Superoxidized States of *Escherichia coli* Sulfite Reductase Heme Protein Subunit†

Lawrence J. Young and Lewis M. Siegel*

Department of Biochemistry, Duke University, Durham, North Carolina 27705

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ABSTRACT: The oxidized forms of resting and sulfite-complexed *Escherichia coli* sulfite reductase heme protein subunit react with near-stoichiometric amounts of porphyrexide to produce what is best characterized as a ferrisiroheme π cation radical. Addition of either sodium ascorbate or NADPH completely regenerates the parent form. Implications of these findings with respect to mechanisms of metal–radical J coupling and catalysis are discussed.

The hemeprotein subunit of *Escherichia coli* sulfite reductase (SiRHF),[1] which catalyses the six-electron reduction of sulfite to sulfide and nitrite to ammonia (Siegel et al., 1982), contains a closely apposed siroheme and 4Fe–4S cluster at its active center (Christner et al., 1981, 1984). Each prosthetic group can accept a single electron added exogenously (Janick & Siegel, 1983a). McRee et al. (1986) have shown that the heme Fe in SiRHP is located 4.4 Å from the nearest cluster Fe and the two metal atoms appear to be bridged by a single amino acid, possibly a cysteine S. A second site of interaction between the heme and the cluster exists in that a cubane sulfide of the cluster is in van der Waals contact with an edge of the siroheme macrocycle. Thus, there appears to be a possibility for multiple pathways of effective "inner-sphere" electron transfer between the prosthetic groups of sulfite reductase [see Gupta et al. (1985, 1987) and Scheidt et al. (1987) for a discussion of spin coupling in noncovalently linked porphyrin dimers]. A second multielectron reductase, the ferredoxin-nitrite reductase of spinach, has been shown to contain a similarly interacting siroheme/4Fe–4S active center (Wilkerson et al., 1983; Siegel et al., 1986).

Siroheme is an iron isobacteriochlorin (iBC; i.e., two adjacent pyrrole rings are partially saturated; Murphy et al., 1973; Scott et al., 1978). It is not yet understood why this particular macrocycle has been selected by nature to participate in the multielectron reductions of sulfite and nitrite. As would be expected, there is little difference in the midpoint potentials for reduction of ferric to ferrous iron in metalloporphyrins, clorins, and isobacteriochlorins (Chang, 1982; Stolzenberg et al., 1980). Perhaps the most novel property of the iBCs is the relative ease with which the macrocycle can be oxidized to generate a π-cation-radical species (Stolzenberg et al., 1979, 1980; Richardson et al., 1979; Chang et al., 1981; Chang, 1984; Fujita et al., 1985). The reduction potential of the iBC cation radical, either as a free base or a complex with Zn(II), Co(III), Fe(III), Co^{II}·NO, or Fe^{II}·NO, is typically 250–300 mV lower than its chlorin analogue and as much as 500 mV less than the corresponding porphyrin species. The explanation of this phenomenon has to do with the fact that due to reduction of a pair of pyrroles to the corresponding pyrrolines, the HOMO for an iBC (an a_1u orbital) is destabilized relative to those of its chlorin (a_1u) and porphyrin (a_2u) counterparts. In the pyridine Fe^{II}·CO complexes of OEP, etiochlorin, and DMOEiBC, oxidation of the iBC ring occurs before the metal and produces a ferrous carbonyl π-cation species. However, with the porphyrin and chlorin complexes, the HOMO is metal-centered and oxidation leads to formation of a ferric iron with concomitant dissociation of the CO. A variety of different techniques have been used to show that in all instances the iBC oxidation product in the model systems is ring-centered.

Up to now there has been no evidence that a similar ring-centered oxidation could occur in the protein-bound siroheme moiety of the sulfite or nitrite reductases. Oxidation of the ring itself during particular steps in the catalytic cycle could be a source of "extra" electrons for the multielectron reduction reactions catalyzed by these enzymes. We present in this paper evidence that a "superoxidized" form of SiRHP can be generated by the addition of 1.25–1.50 equiv/heme of the oxidant porphyrexide[2] to the native uncomplexed enzyme and to its sulfite complex. In both cases, the optical and EPR properties of the superoxidized species are those anticipated for a siroheme π cation radical coupled to a ferric iron. The reaction is reversible in that the superoxidized species generated in this fashion can be reduced to the starting material with an excess of either sodium ascorbate or NADPH. The nitrite and cyanide adducts of the resting enzyme have also been examined, but the reaction with these ligands is not clean and generates modified forms of the protein. We have also observed that, if potassium ferricyanide is used, the oxidation is not complete and some irreversible modification of SiRHP occurs.

Materials and Methods

NADPH-sulfite oxidoreductase and its heme protein subunit were isolated from *E. coli* B by a minor modification[3] of the procedure of Siegel et al. (1973) and Siegel and Davis (1974). In brief, this amounts to removing the nucleic acids with

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* Address correspondence to the Department of Biochemistry, Duke University Medical Center and VA Hospital, Durham, NC 27705.

[1] Abbreviations: OEP, octaethylporphyrin; DMOEiBC, dimethyloctaethylisobacteriochlorin; iBC, isobacteriochlorin; MO, molecular orbital; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; EPR, electron paramagnetic resonance; ENDOR, external nuclear double resonance; EDTA ethylenediaminetetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; SiRHP, heme protein subunit from *Escherichia coli* sulfite reductase; NHE, normal hydrogen electrode; HiPiP, a high-potential iron protein; HRP, horseradish peroxidase; CPO, chloroperoxidase.

