Evidence for Siroheme–Fe_4S_4 Interaction in Spinach Ferredoxin–Sulfite Reductase†

Rick J. Krueger‡ and Lewis M. Siegel*

ABSTRACT: Spinach ferredoxin–sulfite reductase (SiR) contains one siroheme and one Fe_4S_4 center per polypeptide subunit. The heme is entirely in the high-spin Fe^{3+} state in the oxidized enzyme. When SiR is photochemically reduced with ethylenediaminetetraacetate (EDTA)–deazaflavin, the free enzyme and its CN^- and CO complexes show changes in absorption spectra associated with the heme even after the heme has been reduced from the Fe^{3+} to the Fe^{2+} state. With CO– or CN^-–SiR, these spectral changes are associated with the appearance of a classical "$g = 1.94$" type of EPR spectrum characteristic of reduced Fe_4S_4 centers. The line shapes and exact g values of the $g = 1.94$ EPR spectra vary with the nature of the ligand bound to the heme Fe. Photoreduction of free SiR results in production of a novel type of EPR signal, with $g = 2.48$, 2.34, and 2.08 in the fully reduced enzyme; this signal accounts for 0.6 spin per heme. (A small $g = 1.94$ type EPR signal, representing 0.2 spin per heme, is also found.) These data suggest the presence of a strong magnetic interaction between the siroheme and Fe_4S_4 centers in spinach SiR, this interaction giving rise to different EPR signals depending on the spin state of the heme Fe in the reduced enzyme.

Spinach SiR,[1] like NiR, catalyzes the six-electron reductions of SO_3^{2-} to S^{2-} and NO_2^- to NH_3 with either reduced ferredoxin or MV^+ as electron donors (Krueger & Siegel, 1982). Both of these enzymes contain a catalytic unit consisting of one siroheme and one Fe_4S_4 center per polypeptide subunit (Lancaster et al., 1979; Krueger & Siegel, 1982). In each enzyme it has proven difficult to detect a classical "$g = 1.94$" type EPR signal, characteristic of reduced Fe_4S_4 centers, on treatment of enzyme with agents such as MV^+ or $S_2O_4^{2-}$. Such a signal could be produced nearly quantitatively if either enzyme was converted to its CO complex. Similar results have been reported with *Escherichia coli* SiR (Siegel, 1978). Mossbauer studies with the *E. coli* SiR have shown that the siroheme and Fe_4S_4 centers of that enzyme are exchange coupled, probably through a common bridging ligand, and that this coupling is maintained as the enzyme is reduced (Christner et al., 1981). Janick & Siegel (1979) have reported preliminary results which indicate that a novel form of EPR signal is produced on reduction of both the heme and Fe_4S_4 centers of *E. coli* SiR. This signal, with g values at 2.53, 2.29, and 2.07, was considered by Christner et al. (1981) to represent that of the coupled reduced Fe_4S_4 ($S = {}^1/_2$)–ferroheme ($S = 1$ or 2) system.

In the present work, we have investigated optical and EPR spectra of various oxidation states of spinach SiR in both the native enzyme and its complexes with CN^- and CO. We find that a novel EPR signal similar to but not identical with that described in the *E. coli* enzyme is produced on reduction of uncomplexed spinach SiR. Analysis of the spectral changes strongly indicates that interaction between the heme and Fe_4S_4 centers is present in the spinach as well as the *E. coli* SiR.

Experimental Procedures

Spinach SiR was purified by the method of Krueger & Siegel (1982). The 63K form of the enzyme was used, and enzyme concentrations were determined spectroscopically with use of the $E_{587} = 1.78 \times 10^4$ (M heme)$^{-1}$ cm^{-1} determined by Krueger & Siegel (1982). Anaerobic conditions were maintained by alternately evacuating samples and flushing them with Ar gas, which was deoxygenated by passage through a column of BASF R-311 catalyst maintained at 190 °C. CO was deoxygenated by passage through solutions of MV^+. Dfl was a generous gift of Dr. D. Lambeth. All other chemicals were reagent grade and used without further purification.

