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On the Reaction of Ferric Heme Proteins with Nitrite and Sulfite†

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ABSTRACT: Optical and EPR spectroscopy of ferric heme proteins of the porphyrin, oxyporphyrin, and isobacteriochlorin classes has indicated that nitrite reacts with these proteins at the heme iron. Sulfite has been conclusively proven to react only with proteins containing the isobacteriochlorin macrocycle. Quantitative EPR spectroscopy of these nitrite and sulfite adducts showed that most contained a substantial quantity of undetectable heme. It is suggested that protein-induced autoreduction of nitrite (but not sulfite) and a strained and/or uniaxial *g*-tensor are the principal ways by which the silent state is produced.

The iron isobacteriochlorin siroheme (Scott et al., 1978) is the prosthetic group for many of the enzymes involved with the reduction of NO_2^- to NH_3 and of SO_3^{2-} to H_2S. Insofar as the binding of the substrate is concerned, this cofactor requirement appears to be much more stringent for sulfite than for nitrite; i.e., with the possible exception of the cytochrome d_1 of a pair of bacterial dissimilatory nitrite reductases (Sawhney & Nicholas, 1978; Kuronen & Ellfolk, 1972) sulfite has been proven to form a stable ferric complex only with proteins containing the isobacteriochlorin macrocycle. In contrast, oxidized forms of sperm whale myoglobin (Smith & Williams, 1969), human hemoglobin A (Gibson et al., 1969), *Pseudomonas putida* cytochrome P-450 (Sono & Dawson, 1982), *Chaldariomyces fumago* chloroperoxidase (Sono et al., 1986), *Pseudomonas aeruginosa* cytochrome cd_1 (Muhoberac & Wharton, 1983), and *Escherichia coli* cytochrome c_{552}

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(Kajie & Anraku, 1986) are all known to react with nitrite.

The purpose of this investigation was to determine the role of the macrocycle and the protein in the ligation of sulfite and nitrite and then to characterize the products by using optical and EPR spectroscopies. We confirm earlier findings with respect to nitrite-induced optical changes for sperm whale myoglobin, *C. fumago* chloroperoxidase, and *Spinacia oleracea* NiR[1] (Vega & Kamin, 1977) and present new optical and EPR data for bovine liver catalase, horseradish peroxidase, *Thiobacillus denitrificans* cytochrome cd_1, equine heart cytochrome *c*, and *E. coli* sulfite reductase heme protein. NO_2^- ligation to the peroxide-binding enzymes (but not to myoglobin) is markedly sensitive to the pH.

Sulfite shows little or no tendency to bind to any ferriprotoheme protein with the possible exception of horseradish peroxidase at pH 5.7. Sulfite may react with *T. denitrificans* cytochrome cd_1 but only at acidic pH. In confirmation of previous studies sulfite was found to react rapidly with spinach nitrite reductase and slowly with SiRHP (Rueger & Siegel, 1973).

The EPR spectra of many of the proteins studied here in the presence of nitrite show significant amounts of silent heme. Taken together, the optical and EPR data suggest that autoreduction of nitrite cannot be the sole explanation for EPR silence. We postulate that a strained and/or uniaxial *g*-tensor is responsible for most of the undetectable heme. The sulfite adduct of SiRHP also contains a large proportion of EPR-undetectable heme, and at present the perturbed *g*-tensor hypothesis appears to be the only plausible way to account for the silence.

The data also suggest that with sulfite, but not nitrite, the type of pyrrole/pyrroline macrocycle is of importance with respect to both ligation and turnover. With both anions the protein plays an essential role in determining the "active site" stereochemistry of the bound ligand and whether or not turnover occurs subsequent to ligation.

Materials and Methods

NADPH–sulfite oxidoreductase holoenzyme and its heme protein subunit were isolated from *E. coli* B by using a minor modification[2] of the published procedure (Siegel et al., 1973; Siegel & Davis, 1974). Bacteria were grown on minimal media by Grain Processing Corp., 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 Na EDTA, pH 7.7 (standard buffer). The nitrite reductase from *S. oleracea* was prepared from fresh spinach leaves, as previously described (Lancaster et al., 1982). *T. denitrificans* was grown according to a standard method (Baldensperger & Garcia, 1975) and its cytochrome cd_1 isolated (Huynh et al., 1982). The cytochrome P-594 from the same strain was also isolated (Aminuddin & Nicholas, 1973) and was the kind gift of Dr. R. Wahl. *Desulfotomaculatum nigrificans* was grown and its cytochrome P-582 prepared according to the procedures of Seki et al.[3]

Sperm whale myoglobin (type II), horse heart cytochrome *c* (type VI), horseradish peroxidase (type VI), horseradish peroxidase acidic and basic isoenzymes, *C. fumago* chloroperoxidase, and bovine liver catalase were purchased from Sigma Chemical Co. The activities and spectral properties of these commercial protein preparations were comparable to those found for freshly isolated material and showed no significant variability from lot to lot. All chemicals were of reagent or analytical grade and unless noted otherwise were not purified further.

Optical quantitations of the proteins used in this study were based on the following extinction coefficients for the oxidized form (expressed as mM^{-1} cm^{-1} at the specified wavelength): SiRHP, 18.1 at 590 nm (Siegel et al., 1982); NiR, 76 at 386 nm (Vega & Kamin, 1977); cytochrome cd_1, 312 at 407 nm (Huynh et al., 1983); Mb, 3.3 at 632 nm (Smith & Williams, 1968); HRP, 11.3 at 497 nm (Ohlsson & Paul, 1976); catalase, 8.1 at 622 nm (Kirkman et al., 1987); CPO, 13.7 at 515 nm (Sono et al., 1985); and cytochrome *c*, 8.4 at 550 nm (Margalit & Schejter, 1970). In the case of catalase, the extinction coefficient is expressed in units per heme, while with cytochrome cd_1 the units are per molecule (2 heme *d* per molecule).

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. Samples were typically run at 10 K with temperature control provided by an Air Products Heli-Tran refrigeration unit. Optical spectra were recorded on either an Aminco DW-2 UV–vis spectrophotometer or a Perkin-Elmer Lambda 9 UV–vis–NIR spectrophotometer.

The optical and EPR experiments reported here were carried out in air-saturated buffer with incubations either at ambient room temperature (21–25 °C) or in the sample compartments of the optical spectrophotometers that if not thermostated gave equilibrium temperatures of 26.5 (PE) and 30.5 °C (Aminco). No matter what the length of the experiment, controls (without added nitrite or sulfite) were always done, and in no instance were significant differences found between the optical spectrum at the start and end of a particular run. Many of the experiments were done "in tandem", which means that the optical and EPR samples were taken from the same reaction vessel. This methodology is to be contrasted with "in parallel" and "in sequence", which involve two reaction vessels at the same time or at different times, respectively.

Many of the experiments used 0.1 M potassium phosphate plus 0.1 mM EDTA, pH 7.7, as the supporting buffer so as to allow comparison to SiRHP and NiR with respect to ionic strength and the protonation state of the sulfite. If a different buffer was used, then this has been noted. Optical and EPR experiments were done in triplicate and duplicate, respectively, except for those with *T. denitrificans* cytochrome cd_1, which, due to limited amounts of the enzyme, were done twice and once, respectively. The results of different runs were identical within the limits of experimental error (optical ±1%, EPR ±15%).

The method used to quantitate the nitrite-induced low-spin species involved first the determination of the heme protein concentration by optical spectroscopy prior to addition of nitrite. EPR spectra were then obtained on this same sample (before and after addition of nitrite) and the various signals quantitated (Aasa & Vanngard, 1975; DeVries & Albracht, 1979) by using a solution of CuEDTA (2.40 mM $CuSO_4 \cdot 5H_2O$ in Bicine–EDTA, pH 9.4) as a reference. A number

[1] Abbreviations: SiRHP, the heme protein subunit of the NADPH–sulfite oxidoreductase from *E. coli*; iBC, isobacteriochlorin; CT, charge transfer; NiR, nitrite reductase; Mb, myoglobin; HRP, horseradish peroxidase; CPO, chloroperoxidase; Hb, hemoglobin; TPP, tetraphenylporphyrin; OEP, octaethylporphyrin; HP(II) or HP(III), ferrous or ferric heme protein; HALS, highly anisotropic low spin; EDTA, ethylenediaminetetraacetic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; SB, standard buffer.