[2] IUPAC nomenclature for the reduced form of porphyrexide: 4-amino-2,5-dihydro-2-imino-5,5-dimethyl-1*H*-imidazol-1-yloxy.

[3] Young, L. J., & Siegel, L. M. *Biochemistry* (in press).

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poly(ethyleneimine) instead of protamine sulfate and performing an additional chromatography of partially purified SiRHP on benzyl–DEAE cellulose. Bacteria were grown in bulk (80–90 kg) on minimal media (Grain Processing Corp., Muscatine, IA) and stored as 2.5-kg cakes at 77 K until used. Following isolation, the heme protein subunit was concentrated to 100 μM in heme and stored at −20 °C in 0.1 M potassium phosphate plus 0.1 mM EDTA, pH 7.7 (standard buffer). Protein concentrations were determined by measuring the absorption of the oxidized enzyme, using an extinction coefficient of 18.1 mM^{-1} cm^{-1} at 590 nm. All chemicals used were of reagent or analytical grade and unless noted otherwise were not purified further.

Solutions of potassium ferricyanide were prepared by dissolving the salt in standard buffer and then measuring its concentration, using a millimolar extinction coefficient of 1.02 at 420 nm. Invariably, a species giving rise to a shoulder at 400 nm was present and accounted for 5–10% of the absorption at this wavelength. It is most probably the pentacyano–aquo derivative (Michaelis & Smythe, 1931), and because its midpoint potential is almost identical with that of the hexacyanide (E_{m7} = 440 mV), no correction was made for its presence.

Porphyrexide was dissolved in ice-chilled distilled H_2O and standardized by titration against NADPH just prior to each set of experiments. The procedure involved addition of aliquots of the porphyrexide solution to a sample of NADPH (ϵ_{mM} = 6.22 at 340 nm) and then measurement of the loss of intensity at 340 and 346 nm. The use of two wavelengths was necessary because the isosbestic point for the reduced and oxidized forms of porphyrexide was slightly variable. Nonetheless, the values for the concentration of porphyrexide routinely agreed to within 2–4% at the two wavelengths. It was assumed that 1 mol of NADPH would reduce 2 mol of the oxidant. Stoichiometric titration of a solution of porphyrexide with NADPH gave results which were within 20% of that found when the reverse procedure was done (vide supra). When stored on ice, this standardized solution of porphyrexide was stable for about 6 h, but if left at room temperature (20–25 °C), up to 50% of the original intensity at 412 nm could be lost. All experiments with porphyrexide and SiRHP were performed in 0.1 M potassium phosphate, pH 7.7, without EDTA because it was found that porphyrexide [E_{m7} = 722 mV; Meckstroth et al. (1981)] could oxidize EDTA.

Two techniques were used to examine the reaction of air-saturated solutions of the enzyme with this oxidant, e.g., substoichiometric titration with 0.1–0.2 equiv of porphyrexide/heme per aliquot or a bolus addition of 0.75–3.0 equiv. It is necessary that all experiments be done at 5 °C in order to minimize what appears to be an "autoreductive" back-reaction, which is particularly evident when a bolus of porphyrexide is used at 25 °C; i.e., with no added reductant the porphyrexide-induced loss of α-band intensity is reversed, with a $t_{1/2}$ of 15–20 min. By lowering the temperature to 5 °C the rate of the autoreduction is diminished by nearly an order of magnitude. If the titration methodology was used, the back-reaction was slow enough to be ignored in the time frame of these experiments; i.e., isosbestic points could be held during the course of a 15–25-min run.

Reversal of the porphyrexide effect was achieved by addition of solid sodium ascorbate or NADPH to give a final concentration of 1–10 mM. Lesser amounts (0.1–1 mM) could be used, but the rate of the reduction was proportionately slower. For the purposes of this investigation, the presence of excess reductant is of no consequence.

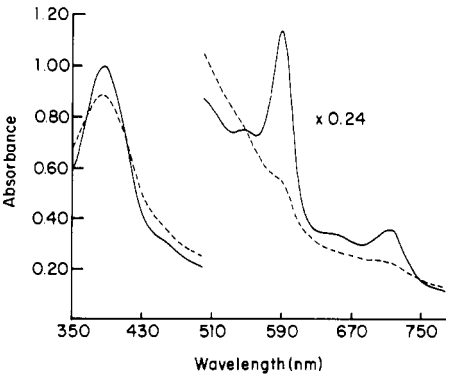

FIGURE 1: Optical spectrum of 15.1 μM SiRHP before (solid) and after (dash) addition of 1.25 equiv of porphyrexide at 5 °C. The sample was dissolved in 0.1 M potassium phosphate buffer, pH 7.7 (this buffer is used in all experiments involving porphyrexide and henceforth will be called the "reference buffer"). The spectrum of the porphyrexide-treated enzyme was recorded 30 s after addition of the oxidant and has not been corrected for the 2% dilution. When excess ascorbate was added to the superoxidized enzyme, the spectrum of the parent form (solid) was found.

The various derivatives of SiRHP were prepared with the ligand in excess either by prolonged (16–48-h) incubation at room temperature (the sulfite and nitrite complexes) or by rapid (5–10-min) photoreduction of SiRHP using deazalumiflavin–EDTA followed within several minutes by air reoxidation (cyanide complex). Deazalumiflavin was synthesized for us by Dr. S. Ghisla of the University of Konstanz. Samples were freed of EDTA, deazalumiflavin, and excess ligand by passage down a column of G-25 Sephadex equilibrated in phosphate buffer (T = 3 °C), whereupon the protein was placed on ice and then immediately used.