Absorption spectra were measured, unless otherwise indicated, in silica cells of 1-cm path length at 22 °C with an Aminco DW-2 spectrophotometer with 2-nm band-pass. In some experiments, absorption spectra of solutions contained in EPR tubes were measured in order to follow the progress of a reaction, the final state of which was to be analyzed by EPR spectroscopy. In such cases, the DW-2 spectrophotometer was fitted with an Aminco J4-9618 microcell condensing system, and the EPR tube (3.2-mm internal diameter) was carefully positioned at the most condensed section of the light beam.

EPR spectra were recorded on frozen samples with a Varian Model E9 spectrometer operating at X band with field modulation of 100 KHz. Temperature was maintained by flow of cooled He gas with an Air Products cryostat system. For quantitation of spins, all spectra were recorded under nonsaturating conditions at 20 K, and double integration was performed with a solution of cupric EDTA as standard (Aasa & Vangaard, 1975).

Results

Photoreduction of SiR. Spinach SiR can be readily reduced by irradiation of the enzyme with light from a sealed beam lamp in the presence of Dfl and EDTA, according to the procedure of Massey & Hemmerich (1978). Enzyme reduction can be followed optically and, after freezing of the sample, by EPR spectroscopy.

Figure 1 shows the absorption spectra observed following photoreduction of native SiR for various periods of time under anaerobic conditions. The reduction process exhibits two phases. In the first phase, there is a diminution of absorbance at the Soret (384 nm) and α-band (587 nm) maxima, with

† 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: Dfl, 5′-deazaflavin, Fe_4S_4, tetranuclear iron–sulfur center; MV^+, reduced methylviologen; NiR, nitrite reductase; SiR, sulfite reductase; EDTA, ethylenediaminetetraacetic acid.

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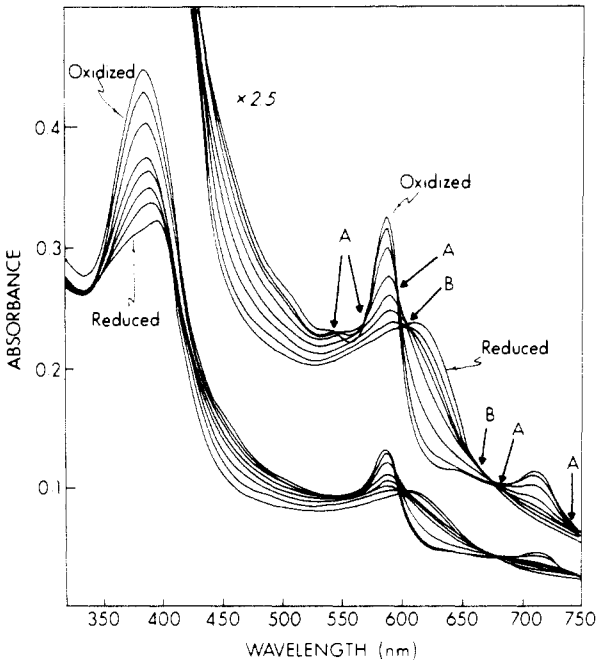

FIGURE 1: Absorption spectra of spinach SiR during photoreduction with EDTA–Dfl. A silica cuvette, 1-cm path length, containing an anaerobic solution of 7.1 μM SiR, 1.8 μM Dfl, 1 mM EDTA, and 0.05 M potassium phosphate buffer (pH 7.75), in a total volume of 0.5 mL, was placed 30 cm from a 200-W General Electric sealed beam lamp and irradiated for successive periods of time to yield 0, 0.5, 1, 2, 3, 4, 5, and 7 min of total irradiation. A Pyrex chromatography tank containing an ice–water slurry was placed between the lamp and the cuvette to minimize heating of the solution. Absorption spectra of the oxidized enzyme and the solution after each period of irradiation are shown. No change in spectrum occurred when the irradiation period was extended beyond 7 min. The spectrum of the species found after 7 min of total irradiation is therefore termed the "Reduced" spectrum in the figure. The spectra recorded after 0, 0.5, and 1 min show a set of apparent isosbestic points marked "A" in the figure. The spectra recorded after 4, 5, and 7 min show a different set of apparent isosbestic points marked "B" in the figure.

