Resolution of the Hemes of Hydroxylamine Oxidoreductase by Redox Potentiometry and Electron Spin Resonance Spectroscopy†

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ABSTRACT: Hydroxylamine oxidoreductase from *Nitrosomonas* catalyzes the oxidation of hydroxylamine to nitrite. It is a multi-heme enzyme containing at least five thermodynamically distinct *c*-type hemes and an apparently unique heme-like moiety known as P-460. The electron spin resonance (ESR) spectra of the enzyme are remarkably complex, indicating several different types of interactions between the redox centers. ESR spectra of the enzyme poised by redox potentiometry at known states of reduction have allowed the following tentative assignments: The reduction of one or two hemes of c_{553} (295 mV) is accompanied by changes in the ESR properties of separate hemes. Three scenarios are discussed: $g = 3.06$ hemes are reduced, with either (i) ESR-silent or (ii) $g = 3.06$ hemes shifting to $g = 2.98$, or (iii) ESR-silent hemes may be reduced while $g = 3.06$ hemes shift to $g = 2.98$. Heme c_{553} (10 mV) has $g = 3.02$ and is very difficult to saturate with microwaves. Hemes c_{559} (0 mV) are a pair of hemes that interact to give rise to signals at 194, 244, 356, and 397 mT at 9 GHz. Hemes c_{553} (−190 mV) are a pair of hemes with $g = 2.98$ that are seen fully only after reduction of heme(s) c_{553} (295 mV). P-460 (−260 mV) has signals near $g = 6$ that arise as hemes c_{553} (10 mV) and c_{559} (0 mV) are reduced. The $g = 6$ signals and a signal near $g = 3.23$ disappear as P-460 goes reduced. Heme c_{553} (−390 mV) is ESR-silent.

The oxidation of ammonia to nitrite, a key part of the nitrogen cycle of the biosphere, is catalyzed by the soil auxotrophic bacterium *Nitrosomonas europeae* (Hooper, 1984; Suzuki, 1984). Hydroxylamine is a key intermediate in the process, although the enzymes responsible for its production from ammonia have not been well characterized [although, see Tsang and Suzuki (1982), Shears and Wood (1985) and Hyman and Wood (1985)]. In contrast, the enzyme responsible for oxidizing hydroxylamine to nitrite, hydroxylamine oxidoreductase, has been purified to homogeneity (Terry & Hooper, 1981). The isolated enzyme is an $\alpha_3\beta_3$ aggregate (M_r 200 000) of a large protein (β) containing six *c*-type hemes and one heme-like P-460 component and a small monoheme *c*-type cytochrome (α) (Hooper, 1984). The $\alpha_3\beta_3$ aggregate thus contains 24 hemes! P-460 is thought to be part of the enzyme active site (Hooper & Terry, 1977).

Optical spectroscopy reveals that the α-band maximum is at approximately 553 nm for five of the *c*-type hemes and at 559 nm for the other two (Hooper & Balny, 1982). At pH 7 these components have the following approximate ratios and midpoint oxidation–reduction potentials: one or perhaps two c_{553} (295 mV), one c_{553} (10 mV), two c_{559} (0 mV), two c_{553} (−190 mV), and one c_{553} (−390 mV). There is also the unusual chromophore known as P-460 with an E_{m7} of −260 mV (Prince et al., 1983).

Hydroxylamine oxidoreductase has remarkably complex electron spin resonance (ESR) properties (Vickery & Hooper, 1981; Lipscomb & Hooper, 1982), with strong indications that the hemes do not behave independently. In this work we report the ESR properties of the enzyme under conditions of known redox poise and define which hemes respond to the redox poise of their neighbors.

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Materials and Methods

Hydroxylamine oxidoreductase was prepared from *Nitrosomonas europeae* by the method of Hooper et al. (1978). Redox potentiometry used similar buffer and redox mediators to the optical experiments (Prince et al., 1983); these were 20 mM 3-(*N*-morpholino)propanesulfonate and 100 mM KCl, pH 7.0, with 40–50 μM of appropriate redox mediators, chosen from the following: 2,3,5,6-tetramethylphenylenediamine (E_{m7} = 275 mV), *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine (E_{m7} = 275 mV), 1,2-naphthoquinone-4-sulfonate (E_{m7} = 215 mV), *N*-methylphenazonium methosulfate (E_{m7} = 85 mV), pyocyanin (E_{m7} = 30 mV), 5,5′-indigodisulfonate (E_{m7} = −125 mV), 2-hydroxy-1,4-naphthoquinone (E_{m7} = −145 mV), 2-hydroxy-1,4-anthraquinone (E_{m7} = −205 mV), benzyl viologen (E_{m7} = −350 mV), and methyl viologen (E_{m7} = −430 mV) [see Prince et al. (1981)].