In a typical experiment, 0.5–1.0 mL of a 10–20 μM solution of enzyme was placed in a quartz cuvette housed in the thermostated cell holder of a Perkin-Elmer λ9 UV–vis NIR spectrophotometer and then reacted with porphyrexide. Some combination optical/EPR experiments were done rigorously in tandem with an aliquot of the enzyme present inside the cuvette; the aliquot was removed and then frozen in liquid nitrogen within 30 s of completion of the optical run. At other times, the optical experiment was conducted with a 500-μL aliquot of enzyme, but EPR samples were taken from a reservoir of identically treated enzyme while the optical spectrum was being recorded. No difference was noted in the results obtained from the two techniques.

EPR spectra were taken with a Bruker ER 200D spectrometer at a modulation amplitude of 10 G, an operating frequency of 9.47–9.48 GHz, and a modulation frequency of 100 kHz. The running temperature was typically 10 K and was controlled by an Air Products Heli-Tran refrigeration unit.

The porphyrexide used in these experiments can be obtained from Fluka, Tridom Chemical Inc., Hauppage, NY. This somewhat unusual oxidant [see Clark (1960) for a structural formula] has previously been used in EPR studies of the high-spin signals of bovine heart cytochrome *c* oxidase (Beinert & Shaw, 1977). It was selected for use in the studies reported here because (i) compared to ferricyanide or iodine, it rapidly reacted with the enzyme with a minimum of side reactions, (ii) its reduced form is EPR-silent and its oxidized form is highly isotropic, and (iii) its midpoint potential is in the range found for oxidation of the model compound octaethylisobacteriochlorin (Chang, 1982).

RESULTS

Porphyrexide Oxidation of Unligated SiRHP. The optical spectrum of resting SiRHP in 0.1 M potassium phosphate, pH

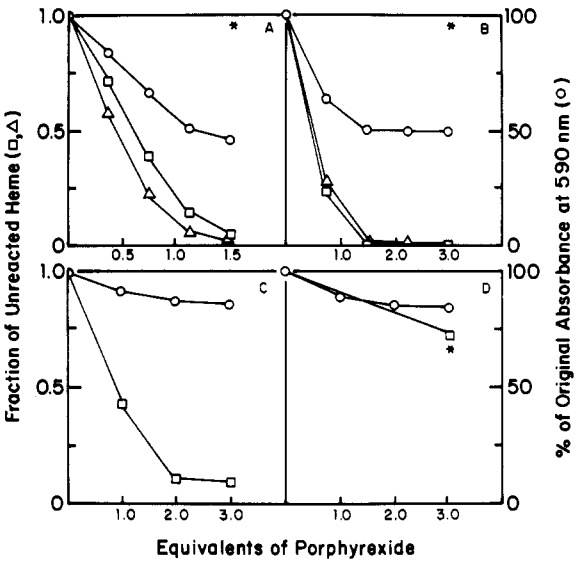

FIGURE 2: Parallel and in tandem optical/EPR experiments of the reaction of porphyrexide with various derivatives of SiRHP. (A) Titration of free SiRHP with porphyrexide as monitored by the loss of the absolute intensity at 590 nm (circle), the change in the relative intensity at 590 nm (square), and the loss of the absolute intensity of the EPR signal at $g = 6.70$ (triangle). The optical experiment was done at 5 °C with 37.5 μM SiRHP dissolved in reference buffer. Samples for the EPR experiment were taken from a reservoir of enzyme treated in parallel (see Materials and Methods). The asterisk indicates the height of the EPR signal after addition of excess ascorbate. (B) Titration of the sulfite complex of SiRHP as monitored by the loss of the absolute intensity at 583 nm (circle), the change in the relative intensity at 583 nm (square), and the loss of the absolute intensity of the sulfite-induced EPR signal with $g_z = 2.71$ (triangle). The optical experiment was done at 5 °C with 22 μM enzyme dissolved in reference buffer; EPR samples were taken as in A. The asterisk indicates the height of the EPR signal after addition of excess ascorbate. (C) Titration of the nitrite complex of SiRHP as monitored by the loss of the absolute intensity at 579 nm (circle) and the loss of the absolute intensities of the nitrite-induced EPR signals with $g_z = 2.91$ and 2.82 (square). The optical experiment was done at 5 °C with 37.5 μM enzyme dissolved in reference buffer; samples for EPR were taken in tandem (see Materials and Methods). (D) Titration of the cyanide complex of SiRHP as monitored by the loss of the absolute intensity at 581 nm (circle) and the loss of the absolute intensities of the cyanide-induced EPR signals with g_z, $g_y = 2.44$, 2.34 and 2.38, 2.29 (square). The optical experiment was done at 5 °C with 15.2 μM enzyme dissolved in reference buffer; EPR samples were taken in tandem. The asterisk indicates the intensity of the EPR signals after treatment with excess ascorbate.