only slight shifts in the positions of these maxima detected. This phase is associated with abolition of the β band at 542 nm and the weak absorption band at 712 nm (the latter band has only been observed in high-spin Fe^{3+} siroheme enzymes and probably represents charge transfer characteristic of this spin and oxidation state). This phase is also associated with an *increased* absorbance in the 545–565-nm region upon enzyme reduction. Isosbestic points for the first reduction phase are observed at 544, 567, 595, 683, and 740 nm (these points are marked A in Figure 1). EPR spectra of SiR reduced during this phase of the optical spectral change show decreased intensities of the high-spin ferriheme resonances at $g = 6.43$, 5.46, and 1.99 characteristic of the oxidized enzyme (Krueger & Siegel, 1982); but no new EPR signals appear during this stage of reduction.

In the second phase of photoreduction, there is a marked shift in the absorption wavelength maxima in the Soret and α-band regions, with the formation of new, relatively broad maxima at 396 and 612 nm, respectively, in the fully reduced SiR. This phase is associated with the presence of a new set of isosbestic points, at 601 and 655 nm (these points are marked B in Figure 1). It should be noted that there is a loss of isosbesticity observed between the first and second phases, a result which might indicate either multiplicity of reduced species or that the potentials for two species which are being sequentially reduced are sufficiently close together that complete separation of the two successive reduction processes cannot occur.

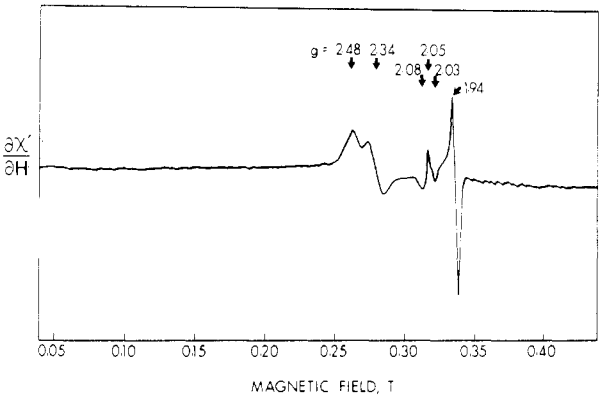

FIGURE 2: EPR spectrum of fully reduced spinach SiR. An EPR tube containing 0.2 mL of an anaerobic solution of 40 μM SiR, 10 μM Dfl, 2.5 mM EDTA, and 0.05 M potassium phosphate buffer (pH 7.75) was irradiated for successive intervals of 1 min each by the procedure described in Figure 1. Absorption spectra of the solution were recorded, and the irradiation was continued until no further change occurred (7 min of irradiation; the spectrum was qualitatively identical with that termed "Reduced" in Figure 1). The solution was then frozen in liquid N_2 and the EPR spectrum recorded. Conditions of measurement: temperature 20 K; microwave frequency 9.13 GHz; microwave power 50 mW; modulation amplitude 1 mT.

The second phase of reduction is associated with appearance of two sets of EPR signals. The two sets of signals increase roughly in parallel on photoreduction. The EPR spectrum of fully reduced SiR is shown in Figure 2. One set of resonances, at $g = 2.04$–5, 1.936, and 1.917, is characteristic of a reduced Fe_4S_4 center. Curiously, the low field resonance of this spectrum exhibits two peaks, at $g = 2.048$ and 2.038 (Figure 3), possibly indicating multiple states of the Fe_4S_4 centers in the SiR preparation. Quantitation of the $g = 1.94$ type of spectrum shows that it represents a minority enzyme species, with 0.2 spin per heme in the fully reduced uncomplexed enzyme.

The second set of resonances in the fully reduced enzyme exhibits g values at 2.48, 2.34, and 2.08. These resonances vary together as the temperature and microwave power are changed; these signals are much less readily saturated with microwave power at 10 K than the set of resonances associated with the $g = 1.94$ type of spectrum. Quantitation of the $g = 2.34$ type of spectrum yields 0.6 spin per heme in the fully reduced uncomplexed enzyme.