For some experiments the enzyme was recovered from the ESR tubes, washed and concentrated in an Amicon ultrafiltration cell using a PM30 filter, and reused. No differences in the thermodynamic behavior, or in signal line shape, were noticed after this treatment. Samples were withdrawn anaerobically, transferred to argon-flushed ESR tubes, and frozen in liquid nitrogen chilled isooctane. ESR spectra were recorded with a Varian E-109 spectrometer equipped with an Oxford flowing helium cryostat. Microwave frequency was measured with a EIP Model 548A microwave frequency counter. Pitch and metmyoglobin fluoride were used as ESR *g*-value standards.

Results

The results of the ESR titrations are described in five steps, as outlined in Table I. Hemes are expected to be ESR detectable in their oxidized (Fe^{3+}) form, so all the hemes would be expected to be visible in the sample poised at E_h = 406 mV. The subsequent samples were chosen to sequentially reduce the hemes and P-460, with the prediction that the ESR spectra

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Table I: Redox States of the Hemes of Hydroxylamine Oxidoreductase at the Potentials Used in Figures 1 and 2

	redox center[a]					
E_h (mV)	c_{553} (E_{m7} = 295 mV)	c_{553} (E_{m7} = 10 mV)	c_{559} (E_{m7} = 0 mV)	c_{553} (E_{m7} = −190 mV)	P-460 (E_{m7} = −260 mV)	c_{553} (E_{m7} = −290 mV)
406	Ox.	Ox.	Ox.	Ox.	Ox.	Ox.
150	Red.	Ox.	Ox.	Ox.	Ox.	Ox.
−100	Red.	Red.	Red.	Ox.	Ox.	Ox.
−215	Red.	Red.	Red.	Red.	Ox.	Ox.
−315	Red.	Red.	Red.	Red.	Red.	Ox.

[a] Based on Prince et al. (1983).

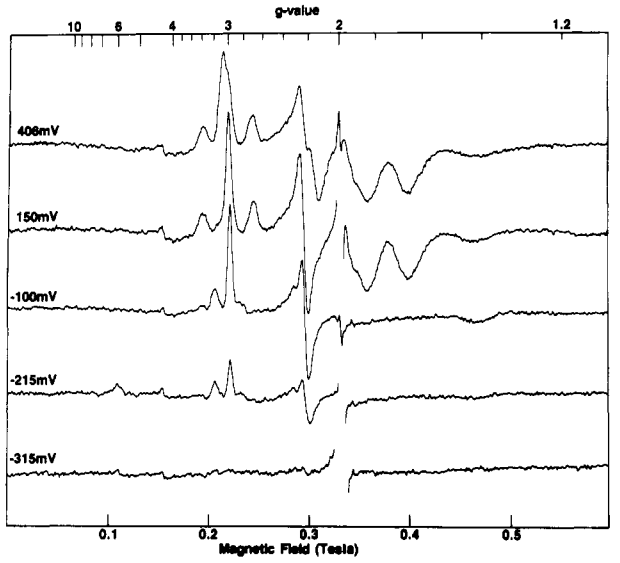

FIGURE 1: Representative ESR spectra of hydroxylamine oxidoreductase. The enzyme was present at 6 mg/mL (800 μM heme), and spectra were recorded at 9.5 K with 1 mW of applied microwave power and a modulation amplitude of 1.25 mT.

