen–sulfite reductase catalytic activities were determined as Siegel and Davis (1974) have described.

All chemicals used were of reagent or analytical grade and unless noted otherwise were not purified further. Salts were typically added in solid form to buffered protein solutions. When needed, neutralized solutions of KCN or $NaHSO_3$ + EDTA + mannitol were prepared on ice immediately before use.

In order to achieve anaerobiosis, ultrahigh purity argon (Alphagas 99.999%) was passed through a column of BASF catalyst and then to an all-glass gas train to which a modified quartz Thunberg cuvette was attached by a 6-in. length of butyl rubber tubing. After a 15-min preflush and 10, 4-min flush–evacuation cycles, the cuvette was sealed off under positive argon pressure. Reduction was achieved photochemically by using 10 mM EDTA plus either deazaflavin or deazalumiflavin at one-fifth the concentration of protein (Massey & Hemmerich, 1978). The extent of reduction was determined by optical spectroscopy, and when judged complete, the sample was reoxidized by exposure to air. All of these operations were done at 0–5 °C.

pH titrations were performed by using a difference spectroscopic technique with the reference cuvette containing an aliquot of the alkaline-reoxidized enzyme (pH 10.5) that had been rapidly readjusted to pH 7.6. The sample was titrated by using aliquots of 1 M KH_2PO_4 with pH readings taken from a Radiometer PHM-62 standard pH meter attached to a Lazar Research Lab PHR146 microelectrode. Cuvettes were housed in cell holders thermostated at 5 °C in the cell compartment of a Perkin-Elmer λ-9 optical spectrophotometer purged with CO_2-free air.

EPR samples from nonanaerobic, optical experiments were frozen in liquid N_2 within 30 s of completion of a given optical measurement. When anaerobic sampling was required, a modified version of the titration cuvette described by Palmer (1977) was used. EPR spectra were taken with a Bruker ER

[1] Abbreviations: SiRHP, *Escherichia coli* NADPH–sulfite reductase heme protein subunit; NiR, nitrite reductase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CT, charge transfer; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] Resting enzyme is defined as the end product of the isolation procedure and is characterized by a ferric, $S = {}^5/_2$, heme iron and an oxidized, $S = 0$, 4Fe-4S cluster.

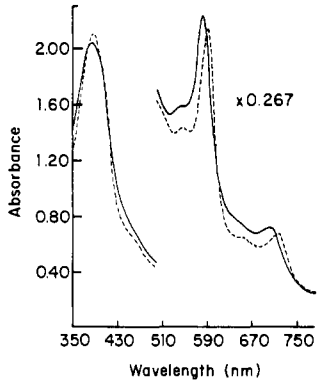

FIGURE 1: Optical spectrum of 28.3 μM SiRHP dissolved in CE buffer, pH 9.9, before (dashed line) or after (solid line) reduction and reoxidation. These and all other optical spectra were taken at 5 °C.

200D spectrometer at an operating frequency of 9.47–9.48 GHz., modulation amplitude of 10 G, and modulation frequency of 100 KHz, at 10–12 K. Sample temperatures were controlled with an Air Products Heli-Trans refrigeration unit.

Quantitation of high-spin EPR signals was done by comparing the area under the g_x feature to that of a sample of SiRHP of a known concentration. The double integral of the latter quantitated to 0.95 spin/heme against a CuETDA standard. It was found that D (one of the zero field splitting parameters) was the same for all high-spin signals except the one at lowest field (see Results). In the one instance where a deconvolution was necessary (see Figure 5), a manual method was employed in which a Gaussian line shape was assumed, with the line width determined from a sample in which there was minimal interference from other conformeric components.

RESULTS

Effects of Alkali on Resting SiRHP. The optical spectrum of resting SiRHP in standard buffer, with its Soret, α, and CT bands at 387, 590, and 714 nm, respectively, is similar to that observed for the ferric, high-spin chloride derivative of octaethylisobacteriochlorin (Stolzenberg et al., 1981). Alkalinization of the enzyme to pH 9.9 (Figure 1) (and in fact as high as pH 10.5) leaves the spectrum essentially unchanged, with amost complete restoration of the optical spectrum of the original species obtained following readjustment of the pH to 7.7. However, if the pH is raised to 12.1, there is a 30% loss of the Soret- and α-band absorptivities and a concomitant 25-nm hypsochromic shift of both band maxima. This very alkaline form of the enzyme does not revert to the normal resting form after acidification to pH 7.7 with 1 M KH_2PO_4.

The ferriheme EPR spectrum of a sample of resting SiRHP at pH 9.9 is slightly more axial than that of the enzyme at pH 7.7. After readjustment of the pH of this sample to 7.6, the spectrum is almost identical with that of the parent form (Figure 3F); i.e., $g_{x,y,z}$ = 6.70, 5.24, and 1.98, and the integrated intensities of the g_x features agree to within 5%.

Effects of Alkaline Reduction/Reoxidation on Resting SiRHP. (a) Optical Spectroscopy. In contrast to the nonperturbing effect of moderate alkalinization, a cycle of reduction and reoxidation in 0.1 M CHES + 0.01 M EDTA buffer (CE buffer), pH 9.9, significantly alters the optical spectrum of the resting form (Figure 1, Table I). Comparison of the optical spectrum of this species after the pH is readjusted back to 7.6 to that of the resting enzyme shows that 5–10% of the heme has been lost and that a further 5–10% has been modified to produce a species which has an α band that is blue-shifted 2 nm from the resting position. If the sample is left on ice for 16–18 h, at least 50% of the blue-shifted form will revert to the resting form.[3]

Table I: Optical Absorption Data for Ligand Complexes of SiRHP

species	absorption maxima [λ (ϵ_{mM})] Soret	α	CT
resting pH 7.7	387 (65.6)	590 (18.1)	714 (5.7)
activated pH 9.9	385 (63.7)[a]	582 (18.8)	699 (6.1)
+selenite	391 (67.8)	593 (16.4)	722 (5.6)
+nitrite	400 (57.1)	576 (20.9)	730 (sh)[b]
+azide	398 (60.9)	583 (17.9)	730 (sh)
+cyanide	406 (59.2)	581 (20.4)	740 (sh)
+sulfite	408 (58.7)	583.5 (19.7)	
	392 (sh)		

[a] All extinction coefficients for the activated enzyme are calculated by comparison to the resting enzyme at pH 7.7 with a correction for a 10% loss of heme (accuracy is no better than ±5%). [b] sh = shoulder.