The $g = 2.34$ type spectrum was also observed when SiR was photoreduced in the presence of an excess of spinach ferredoxin (2 per heme). Thus the new species is seen even in the presence of the physiological electron donor for SiR. Oxidation of the photoreduced enzyme with $Fe(CN_6)^{3-}$ or O_2 results in the disappearance of both the $g = 2.34$ and $g = 1.94$ type signals and regeneration of the original EPR and optical spectrum characteristic of the oxidized enzyme.

SiR–Cyanide Complex. Photoreduction of SiR in the presence of 1 mM KCN yielded a series of absorption spectra (not shown) characteristic of uncomplexed oxidized enzyme, reduced cyanide complexes, and mixtures of these species. No spectrum characteristic of the oxidized SiR–cyanide complex is seen during the photoreduction. This result is in agreement with the catalytic data of Krueger & Siegel (1982) which indicate that cyanide readily interacts with spinach SiR only under reducing conditions.[2]

[2] The ability of CN^- to bind rapidly only to reduced enzyme is also a property of *E. coli* SiR (Rueger & Siegel, 1976). In this case it has been shown that the barrier to CN^- binding in the oxidized enzyme is kinetic rather than thermodynamic (Siegel et al., 1982).

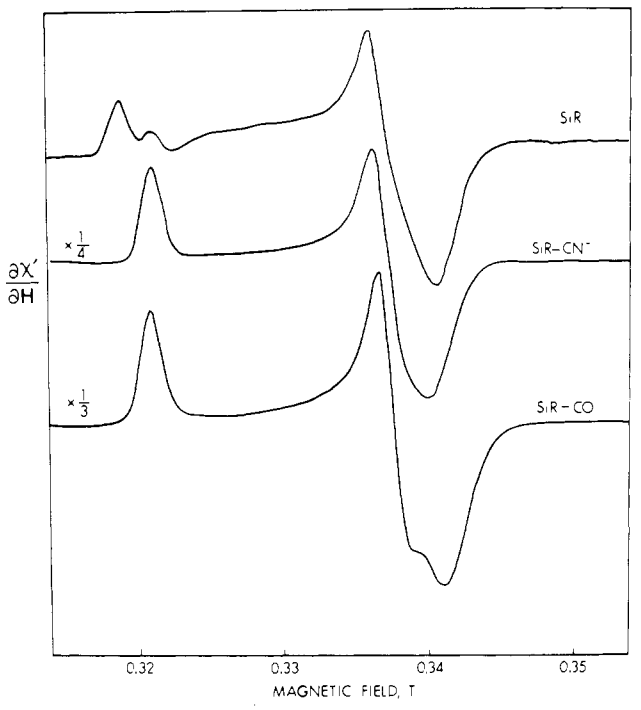

FIGURE 3: EPR spectra of reduced Fe_4S_4 centers of spinach SiR (g = 1.94 type) and its complexes with cyanide and CO. (Upper spectrum) Reduced SiR, prepared as described in Figure 2. (Middle spectrum) Reduced SiR–CN^- complex, prepared as described in Figure 5. (Lower spectrum) Reduced SiR–CO complex, prepared as follows: An EPR tube containing 0.2 mL of an anaerobic solution of 40 μM SiR, 0.6 mM CO, 10 μM Dfl, 2.5 mM EDTA, and 0.05 M potassium phosphate buffer (pH 7.75) was irradiated for 1-min intervals until fully reduced by the procedure described in Figure 2. The solution was then incubated without irradiation at 22 °C for 20 min in order to permit CO complex formation to occur. The irradiation and incubation procedures were then repeated. After the second incubation, an absorption spectrum of the solution showed the enzyme to be in the form of the fully reduced SiR–CO complex. The solution was frozen and the EPR spectrum recorded. Conditions of measurement: temperature 20 K; microwave frequency 9.13 GHz; microwave power 50 mW; modulation amplitude 1 mT.