7.7 and 5 °C, is markedly perturbed within seconds of a bolus addition of 1.25 equiv/heme of a standardized solution of porphyrexide (Figure 1). When compared to the oxidized enzyme, the molar absorptivities of the Soret (384 nm), α (588 nm), and CT (710 nm) bands have been diminished by 8.5%, 48%, and 35%, respectively, with the respective band positions reflecting 3-, 2-, and 4-nm blue shifts from their positions in the oxidized enzyme. If a further 1.25 equiv is introduced, there is a very slight (1–2% of the initial value) decrease of the α and CT intensities but with no further change in position. The conditions of this experiment are such that the SiRHP autoreduction phenomenon described under Materials and Methods is minimized, the α-band absorptivity increasing at a rate of only 3.6×10^{-2} mM^{-1} cm^{-1} min^{-1}. Titration of SiRHP with substoichiometric aliquots of porphyrexide gives a comparable endpoint spectrum, with a pair of isosbestic points at 550 and 738 nm holding for 90–95% of the experiment.

While resting SiRHP exhibits an EPR spectrum characteristic of a high-spin ferric heme, with g values of 6.70, 5.24, and 1.98 (Siegel et al., 1982), the prophyrexide-treated enzyme

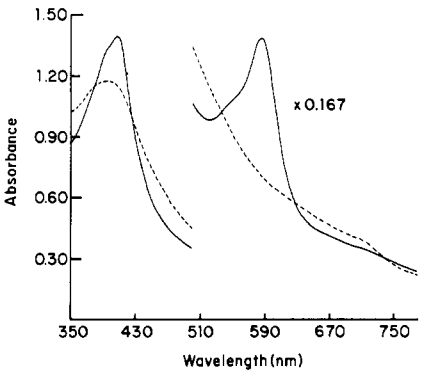

FIGURE 3: Optical spectrum of 22.0 μM SiRHP·SO_3^{2+} before (solid) and after (dash) addition of 1.5 equiv of porphyrexide at 5 °C. The spectrum of the porphyrexide-treated enzyme was taken 1 min after addition of the oxidant and has not been corrected for a 1% dilution. When excess ascorbate was added, the spectrum of the parent form (solid) was produced.

is EPR-silent. Results of a parallel optical/EPR titration experiment are illustrated in Figure 2A. The oxidation of SiRHP was followed by measuring the loss of intensity of the rhombic high-spin EPR signal and of the 590-nm α band. An end point for the raw optical data of Figure 2A was estimated from the intensity of the high-spin EPR absorption at 1.5 equiv of porphyrexide. With a 1:1 correlation between the two then being assumed, a replot of the optical data (expressed as $\Delta A/\Delta A_\infty$) shows no coincidence with the EPR data at all points on the two curves. At present we have no facile explanation for this discrepancy, but it might be due to effects of temperature on midpoint potentials. An EPR signal due to unreacted porphyrexide was seen only in the 1.13- and 1.5-equiv samples, and at no level of oxidation was there evidence of a second radical species.

It is possible to completely regenerate the optical spectrum and over 95% of the EPR signal intensity of resting SiRHP by the addition of a 100-fold excess of sodium ascorbate or NADPH. The ascorbate-induced reversion of the optical spectrum is complete in the time of manual mixing and recording of the spectrum (~1 min), while the reaction with a similar amount of NADPH is roughly an order of magnitude slower (there is no effect of ascorbate or NADPH on the optical spectrum of resting SiRHP). The methyl viologen sulfite reductase activity of resting SiRHP is not altered after treatment with porphyrexide plus ascorbate.

Porphyrexide Oxidation of the Sulfite Complex of SiRHP. SiRHP forms a complex with its substrate sulfite in which the siroheme appears to be in the low-spin ferric state (Siegel et al., 1982; Day et al., 1987). Figure 3 shows that porphyrexide produces changes in the optical spectrum of this sulfite adduct that are similar to those described for the free enzyme; i.e., the Soret and α bands show a loss of intensity of 15% and 47%, respectively, accompanied by a 13-nm blue shift of the Soret band maximum from 407 to 394 nm. There is also a 4-nm blue shift of the long-wavelength charge-transfer feature (from 714 to 710 nm) and a 1% increase in its absorptivity, neither of which is due to the presence of unligated enzyme. These results were obtained with 1.5 equiv of porphyrexide/heme and were unchanged after addition of a further 1.5 equiv. If instead of using a single bolus of oxidant, one makes sequential additions of 0.15 porphyrexide equiv/heme, a comparable end-point spectrum is obtained, with a pair of isosbestic points at 530 and 625 nm observed for 90% of the titration. As was observed with the free enzyme, excess ascorbate completely regenerates the optical spectrum of the starting material (ascorbate shows no reaction with the resting sulfite adduct[4]).

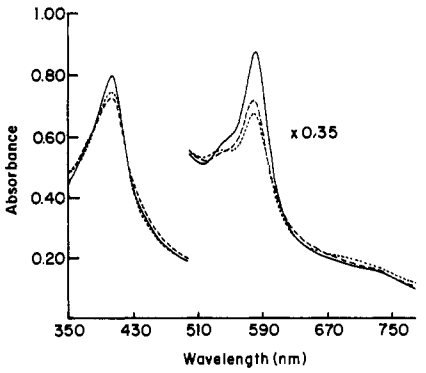

FIGURE 4: Optical spectrum of 15.2 μM SiRHP·CN^- before (solid) and after (dash) addition of 3 equiv of porphyrexide at 5 °C. The spectrum of the superoxidized complex was taken 30 s after the addition of the oxidant. The final trace (dash–dot) was recorded 20 min after adding an excess of ascorbate.