[2] L. J. Young and L. M. Siegel, *Biochemistry* (submitted for publication).

[3] Y. Seki and L. M. Siegel, unpublished results.

Table I: Optical Absorption Data of the Resting, Nitrite, and Sulfite Forms of Ferric Heme Proteins

species	absorption maxima [λ (e_{mM})]			
	Soret	β	α[a]	CT
Mb	409 (117)		502 (7.8)	632 (3.3)
Mb·NO_2^-	411	535 (5.9)	570 (4.2)	627 (3.5)
HRP	402 (102)		497 (11.3)	640 (3.3)
HRP·NO_2^-	416	532 (11.1)	563 (9.7)	
cat	407 (93)	500 (12.2)	536 (9.8)	618 (8.1)
cat·NO_2^-	408	536 (12.7)	575 (11.8)	618 (8.6)
CPO	399 (91)	514 (13.4)	540 (12.1)	650 (5.2)
CPO·NO_2^-	426	544 (12.3)	577 (9.6)	645 (2.6)
cytcd_1[b]	407 (312)		641 (49.0)	
cytcd_1·NO_2^-	407		627 (47.2)	
cytcd_1·SO_3^{2-}	415 (298)		632 (41.1)	
cyt$c$	408 (104)		530 (11.7)	
cyt$c$·NO_2^-	409		530[c] (11.8)	
NiR	386 (76)		573 (14.7)	691 (4.8)
NiR·NO_2^-	396	528 (14.5)	567 (16.6)	
NiR·SO_3^{2-}	404 (72)		575 (16.9)	
SiRHP	387 (65.6)	545 (12.0)	590 (18.1)	714 (6.0)
SiRHP·NO_2^-	398	545 (14.3)	579 (19.0)	
SiRHP·SO_3^{2-}	408 (57.1)	545 (14.8)	583 (19.3)	

[a] This denotes the wavelength of maximum intensity in the 500–700-nm range. [b] Only the data for the cytochrome d_1 component in the 500–700-nm range are listed in this table. [c] There is a shoulder at 560 nm in the spectrum, with an extinction coefficient of 7.8 mM^{-1} cm^{-1}.

Table II: EPR Parameters for Ferric Heme Protein Nitrite Complexes

sample	pH	g_z	g_y	g_x[a]	low spin[b]	high spin	silent
Mb	7.7	3.11	2.30	1.00	0.19	0.14	0.44
		2.97	2.18	1.56	0.23		
Mb	6.7	3.13	2.30	0.95	0.11	0.27	0.46
		3.02	2.18	1.47	0.16		
Mb	5.4	3.10	2.18	1.28	0.31	0.31	0.38
HRP	5.4	3.08	2.18	1.33	0.82		0.18
HRP	7.7	2.99	2.24	1.31	0.78		0.22
cat	5.4					0.50	0.50
CPO	5.4	2.60	2.28	1.83	0.33		0.58
		2.54	2.28	1.85	0.09		
cytcd_1	7.7	3.02			0.26	0.02	0.72
cyt$c$	5.4	3.14			0.08	0.01	0.80
		2.94	2.12	1.69	0.11		
SiRHP	7.7	2.93			0.10	0.20	0.60
		2.84			0.10		
NiR[c]	7.7						1.00

[a] All g_x values except those for CPO and HRP, pH 7.7, have been calculated from the assumption that the sum of the squares of the g values is 16.018. [b] Quantitation of the amount of low-spin, high-spin, and silent forms is based on the assumption that their sum is 1 spin/heme. [c] There may be from 0 to 0.1 spin/heme of an unreactive low-spin species present in both control and nitrite-treated enzyme.

of standards have validated this quantitation procedure: ferric SiRHP (a rhombic high spin), 0.98 spin/heme; ferric SiRHP–CN^- (a rhombic low spin) 0.98 spin/heme; ferric Mb, pH 7.7 (an axial high spin), 0.94 spin/heme; ferric cytochrome c, pH 5.4 (a rhombic low spin), 0.94 spin/heme; and ferric CPO, pH 5.4 (a rhombic low spin), 0.90 spin/heme.

Results

Most of the optical and EPR data have been presented in summary form in Tables I and II. The Soret band was not included in the first four figures due to the high concentration of the protein sample and/or the presence of nitrite (which shows a significant absorption in the 350–400-nm range). Figures 1–4 were selected to illustrate the effects of nitrite on the two types of protoporphyrin proteins (O_2 binding and H_2O_2 binding) and those of the oxyporphyrin and isobacteriochlorin classes.

Optical Spectroscopy of Nitrite Complexes. (a) Ferric Porphyrin and Oxyporphyrin Metalloproteins. The reaction

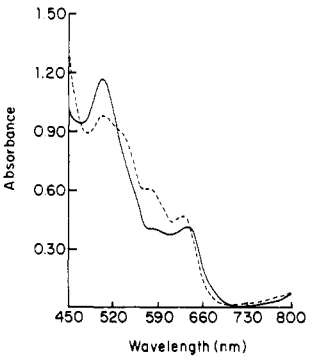

FIGURE 1: Optical spectra of metMb in 0.1 M SB, pH 6.7, 25 °C, before (solid) and 30 min after (dash) addition of sodium nitrite to 100 mM. The protein concentration is 143 μM in heme.

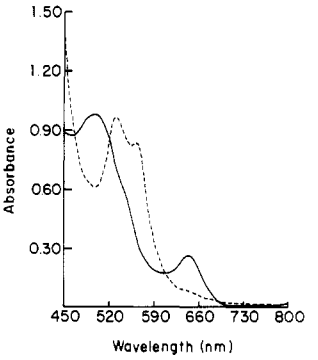

FIGURE 2: Optical spectra of metHRP in 0.1 M SB, pH 5.4, 25 °C, before (solid) and 1 min after (dash) addition of sodium nitrite to 100 mM. The protein concentration is 87 μM in heme.

of sperm whale myoglobin with 100 mM nitrite is more than 99% complete in less than 1 min and is insensitive to pH in the range from 5.4 to 7.7. The spectral changes (Figure 1, Table I) are like those seen previously (Smith & Williams, 1968; Beetlestone & George, 1964) and suggest formation of new low- and high-spin forms. The small feature at 585 nm in the control spectrum is removed by treatment with ferricyanide and is logically attributed to a small amount of oxymyoglobin (Antonini & Brunori, 1971).

Ligation of NO_2^- to horseradish peroxidase is very sensitive to pH. The optical spectrum of the oxidized enzyme showed no change after 13 h at 30 °C in 20 mM $NaNO_2$, pH 7.7, and only a 3% loss of CT intensity after 16 h at 25 °C in 100 mM NO_2^-. However, at pH 5.4 with NO_2^- at 100 mM, there is a striking change in the optical spectrum that is more than 95% complete in 1 min (Figure 2, Table I) and suggests formation of only low-spin species.

This early form is not stable and with time changes to what appears to be a mixture of the ferric NO species (Yonetani et al., 1972) and a verdoheme-like compound, the latter having an essentially featureless spectrum between 500 and 700 nm, with a short-wavelength maximum at 465 nm. The reaction that produces these two species appears to be polyphasic, with a rate that increases with a lowering of pH and/or an increase in the nitrite concentration.

Nitrite binding was incomplete in the pH range from 7.7 to 6.5 but did give a single product as indicated by a set of isosbestic points. However, from pH 6.5 to 5.4, a drift of the isosbestic points was seen along with a loss of peak to trough intensity at the 419–400-nm wavelength pair. This loss of isosbesticity was followed at pH 5.4 with conditions as in Figure 2, and a $t_{1/2}$ of 1.3 h was measured.