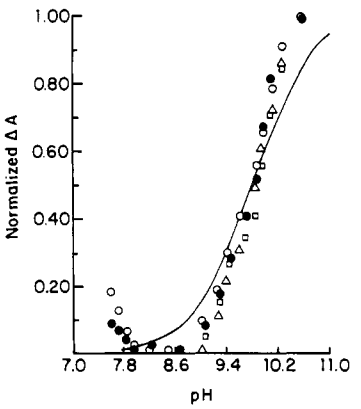

FIGURE 2: pH titration using optical difference spectroscopy of two different samples of 13.9 μM SiRHP dissolved in CE buffer. Enzyme subjected to a cycle of reduction and reoxidation at pH 10.5 was then titrated with 5–10-μL aliquots of 1 M KH_2PO_4 (distilled H_2O for the reference). Each addition was followed by an optical scan from 800 to 350 nm. Data points were taken at the 690–722-nm wavelength pair (square and triangle) and the 575–596-nm wavelength pair (open circle and filled circle).

These data appear to show that the alteration in the optical spectrum achieved when resting SiRHP is subjected to a reduction/oxidation cycle at alkaline pH is a reversible phenomenon involving 90 ± 5% of the resting conformers. We have also found that this protocol (i) preserves the resting enzyme's methylviologen–sulfite reductase activity, (ii) produces an EPR signal in the doubly reduced enzyme which indicates that the ferrous siroheme is coupled to the reduced iron–sulfur cluster [see Janick and Siegel (1982)], and (iii) gives rise to similar optical spectral changes in the intact holoenzyme.

Figure 2 shows the results of two titration experiments in which enzyme samples were subjected to a cycle of reduction/reoxidation at pH 10.5 and then rapidly brought to the indicated pH values by addition of 1 M KH_2PO_4. The changes in the α and CT bands appear to be complete at about pH 9.0. The fit of the data to the theoretical curve for a single pK of 9.8–9.9 is barley credible. Although a marginally better agreement of theory and data could be achieved by shifting the curve to the right, there are good reasons for not doing this, which we will elaborate on presently. In any case, at pH's above 10, the slope is steeper than predicted, indicating that more than a single process may be occurring (the upward drift of the data below pH 7.8 is due to slight differences in the

[3] Even at pH 7.7 a cycle of reduction and reoxidation will produce a small amount of this species (L. J. Young, P. A. Janick, and L. M. Siegel, unpublished results).

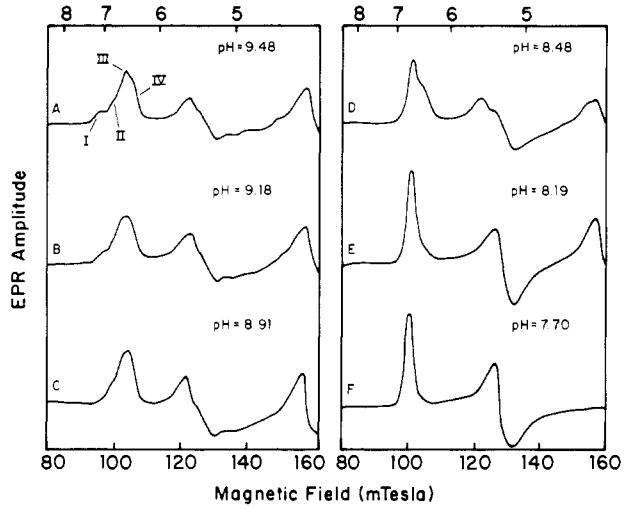

FIGURE 3: EPR spectra of acidified samples of activated 13.9 μM SiRHP dissolved in CE buffer, pH 9.9. After titration to the desired pH and optical spectroscopy, aliquots were immediately frozen in liquid N_2. Spectra were taken at 10 K, 20-mW power, and a gain of 6.3 $\times$ 10^5. A g-value scale is at the top of this figure and that of Figures 4, 5B, and 8.

end-point pH of sample and reference and hence differing amounts of the blue-shifted species).

(*b*) *Activation in Alkaline Reduced/Reoxidized SiRHP.* A variety of ligands react more rapidly with the alkaline reduced/reoxidized form of the enzyme when compared to what has been observed with the resting species at pH 7.7 (Janick et al., 1983; Janick & Siegel, 1983). This phenomenon, which we will term "activation", is most strikingly seen in the reaction of this activated form of the enzyme with azide (Figures 5–7, Table I), a ligand that shows no affinity at all for resting SiRHP (henceforth the alkaline reduced/reoxidized SiRHP will be called activated SiRHP). We will show in a later section that sulfite, cyanide, and nitrite have observed rate constants for ligation to the activated enzyme that are 3 orders of magnitude greater than those seen with the resting form, and we will present evidence for complex formation between hydroxylamine or selenite and SiRHP. It is of consequence that activated holoenzyme also demonstrates the ability to rapidly bind both selenite and azide.