The sulfite complex of SiRHP is largely EPR-silent in spite of the fact that this enzyme species is in an $S = {}^1/_2$ state, as determined by magnetic susceptibility (Day et al., 1987). There is a small, sulfite-induced, low-spin ferriheme EPR[5] signal with $g_z = 2.71$, which accounts for only 0.1 spin/heme. Figure 2B shows that the loss in intensity of this EPR signal closely paralleled the loss in intensity of the optical absorption and, furthermore, that the porphyrexide-treated sulfite complex is EPR-silent (about 5% of the enzyme in this sample is not complexed with sulfite and its high-spin EPR signal responds to addition of porphyrexide as expected for free SiRHP. At all levels of oxidant there is a small derivative-type feature at $g = 2.03$, which accounts for only 0.01–0.02 spin/heme). The amount of a radical signal due to excess porphyrexide follows a trend like that found with the unligated enzyme but is always greater at comparable porphyrexide/heme ratios. When excess ascorbate was added to enzyme that had been reacted with 3 equiv of oxidant, there was nearly complete restoration of the EPR spectrum of the original SiRHP–sulfite complex.

Reaction of Porphyrexide with SiRHP–Cyanide and –Nitrite Complexes. There appears to be a clear difference in the reactivity of porphyrexide with the low-spin, cyanide complex of ferric SiRHP (Siegel et al., 1982) when compared to the unligated and sulfite-complexed forms. Figure 4 shows that addition of 3 equiv of porphyrexide (in sequential amounts of 1 equiv/heme) to the cyanide complex causes a partial bleaching of the Soret and α bands (6% and 15%, respectively) and small blue shifts of the band maxima of 0.5 and 1.5 nm, respectively. However, no isosbesticity was observed during the course of the titration, and as shown in Figure 4, excess ascorbate did not regenerate the starting species (control experiments demonstrated that ascorbate did not change the optical spectrum of the resting cyanide species). Figure 2D shows that the EPR signature of the SiRHP–cyanide complex is reduced in intensity by only 25% after the addition of 3 equiv of prophyrexide/heme and that ascorbate does not restore the original intensity. Furthermore, the amount of unreacted porphyrexide is much less (by a factor of ~3) than what would have been found with the unligated and sulfite-bound enzyme under similar conditions.

We have recently shown (Young & Siegel, 1988) that 100 mM NO_2^- slowly reacts with 40–120 μM resting SiRHP and after a 16–18-h incubation a mixture is produced consisting of a low-spin ferric nitrite species (65–80%), unligated enzyme (20%), and a diamagnetic ferric nitrosyl form (0–15%). The experiments described here use the product of this 16-h incubation, which we will term the SiRHP "nitrite adduct". Figure 5 shows that addition of 3 equiv of porphyrexide (in sequential amounts of 1 equiv/heme) to this incubation product results in a partial bleaching of the α- (16%) and Soret- (7%) band optical intensities, a 1-nm blue shift in the position of the former to 578 nm, and 1-nm red shift of the latter to 398 nm. The mixture of starting species precluded the observation of isosbesticity.

The EPR spectrum of the nitrite adduct prepared as outlined above exhibits two highly anisotropic low-spin EPR signals of approximately equal intensity with g_z values of 2.91 and 2.82 (Young & Siegel, 1988). Figure 2C shows that this pair of low-spin EPR signals are nearly completely abolished at 2 porphyrexide equiv/heme. At this level of oxidation, the amount of unreacted porphyrexide is about an order of magnitude less than that seen with the unliganded and sulfite-complexed enzyme. Also, nearly 75% of the original uncomplexed enzyme (0.17 spin/heme) remains, and this amount is not reduced by the addition of a further 1 equiv of oxidant.

Janick et al. (1983b) reported that treatment of a ferrous SiRHP·NO complex with ferricyanide followed by addition of excess ascorbate resulted in the regeneration of the starting species with its characteristic optical spectrum (α band at 599 nm, Soret band at 400 nm). Figure 5 shows that addition of excess ascorbate to the porphyrexide-treated nitrite adduct produces a species with a Soret band at 392 nm and an α band at 593 nm. A similar species could be obtained by reaction of the nitrite adduct with ascorbate but no porphyrexide; however, the relative intensities of the α bands of the resting/ascorbate couple and the resting plus porphyrexide/ascorbate couple were not the same, e.g., A_{593}/A_{579} (resting) = 0.94 compared to A_{593}/A_{579} (resting plus porphyrexide) = 1.04.

The EPR signature of the ascorbate-reduced, porphyrexide-oxidized nitrite adduct contains a ferrous nitrosyl species, which quantitates to 0.75 spin/heme, a small radical signal at $g = 2.01, 2.00$, which accounts for about 0.02 spin/heme, and a high-spin signal of the unligated enzyme at 0.17 spin/heme.[6]

[4] We have examined the nonenzymatic reaction between porphyrexide and the heme ligands studied in this work. Several seconds after mixing 24 μM porphyrexide and 50 μM sulfite at pH 7.7, an end point is observed at 50% of the original absorption at 412 nm, the λ_{max} of the free porphyrexide. We have not investigated the kinetics of this reaction, but it must be that, with the experimental conditions used here, either the concentration of free sulfite is low or the reaction of porphyrexide with the enzyme is faster than with free sulfite, or both. Porphyrexide will also react with KCN and $NaNO_2$. The former case appears to be a reduction with a rate ~3 orders of magnitude less than that found for sulfite. In contrast, the nitrite reaction has more of the characteristics of an addition; i.e., instead of a simple "reductive" bleaching at 412 nm, there is a shift of the maximum to 385 nm with an isosbestic point at 397 nm that occurs at a rate which is 3 orders of magnitude slower than the cyanide reaction.