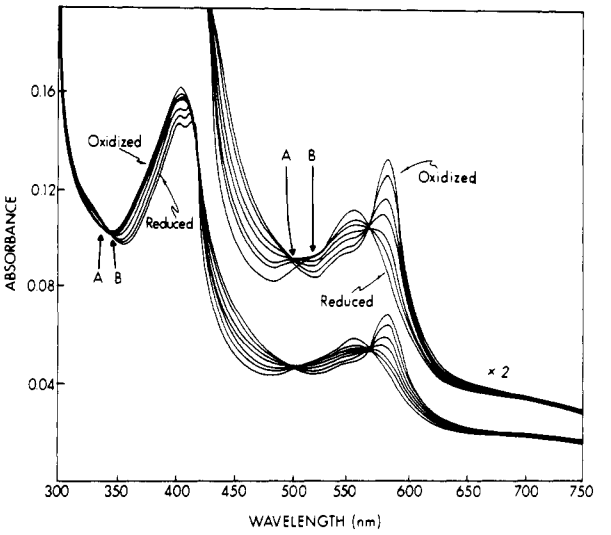

FIGURE 4: Absorption spectra of spinach SiR–cyanide complex. A silica cuvette, 1-cm path length, containing an anaerobic solution of 3.0 μM SiR, 1 mM KCN, 0.75 μM Dfl, 1 mM EDTA, and 0.05 M potassium phosphate buffer (pH 7.75), in a total volume of 0.5 mL, was irradiated by the procedure described in Figure 1 until no further change in absorption spectrum occurred. The resulting spectrum was identical with that labeled "Reduced". Small amounts of air were then injected into the solution until no further change in spectrum was observed. The resulting spectrum is labeled "Oxidized". The cuvette was then subjected to irradiation for successive 0.5–1-min intervals in order to photoreduce the SiR–CN^- complex. Absorption spectra of the solution were recorded after each period of irradiation until no further change occurred (4 min of irradiation in this experiment). The final spectrum is labeled "Reduced" in the figure. The spectra recorded during the early periods of irradiation show a set of isosbestic points marked "A", and those recorded later in the irradiation procedure show isosbestic points marked "B".

An absorption spectrum characteristic of the fully reduced SiR–CN^- complex is shown in Figure 4. The spectrum exhibits a split Soret band at 399 and 412 nm and a maximum at 552 nm. [The split Soret band has also been reported in spectra of reduced *E. coli* SiR–CN^- complex (Siegel et al., 1973).] EPR spectra of the fully reduced spinach SiR–CN^- complex show an intense g = 1.94 type of signal, quantitated at 0.9 spin per heme, at g = 2.034, 1.936, and 1.926 (Figure 3). No g = 2.34 type of EPR signal can be detected in the reduced SiR–CN^- complex (Figure 5).

The reduced SiR–CN^- complex can be reoxidized with $Fe(CN_6)^{3-}$ to yield a low-spin ferriheme species with the optical spectrum (wavelength maxima at 403 and 579 nm) shown in Figure 4 and the EPR spectrum (g = 2.40, 2.31, and 1.71) shown in Figure 5. [Both the optical and EPR spectra of the oxidized spinach SiR–CN^- complex are similar to the spectra reported by Rueger & Siegel (1976) for the *E. coli* SiR–CN^- complex.]

As shown in Figure 4, the oxidized SiR–CN^- complex can readily be photoreduced back to the fully reduced complex. This photoreduction, followed optically, exhibits two distinct phases, with no overlap between the phases like that seen upon reduction of the uncomplexed SiR. This result is consistent with the presence of only two reducible species with substantially different potentials (the first species reduced with much more positive potential than the second) in the SiR–CN^- complex. The first phase of reduction exhibits isosbestic points at 495 and 336 nm and is associated with disappearance of the low-spin ferriheme EPR signal but not the appearance of the reduced Fe_4S_4 center signal. The second phase exhibits isosbestic points at 515 and 347 nm and is associated with appearance of the reduced Fe_4S_4 center EPR signal after reduction of the ferriheme has been completed.