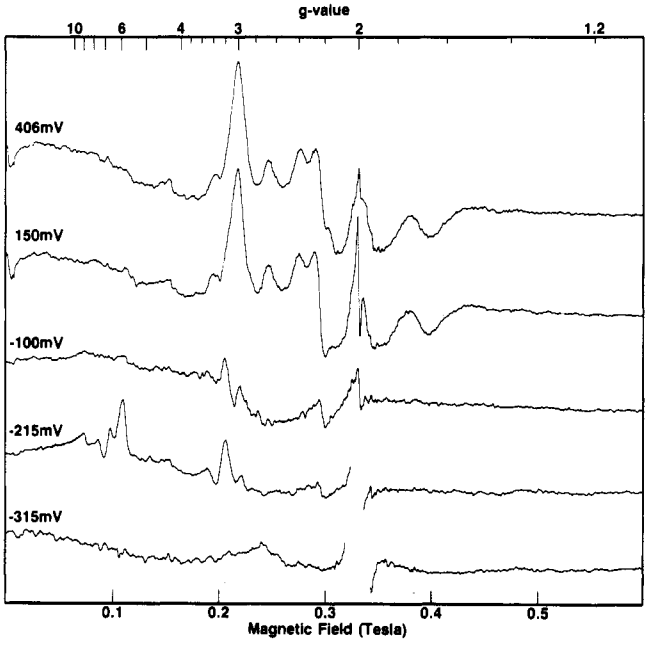

FIGURE 2: Representative ESR spectra of hydroxylamine oxidoreductase. These are the same samples as in Figure 1 but measured at 5.5 K with 50 mW of applied microwave power.

would become correspondingly simpler.

Figure 1 shows spectra of the enzyme measured at conditions usually optimal for the observation of oxidized hemes. They are very similar to those shown by Lipscomb and Hooper, although our interpretation is now a little different. Figure

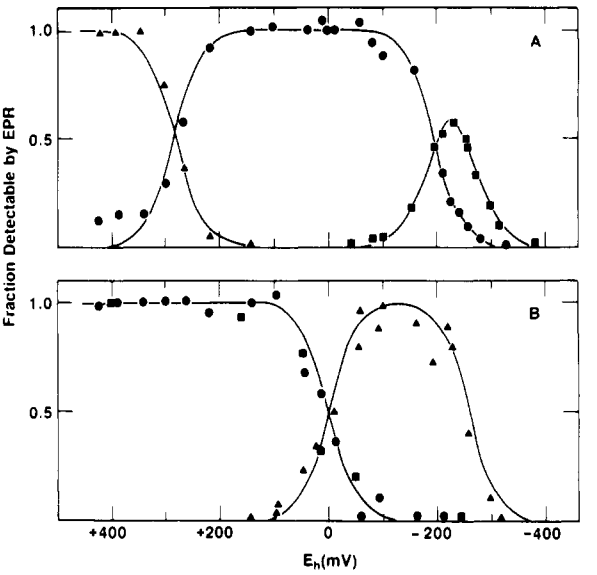

FIGURE 3: Redox titrations of the hemes of hydroxylamine oxidoreductase. This figure shows data from several titrations similar to those illustrated in Figures 1 and 2, with three different batches of enzyme. (A) (▲) g = 3.06 and (●) g = 2.98, both measured as in Figure 1; (■) high-spin signals measured as in Figure 4. (B) (▲) g = 3.20 and (●) 397 mT, both measured as in Figure 1; (■) g = 3.0 measured as in Figure 2. The lines drawn through the points are standard Nernst curves, except for the case of the high-spin component discussed in the text. Discussions of such curves can be found in Clark (1972) It should be noted that the data and lines have been normalized to the maximum seen, except for the high-spin features discussed in the text. The spectra have proven far too complex for reliable spin quantitation.

2 shows the same samples viewed at significantly lower temperature and higher power, conditions that might be expected to saturate signals from typical hemes.

E_h = *406 mV.* At high potential (E_h = 406 mV), the enzyme is essentially fully oxidized (Table I). The ESR spectrum (at 9.5 K, 1 mW of applied power) is dominated by a signal with g = 3.06, 2.14, and 1.35. A second signal is observed with g = 2.98, 2.24, and 1.41. There are also prominent features at 194, 244, 356, and 397 mT (apparent g values of 3.43, 2.72, 1.87, and 1.67), which are known to shift their apparent g value at other microwave frequencies [see Hooper (1984)], suggesting that they reflect some sort of spin-coupling phenomenon. At first glance the ESR spectrum of E_h = 406 mV in Figure 2 looks rather similar to that in Figure 1, but in fact, the prominent features are at g = 3.02 and 2.24 rather than 3.06 and 2.14. There is also a pronounced feature at g = 2.4, and the features at 194, 244, 356, and 397 mT discussed above are still seen.