The spectral perturbations seen when nitrite is added to ferric bovine liver catalase are a mixture of those found with

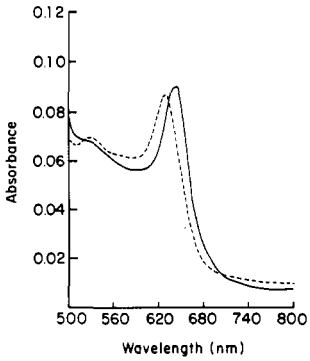

FIGURE 3: Optical spectra of metcd_1 in 0.1 M sodium citrate, pH 5.7, 30 °C, before (solid) and 21.6 h after (dash) addition of sodium nitrite to 20 mM with a heme d_1 concentration of 1.8 μM.

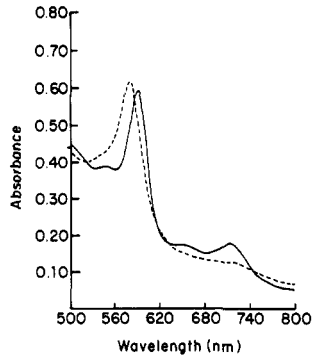

FIGURE 4: Optical spectra of metSiRHP in 0.1 M SB, pH 7.7, 30 °C, before (solid) and 23.5 h after (dash) addition of sodium nitrite to 100 mM. The protein concentration was 33 μM in heme.

Mb and HRP (Table I). The response to changes of the pH mimics that of HRP, e.g., no reaction at pH 7.7 after 13 h in 20 mM NO_2^- and immediate (complete within 1 min) reaction at pH 5.6 in 100 mM NO_2^-, with formation of new low- and high-spin species.[4]

The spectral changes and pH dependency of the reaction of nitrite with CPO are comparable to those of catalase, e.g., rapid reaction at pH 5.4 to give a mixture of low- and high-spin forms (Table I). The nitrite reaction at alkaline pH is complicated by the fact that in the absence of nitrite the enzyme undergoes a spin-state transition (from high spin to low spin), which occurs with a half-time of about 0.5 h at pH 7.3. Addition of 125 mM nitrite to the low-spin forth, followed by incubation for 30 min at 25 °C, pH 7.5, produces a small absorbance change in the 500–700-nm region, diminishes the Soret intensity by 30%, and increases the δ-band absorption by 25%. After a further 16 h, these perturbations are slightly accentuated. We have also observed that unlike HRP the pH 5.4 nitrite form of CPO is quite stable, with only a 3% change of the α/β ratio seen after a 16-h incubation at 25 °C.

Cytochrome cd_1 isolated from *T. denitrificans* appears to react with nitrite at both pH 5.7 (Figure 3, Table I) and pH 7.7. As yet we cannot say whether the difference in position of the α band of the nitrite adduct at pH 5.7 (627 nm) compared to that at pH 7.7 (636 nm) reflects an effect of pH, the extent of NO_2^- binding, or both. It does seem safe to say that nitrite reacts more quickly with the d_1 heme at lower pH ($t_{1/2}$ of minutes as opposed to hours) and that at neither pH is there any optical evidence for an effect of nitrite on the cytochrome *c* component.

Horse heart cytochrome *c* shows no reaction with 100 mM NO_2^- at either pH 7.7 or pH 13, but at pH 5.4 small reproducible changes are seen (Table I) that are complete within 1 min of addition of nitrite. The spectral perturbations are exquisitely sensitive to the constitution of the buffer in that if instead of using 0.1 M citrate plus 0.1 mM EDTA, pH 5.4 one uses only 0.1 M citrate, the rate of the reaction is markedly slowed.

(*b*) *Ferric Isobacteriochlorin Metalloproteins.* In agreement with previous findings (Vega & Kamin, 1977), spinach NiR was observed to react quickly (more than 99% complete after 1 min) with 20 mM NO_2^- in standard buffer at 25 °C (Table I). On the other hand, cytochromes P-594 (from *T. denitrificans*) and P-582 (from *D. nigrificans*) showed no evidence of nitrite ligation after incubation at 25 °C for 16 h in the presence of 20 mM $NaNO_2$. These proteins are thought to have a role in sulfite metabolism, the former possibly acting as a sulfide oxidase (Aminuddin & Nicholas, 1973), the latter as a respiratory sulfite reductase (Trudinger, 1970).

As is the case for ligands such as CN^- and SO_3^{2-}, the reaction of ferric SiRHP with NO_2^- at pH 7.7 is very slow. Nonetheless, after 24 h at 25 °C, there is definite evidence of a nitrite effect on the optical spectrum of the resting enzyme (Figure 4, Table I). The binding kinetics (obtained from an experiment using 65 μM enzyme plus 100 mM nitrite at 25 °C) suggest that at least three phases are present with rate constants and amplitudes of 2×10^{-2} min^{-1} (5%), 1.8×10^{-3} min^{-1} (70%), and $<10^{-4}$ min^{-1} (25%). It is noteworthy that the amount (about 20%) of residual uncomplexed enzyme present after a 16–24-h incubation is only slightly diminished after a further 24 h.

Optical Spectroscopy of Sulfite Complexes. Compared to the spectral changes seen with NO_2^-, those observed for SO_3^{2-} (in all cases present at 20 mM in 0.1 M standard buffer with a temperature of 25–30 °C) are rather unremarkable.[5] Mb and HRP show no significant modification of the shape of the optical spectrum at pH 5.7 or 7.7 after a 12-h incubation at 30 °C, with only a small (1%) increase of the Soret band intensity for myoglobin at both pHs and peroxidase at pH 7.7. The pH 5.7 peroxidase sample undergoes a 10% loss in absorption of the Soret, 498-nm, and 640-nm bands.

At pH 7.7 oxidized bovine liver catalase showed no evidence of a sulfite effect after a 20-h incubation at 30 °C, while at pH 5.7 there was a 2-nm blue shift of the 622-nm ferric CT band plus a 3% increase of its intensity and that of the Soret band.

The reaction of chloroperoxidase with sulfite at pH 5.4 is characterized (after a 16-h incubation at 25 °C) by a generalized loss of sample intensity (10% at any wavelength), while at pH 7.7 the spectral perturbations are almost identical with those induced by nitrite. As noted by others (McCord & Fridovich, 1969), it has been observed here that sulfite will slowly reduce horse heart cytochrome *c*.

T. denitrificans cytochrome cd_1 is thought to be able to form a complex with sulfite (Huynh et al., 1982), but we found only very small changes in the Soret and α bands after a 12.5-h

[4] All experiments with catalase were done with enzyme that had been exhaustively dialyzed against standard buffer and then centrifuged to remove insoluble material. There is some variability with respect to the stability of this commercial preparation in that some batches of the enzyme can become turbid in acidified buffer if left at room temperature for more than a few minutes. By chilling the sample on ice and running the optical spectra at 5 °C, the problem can be eliminated.

[5] No evidence was found for a reaction of sulfite with the ferrous forms of sperm whale myoglobin, horseradish peroxidase, or *C. fumago* chloroperoxidase after 30 min in pH 7.7 standard buffer at 25 °C. Due to limited supplies of the enzyme, the reaction of sulfite with ferrous cytochrome cd_1 from *T. denitrificans* was not examined. In agreement with previous studies sulfite was found to react rapidly with the two-electron-reduced forms of spinach NiR and *E. coli* SiRHP.

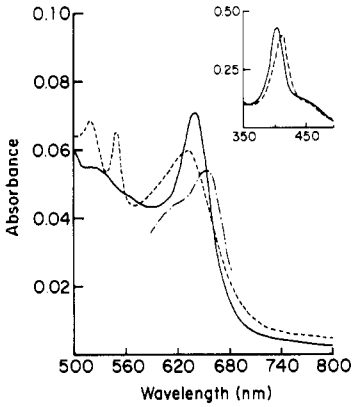

FIGURE 5: Optical spectra of 1.4 μM metcd_1 before (solid) and 19.8 h after (dash) addition of sodium bisulfite to 20 mM. The sample was incubated at 30 °C in 0.1 M sodium citrate, pH 5.7. The insert shows the Soret bands, while the other spectrum (dash-dot) is that of the fully reduced but unligated enzyme with only the d_1 component pictured.