It can be noted from Figure 4 that the second phase of photoreduction, associated specifically with Fe_4S_4 reduction by EPR analysis, yields marked qualitative changes in the enzyme absorption spectrum which are not like the rather general "bleaching" of absorbance in the visible wavelength region normally seen on reduction of iron–sulfur center proteins.

SiR–CO Complex. Krueger & Siegel (1982) have shown that CO slowly forms a complex with spinach SiR in which the MV^+–SO_3^{2-} reductase activity of the enzyme disappears. Inhibition by CO required the presence of a reducing agent. When SiR was irradiated in the presence of EDTA, Dfl, and CO, a complex series of optical spectra (not shown), involving oxidized and reduced uncomplexed SiR as well as CO complex, were observed until CO complex formation was complete. The optical spectrum of the fully formed photoreduced spinach SiR–CO complex exhibits a pattern of absorbance bands (intense α band in the 590–610-nm region, smaller β band in the 540–550-nm region, and a Soret at approximately 400 nm) characteristic of siroheme–CO complexes both in solution (Murphy et al., 1974) and in all siroheme-containing enzymes studied to date (Siegel et al., 1978). Figure 6 shows that in fully reduced spinach SiR–CO complex, the α-band maximum is at 607 nm, the β-band maximum is at 559 nm, and the Soret is at 401 nm. This pattern indicates that CO is binding at the heme Fe in SiR and the other siroheme enzymes.

The EPR spectrum of the fully reduced SiR–CO complex

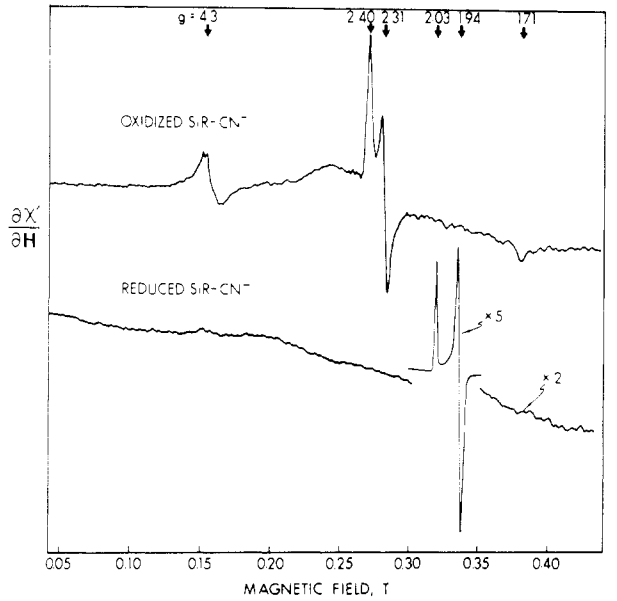

FIGURE 5: EPR spectra of oxidized and fully reduced spinach SiR–cyanide complex. An EPR tube containing 0.2 mL of an anaerobic solution of 40 μM SiR, 1 mM KCN, 10 μM Dfl, 2.5 mM EDTA, and 0.05 M potassium phosphate buffer (pH 7.75) was irradiated for 1-min intervals by the procedure of Figure 1 until reduction of the enzyme, as determined from its absorption spectrum, was complete (3 min of irradiation in this experiment). The sample was frozen and the EPR spectrum (lower spectrum) recorded at 20 K. The sample was then thawed and reoxidized by addition of 5 μL of 5 mM K_3FeCN_6. An absorption spectrum of the sample showed that it was completely in the form of the oxidized SiR–CN^- complex. The sample was then frozen at the EPR spectrum (upper spectrum) recorded at 25 K. Conditions of measurement: microwave frequency 9.13 GHz; microwave power, 50 mW; modulation amplitude 1 mT.