(*c*) *EPR Spectroscopy of Activated SiRHP.* The EPR signature of pH 9.5–9.9 activated SiRHP is complex and appears to be composed of four rhombic, high-spin conformers with g_x values of 7.11, 6.85, 6.63, and 6.45, which have been designated conformers I–IV, respectively (Figure 3) (not shown is the doubly reduced pH 9.5 form, which has g values of 2.54, 2.28, and 2.065 as opposed to 2.53, 2.29, and 2.07 at pH 7.7). The g_x intensity of pH 9.5 activated SiRHP appears to be 30% lower than that of the resting pH 7.7 form, even after correction for a 10% loss of heme. Some 15–20% can be accounted for by the presence of residual doubly reduced SiRHP because at this pH it is difficult to reoxidize the enzyme with dioxygen. (We cannot rule out the presence of some singly reduced enzyme because there is no way to quantitate it.) Another 10% is due to a smaller value of D (the axial zero field splitting parameter) for conformer I, as comparison of the total intensities in the two spectra of Figure 4 might suggest. This change in D (for conformer I and not for conformers II–IV) has been confirmed by observing that the relative intensity of conformer I to conformer II increases as the temperature is decreased. Although a value for D was not determined, a correction factor of 2 was obtained for the measured intensity of conformer I at 10 K. With these corrections the pH 9.5 activated form has 0.88 spin/1.0 spin of resting pH 7.7 enzyme. This extent of agreement is within the 15% error margin of any EPR quantitation performed in this study, but it might possibly be improved upon if singly reduced SiRHP could be quantitated.

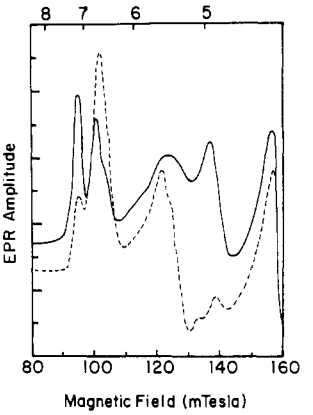

FIGURE 4: EPR spectrum of a 13.9 μM sample of pH 9.9 activated enzyme dissolved in CE buffer before (solid line) and after (dashed line) addition of KCl to 0.2 M. The data were recorded at 10 K, 20-mW power, and a gain of 6.3 $\times$ 10^5.

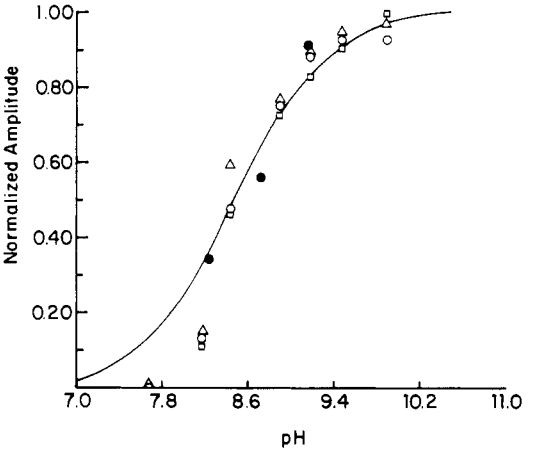

FIGURE 5: Normalized plot of the azide reactivity and the percentage of conformer III as a function of pH. Different samples of 13.9 μM activated enzyme dissolved in CE buffer were monitored for their "initial" reactivity (see text) with 100 mM sodium azide by optical (open circle) or EPR (triangle) spectroscopy. Residual CT band intensity (at 5 °C and 10 min after the pH was adjusted) was used in the former and the residual high-spin EPR signal (at 10 K, 20-mW power, a gain of 6.3 $\times$ 10^5 and 12–19 min after the pH was adjusted) in the latter. Similar EPR parameters were used when the intensity of conformer III (square) was measured with all samples taken 4–6 minutes after the pH was adjusted. The extrapolated "zero time" azide reactivity (filled circle) was determined from the kinetic data of Figure 6B.

Figure 3 illustrates the effect of pH on the intensities of the four high-spin conformers. In this experiment, activated enzyme was prepared at pH 9.9, and aliquots were adjusted to the indicated pH values by addition of 1 M KH_2PO_4. The data have been normalized to the average intensity of the pH 9.9–8.2 samples (3045 $\pm$ 130 intensity units), which is computed from the areas under the low-field peaks, after correction for the different D of conformer I and the amount of doubly reduced enzyme (the spectrum of the pH 9.9 sample is not shown in Figure 3). A plot of the intensity of the g_x feature of conformer III (Figure 5) or of the observed shifts in the values of g_x for conformers III and IV as a function of pH are quite well correlated to a process having a pK of 8.5. [A pH profile for conformer III was constructed with the assumption that at pH 9.9 the intensity was at a minimum and at pH 8.2

it was 90% of the maximum. The logic derives from the comparison of the pH 8.2 spectrum (Figure 3E) to the one at pH 7.7 (Figure 3F) and from the actual intensity at $g = 6.72$ in the spectrum at pH 9.9.] The shifts in g_x for conformers III and IV are to higher field (lower g values) with increasing pH, moving from 6.72 to 6.61 for the former and from 6.52 to 6.40 for the latter. The data of Figure 3 also show that at pH 8.5 conformer IV has a maximum intensity, while conformers I and II are essentially eliminated; i.e., as the pH is lowered from 9.5 to 8.5 conformers I and II transfer intensity to conformers III and IV, while below pH 8.5 the intensity shift is from conformer IV to conformer III.

The distribution of intensity for the various conformers can be affected by several variables. Using enzyme dissolved in 0.1 M CHES + 0.01 M EDTA + 0.02 M potassium phosphate buffer (CEP buffer), pH 9.5, as the standard, we have found that increasing the ionic strength to 0.8–1.2 by addition of "ampholytic" buffers produces a shift of intensity from conformer IV to conformers I and II. A high concentration of phosphate (more than 0.1 M) appears to favor conformer I over conformer II but only if no CHES buffer is present. Anions like chloride (Figure 4) and nitrate will increase the amount of conformer I. Neither anion has a remarkable effect on the room temperature optical spectrum of the activated enzyme; the same is true if the ionic strength or the phosphate concentration is increased.