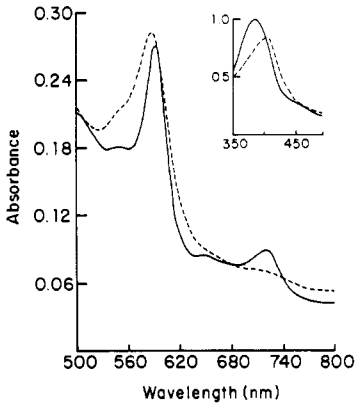

FIGURE 6: Optical spectra of 14.9 μM metSiRHP in 0.1 M SB, pH 7.7, before (solid) and 19.8 h after (dash) addition of sodium bisulfite to 20 mM at 25 °C. The insert shows the Soret bands.

incubation in 20 mM $NaHSO_3$ at pH 7.7. The experiment was repeated at pH 5.7 (Figure 5, Table I) and showed complete reduction of the *c*-type hemes plus a change in the shape, intensity, and band maximum for cytochrome d_1. The half-time for reduction of cytochrome *c* was about 1.5 h, measured by using the 407–420-nm wavelength pair.

The oxidized forms of cytochromes P-582 and P-594 do not appear to react with sulfite following incubation for several hours at room temperature in neutral pH buffer with the anion in large excess.[6,7]

It has previously been observed (Vega & Kamin, 1977) and is confirmed here that at pH 7.7 spinach NiR rapidly (more than 99% complete in less than 1 min) reacts with 20 mM sulfite (Table I). Sulfite has been shown (Rueger & Siegel, 1976) to slowly combine with SiRHP at pH 7.7, and again this is verified here (Figure 6, Table I). The binding reaction is polyphasic with a rapid phase (half-time of 1.8 h) accounting for 20% of the intensity change at the 380–421-nm wavelength pair and two slower phases responsible for the rest; e.g., even after 4 days about 5% of the resting intensity of the 714-nm band remains, while the remaining 75% can be attributed to a reaction with a half-time of about 3.8 h.

EPR Spectroscopy of Nitrite Complexes. (a) Ferric Porphyrin and Oxyporphyrin Metalloproteins. Nitrite significantly reduced the intensity of the axial high-spin signal of

[6] Y. Seki, S. Seki, and L. M. Siegel, unpublished results.
[7] R. Wahl and L. M. Siegel, unpublished results.

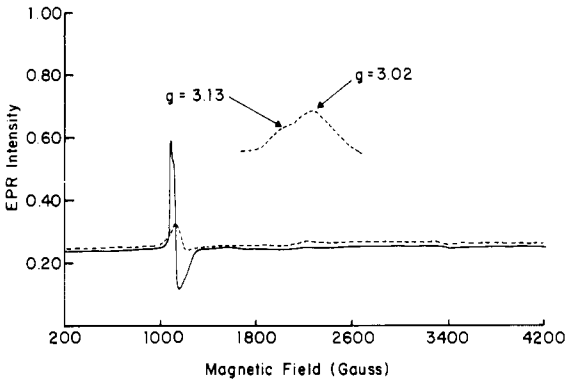

FIGURE 7: EPR spectra of the same samples used in Figure 1. The full-scale spectra are run at 20-mW power and a gain of 6.3×10^3, while the expanded spectrum of the nitrite adduct was taken at 2600-G center field, 1600-G sweep width, 10-mW power, and 5×10^5 gain.

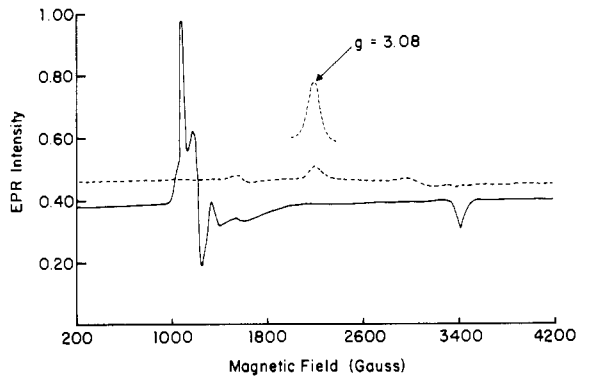

FIGURE 8: EPR spectra of the same samples used in Figure 2. All spectra are full scale at a power of 10 mW and a gain of 10^5 in the nonexpanded run. The expanded run of the nitrite adduct was done at a power of 2.5 mW and a gain of 4×10^5.

Mb at pH 5.4, 6.7, and 7.7 and produced two new low-spin forms (Figure 7, Table II). There appear to be pH-dependent shifts in the distribution of *g* values of the various low-spin species, but of more significance is the fact that in all cases about 40% of the heme is EPR silent.

In contrast to what was seen with Mb, nitrite abolished the high-spin signals of HRP but did produce a new low-spin form (Figure 8, Table II) that was distinct from a pair of species at $g = 3.42$ and 3.18 (in total <10% of a heme) in the pH 5.4 control spectrum. After a 16-h incubation at 25 °C, the nitrite adduct showed a significant loss (70–80%) of the $g = 3.08$ intensity, concomitant with the optical changes mentioned earlier. Of further interest is the finding that if instead of adding NO_2^- to a preacidified sample one brings a pH 7.7 solution of enzyme (containing 100 mM NO_2^-) to pH 4.4 (with 1 M H_3PO_4), the intensity of the nitrite-induced low-spin species is only 0.34 spin and a "matrix" NO EPR signal is found that absorbs at $g = 1.97$.

As mentioned earlier, the optical spectra of horseradish peroxidase plus nitrite at pH 7.7 show little or no difference from those of the oxidized controls after a 13-h incubation at 30 °C. However, the EPR spectrum of the 100 mM nitrite sample has a low-spin signal with slightly different *g* values (Table II) but which quantitates to about the same number of spins per heme (a 20 mM nitrite sample gives only 0.52 spin/heme). Similar results were found at pH 7.7 with 100 mM nitrite and the purified acidic or basic isoenzymes.

At pH 5.6 in standard buffer, oxidized bovine liver catalase showed one major and two minor high-spin conformers, as observed previously (Peisach et al., 1971). Addition of NO_2^- to 100 mM abolished a $g = 7.97$ species and diminished the

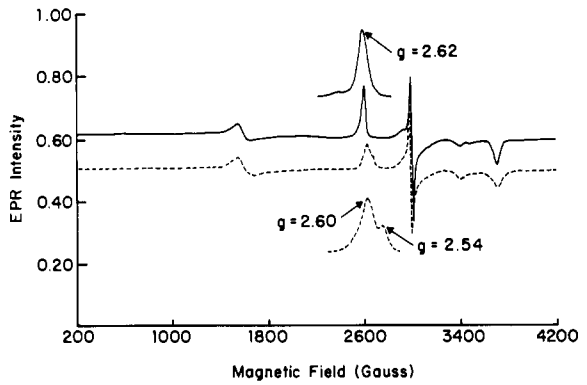

FIGURE 9: EPR spectra of metCPO in 0.1 M SB, pH 5.4, before (solid) and 16 h after (dash) addition of sodium nitrite to 100 mM. The sample was 66 μM in heme and incubated at 25 °C prior to taking the nitrite specimen. All spectra were recorded at 2.5-mW power and gains of 1.25×10^5 and 1.6×10^5 for the controls and 1.6×10^5 and 3.2×10^5 for the nitrite species. The expanded spectra were run at a center field of 3200 G and a sweep width of 1600 G.