E_h = *150 mV.* As the potential is lowered to 150 mV, where about 20% of the total absorbance change at 553 and 559 nm is seen in the optical spectra (Prince et al., 1983), the ESR spectra show a progressive decrease in the signal at g = 3.06 and an increase of the g = 2.98, 2.24, and 1.41 signal. This

effect titrates with the reduction of the highest potential heme (c_{553}, 295 mV), as shown in Figure 3.

It is noteworthy that the spectrum has changed little from that at 406 mV when recorded at 5.5 K and 50 mW of applied power (Figure 2). A small signal at $g = 2.14$, probably associated with $g = 3.06$, disappears, but there is no significant change near $g = 3$.

$E_h = -100$ mV. At this potential we expect two more hemes to have gone reduced, c_{553} (10 mV) and c_{559} (0 mV) (see Table I). They account for a further 13 and 20% of the heme α-band absorbancy (Prince et al., 1983). The reduction of these hemes is accompanied by the simultaneous disappearance of the features assigned above to a spin-coupled system, together with the $g = 3.02$ signal seen at 5.5 K, 50 mW (Figure 3). Clearly accompanying the reduction of these hemes is the appearance of new ESR features at 187 (5.5 K), 206, 231, and 287 mT (apparent g values of 3.53, 3.23, 2.88, and 2.32), as shown in Figures 1–3. The origin of these rather weak signals is obscure.

$E_h = -215$ mV. This potential was chosen as approximately midway between the midpoint potentials of heme c_{553} (−190 mV) and P-460 (−260 mV); at this potential the former would be about 72% reduced and the latter about 86% oxidized. Heme c_{553} (−190 mV) accounts for about 33% of the total α-band absorbancy (Prince et al., 1983), and a simple interpretation of the relative absorbancies suggests that there may be two of these hemes per enzyme (Prince et al., 1983). The reduction of heme c_{553} (−190 mV) is associated with the disappearance of the ESR signal at $g = 2.98$ (Figures 1 and 3). This signal is not seen to any extent at 5.5 K, 50 mW (Figure 2).

A dramatic feature is seen at this potential at 5.5 K, 50 mW, in the $g = 10$–6 region of the spectrum (Figure 2); it is shown more clearly in Figure 4. All the features of this spectrum seem to titrate together, and as shown in Figures 2 and 3, they are seen only when heme c_{553} (−190 mV) is reduced but P-460 oxidized. Perhaps the simplest interpretation of this is that the signals near $g = 6$ arise from P-460, but only when heme c_{553} (−190 mV) is reduced. For the system

$$\text{oxidized heme } c_{553} + e^- \overset{E_{m1}}{\rightleftharpoons} \text{reduced heme } c_{553}$$

$$\text{oxidized P-460} + e^- \overset{E_{m2}}{\rightleftharpoons} \text{reduced P-460}$$

the fraction of the sample that has the former reduced and the latter oxidized may be calculated from

$$[10^{(E_h - E_{m1})/59} + 1]^{-1} \times [10^{(E_{m2} - E_h)/59} + 1]^{-1}$$

This estimates that the maximal fraction of signal detectable, which would be at $E_h = -225$ mV, would be 0.59 if there were one P-460 per enzyme interacting with a single heme c_{553} (−190 mV). The situation would be a little more complicated if P-460 interacted with either of two c_{553} (−190 mV) hemes, but since the data are not adequate to distinguish such models, they are not treated further here.

The line shapes of the signals in Figure 4 are somewhat different from those reported in the absence of redox mediators by Lipscomb and Hooper (1982), but the ambient redox potential required for their observations suggests that they are alternative manifestations of the same underlying species.