Rapid temperature cycling (1–2 min) between 298 and 10 K does not perturb either the EPR or optical spectrum of the pH 9.5 activated enzyme, as is also true for prolonged storage (8–12 months) at 77 K. However, the chloride and nitrate species will revert to the pH 9.5 control after 7–8 months at 77 K.

Variability in conformer patterns during pH titrations can be minimized by appreciating that (i) there is a large dpK_a/dT for CHES (−0.027/°C) compared to phosphate (−0.0028/°C) and (ii) the mixing technique used when KH_2PO_4 is added is critical; i.e., direct "syringe injection" of a small aliquot of acidic phosphate into a solution of enzyme followed by agitation produced a local pH effect and did not give a reproducible optical spectrum for a given final solution pH. This did not occur if the enzyme solution was rapidly mixed by inversion, with a droplet of acid layered on the wall of the cuvette. Although the latter technique gave reproducible optical spectra, we cannot be sure that mixing was perfect, and for this reason we did not feel justified in moving the optical pH titration curve to the right.

Relaxation Phenomenon of Activated SiRHP. With identification of and control over a number of critical variables having been achieved, it became possible to appreciate that yet another process could affect the conformer distribution of activated SiRHP. The optical and EPR spectra of activated SiRHP at a given pH will with time change back to those of resting SiRHP at that pH. The rate of this relaxation is a function of the starting pH and increases as the pH is lowered. We have used the reaction of the enzyme with azide to follow the relaxation because azide shows no reaction with the resting enzyme. In Figure 5 is shown the effect of pH on the initial azide reactivity ("initial" refers to the extent of reaction with azide measured immediately after the pH is adjusted to the indicated value). If the sample is monitored as a function of time after adjustment of the pH, data like those of Figure 6A are obtained, which graphically illustrate how the activated enzyme can relax back to the nonbinding resting form. Figure 6B shows that the rate of this process is dependent on the initial pH, and indeed a plot of the log of the rate constants (corrected for the amount of acidic form by using a pK of 8.5) against pH has a slope of 0.9.

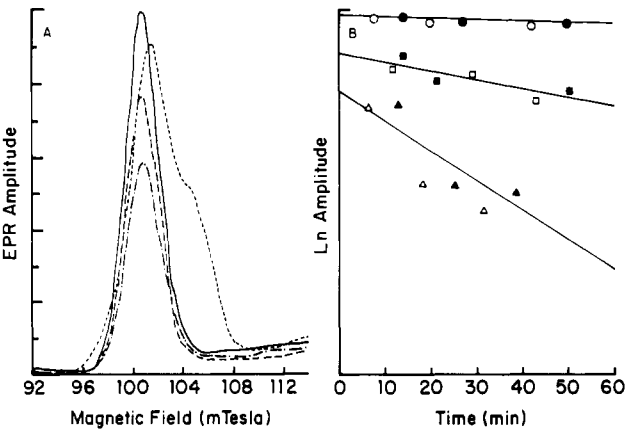

FIGURE 6: (A) EPR spectra of a sample of pH 8.75 activated enzyme dissolved in CE buffer (dotted line) at 19 (dash-dot line), 29 (dashed line), and 50 (solid line) min after the addition of 100 mM sodium azide. EPR parameters were as in Figure 5 except for a gain 5×10^5. (B) Normalized, least-squares, semilog plot of the extent of reaction of 13.9 μM activated enzyme dissolved in CE buffer with 100 mM sodium azide as a function of time of incubation of the unliganded, activated enyzme at pH 9.2 (circle), pH 8.75 (square), and pH 8.25 (triangle). Optical (open symbols) and EPR (filled symbols) data were obtained by using the same procedures and operating parameters as in Figure 5 but for a gain of 5×10^5.

It was of interest to determine if there was any correlation between the optical spectrum of the activated enzyme after adjustment to a given pH and the extent of reaction with azide. When the pH was raised from 9.9 to 10.5, this appeared to be so; i.e., the increased reactivity at pH 10.5 was associated with a spectral change, both of which were reversed on restoration of the starting pH. However, if the pH was rapidly decreased from 9.9 to 8.2 and then returned to pH 9.9 (total time <1 min), the optical spectrum was that of the pH 8.2 activated enzyme, but the azide reactivity was that of pH 9.9 activated enzyme. Similar results were found at pH 8.7 and 9.3. In a separate experiment it was shown that the EPR conformer patterns of the samples "cycled" between pH 9.9 and pH 8.2 or pH 8.7 were like those of the pH 9.9 starting material.

Reaction of Activated SiRHP with Ligands. (a) Nitrite, Hydroxylamine, and Selenite Complexes. Resting SiRHP has recently been shown (Young & Siegel, 1988) to slowly react with 100 mM NO_2^-, with a rate constant for the major (70%) component in the polyphasic reaction of 0.002 min^{-1}. This rate constant is increased by 3 orders of magnitude with the pH 9.9 activated enzyme. The optical and EPR spectral data for the pH 9.9 nitrite complex (Table I) are similar to those of the resting nitrite adduct except that (i) the α band is at 576 nm, not 579 nm, and there is no ferric high-spin marker band at 714 nm (these changes indicate essentially complete reaction of the activated enzyme with nitrite) and (ii) there is never more than 0.05 spin/heme of the nitrite-induced low-spin EPR signal seen in the resting enzyme at pH 7.7, which typically quantitates to about 0.2 spin/heme.

The details of the reaction of hydroxylamine with the activated heme protein will be described in a future publication. In brief, at a concentration of 20 mM it reacts rapidly ($t_{1/2}$ < 15 s) with a 20 μM solution of enzyme to produce a complex with a spectral form like that of the ferrous NO species (Janick et al., 1983) except that the α band is at 594 nm, not 599 nm.