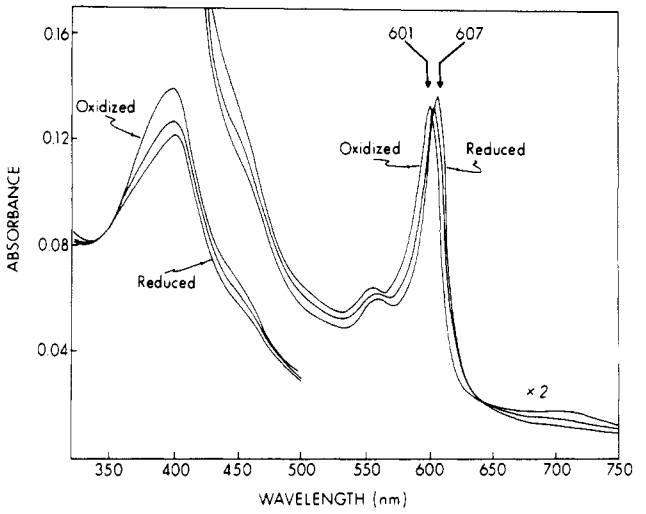

FIGURE 6: Absorption spectra of SiR–CO complex. A silica cuvette, 1-cm path length, containing an anaerobic solution of 2.6 μM SiR, 0.4 mM CO, 0.65 μM Dfl, 1 mM EDTA, and 0.05 M potassium phosphate buffer (pH 7.75), in a total volume of 0.5 mL, was irradiated by the procedure described in Figure 1 for a total of 10 min, and the cuvette was then incubated in the dark at 22 °C to allow CO complex formation to occur. The absorption spectrum was recorded, and small amounts of air were then injected into the solution until no further increase in A_{400} was detected. The spectrum of the resulting SiR–CO complex is labeled "Oxidized". The cuvette was then irradiated for two successive intervals of 5 min each. After each interval the spectrum was recorded and is shown in the figure. No further change in spectrum occurred when the period of irradiation was extended past a total of 10 min. The spectrum resulting after the second 5-min irradiation period is therefore labeled "Reduced".

(Figure 3) exhibits resonances with g = 2.035, 1.934, and 1.915. The reduced Fe_4S_4 center spectrum quantitates at 0.9 spin per heme. No g = 2.34 type of EPR signal is detected in the fully reduced SiR–CO complex. It is evident from Figure 3 that the line shape and g values of the reduced Fe_4S_4 center EPR spectrum differ depending upon whether the SiR heme is ligated with CN^- or CO.

When the fully reduced SiR–CO complex is mixed with O_2, the optical spectrum shows shifts in the α- and β-band maxima to 601 and 554 nm, respectively, together with a marked increase in absorbance in the Soret band region, accompanied by a small shift in the Soret band maximum wavelength to 399 nm. The EPR spectrum of such reoxidized SiR–CO yields no signals at all other than a small signal at g = 4.3, probably due to a small amount of adventitious iron, and a variable amount of high-spin ferriheme (<0.1 spin per heme) due to dissociation of CO from the oxidized enzyme [see Murphy et al. (1974)]. These results suggest that O_2 oxidizes the reduced Fe_4S_4 center of the photoreduced SiR–CO complex, while the heme, complexed to CO, remains in the Fe^{2+} state. Photoreduction of the reoxidized SiR–CO complex results in a reversal of the shifts in the optical spectrum seen on reoxidation of the initially photoreduced SiR–CO complex and in quantitative regeneration of the reduced Fe_4S_4 center EPR spectrum (Figure 6).

Discussion

Optical and EPR spectra recorded at various times during photoreduction of spinach SiR show that the enzyme is reduced in at least two sequential steps. The steps are essentially independent, i.e., the potentials of the reducible groups are very different when SiR is complexed with CN^-, a potent inhibitor of SiR catalytic activity (Krueger & Siegel, 1982), but there seems to be some overlap of the reduction steps in the uncomplexed enzyme. Christner et al. (1981) have cited data which indicate that the heme and Fe_4S_4 potentials in *E. coli* SiR differ by about 70 mV.

CN^- complexation to SiR results in a change in the heme spin state to low spin in the oxidized enzyme; this suggests (but does not prove) that CN^- is directly ligated to the heme Fe. Reduction of the SiR–CN^- complex results first in reduction of the ferriheme to a diamagnetic ferroheme species and then in reduction of the Fe_4S_4 center to yield a typical g = 1.94 type EPR spectrum. Reduction of the Fe_4S_4 in SiR–CN^- is associated with marked shifts in the absorption bands of the enzyme, even though the heme is already in the Fe^{2+} state.