[5] Due to the unusual nature of the sulfite-induced EPR signal we cannot be certain of our quantitation. Thus, the close correlation between the optical and EPR titrations may be entirely fortuitous instead of simply reflecting that EPR-silent and EPR-active sulfite species show similar midpoint potentials. An optical–EPR correlation does not form an essential part of our arguments concerning the etiology of sulfite-induced optical perturbations and/or EPR silence. We used second harmonic mode EPR spectroscopy, as outlined by Salerno et al. (1986), in an attempt to better visualize the signal and found a ramp-shaped g_z feature that extended to almost 10000 G with no well-defined (cf. cytochrome *c*) turning points. Collaborative studies with the laboratories of Drs. P. Debrunner, W. R. Dunham, and E. P. Day are currently in progress.

[6] The position of the α band in the optical spectrum of ferrous NO complexes of SiRHP appears to be sensitive to the experimental protcol used in their preparation, and we are actively investigating the etiology of this phenomenon.

Discussion

The results presented in this paper show for the first time that the siroheme moiety of an enzyme can be reversibly converted to a superoxidized state and that this oxidation can also occur in a complex between the enzymic heme and its physiological substrate.

What is the chemical nature of the superoxidized state of SiRHP? The sites at which porphyrexide could effect oxidation of the enzyme include the following: (i) the isobacteriochlorin ring, (ii) the iron associated with the ring, (iii) a sulfur or iron in the 4Fe–4S cluster; (iv) an axial ligand either endogenous or exogenous (e.g., sulfite), (v) specific amino acid residues situated outside the inner coordination sphere of the heme iron, (vi) "nonspecific" amino acid residues, and (vii) some combination of the above. The changes that we have observed in the optical spectra of Figures 1 and 3 are profound and are in keeping with the notion that the primary site of oxidation is at or near the heme. The finding that the superoxidized states are EPR-silent is not particularly helpful in differentiating among the first four possibilities since non-Kramers' systems [e.g., Fe(IV)], exchange interactions between Kramers' systems [e.g., Fe(III) and a π cation of the iBC macrocycle], and dipolar broadening can all produce this condition.[7]

Fortunately, there have been a considerable number of model system studies which suggest quite strongly that the product of oxidation of both free and sulfite-bound SiRHP is a π cation radical which is exchange coupled to a ferric iron. If this is so, then it is the first example of a coupled ferriheme π cation radical in a protein. With but one exception, studies with Fe or Co porphyrins (Felton et al., 1971, 1973; Phillippi & Goff, 1982; Goff & Phillippi; 1983) and iBCs (Stolzenberg et al., 1979, 1980; Richardson et al., 1979; Chang et al., 1981; Fujita et al., 1985) have shown that formation of a π-cation species is accompanied by a diminution of the Soret- and α-band extinctions, a blue shift in the Soret maximum, and an increase in absorptivity at long (>750 nm) wavelengths when compared to the spectral profile of its parent form. These are precisely the changes seen with superoxidized SiRHP.

Chang et al. (1982) pointed out that the axial ligation state of the iron may have a critical and possibly deciding role in determining whether an oxidation is ring- or metal-centered. In a similar vein, Phillippi and Goff (1982) suggested that their demonstration of the absence of a metal-centered oxidation in porphyrin complexes with weak-field axial ligands indicated that oxo or hydroxo ligands would promote formation of ferryl ion porphyrins. This hypothesis has recently been confirmed (Groves et al., 1985; Lee et al., 1985), although it is also of interest that low-spin, bis(imidazole)Fe(III)TPP π-cation species are found if imidazole is added to the chloro π cation at low temperatures (Goff & Phillippi, 1983); i.e., it may be that the ligand field strength is not the only important factor.

Our impression that the superoxidized forms of the free and sulfite-bound enzyme are π-cation species is supported by the preliminary results of resonance Raman experiments,[8] which show a shift of the siroheme oxidation-state marker (ν_4) of the free enzyme to lower frequency plus the presence of several new features in the spectrum after reaction with porphyrexide. Such a shift is not expected to occur if the ferric iron was oxidized (the shift should be to a higher frequency). In addition, both ENDOR (Cline et al., 1985) and EPR[3] experiments give no evidence for there being a water at the distal coordination site of the heme, which could facilitate formation of a ferryl–oxo species.

We consider it improbable that an axial ligand(s) has been oxidized since the changes observed in the electronic spectrum are unlike anything yet reported for complexes of the enzyme with added ligands; i.e., unless the unpaired "axial" spin is significantly delocalized, such a simple change in the strength of the ligand field and/or the electronegativity of the iron should not produce such a striking change in the spectrum.

Recent X-ray crystallographic studies of ferriheme SiRHP (McRee et al., 1986) have shown that the 4Fe–4S cluster is in such intimate contact with the siroheme that one edge of the macrocycle is in van der Waals contact with one of the cubane sulfur atoms of the cluster. There also appears to be a ligand bridging between the heme Fe and one of the cluster Fe atoms. For these reasons it is not surprising that the optical spectrum of both free and ligated forms of SiRHP is sensitive to the oxidation state of the cluster (Janick & Siegel, 1983a). The most constant finding on reduction of the cluster is a red shift of the α band with little change in shape or intensity. The Soret band can move to higher or lower wavelengths and usually loses absorptivity. It could be postulated then that porphyrexide is producing a superoxidized HiPiP-type cluster, which through a heme–cluster interaction shifts the entire spectrum to the blue. However, a cluster-based oxidation yields no explanation for the highly bleached and nearly featureless heme absorption in the region of the α band and the increased absorptivity at long wavelengths that are characteristic spectral properties of π cation radicals.