Sodium selenite (a substrate analogue of sulfite) at a concentration of 25 mM significantly alters the optical spectrum of the pH 9.9 activated enzyme (Table I). The changes are

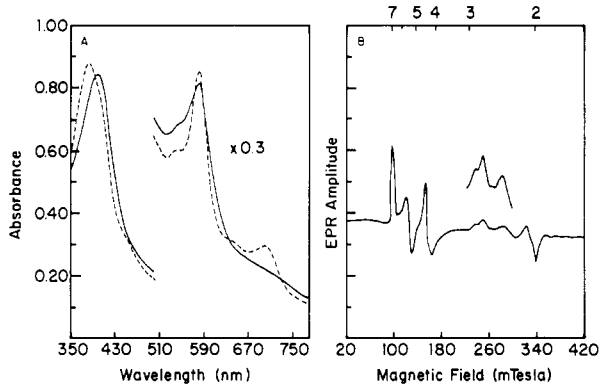

FIGURE 7: (A) Optical spectrum of a solution of 13.9 μM pH 9.9 activated enzyme dissolved in CE buffer before (solid line) and 1 min after (dashed line) addition of solid NaN_3 to 100 mM. (B) EPR spectrum of a 57 μM sample of activated enzyme dissolved in CEP buffer, pH 9.5, plus 100 mM sodium azide. The spectrum was taken at 10 K, 20-mW power, and a gain of 1.25×10^5. The large derivative feature around $g = 2$ is a cavity contaminant and is also evident in Figure 8.

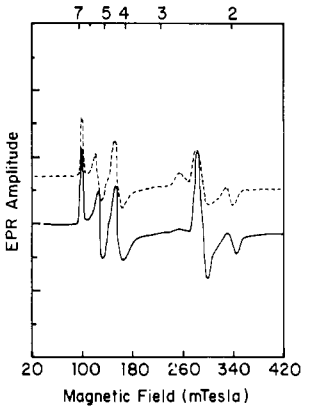

FIGURE 8: EPR spectra of a single sample of pH 9.5 activated enzyme dissolved in CEP buffer in the presence of 3 mM (solid line) and 14 mM (dashed line) KCN. The original enzyme concentration was 72 μM, diluted to 65 μM after addition of the second aliquot of cyanide. Data were taken at 10 K, 20-mW power, and a gain of 1.25×10^5.

complete after 1 min and are stable on readjustment of the pH to 7.7. The EPR spectrum of the selenite adduct is unusual in that except for a small amount of conformer IV (0.01–0.02 spin) the high-spin conformers found in the unreacted enzyme have collapsed into a single species (0.91 spin) with g values identical with those of the pH 7.7 resting form.

(*b*) *Azide Complex.* Addition of 20–100 mM azide to activated enzyme at pH 9.5–9.9 rapidly (reaction complete in <1 minute) produces a species with optical (Figure 7A, Table I) and EPR (Figure 7B) features characteristic of a low-spin ferric complex. When the pH is lowered to 7.7 (with the sample kept on ice), a shift of the α band to 586 nm is observed. This red shift is due to a higher K_d at pH 7.7, which is probably due to the "relaxation" process (as opposed to just the change in the pH). If the same sample is warmed to 25 °C, the azide completely dissociates within 15 min. This pH-induced release of an anionic ligand (with the total azide concentration at 0.1 M) from the activated enzyme is unique to azide.

The EPR spectrum of the pH 9.5 azide complex shows a considerable loss of the intensity of the high-spin signals and formation of a pair of low-spin species. The transitions at g_z = 2.82 and 2.69 appear to have g_y components at 2.32 and 2.38, respectively (the minor resonance at g_z = 2.57 is not reproducibly observed). Quantitation of the major low-spin species shows them to be of roughly equal intensity and equivalent to 0.3 spin/heme in total. After this fraction is combined with that for the residual high-spin signal, 0.6 spin remains undetectable by EPR. In separate experiments it has been found that the most rhombic EPR conformers react with azide first, followed then by conformer IV. This conformeric selectivity is also observed when cyanide and nitrite react with the activated enzyme. The residual high-spin EPR signal has g values like those of the resting enzyme and at pH 9.5 quantitates to the amount of nonreactive enzyme (0.1 spin/heme) that would be expected if the activation process had a pK of 8.5.

This suggestion finds support in the observation that the equilibrium optical spectrum at pH 9.5 was the same with 20, 100, or 500 mM azide (i.e., the residual intensity at 714 nm could not be abolished by increasing the azide concentration). The results of a titration experiment, performed by adding 100 mM azide to samples of activated enzyme at a given pH and then recording the optical spectrum at 278 K and the EPR spectrum at 10 K, verify the hypothesis (Figure 5). The data fit the curve for a pK of 8.5 quite well, whether it is the residual intensity of the EPR high-spin species or the optical CT band that is used to measure the extent of the reaction (deviations at low pH are reasonably attributed to the relaxation phenomenon).

(*c*) *Cyanide Complex.* It is known that cyanide will form a complex with both oxidized and reduced resting enzyme at pH 7.7 (Janick et al., 1983). The optical spectrum of the cyanide complex of pH 9.9 activated enzyme (Table I) is not greatly different from that of the pH 7.7 resting enzyme; however, with cyanide at 100 mM, the rate constant of 10 min^{-1} is 4 orders of magnitude greater. The reaction of 1 mM cyanide with activated SiRHP at pH 9.5 is biphasic and shows a k_{obsd} of 1.2 and 0.2 min^{-1} for the fast (65%) and slow (35%) phases, respectively. These experiments were done at 5 °C by using optical difference spectroscopy and a monitoring wavelength of 380 nm. Both KCl and Na_2SO_4 (I = 0.2) shifted the phase distributions to favor the slower one, giving values of 82% for Cl^- and 65% for SO_4^{2-}, with neither anion altering the rate constants (the Kezdy–Swinbourne approximation was used to determine end points in this set of experiments and also those with sulfite).