$E_h = -315$ mV. At this potential only a single heme c_{553} (−390 mV), accounting for approximately 12% of the α-band absorption, remains oxidized. Surprisingly, there are no ESR signals indicative of low- or high-spin hemes at this potential. As the potential was lowered through the region where P-460 goes reduced, the features of Figure 4 and the signal with apparent g value of 3.2 (206 mT, Figure 1) titrate out as if

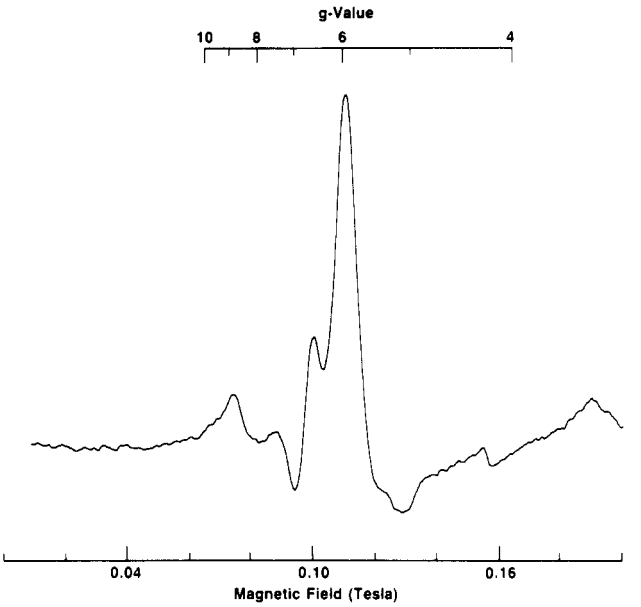

FIGURE 4: High-spin ESR signals of hydroxylamine oxidoreductase. The enzyme was present at 6 mg/mL in the presence of 20 μM 2-hydroxy-1,4-anthraquinone, with the E_h poised at −217 mV. The spectrum was measured at 5 K with 50 mW of applied power and a modulation amplitude of 1.5 mT.

their observation is dependent on the presence of oxidized P-460 (Figure 3). Similarly, the features at 231 and 287 mT disappear in this region, although these signals are too small for reliable titration. All the latter signals grew in as hemes c_{553} (10 mV) and c_{559} (0 mV) went reduced, as discussed above and shown in Figure 3.

Discussion

Hydroxylamine oxidoreductase has a remarkably complicated complement of ESR-detectable chromophores, but redox potentiometry together with appropriate use of the ESR spectrometer variables allows some simplification to be imposed. The ESR data seem compatible with the inferences drawn on the thermodynamic properties of the hemes determined optically (Prince et al., 1983), and we have used those data as the basis for our tentative assignments in this paper. Note, however, that because we do not know the true values for oxidized *minus* reduced extinction coefficients for the hemes, there is considerable uncertainty as to the number of hemes titrating in each thermodynamic category. Indeed, the total number of hemes in the enzyme is still the subject of some debate. We interpreted our optical titrations on the basis of seven hemes per P-460, but alternative scenarios with a different stoichiometry could not be ruled out. With this in mind, our results will be discussed below in an attempt to correlate ESR spectra with optically detected hemes.

Heme c_{553} (295 mV). The simplest interpretation of the present data is that there is one or two of these hemes per enzyme pair and that the oxidized form has $g_z = 3.06$, $g_y = 2.14$, and $g_x = 1.35$. This is in keeping with earlier conclusions (Lipscomb & Hooper, 1982; Prince et al., 1983). Optimal ESR conditions seem to be near 10 K with a few milliwatts of applied microwave power.

As shown in Figure 3, this ESR signal titrates with the appropriate E_{m7}, but its disappearance is correlated with the *appearance* of signal at $g = 2.98$. This trend has been noticed before (Lipscomb & Hooper, 1982). As we shall discuss below, the disappearance of this latter signal titrates with the reduction of heme c_{553} (−190 mV). But what causes its appearance as heme c_{553} (295 mV) is reduced? We cannot