It should also be clear that given the present data, there is little that can be said with certainty about the midpoint potentials of oxidation in free and sulfite-bound SiRHP, except that they are more negative than 720 mV vs NHE. This is due to the fact that oxidants[9] appear to be able to react with the protein in a nonspecific manner. This is especially so in the case of the SiRHP cyanide and nitrite species.

Porphyrexide reacts much differently with these two forms of the enzyme when compared to the free and sulfite-complexed species, and these differences can be summarized as follows: (i) a failure to bleach the α band of the parent form, (ii) a significantly lower level of free porphyrexide at comparable heme/oxidant ratios, (iii) a high level of unliganded SiRHP in the nitrite adduct when porphyrexide is in 3-fold excess, and (iv) a failure to regenerate the amount of heme present in the starting material after addition of ascorbate. This set of findings indicates that porphyrexide is interacting with an oxidizable site(s) on the protein in addition to the

[7] Some $S = 2$ systems like the cytochrome a_3–Cu a_3 couple of cytochrome c oxidase are EPR-detectable (Hagen, 1982), but the absence of an EPR signal in no way eliminates this spin state from consideration here.

[8] S. Han, L. J. Young, T. G. Spiro, and L. M. Siegel, unpublished results.

[9] The reaction of ferricyanide with the SiRHP species examined here can be characterized as being much less complete than the reaction with porphyrexide at comparable oxidant/heme ratios, and showing a variable degree of reversibility. The unliganded and cyanide-complexed forms of the enzyme demonstrate only these responses to ferricyanide. Addition of 1–3 equiv of ferricyanide to the nitrite adduct appears to accelerate the rate at which nitrite dissociates from the enzyme. Treatment of the sulfite complex with 5 equiv of ferricyanide produced a species with an optical spectrum significantly different from that found with 1.25 equiv of porphyrexide (Figure 3); i.e., the α band was blue-shifted by 2 nm to 581 nm but diminished in intensity by only 16%, while the Soret band was unchanged in position but showed an increase of absorptivity of 1%. A pair of isosbestic points at 520 and 628 nm were lost at 1.0–1.5 equiv of ferricyanide/heme. The EPR spectrum of the resting sulfite adduct is affected by ferricyanide in a manner not greatly dissimilar from that found when porphyrexide is the oxidant; e.g., at 1 equiv of ferricyanide/heme only about 25% of the original sulfite EPR signal is present, but without free ferricyanide.

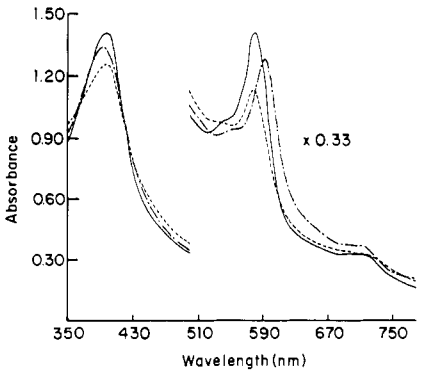

FIGURE 5: Optical spectrum of 37.5 μM SiRHP·NO_2^- before (solid) and after (dash) addition of 3 equiv of porphyrexide at 5 °C. The spectrum of the superoxidized species was recorded 23 min after the start of the titration experiment and has not been corrected for a 4% dilution factor. The final trace (dash–dot) was taken 10 min after the addition of excess ascorbate.

siroheme and could also be combining with free cyanide and nitrite. These phenomena could arise from a change in the E_m for ring oxidation and/or a different protein conformation that either exposes an alternate site(s) or changes the accessibility of the ring to the oxidant.

Whether or not porphyrexide will react with free ligand or with the protein will depend on the relative values of the rate constants for these processes. Results of kinetic studies on the reaction of porphyrexide with the ligands show the ratio of rate constants to be 10^6(sulfite):10^3(cyanide):1(nitrite). On the basis of the experimental protocol used to prepare individual samples, the amounts of free sulfite and nitrite should be comparable and almost an order of magnitude greater than the amount of free cyanide. Yet, the quantity of free porphyrexide is largest with the sulfite form, while the nitrite and cyanide species show roughly equivalent amounts, but up to an order of magnitude lower than with sulfite. Such data cannot be explained solely by reaction of porphyrexide with free ligand and suggest that, in addition to producing spectrally detectable, superoxidized species, oxidants can react with the protein in a nonspecific manner, the extent of which depends on both the oxidant and the particular SiRHP species that is being oxidized.