The EPR spectrum of the pH 9.5 enzyme in the presence of 3 mM KCN is shown in Figure 8 (the amount of residual high spin is slightly less than expected from the results of the experiments with azide). Four low-spin species are present at g_z = 2.67, 2.55, 2.42, and 2.36, with the two high-field resonances showing common values for g_y and g_x of 2.32 and 1.65, respectively. Further addition of cyanide to 14 mM gives an unusual result; i.e., there is a 60% attenuation of the g = 2.42 and 2.36 intensities and an approximately 3-fold increase in the amount of the two low-field species.

These data suggested that an excess cyanide could affect the distribution of low-spin CN^- EPR conformers. In order to ensure complete reaction of the enzyme with cyanide (thereby eliminating possible artifacts due to partitioning into the residual high-spin ferriheme), the reduced pH 7.7 enzyme was reacted with cyanide, reoxidized, and exchanged into pH 9.5 CEP buffer by passage down a Sephadex G-25 column. The sample was then reduced, reoxidized, and titrated with aliquots of 100 mM KCN to give the results shown in Figure 9. A higher concentration of CN^- appears to favor the g = 2.67 form (there is no g = 2.55 species) over the two at higher field (the pH 7.7 cyanide control showed 0.98 spin/heme of a species with g values of 2.39, 2.33, and 1.67).

It is difficult to quantitate the g = 2.67 resonance because the g_y and g_x features are not discernible and g_z is significantly

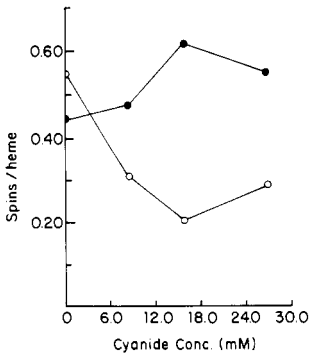

FIGURE 9: Cyanide titration of a 18 μM sample of the cyanide complex of activated SiRHP. The cyanide was complexed at pH 7.7 in standard buffer, exchanged into CEP buffer, and then reduced and reoxidized at pH 9.5. Titration was with aliquots of 100 mM KCN (buffered at pH 9.5), and the data were recorded at 10 K, 10-mW power, and a gain of 6.3×10^5. Open and filled circles refer to the high- and low-field species, respectively.

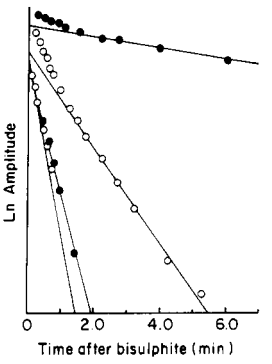

FIGURE 10: Normalized semilogarithmic plot of the reaction between 13.5 μM pH 9.5 activated enzyme dissolved in CEP buffer and 20 mM $NaHSO_3$ with (open circle) and without (filled circle) 200 mM KCl. Due to the addition of chloride, the concentration of enzyme was 10% lower than in the control run. Data were taken at 5 °C, 10–15 s after introduction of an aliquot of unbuffered 200 mM $NaHSO_3$.

less than 3. If one assumes that initially the sum of the intensities of the three low-spin forms quantitates to 1 spin/heme, then the change in intensity of the low-field resonance can be estimated; i.e., the height of the $g = 2.67$ feature prior to addition of cyanide is set equal to 0.45 spin/heme (the residual after the amount of the other two species is subtracted) and then quantified on the basis of the change in its height. If the sum of all species remains equal to unity, then there is a shortfall of 20% at each concentration of added cyanide. This suggests that the initial estimate of 0.45 spin/heme for the low-field form is too high and that therefore at least one EPR-silent species has to be present, accounting for, at the minimum, 0.2 spin/heme.

(*d*) *Sulfite Complex.* The optical and EPR spectra of the pH 9.9 sulfite adduct of the activated enzyme (Table I) are similar to those of the pH 7.7 resting enzyme (Janick & Siegel, 1983) except that (i) the quantity of sulfite-induced, EPR-detectable low-spin heme (Young & Siegel, 1988; Day et al., 1988) is higher (0.1 spin/heme) in the resting species, with the ratio of the two being about 3:1, and (ii) the α band is red-shifted by 0.5 nm in the activated enzyme.

The kinetics of sulfite ligation to the activated heme protein are sensitive to the presence of anions such as chloride, nitrate, and sulfate. The data were taken at 5 °C by using pH 9.5 enzyme, with the reaction initiated by addition of an aliquot of 0.2 M $NaHSO_3$ (pH 5.1) and followed by measuring the loss of absorbance at 380 nm against a reference cuvette containing enzyme. The effect of 0.2 M chloride on the reaction of sodium bisulfite with the activated heme protein is striking (Figure 10). The rate constant for the fast phase (which is about 3 orders of magnitude greater than in the resting enzyme) is slightly diminished from 2.3 to 2.0 min^{-1}, but an order of magnitude difference exists in the slow phase, e.g., 0.74 min^{-1} compared to 0.08 min^{-1}. Concomitantly, the intensities change from roughly equal amounts for each phase in the control to 30% fast component and 70% slow component. Nitrate gives a result like that seen with chloride except that the percentage of the slower phase (85%) is greater. These effects are not simply the result of a change in the ionic strength, since 0.1 M Na_2SO_4 diminishes the amount of fast phase by 10% and the value for each rate constant by a factor of 2.

Discussion

Conformational Substrates of SiRHP. Our data clearly show that the protocol used to produce multiple high-spin EPR conformers of SiRHP involves the majority of heme protein molecules and also fulfills the critical criterion of reversibility. With reference to the question of whether or not the present observation of conformational heterogeneity in SiRHP is the result of a particular set of experimental conditions, the results of a recent study (Janick & Siegel, 1982) are pertinent. In standard buffer, the two-electron-reduced resting enzyme was found to have four EPR-detectable species amounting to 0.82 spin per optically detectable heme: a $g = 1.94$ conformer (0.03 spin); a $S = {}^1/_2$ conformer (0.63 spin); and two $S = {}^3/_2$ conformers (0.16 spin). Addition of Cl^- caused a shift in the distribution to favor the $S = {}^3/_2$ species.