accurately quantitate the number of spins involved in this process, but clearly, three possibilities could explain the data: (i) $g = 3.06$ hemes are reduced, allowing previously ESR-silent hemes to become visible at $g = 2.98$; (ii) some $g = 3.06$ hemes become reduced, and hence ESR silent, while others shift their apparent g value to 2.98; (iii) an ESR-silent heme becomes reduced, shifting the apparent g value of the ESR visible hemes from 3.06 to 2.98. The former receives some support from our ESR data, since the latter two would both predict that the prominent features of the fully oxidized enzyme might be only apparent g values, and hence frequency dependent; this is not seen [see Hooper (1984)]. On the other hand, if the changes postulated to cause alternatives ii and iii were protein conformational changes rather than magnetic interactions, the ESR signals might always represent true g values, and hence be frequency independent. Indeed, preliminary Mössbauer data (V. Papastimios, M. Collins, K. K. Andersson, J. D. Lipscomb, E. Munck, and A. B. Hooper, unpublished results) lend some support to the idea that the heme(s) going reduced with an E_{m7} of 295 mV may not have been detectable by ESR, lending weight to the notion that the interchange between ESR signals with $g_z = 3.06$ and 2.98 indeed reflects some conformational change in the protein and that, in fact, the ESR signals always arise from the heme c_{553} (−190 mV). Clearly although these experiments place some constraints on possible explanations, they cannot completely discriminate between them.

Hemes c_{553} (10 mV) and c_{559} (0 mV). The redox properties of these hemes are so similar that it is impossible to discriminate between them solely on the potentiometric data. On the basis of assumed extinction coefficients, we have suggested that an $\alpha\beta$ pair of the enzyme contains one heme c_{553} (10 mV) and two heme c_{559} (0 mV) (Prince et al., 1983). Two ESR features can be ascribed to these redox species: a signal with apparent $g_z = 3.02$, $g_y = 2.24$, and g_x undetected, but predicted to be near $g = 1.36$, which is very difficult to saturate, and a signal attributed to a spin interaction with features at 194, 244, 356, and 397 mT at 9.226 GHz. The latter are known to be frequency dependent, and hence not true g values (Hooper, 1984). Since the spin-coupled system probably involves two hemes, it is tempting to assign it as arising from the two c_{559} (0 mV) hemes. This would lead to the assignment of the $g_z = 3.02$ and $g_y = 2.24$, (g_x 1.36 if the sum of the squares of the g values is typical for a low-spin heme) signal to heme c_{553} (10 mV).

The reduction of these two hemes is also accompanied by the loss of the signal at 282 mT (apparent g value of 2.4) seen at 5.5 K, 50 mW applied power. The origin of this signal, and the location of any companion spectral features, remains obscure.

Heme(s) c_{553} (−190 mV). Optical spectroscopy suggests that there are two of these hemes per $\alpha\beta$ enzyme pair (Prince et al., 1983). The ESR spectrum of the species is $g_z = 2.98$, $g_y = 2.24$, and $g_x = 1.41$, but as discussed above, this is only seen when heme c_{553} (295 mV) is reduced. Also, as discussed above, it is not clear whether heme c_{553} (−190 mV) is ESR-silent when heme c_{553} (295 mV) is oxidized or displays a shifted ESR spectrum.

As shown in Figures 1 and 3, it has not proven possible to completely eliminate the signal at $g = 2.98$ at high potentials; 12–20% of the signal always remains [see also Lipscomb and Hooper (1982)]. It is possible that this remainder represents an additional c-type heme not previously accounted for in the optical spectrum or that it reflects a small population of the enzyme that has lost the highest potential heme. It is also possible that the remainder is actually a remnant at this higher temperature (Figure 1) of the prominent signal at $g = 3.02$ seen at 5.5 K with 50 mW of applied power.

P-460 (−260 mV). This redox species seems to be unique to hydroxylamine oxidoreductase. It is a heme-like moiety, although its optical, Mössbauer, and ESR parameters are unique (see Andersson et al. (1984)]. Lipscomb and Hooper (1982) assigned signals at $g = 6.4$ and 5.6 to this species in a dithionite reductive titration using no redox mediators. As in the experiments reported here, those signals were only seen over a narrow range of potentials when most of the hemes were reduced. Three separate preparations of the enzyme used in our experiments gave spectra similar to those of Figure 4. All such experiments used redox mediators, but varying the concentration of the redox dyes from just 20 μM 2-hydroxy-1,4-anthraquinone ($E_{m7} = -205$ mV; Prince et al., 1981) to 200 μM of all the mediators listed under Materials and Methods (approximately 100 μM enzyme) had no effect on the line shape of the signals (cf. Figures 1 and 4). We are thus not sure what causes the difference in the spectra. Clearly, one very informative experiment will be to examine the signals of Figure 4 at a higher microwave frequency to see whether they are true high-spin g values or perhaps some interacting system.