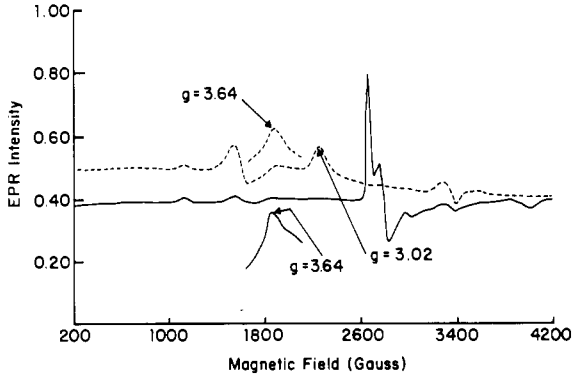

FIGURE 10: EPR spectra of metcd_1 before (solid) and 21.5 h after (dash) addition of sodium nitrite to 90 mM. The sample was incubated at 25 °C with addition of nitrite causing a 9% dilution of the 98 μM (in heme d_1) starting material. The full-scale spectra were run at 2.5 mW with gains of 2×10^5 and 5×10^5 for the control and nitrite samples, respectively. The expanded spectra were at 100-mW power and gains of 4×10^5 and 5×10^5, respectively.

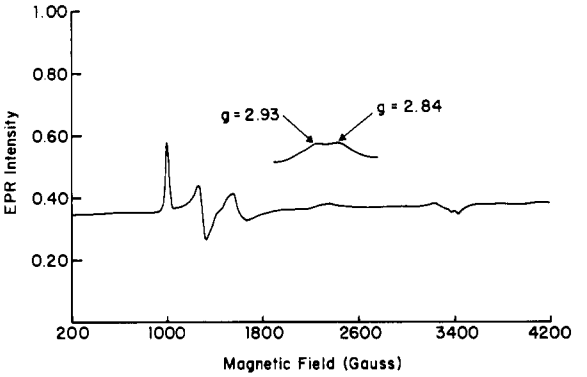

FIGURE 11: EPR spectra of metSiRHP 15 h after addition of sodium nitrite to 100 mM. The 60 μM sample was incubated at 25 °C prior to taking the EPR specimen. The full-scale spectrum was taken at 2.5-mW power and 2.5×10^5 gain, while the expanded spectrum was at 10-mW power, 5×10^5 gain, a center field of 2600 G, and a sweep width of 1600 G.

g_x amplitude of the resting $g = 6.89$ (now at $g = 6.74$) and $g = 8.57$ forms by 50%. There was no EPR evidence of a new low-spin adduct. If one asumes no change in the transition factor or the axial zero field splitting parameter (D) for the major form, it appears that 50% of the heme is unaccounted for (Table II).

At pH 7.7 there is no effect of nitrite on the integrated intensity of the various high-spin species; however, it does affect their distributions. As yet we do not know whether this is specific to nitrite or if it can be mimicked by other anions.

The g values for the major (in this sample a minor species was present at $g = 2.71$, 2.30, and 1.94) low-spin form of ferric CPO at pH 5.4 (Figure 9) were as noted by others (Rutter et al., 1984) and amounted to 0.81 and 0.09 spin/heme, respectively. Addition of 100 mM nitrite plus a 16-h incubation produced two new low spins and left about 60% of the heme unaccounted for (Figure 9, Table II).

Some variability has been seen with respect to the distribution between the major and minor forms of chloroperoxidase and its nitrite complex, and for the moment this is thought to be reflective of the methodology used to isolate the enzyme. However, the sum of the intensities of the various forms was found to be the same within the limit (±15%) of experimental error, e.g., 0.90 vs 0.83 spin/heme for CPO and 0.42 vs 0.48 spin/heme for CPO plus nitrite in two different runs.

The 100 mM nitrite produced two low-spin signals and a substantial amount of EPR-silent heme when added to horse heart cytochrome c dissolved in 0.1 M citrate plus 0.1 mM EDTA, pH 5.4 (Table II). When this sample was rerun after being frozen in liquid N_2 for 5 months, its spectrum was like that of the oxidized protein. Interestingly, a simple change of the buffer to either 0.1 M citrate or 0.1 M phosphate (both at pH 5.4) gave a mixture of the oxidized spectrum plus a broad rhombic signal with $g_z = 2.71$ and $g_y = 2.47$.

The EPR spectra of Figure 10 are from a single sample of *T. denitrificans* cytochrome cd_1 which demonstrated g values and saturation characteristics for the two cytochromes of the resting enzyme that were similar to those previously found (Huynh et al., 1982). Two minutes after nitrite was added to 90 mM only a slight (5%) attenuation and increased axiality of cytochrome d_1 were observed. Further incubation still produced no change in the heme c component, but the heme d_1 signature was completely eliminated and a new low-spin species was found (Figure 10, Table II).

(*b*) *Ferric Isobacteriochlorin Metalloproteins.* The EPR spectrum of resting spinach nitrite reductase was completely abolished 2 min after addition of 20 mM NO_2^- and no new low-spin species were detected (Table II).

In the absence of nitrite, the EPR spectrum of SiRHP shows a rhombic high-spin signal with g values of 6.69–6.72, 5.24, and 1.97–1.98. Nitrite abolished about 80% of the resting high-spin signal, produced two new low-spin species with $g_z = 2.93$ and 2.84 (Figure 11, Table II), and left the residual high-spin signal unchanged. Due to the unusual characteristics of the low-spin signals, quantitation using established methodologies may have a significant (25–50%) error associated with it but still leaves a considerable (up to 60%) amount of EPR-undetectable heme.

EPR Spectroscopy of Sulfite Complexes. As anticipated from the optical data, the EPR spectra of Mb at pH 5.4 and 7.7 or peroxidase at pH 7.7 were the same with or without sulfite. The pH 5.7 HRP sample showed a diminished intensity of all three high-spin forms in the presence of sulfite that amounted to 0.25 spin/heme (the $g \sim 2$ peaks were used as indicators). Except for producing minor changes in the rhombicity of the major high-spin forms, SO_3^{2-} had no effect on the spectrum of catalase at either pH 5.4 or 7.7.

In agreement with earlier reports (Vega & Kamin, 1977) the EPR spectrum of the sulfite adduct of spinach nitrite reductase showed elimination of the high-spin resonance of the resting enzyme and no evidence of a sulfite-induced low-spin species.

The sulfite adduct of SiRHP typically produced an EPR spectrum in which, as with spinach nitrite reductase, there was

Table III: B and Q Band Maxima for NO^+ and NO Complexes of Ferric and Ferrous Heme Proteins

complex[a]	B	Q_α	Q_β	ref
Mb(III)NO^+ (SW)	411	570	535	*b*, this work
Mb(III)NO (SW)		575	537	*c*
Mb(II)NO (SW, EQ)	420	580	548	*c*, this work
HRP(III)NO^+	416	563	532	this work
HRP(III)NO	419	568	533	*d*
HRP(II)NO	421	570	542	*d*
CPO(III)NO^+	426	577	544	*e*, this work
CPO(III)NO	437	577	545	*e*
CPO(II)NO	440	585	560	*f*
cat(III)NO^+ (BL)	408	575	536	this work
cat(III)NO (BL)		576	540	*c*

[a] Abbreviations: SW, sperm whale; EQ, equine; BL, bovine liver. [b] Beetlestone and George (1964). [c] Ehrenberg and Szczepkowski (1960). [d] Yonetani et al. (1972). [e] Sono et al. (1986). [f] Sono et al. (1985).

loss of the high-spin signal of the resting enzyme, but instead of EPR silence an absorption-type EPR signal with g_z at 2.71–2.73 was frequently seen. An approximate estimate for the amount of this species (by the same quantitation procedure as for the nitrite complex) is 0.2 spin/heme.

Discussion

Our data appear to show that nitrite binds to most (8 of 10) of the proteins examined, regardless of the prosthetic group or proximal ligand, and produces both EPR-active and EPR-inactive forms, while sulfite has an affinity for only isobacteriochlorin metalloproteins. However, there are but a few probes which probe directly that binding of a ligand at the active site of a metalloprotein has occurred. When done in tandem, optical and EPR spectroscopy can give compelling indirect evidence for such ligation and in some instances provide information about the chemical nature of the adduct.