When SiR is complexed with CO (another potent inhibitor of catalytic activity), the resulting optical spectrum is qualitatively similar to that characteristic of free siroheme–CO in aqueous solution (Murphy et al., 1974). Thus it appears that CO, like CN^-, is probably ligated directly to the heme Fe of SiR. It is a characteristic of hemoprotein–CO complexes, unlike CN^- complexes, that CO remains tightly bound to the heme only when the heme is in the Fe^{2+} state; i.e., CO tends to stabilize the ferroheme state. CO dissociation from siroheme enzymes is generally rather slow (Murphy et al., 1974; Vega & Kamin, 1977), so that O_2 can be used to oxidize the reduced Fe_4S_4 center of photochemically reduced SiR–CO complex without substantial reoxidation of ferroheme–CO. In such enzyme, there are marked shifts in the optical bands of the SiR ferroheme–CO on oxidation and reduction of the Fe_4S_4 center.

The optical changes seen in the SiR–CN^- and SiR–CO spectra on changes in Fe_4S_4 oxidation state are quite different from the rather general "bleaching" in the visible wavelength region usually observed on reduction of iron–sulfur proteins. Furthermore, the detailed line shapes and g values of the reduced Fe_4S_4 center spectra of SiR vary depending upon

whether the heme is ligated with CN^- or CO. These results suggest that each prosthetic group of SiR is sensitive to the ligation and/or oxidation state of the other group; i.e., there is some sort of interaction between these groups in the SiR catalytic center. Marked changes in the nature of the $g = 1.94$ type EPR spectrum of the fully reduced SiR–CN^- and –CO complexes (e.g., to the $g = 2.34$ type of spectrum) would not be expected, even if the centers were tightly linked, as has been proposed for *E. coli* SiR (Christner et al., 1981), since the ferroheme in these complexes is expected to be $S = 0$.

Mossbauer studies of *E. coli* SiR indicate that electrons in that enzyme are sequentially added to the heme and Fe_4S_4 centers (Christner et al., 1981). A novel EPR signal with three g values above 2.0 has been described by Janick & Siegel (1979) in the fully reduced *E. coli* SiR. The combined EPR and optical spectra of uncomplexed spinach SiR (e.g., loss of the high-spin ferriheme EPR signal and disappearance of the characteristic charge-transfer band at 712 nm) indicate that in this enzyme, too, the ferriheme is reduced prior to the Fe_4S_4 center. Full reduction of the spinach enzyme leads to the appearance of a novel EPR spectrum, with $g = 2.48$, 2.34, and 2.08, similar to but not identical with that described in the *E. coli* SiR. This novel signal represents a majority of the species of the enzyme, a minor proportion of $g = 1.94$ type signal also being seen. In light of the *E. coli* results, it seems likely that this novel EPR signal is due to a coupled reduced Fe_4S_4–ferroheme ($S = 1$ or 2) system in spinach SiR as well. [The finding of a significant amount of $g = 1.94$ type EPR signal in fully reduced spinach SiR (as well as two distinct features in the $g = 2.04$ region of this spectrum) suggests that there may be more than one state of the siroheme–Fe_4S_4 system in the spinach enzyme preparation.]

Taken together with observations that $g = 1.94$ type EPR spectra are not normally observed on reduction of plant NiRs, but that such spectra are readily observed on addition of heme ligands such as CO (Cammack et al., 1978; Lancaster et al., 1979), and the preliminary finding that the Mossbauer spectra of oxidized spinach NiR is qualitatively similar to that of oxidized *E. coli* SiR, the data presented in this work suggest that the siroheme–Fe_4S_4 interaction, perhaps by direct coupling through a bridging ligand, is a general feature of both types of "multielectron reductase" enzymes. There are a number of possible implications of such coupling for mechanisms of catalysis in this class of enzymes.

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