It is not clear why the high-spin signals attributed to P-460 are not seen in the fully oxidized enzyme, but it is clear from Figure 3 that the component is only detected as a high-spin species when heme c_{553} (−190 mV) is reduced but P-460 is still oxidized. The signals that appear at 189, 206, 231, and 287 mT (apparent g values of 3.53, 3.23, 2.88, and 2.32) upon the reduction of hemes c_{553} (10 mV) and c_{559} (0 mV) all seem to disappear with the reduction of P-460 and so may be manifestations of some alternative form(s) of the center.

A chromophore that shares some optical and Mössbauer properties with the P-460 of hydroxylamine oxidoreductase is found attached to a small protein, M_r 17000. Although Miller et al. (1984) report that the small protein does not cross-react immunologically with the oxidoreductase, the aggregate of spectral and chemical evidence suggests that the two proteins contain the same P-460 chromophore. In the isolated state, P-460 of the small protein exhibits ESR parameters of $g = 6.15$, 5.70, and 2.0 (Andersson et al., 1984). These are different from those attributed to P-460 in partially reduced hydroxylamine oxidoreductase [Figure 4 and Lipscomb and Hooper (1982)].

One additional complication deserves mention. In the optical experiments reported earlier (Prince et al., 1983) we were unsure of the optical spectrum of P-460 in the enzyme. Some changes at 553 nm occurred in the same redox potential range as the reduction of P-460, which we were measuring at 460 nm. At the time we were unsure as to whether the changes at 553 nm might reflect an α-band of P-460 or perhaps another heme. With the finding of so many changes concomitant with the reduction of P-460, we raise the possibility that there may be an additional heme c_{553} with $E_{m7} = -260$ mV in the enzyme, although this is by no means unequivocally demonstrated by the data.

Heme c_{553} (−390 mV). This very low potential heme has not yet been detected by ESR.

ACKNOWLEDGMENTS

We thank Celine Lyman for growth of bacteria and K. K. Andersson for purification of the enzyme.

Registry No. P-460, 62395-29-3; cytochrome *c*-553, 12624-01-0; cytochrome *c*-559, 106266-27-7; hydroxylamine oxidase, 9075-43-8; heme *c*, 26598-29-8.

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CORRECTIONS

Thermodynamic Analysis of the Lactose Repressor–Operator DNA Interaction, by Peggy A. Whitson, John S. Olson, and Kathleen Shive Matthews*, Volume 25, Number 13, July 1, 1986, pages 3852–3858.

Page 3852. The title footnote should read as follows: This work was supported by grants from the National Institutes of Health (GM 22441 to K.S.M. and GM 35649 to J.S.O.) and the Robert A. Welch Foundation (C-576 to K.S.M. and C-612 to J.S.O.).

Page 3855. In column 2, the sentence beginning on line 12 should read as follows: The activation energies were 2.0 (>12 °C) and 8.0 kcal/mol (<12 °C).

An Ordered Addition, Essential Activation Model of the Tissue Factor Pathway of Coagulation: Evidence for a Conformational Cage, by Yale Nemerson* and Rodney Gentry, Volume 25, Number 14, July 15, 1986, pages 4020–4033.

Page 4022. Owing to a programming error, the graph in the right panel of Figure 2 is in error. The velocity in fact does not pass through a maximum as a function of substrate concentration. However, under the conditions of this system in which $k_{cat} \gg k_{-1}$, the $K_{1/2}$ for enzyme or activator titration varies directly with substrate concentration. As this is contrary to our experimental observations, we still reject the steady-state model III.

Electrostatic Analysis of the Interaction of Cytochrome *c* with Native and Dimethyl Ester Heme Substituted Cytochrome b_5, by Marcia R. Mauk, A. Grant Mauk,* Patricia C. Weber, and James B. Matthew*, Volume 25, Number 22, November 4, 1986, pages 7085–7091.

Page 7089. The legend for Figure 5A should read as follows: (A) Stereo diagram of positive electrostatic potential surfaces (4 kT) calculated for cytochrome *c* (upper left) and negative potential surfaces for trypsin-solubilized native cytochrome b_5 (left) and DME-cytochrome b_5 (right) at pH 7.6, I = 4 mM, 25 °C. The side chain of His-26 is included.

Page 7090. The labeling of parts A and B of Figure 6 should be reversed.