The discussion that follows will focus on the evidence from optical and EPR spectroscopy for ligation of nitrite as opposed to nitric oxide when nitrite is added to neutral or slightly acidic heme protein solutions, for the mechanisms whereby EPR silence is produced when nitrite is added to neutral or slightly acidic solutions of heme proteins, for the role of the protein in the production of uniaxial *g*-tensors with and without *g*-strain, and for the role of the protein and the heme macrocycle in the ligation of nitrite and sulfite.

Evidence for Ligation of Nitrite. (*a*) *Optical Spectroscopy.* The room temperature optical spectra of the NO_2^- forms of ferric Mb, HRP, CPO, and catalase do by themselves give important information concerning the oxidation and spin state of a complex. It was first observed by Gouterman (1959) that a decrease of the electronegativity of the metal ion in a homologous series of metalloporphyrins will yield a bathochromic shift of B and Q bands. Thus, the series HP(III)NO^+, HP(III)NO, and HP(II)NO (where for the purposes of this discussion NO^+ is taken to be equivalent to NO_2^-) can be anticipated to show such an effect, and indeed this is what is found (Table III). Furthermore, it has already been observed that the protoporphyrin groups of heme proteins have ferric nitrosonium and ferrous nitrosyl species with an α/β ratio less than unity, while the opposite is true for the ferric nitrosyl form (Ehrenberg & Szczepowski, 1960; Yonetani et al., 1970; Sono et al., 1985). All of these findings support the hypothesis that nitrite (or nitrosonium), not NO, is bound to the heme iron.

The data of Tables III and IV suggest that at pH 5.7 the cytochrome d_1 of the *T. denitrificans* enzyme is ligated to nitrite, not nitric oxide, because the α band is at 627 nm, which is 7 nm to the blue of the cytochrome d_1 α band of the ferric nitrosyl complex of the *P. aeruginosa* protein.

The spectrum of horse heart cytochrome *c* plus 100 mM nitrite at pH 5.4 and 25 °C shows very slight changes when compared to that of the control, which do not resemble those seen in the ferric NO form of the protein but instead are akin to what is found with the azide complex (Ehrenberg & Szczepkowski, 1960).

It is conceivable that with spinach NiR and SiRHP there will be four distinct optical forms in the homologous series corresponding to HP(III)NO^+, HP(III)NO, HP(II)NO, and reduced HP(II)NO, the latter referring to a situation in which the 4Fe-4S cluster is reduced. It is not possible on the basis of optical data alone to specify the nature of the nitrite adduct of NiR, although its α band is considerably blue-shifted from the species formed with NO_2^- and excess dithionite (Vega & Kamin, 1977). The HP(II)NO and HP(III)NO forms of SiRHP are reported to have α bands at 599 and 581 nm, respectively, with the ferric nitrosyl species formed either by prolonged incubation of the oxidized enzyme under NO or by oxidation of the HP(II)NO complex with approximately 1 equiv of ferricyanide (Janick et al., 1983). The α band of the nitrite adduct studied here is at 579 nm; however, as will be discussed later, we do not how much of the "nitrite adduct" is in the low-spin ferric state with nitrite as the ligand.

(*b*) *EPR Spectroscopy.* Most of the NO_2^--induced EPR signals we observe are of the rhombic low-spin type; however, several display a HALS spectrum that is characterized by a prominent g_z "absorption" type feature and broad, low-intensity g_y and g_x components. The high-spin signals are typically unremarkable with very small changes in rhombicity being the most frequent finding. Interestingly, it is observed that with each heme protein nitrite complex examined instead of there being just a mixture of low- and high-spin moieties, there is always EPR-silent material present.

Origin of the EPR-Silent Heme. (*a*) *Autoreduction.* Autoreduction has been observed for the nitrite complex of *P. putida* cytochrome P-450 (Sono & Dawson, 1982) and *C. fumago* CPO (Sono et al., 1986) as well as for the ferric NO complexes of equine Hb (Ehrenberg & Szczepkowski, 1960), *P. aeruginosa* cd_1 (Shimada & Orii, 1975), and sperm whale Mb (Benko et al., 1983). The reductions at 25 °C are slow (half-time of 1–2 h) and irreversible and give end products identical with those of the ferric NO and ferrous NO complexes, respectively.

One explanation for how a ferric (but not a ferrous) NO complex could be spontaneously produced in a solution of the ferric heme protein plus nitrite is by the acid-catalyzed decomposition of nitrite, e.g., $3HNO_2 \rightarrow 2NO + HNO_3 + H_2O$. Indeed, the optical spectra of HRP and catalase samples prepared by acidification of a pH 7.7 nitrite-containing solution of ferric enzyme with large (20% of sample volume) aliquots of 1 M H_3PO_4 show α and β bands typical of those for ferric NO forms (see Table II). The EPR spectrum of a sample of HRP so treated shows a 60% loss of the nitrite-induced, low-spin signal and an intense matrix NO signal. These results are significantly different when compared to those found on addition of nitrite to a preacidified preparation, the procedure used in this work. One could also speculate that NO might be produced from nitrite as a result of a change in pH that occurred upon freezing the sample prior to EPR analysis. However, we have found that the optical spectra of all of the nitrite adducts examined here demonstrate reversibility to a (298–77–10–298) K temperature cycle, thus making it unlikely that NO is produced in this fashion.

Another explanation for EPR silence would be "protein-mediated" autoreduction (here the protein facilitates reduction

of the nitrite) occurring either as a result of the drop in temperature because of freezing the sample in liquid N_2 or as a consequence of the methodology used to prepare the sample prior to freezing it. The high activation energy of autoreduction and the reversibility of temperature-cycling argue against a freezing artifact.

The second possibility can occur with many of the proteins studied here if the samples are allowed to sit at room temperature for a sufficient period of time. This is illustrated by the spectral changes observed after overnight reaction of pH 5.4 HRP with 20–100 mM nitrite at 25 °C. At present, the mechanism of this autoreduction of nitrite is unknown, but it is noteworthy that (i) the reaction is slow ($t_{1/2}$ of 1.3 h) with a rate which increases with either a decrease of pH or an increase in the nitrite concentration, (ii) optical and EPR data indicate that there is reduction of nitrite to NO with concomitant production of verdoheme, and (iii) since *P. putida* cytochrome P-450 (Sono & Dawson, 1982), *C. fumago* CPO (Sono et al., 1986), and HRP demonstrate the phenomenon, the proximal ligand cannot be the only important variable, but rather it may be that with cysteinate as compared to imidazolate the porphyrin is spared immediate degradation.[8]

It is emphasized that on the basis of optical and EPR experimental data alone it is not possible to rule out the presence of some percentage of ferric NO species produced during the room temperature preparation of any sample of protein we have studied. We believe that on consideration of the data no case can be made for a diamagnetic nitrosyl complex accounting for all of the EPR-silent heme in proteins listed in Table III. Susceptibility studies (Scheler et al., 1957) have shown that at 20 °C and pH 6.5 sperm whale metMb–NO_2^- is a 0.24:0.76 mixture of low- and high-spin forms; i.e., there was no evidence for significant amounts of diamagnetic material. This ratio is in good agreement with our value of 0.29:0.71 at pH 6.7 and 25 °C and supports the hypothesis of small amounts of ferric NO species in the case of Mb. For the other three proteins, it is the absence of optical features solely attributable to the ferric NO form that makes a case; e.g., in all instances the band maxima of the nitrite adducts are significantly different from those of the diamagnetic NO species.

However, with *T. denitrificans* nitrite reductase, *S. oleracea* nitrite reductase, and *E. coli* sulfite reductase heme protein subunit, the situation is not so well-defined. This is so because with the first of these proteins we are relying on heuristic arguments with respect to the more fully characterized *P. aeruginosa* protein, while for the other two enzymes there are not enough published data to permit the optical or EPR end points of the ferric nitrite species to be specified. That is to say, all three proteins could (but not necessarily will) contain significant amounts of a diamagnetic ferric nitrosyl complex. An independent measure of the amount of such a species in any heme protein nitrite adduct can be done with probes such as IR, MCD, or magnetic susceptibility. Results of in tandem optical/EPR/susceptibility experiments with SiRHP plus sulfite as well as for SiRHP, spinach NiR, and *C. fumago* CPO plus nitrite[9] show that in each instance the major EPR-silent species is not diamagnetic but rather paramagnetic, with $S = {}^1/_2$.