It is apparent that one cannot compare the conformer distribution of the doubly reduced pH 7.7 resting enzyme described previously to that of the pH 9.9 activated enzyme described here since the latter is pH sensitive. What can be said is that resting and activated forms of SiRHP contain multiple active-site conformers, the distribution of which can be perturbed by chloride. It would be surprising if no relationship existed between these procedurely defined entities, but the quantitative aspects of such remain to be developed.

Optical and EPR Spectroscopy of Activated SiRHP. The blue shift of Soret-, α-, and CT-band maxima seen in the activated form of the enzyme unfortunately cannot be used to decide the origin of the change in the electronic properties of the chromophore. The shift could arise from a change in the polarity of the siroheme "solvent milieu" (Bayliss & McCrae, 1954; McCrae, 1957), but it is not possible to specify whether the dielectric constant would have increased or decreased. Furthermore, a change in the net interaction among the π and σ orbitals of the metal, axial ligand, and macrocycle, which widens the gap between the a_1u, a_2u (π), and e_g (π^*) orbitals of the siroheme and is unrelated to any solvent effect, could also account for the differences.

One of the more interesting results is that those conformers which display the most rhombicity are the first to react with added ligands. Given that by increasing the pH from 9.5 to 10.4 one can shift intensity from conformers III and IV to conformers I and II and increase the extent of ligand binding, it is tempting to ascribe these changes in the EPR high-spin rhombicity, ligand reactivity, and optical spectrum to a more solvent-exposed active site. Further studies are required to determine whether or not this hypothesis is correct.

There is no doubt, however, that the conformer distribution of the activated enzyme can be modified by certain anions. This occurs in the unliganded enzyme with nitrate, chloride, and sulfate. It occurs in the cyanide species by variation in the concentration of cyanide added to enzyme whose heme is

completely reacted with cyanide. This modification also occurs in the fluoride complex.[4] Taken together these data suggest that there may be a secondary anion-binding site, the occupation of which perturbs the heme. The question then arises as to the location of the site and whether it is present in forms of the enzyme other than the activated species. The preliminary results of optical/electrochemical experiments[5] point to a most intriguing possibility; i.e., Cl^- has been found to perturb the optical spectrum of both one- and two-electron-reduced SiRHP at pH 7.7 and to concomitantly lower the E_m of the 4Fe-4S cluster by 60 mV, leaving the E_m of the heme unchanged. It is also known that the optical spectrum of the heme (with or without a ligand) is affected by the reduction state of the cluster (Jancik & Siegel, 1982, 1983). Thus, it may be that the site of chloride action is at or near the cluster and is present in both reduced and activated forms of the enzyme.

Spinacea oleracea NiR shares common prosthetic groups with SiRHP, but its resting form reacts more rapidly with exogenous ligands (Vega & Kamin, 1977). When compared to resting SiRHP, resting spinach NiR, like activated SiRHP, has a blue-shifted Soret (386 nm), α (573 nm), and CT (690 nm) band, as well as a more rhombic EPR signal (g = 6.8, 5.1, and 1.93). The opinion has been expressed (Stolzenberg et al., 1981) that spectral differences between spinach NiR and SiRHP are due to nonidentical "proximal" heme ligands. The similarities noted just above and the finding that the EPR conformer distribution of the SiRHP cyanide complex can be changed just by altering the amount of cyanide in the supporting buffer (Figures 8 and 9) cast doubt on this hypothesis. This is especially so since some of the low-spin cyanide and sulfide[6] complexes of activated SiRHP show g values that are just like those of NiR: for sulfide $g_{z,y,x}$ = 2.50, 2.45, and 1.68 (SiRHP) vs $g_{z,y,x}$ = 2.49, 2.42, and 1.77 (NiR); for cyanide g_z = 2.67 (SiRHP) and $g_{z,y}$ = 2.71 and 2.35 (NiR).

It has been found (Young & Siegel, 1988; Day et al., 1988) that many heme proteins of the porphyrin, oxyporphyrin (porphyrindione), and isobacteriochlorin classes can have significant quantities (0.4–1.0 spin/heme) of EPR-undetectable material following reaction with either nitrite or sulfite. The activated form of SiRHP has more EPR-silent heme in its nitrite and sulfite complexes than that found in the resting adducts. There is also evidence that EPR-silent low spins may be present with azide and cyanide. Given the differences in ligand field strength of these four anions, it seems probable that for SiRHP at least the mechanism of the silence, whether it be uniaxiality of the g-tensor, extreme "g-strain", or an as yet unknown process, must involve other factors.

Relaxation and Activation Phenomena. The data concerning the relaxation phenomenon are currently best explained by the following set of reactions:

$$E_{ox}^{+} + 2e + OH^- \rightarrow E_{red}^{2-} + H_2O \quad (k = \text{fast}) \quad (1)$$

$$E_{red}^{2-} + O_2 + 2H^+ \rightarrow E_{ac}^{-}/E_{ox}^{+} + H_2O_2 \quad (pK = 8.5;\ k_{ox} = \text{med}) \quad (2)$$

$$E_{ac}^{-} + H^+ \rightarrow E_{ac} \quad (k = \text{fast}) \quad (3)$$

$$E_{ac} + H^+ \rightarrow E_{ox}^{+} \quad (k \propto pH) \quad (4)$$

E_{ox}^{+}, E_{red}^{2-}, E_{ac}^{-} are the resting oxidized, resting reduced, and activated forms of SiRHP at pH 9.9. E_{ac} is an activated form of the enzyme that has an optical spectrum like that of the resting form but shows enhanced ligand reactivity. As mentioned earlier, it is possible to increase the extent of ligand binding of the pH 9.9 activated form by the addition of base. This implies that a reaction of the type $E_{ox}^{+} + OH^- \rightarrow E_{ac}$ (or E_{ac}^{-}) should occur, and indeed preliminary experiments show this to be true. However, during the prolonged (24–36 h) incubation of resting enzyme at pH 9.9, there is marked (30%) destruction of the heme.