If our interpretation that we are dealing with Fe(III) siroheme π cation radicals is correct, then the superoxidized species of unligated and sulfite-bound SiRHP provide a unique opportunity to study the effects of axial ligation and spin state of the iron on the magnitude (and hence the mechanism) of the exchange coupling between itself and an oxidized macrocycle. To date the two proteins studied most intensively with respect to such questions are the compound I forms of HRP (Schulz et al., 1984) and *Chaldariomyces fumago* CPO (Rutter et al., 1984) with $\mathbf{J} \sim 1.02$ D ($D/k = 52$ K) in the former and $\mathbf{J} \leq 0.1$ D ($D/k = 35$ K) in the latter ($\mathbf{J}$ is a tensor describing the spin–spin interaction, the isotropic term of which usually predominates). It is not known how the metal and radical are coupled given the lack of overlap of the porphyrin orbitals with the metal orbitals that harbor the unpaired electrons. Schulz et al. (1984) suggested that, for HRP compound I, admixture of the iron $4p_z$ orbital into the axial bond or admixture of some t_{2g} character into the radical orbital could facilitate the coupling but assumed that the porphyrin MO was of a_{2u} symmetry. More recent studies by Morishima et al. (1986) suggest that in fact both a_{1u} and a_{2u} porphyrin MOs are involved. Whatever the case may be, this overlap could occur if the symmetry of the ring was reduced from D_{4h} via an S_4 ruffle (Scholz et al., 1982). Since the isobacteriochlorin macrocycle is inherently ruffled (Suh et al., 1984), it is possible that $\mathbf{J}$ will be large in the low-spin sulfite complex. It will also be of interest to see what effect the change in axial ligation and spin state will have on the magnitude of $\mathbf{J}$ on going from the sulfite-bound to the free enzyme (assuming that formation of a π cation does not alter the spin state of the iron).

Insofar as catalysis is concerned, the significance of our findings relates primarily to a demonstration that the resting ferric protein, as well as some of its ligand complexes, can be oxidized and that, in the case of the free and sulfite-bound moieties, the isobacteriochlorin ring appears to be the site of oxidation. At present we do not have any evidence that the π cation forms of free and sulfite-bound SiRHP have any role in the catalytic reduction of sulfite to sulfide. However, it may be that the ease of oxidation of the ring and the close proximity of the 4Fe–4S cluster to the siroheme will facilitate a mechanistic process in which there can be a very rapid transfer of up to three reducing equivalents to certain key bound intermediates. To put it another way, the facile reduction of bound substrate will circumvent the need to stabilize thermodynamically unstable intermediates such as sulfoxylate (or sulfur monoxide) during the multielectron reduction process. These ideas can be readily extended to the catalytic cycle for the more thermodynamically favorable reduction of nitrite to ammonia, where the reduction of the stable intermediate NO to the stable intermediate NH_2OH is a three-electron reduction.

Registry No. Siroheme, 52553-42-1; porphyrexide, 15622-62-5; sulfite reductase, 37256-51-2.

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Polypeptide Domains of ADP-Ribosyltransferase Obtained by Digestion with Plasmin†

Kalman G. Buki‡ and Ernest Kun*,§
Department of Pharmacology and the Cardiovascular Research Institute, School of Medicine, University of California, San Francisco, San Francisco, California 94143-0130

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ABSTRACT: Proteolysis by plasmin inactivates bovine ADP-ribosyltransferase; therefore, enzymatic activity depends exclusively on the intact enzyme molecule. The transferase was hydrolyzed by plasmin to four major polypeptides, which were characterized by affinity chromatography and N-terminal sequencing. Based on the cDNA sequence for human ADP-ribosyltransferase enzyme [Uchida, K., Morita, T., Sato, T., Ogura, T., Yamashita, R., Noguchi, S., Suzuki, H., Nyunoya, H., Miwa, M., & Sugimura, T. (1987) *Biochem. Biophys. Res. Commun. 148*, 617–622], a polypeptide map of the bovine enzyme was constructed by superposing the experimentally determined N-terminal sequences of the isolated polypeptides on the human sequence deduced from its cDNA. Two polypeptides, the N-terminal peptide (M_r 29 000) and the polypeptide adjacent to it (M_r 36 000), exhibited binding affinities toward DNA, whereas the C-terminal peptide (M_r 56 000), which accounts for the rest of the transferase protein, bound to the benzamide–Sepharose affinity matrix, indicating that it contains the NAD^+-binding site. The fourth polypeptide (M_r 42 000) represents the C-terminal end of the larger C-terminal fragment (M_r 56 000) and was formed by a single enzymatic cut by plasmin of the polypeptide of M_r 56 000. The polypeptide of M_r 42 000 still retained the NAD^+-binding site. The plasmin-catalyzed cleavage of the polypeptide of M_r 56 000–42 000 was greatly accelerated by the specific ligand NAD^+. Out of a total of 96 amino acid residues sequenced here, there were only 6 conservative replacements between human and bovine ADP-ribosyltransferase.

ADP-ribosyltransferase [ADPRT,[1] poly(ADP-ribose) polymerase, EC 2.4.2.30] is a DNA-associated nuclear enzyme that synthesizes protein-bound homopolymers of ADP-ribose exhibiting helical conformation (Minaga & Kun 1983a,b) utilizing NAD^+ as a substrate. The enzyme itself is the primary acceptor protein for ADP-ribose (Bauer et al., 1986). The purified enzyme requires double-stranded DNA for activity and consists of a single large polypeptide chain of M_r

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* Correspondence should be addressed to this author.
‡ Visiting Scientist from the Semmelweis University School of Medicine, Budapest, Hungary.
§ Recipient of the Research Career Award of the U.S. Public Health Service.

[1] Abbreviations: ADPRT, adenosine diphosphoribosyltransferase, poly(ADP-ribose) polymerase (EC 2.4.2.30); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; PTH-, phenylthiohydantoinyl-; BSA, bovine serum albumin; bp, base pairs, HEPES, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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