(*b*) *Other Causes of EPR Silence.* Dipolar broadening, a very fast electron spin relaxation rate, and bimetallic antiferromagnetic coupling either are not possible or have not yet been observed in any of the paramagnetic ferric ligand complexes studied here.[10] A ferryl iron (with $S = 1$ or 2) is also conceivable; however, the reaction by which this species would be produced is rather convoluted and to date has no chemical precedent. A uniaxial *g*-tensor or *g*-strain thus remains as the most reasonable choice to explain the silence. It is worth mentioning that our results show that the EPR-silent species does not arise because a protein has a spin-state equilibrium with a low compensation temperature (Beetlestone & George, 1964; Neya et al., 1985; Browert & Stillman, 1984); e.g., both Mb (low T_c) and CPO (high T_c) show silence.

Theory of the Uniaxial State. As recently discussed by Salerno and Palmer (Salerno, 1984, 1985; Palmer, 1985), if the rhombic ligand field parameter (V/λ) is at or near zero, then as the axial ligand field parameter (Δ/λ) increases, g_z will asymptotically approach 4.0 while g_y and g_x approach zero. In the limit, this will translate into a situation where the hybrid orbital containing the unpaired spin shows equivalent amounts of d_{yz} and d_{xz} character and no proportion of d_{xy} (Taylor, 1977). Since the transition probability at g_z is proportional to $g_x^2 + g_y^2$, there will be no observable EPR signal. As yet it has not been possible to construct a model system that demonstrates these predictions of theory, but it is well appreciated that there is a group of proteins and model complexes giving EPR signatures which approach the theoretical limit, e.g., the "highly anisotropic low spin" or HALS resonances.

In an effort to explain HALS-type EPR spectra, Palmer has postulated that for the nonhindered bis(imidazole) complexes it is the relative orientation of the average imidazole planes that defines the *g*-tensor anisotropy of the ligand field. His contention has its origins in the fact that in bis(1-methylimidazole) protoheme and cytochrome b_5 the imidazoles are parallel, and even though the "in-plane imidazole projections" of the former are onto a pyrrolic axis but in the latter are onto a methynic axis, nearly identical rhombic-type EPR spectra are seen. However, in bis(2-methylimidazole) TPP the imidazoles are perpendicular and project onto the methynic axes, giving an EPR spectrum with *g* values of 3.4, 1.67, and 1.19 (Migita & Iwaizumi, 1981; Walker & Bolke, 1984). These findings suggest that for a given pair of identical axial ligands it is their relative orientation that is important in determining *g*-tensor anisotropy.[11]

If this hypothesis is correct, then with identical axial ligands the uniaxial state requires rigorous orthogonality of an appropriate ligand plane. However, when the ligands are nonidentical, the situation is more complicated since the intrinsic bonding characteristics of the axial ligands (π donor, π acceptor, and σ donor) must be correctly modulated, so that the "electromagnetic symmetry" of the ligands maintains a degeneracy of d_{xz} and d_{yz}. It is here that the protein could play a critical role; i.e., by protein modulation of the effective electronegativity and orbital overlap of the ligating atoms in relation to d_{xz} and d_{yz}, the particular electromagnetic symmetry requirements could be met. This fine tuning might be achieved by adjusting the lengths of metal–ligand bonds, by fixing the

[8] The presence of ammonium sulfate in the commercial preparation of CPO used here may explain the failure to observe significant autoreduction of the metCPO–NO_2^- complex under conditions similar to those of Sono (Sono et al., 1986).

[9] E. P. Day, J. Peterson, J. J. Bonvoisin, L. J. Young, J. O. Wilkerson, and L. M. Siegel, *Biochemistry* (in press).

[10] The ready reducibility of the heme iron with ascorbic acid in both ferric NO_2^- and NO complexes of SiRHP and NiR[2] (Janick et al., 1983) makes it extremely unlikely that coupling between a reduced cluster and the heme NO_2^- complex produces EPR silence.

[11] The spin state of heme-bound ligands can also be modulated by steric constraints on the orientation of the axial ligands with respect to the equatorial Fe–N_p bond vectors (Geiger et al., 1984; Scheidt et al., 1982, 1983).

Table IV: Spectral Parameters for Nitrite and Nitrosyl Adducts of *T. denitrificans* and *P. aeruginosa* Cytochrome cd_1

species	band position (nm)		
	α (d_1)	α (c)	β (c)
P. aeruginosa			
oxidized[a]	640	525	
reduced[a]	650	548, 550	522
oxidized + NO[b]	634	560	529
reduced + NO[b]	640	568	534
T. denitrificans			
oxidized	641	526	
reduced	656	548, 550	522
oxidized + NO_2^-	627	526	

[a] Silvestrini et al. (1979). [b] Shimada and Orii (1975).

orientation of ligands both in relation to each other and in relation to the metal and the ring, by modifying the bonding interaction of the ligand with the protein, and by tempering inductive and conjugative ring effects vis à vis ligand interactions with the ring.

Mechanisms of Protein-Induced Uniaxiality and G-Strain. The R and S states of *c*-type cytochromes (HALS-type species) illustrate well the "managerial" role of the protein; e.g., by orienting the ϵ-CH_3 of the axial methionine to a position over the nitrogen of either pyrrole IV or I, the protein forces the unpaired spin to reside primarily in an orbital (d_{yz} by convention) that is situated along the II–IV (R) or I–III (S) pyrrollic axes (Senn et al., 1980). Thus, a pyrrole electron pathway is produced that is unique for each type of protein.

The exquisite sensitivity to buffer composition of the EPR signal in the cytochrome *c*–nitrite complex and the minimal effect of nitrite on the optical spectrum suggest that if uniaxiality is the reason for the silence, then it arises from an indirect effect of nitrite. A uniaxial state could be produced via a reorientation of the π-bonding lone pair of the axial methionine so that it became perpendicular to the plane of the axial histidine, known to be situated along an α–γ methyne axis (Keller & Wüthrich, 1978), and then modulation of the bonding parameters of the axial ligands as outlined above.

Insofar as Mb, CPO, catalase, cytochrome cd_1, SiRHP, NiR, and possibly HRP are concerned, since nitrite is bound to the iron, uniaxiality would be achieved by the protein appropriately adjusting the relative position and electromagnetic symmetry of NO_2^- and the other axial ligand. To the extent that this type of EPR silence has also been observed with ligands such as sulfite and azide,[2,12] it cannot be that it arises from a peculiar reaction of nitrite with these proteins.

As yet we cannot say whether or not a functional significance can be attributed to these phenomena, in the sense of how the observation of a uniaxial or HALS state relates to the catalytic function of an enzyme. A certain "drive to rhombicity" has been observed during the purification of the cytochromes b_{568} and b_{562} from bovine heart succinate cytochrome *c* reductase (Salerno et al., 1986); e.g., the HALS spectrum for cytochrome b_{568} and b_{562} shifts to a rhombic species. We may be observing a similar preparative phenomenon in some fraction of SiRHP, the presence of which is signaled by a sulfite-induced HALS species (g_{max} = 2.7) instead of complete EPR silence.[13]

[12] Footnote deleted in proof.

[13] This low value for g_{max} has been observed (Walker et al., 1984) in Fe^{III}TPP complexes of hindered bis(pyrazole)s and benzotriazoles. In the former there is an implicit change in the proper axis that also occurs when changing from the porphyrin to the isobacteriochlorin ring system. Herein may lie part of the reason for the low g_z value in the HALS-type EPR spectra of the sulfite and nitrite complexes of *E. coli* SiRHP.