It appears that two activated forms of SiRHP exist, but if relaxation takes place, then the enzyme will no longer react rapidly with added ligands. The formation of the E_{ac}^{-} form of the enzyme is controlled by some process with a pK of 8.5 but is not affected by the ligation state of the heme; i.e., production of a complex between a ligand and the activated enzyme cannot pull the binding equilibrium beyond what is dictated by the relation between the prevailing pH and the pK of 8.5.

The kinetic data are compatible with there being increased accessibility of the heme to an entering ligand either due to a repositioning and/or charge change of a "gating" side chain or a general "loosening" of secondary and tertiary structure. There may still be a "ligand size" constraint, since even at a concentration of 1 M imidazole will not bind to the activated enzyme. The hypothesis that activation involves the removal of a gate present in the resting enzyme is perhaps slightly favored given that (i) both azide- and cyanide-binding experiments show that the pK-correlated enhancement of ligation is not heme-linked (regulated by a heme ligation state) and (ii) crystallographic data on the resting enzyme (McRee et al., 1986) portray the distal side of the heme as being unoccupied.

Both alkaline reduction/reoxidation and reduction per se diminish the kinetic barrier for complex formation between the active-site siroheme and sulfite; however, the observed rate constant for binding to the reduced form is still 2 orders of magnitude greater. At least part of this difference may relate to the lower oxidation state of the iron, but it remains to be shown whether reduction at any pH and reduction/reoxidation at alkaline pH remove the structural barrier to ligand binding in the same way; i.e., we do not as yet know with certainty what relation exists among the "activation" site (with its pK of 8.5), the "anion-binding" site (which can perturb the optical and EPR spectra of either resting or activated SiRHP), and the amino acid side chains whose position is sensitive to reduction of the heme and/or the cluster of the resting enzyme (vide infra).

Implications for Catalysis. The absence of a quantitative conversion of the single high-spin species in the resting enzyme to a low-spin form after a cycle of alkaline reduction/reoxidation suggests that there is no aquo ligand at the sixth coordination position and is in keeping with recent ENDOR (Cline et al., 1985) and X-ray (McRee et al., 1986) results. If this position is the site where substrate ligates to the enzyme, then the results of the experiments with azide provide an important clue with respect to the site architecture. The release of azide but not sulfite from the reacidified (and hence deactivated enzyme) indicates that something is crowding the azide out. Furthermore, since the bent end-on bonding mode of N_3^- (McCoy & Caughey, 1970) forces its terminal N to be further from the Fe than the O of either a nonlinear planar SO_2 or a pyramidal SO_3^{2-}, S- not O-bonding is implicated for sulfite. At present we do not know whether the distal steric

[4] Both NiR and SiRHP show the peculiar phenomenon of a fluoride-induced low-spin EPR signal (J. R. Lancaster, J. O. Wilkerson, L. J. Young, and L. M. Siegel, unpublished results).

[5] J. M. Madden and L. M. Siegel, unpublished results.

[6] L. J. Young and L. M., Siegel unpublished results.

constraint is of protein (a mobile pendant side chain) or heme (a ruffled core) origin since the angle between the heme normal (which contains the iron) and the bound, linear azide molecule cannot be specified. Purely electronic heme effects that perturb only the ligation of azide to the resting enzyme seem improbable, since all other ligands that have been examined are bound in both states; i.e., it is the bonding mode of azide that can distinguish it from any other ligand.

Complexes of the resting and/or activated enzyme with ligands such as sulfite, nitrite, azide, and cyanide contain appreciable amounts of EPR-silent heme. It may be that in siroheme-containing proteins the silence has its origin in some intrinsic property of the macrocycle, e.g., ease of distortion of core size and/or extensive ruffling (Strauss et al., 1983) that facilitates the formation of a large number of active-site conformers, and hence produces extreme *g*-strain, due to multiplicity in the interaction between the bound ligand and the metallomacrocycle. This phenomenon of silence has a more practical importance insofar as mechanistic studies of catalysis are concerned because, since the silence in these complexes is not due to diamagnetism or non Kramer's paramagnetism, the absence of an EPR signal in a turnover intermediate is not a sufficient criterion to eliminate the presence of low-spin ferric iron in that intermediate.

Chloride ion profoundly alters the EPR conformer distribution of the activated and doubly reduced resting forms of SiRHP as well as the optical spectrum of both singly and doubly reduced resting SiRHP. Given that chloride has never been directly shown to bind to the iron of any heme protein, although with chloroperoxidase and myeloperoxidase the site is probably close to the heme (Sono et al., 1986; Ikeda-Saito & Prince, 1985), the possibility exists that reduction or alkaline reduction/reoxidation unmasks a secondary anion-binding site which could have a regulatory role in catalysis. Recent experiments in this laboratory with nitrite indicate that both chloride and nitrite can inhibit the release of NO from the ferric NO complex of SiRHP.[6]

In conclusion we believe that the results presented in this paper support and extend the idea that, in addition to bringing the heme and iron–sulfur cluster into close apposition and thus facilitating rapid electron transfer to bound substrate, the protein moiety of SiRHP also plays a critical role in modulating substrate accessibility to the metal centers of the enzyme.

Registry No. Sulfite reductase, 9029-35-0; selenite, 14124-67-5; nitrite, 14797-65-0; azide, 14343-69-2; cyanide, 57-12-5; sulfite, 14265-45-3; hydroxylamine, 7803-49-8.

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