The final point to be discussed in this section is the extent to which *g*-strain contributes to an EPR-silent state; i.e., to what extent are the quantitative disparities between the optical and EPR spectra reflective of only a broad distribution about a given value of *g*? As has been discussed recently (Hagen et al., 1985a,b; Hearshon et al., 1986), it seems clear that a spectroscopic *g* is best described as a "random variable whose statistical properties contain information on the rigidity of the protein...and...in its details is a reflection of protein structure that distributes its spatial coordinates,...the more flexible parts being located on the outside of a molecule". If all of the EPR-silent heme in all of the protein–ligand complexes examined here arises only because of a freezing out of multiple conformers, one would have to postulate either that there is something intrinsically different about nitrite, sulfite, and azide such that a large number of "active-site" conformational substates[14] are produced when these anions interact with the proteins studied here or that extreme *g*-strain can be observed at an active site of any heme protein when the appropriate interactions occur among the metal, axial ligands, macrocycle, the "bulk" protein, and the solvent. It may be that some heme proteins are more disposed toward fulfilling such conditions; i.e., the lower symmetry and increased macrocycle flexibility of the siroheme-containing proteins could favor a multiplicity of states.

Until further experimental data can be obtained, it seems wisest to attribute the silence to some combination of uniaxiality and *g*-strain, the proportions of which may be quite different depending on the particular protein–ligand complex.

Critical Variables in the Ligation and/or Reduction of NO_2^- and SO_3^{2-} by Ferric Heme Proteins. Nitrite is capable of forming a stable adduct with ferric heme proteins regardless of the nature of the macrocycle or the "proximal" ligand and is not dissimilar from other weakly acidic anions insofar as the kinetic component of the binding reaction is concerned (Blanck et al., 1961; Chance, 1952; Dunford & Alberty, 1967; Erman, 1974; Sono et al., 1986). In contrast, sulfite has been proven to bind only to spinach NiR and SiRHP (the data for *T. denitrificans* cytochrome cd_1 is suggestive but not conclusive). Even reduction of the iron in Mb, HRP, and CPO does not lead to ligation of sulfite.

It is not immediately obvious why an iron isobacteriochlorin should be required for binding of sulfite since the affinity for SO_3^{2-} is 3 orders of magnitude more in SiRHP (K_d) than in free siroheme [K_m under conditions of sulfite turnover (Seki et al., 1981)]; i.e., regardless of what the detailed chemistry of the model system reaction is, it is apparent that sulfite binds weakly to siroheme. It may be that all iron pyrrole and pyrrole/pyrroline macrocycles have a low affinity for this anion and that it is the "proteinaceous" milieu of the macrocycle which is of importance; e.g., in SiRHP (and possibly in spinach NiR as well) the "distal" site may be regulating the ligation of sulfite.

What then is the role of the macrocycle in the reduction of nitrite to ammonia or of sulfite to sulfide by these multielectron reductases? Recent results (Murphy et al., 1986) showing that in model $[(tpy)(bpy)M(NO_2^-)]^+$ [M = Os, Ru; tpy = terpyridine; bpy = bipyridine] systems reduction occurs in six sequential one-electron steps and the discovery of a hexaheme *c* nitrite reductase (Kajie & Anraku, 1986) indicate

[14] "Active site" protein conformers are to be distinguished from "global" protein conformers (Elber & Karplus, 1987) in the sense that there need not be a 1:1 correspondence between the two; i.e., significant structural perturbations occurring peripheral to the the active site need not be mirrored at the active site (the converse is also true).

that an iron isobacteriochlorin is not an absolute necessity for production of ammonia from nitrite. However, the evidence to date suggests that for the reduction of sulfite to sulfide such is the case, and we suspect that the facility with which the tetrahydropyrrole forms a π cation (Stolzenberg et al., 1980) is of essential importance in the mechanism by which an assimilatory sulfite reductase catalyzes the turnover of sulfite.

Registry No. NO_2^-, 14797-65-0; SO_3^-, 14265-45-3; NADPH–sulfite oxidoreductase, 9029-35-0.

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Distinct Structural Features of the α and β Subunits of Nitrogenase Molybdenum–Iron Protein of *Clostridium pasteurianum*: An Analysis of Amino Acid Sequences†

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ABSTRACT: Nitrogenase is composed of two separately purified proteins, a molybdenum–iron (MoFe) protein and an iron (Fe) protein. Structural genes (*nifD* and *nifK*) encoding α and β subunits of the MoFe protein of *Clostridium pasteurianum* (Cp) have been cloned and sequenced. The deduced amino acid sequences were analyzed for structures that could be related to the unique properties of the Cp protein, particularly its low capacity to form an active enzyme with a heterologous Fe protein. Cp *nifK* is located immediately downstream from Cp *nifD*, with the start codon of *nifK* overlapping by one base with the stop codon of *nifD*. An open reading frame following *nifK* was identified as *nifE*. The amino acid sequence deduced from *nifK* encompasses the partial amino acid sequences previously reported from the isolated β subunit. Cp *nifK* encodes a polypeptide of 458 amino acid residues (M_r 50 115) whose amino-terminal region is about 50 residues shorter than the otherwise conserved corresponding polypeptides from four other organisms. In contrast, Cp α subunit (*nifD* product) contains an additional stretch of 50 amino acid residues in the 380–430 region, which is unique to the Cp protein. It therefore appears that the combined size of the α and β subunits could be important to nitrogenase function. An analysis of the predicted secondary structure from the amino acid sequence of each subunit from three species (*C. pasteurianum*, *Azotobacter vinelandii*, and *Rhizobium japonicum*) further revealed structural features, including regions adjacent to some of the conserved cysteine residues, differentiating the Cp MoFe protein from others. These different regions may be further tested for correlation with distinct properties of Cp nitrogenase.

The reduction of N_2 to ammonia (nitrogen fixation) is carried out by a number of free-living and symbiotic bacteria, and the reaction is catalyzed by the enzyme nitrogenase [for a recent review, see Orme-Johnson (1985)]. Some organisms contain at least two types of nitrogenase (Bishop et al., 1986; Dilworth et al., 1987), but both types are composed of two separable protein components. The extensively characterized *conventional* nitrogenase (hereinafter referred to as nitrogenase) consists of an iron protein (Fe protein, component II, or dinitrogenase reductase) and a molybdenum–iron protein (MoFe protein, component I, or dinitrogenase), whereas the recently purified *alternative* nitrogenase from *Azotobacter* consists of a different iron protein (Robson et al., 1986; Hales et al., 1986a) and a vanadium–iron protein (Hales et al., 1986b; Eady et al., 1987).

All nitrogenase activities require the presence of both components, and the N_2 reduction site is believed to be located on the MoFe protein. The polypeptide of the Fe protein (a dimer) is encoded by *nifH* (or *nifH1* in *Clostridium pasteurianum*), whereas the polypeptides of the MoFe protein (an $\alpha_2\beta_2$ tetramer) are encoded by *nifD* (α subunit) and *nifK* (β subunit), respectively. Both the Fe protein and the MoFe protein contain iron–sulfur cluster(s), whereas the MoFe protein also contains molybdenum–iron–sulfur clusters that can be extracted as the iron–molybdenum cofactor [see Orme-Johnson (1985)].

In studies concerning the function of nitrogenase, an important aspect is the interaction between the Fe protein and the MoFe protein. Such interactions affect the enzyme's efficiency and relative reactivity toward alternative substrates (Smith et al., 1976; Emerich et al., 1981; Wherland et al., 1981). The physicochemical properties of Fe and MoFe proteins are highly conserved among all N_2-fixing bacteria so far studied, but significant differences also exist, especially between nitrogenase of *C. pasteurianum* (Cp) and the others. Specifically, nitrogenase components (the MoFe protein in particular) of *C. pasteurianum* have a distinctly low capacity to form an active hybrid enzyme with complementary components from other organisms (Smith et al., 1976; Emerich & Burris, 1978; Tsai & Mortenson, 1978; Emerich et al., 1978). *C. pasteurianum* nitrogenase is also less sensitive to H_2 as an inhibitor (Guth & Burris, 1983) and shows a higher specificity for nucleotides (Weston et al., 1983). Recently, a difference in both the relative reduction sequence of the redox

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