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Iron–Mercaptoethanol–Inorganic Sulfide Complex. Possible Model for the Chromophore of Nonheme Iron Proteins*

C. S. Yang† and F. M. Huennekens

ABSTRACT: An iron–mercaptoethanol–inorganic sulfide complex is formed upon mixing $FeCl_3$ with an equal concentration of Na_2S and an excess of 2-mercaptoethanol at pH 9. The absorption spectrum of this complex, characterized by maxima at 325, 412, and 450 mμ, is similar to that of the iron–sulfur chromophore of nonheme iron proteins. Iron and inorganic sulfide are present at a 1:1 ratio in the complex and the iron is in the trivalent state. Studies with analogs of mercaptoethanol suggest that both the sulfhydryl and hydroxyl groups of the parent compound are involved in iron binding, resulting in the formation of a 5-membered chelate ring. Mononuclear and binuclear structures are considered for the complex. The "model complex" is inherently unstable due to intramolecular electron transfer in which Fe^{3+} is reduced to Fe^{2+} by the mercaptoethanol. In the presence of oxygen, the resulting Fe^{2+} is readily oxidized to the ferric state, regenerating the model complex. The combination of these two reactions results in reduction of O_2 and oxidation of mercaptoethanol. The actual O_2 uptake of the system is much faster than would be expected from the above mechanism, and a more efficient process for reduction of O_2 must be involved. At 110°K, the complex system shows an electron paramagnetic resonance signal at $g = 4.1$, accompanied by weaker signals in the central field ($g_{\perp} = 2.01$ and $g_{\parallel} = 1.96$). The latter signals are believed to be associated with a transient species containing an unpaired electron.

Nonheme iron proteins,[1] which are involved in various electron-transfer processes in biological systems, have received intensive study during the past few years (reviewed by Malkin and Rabinowitz, 1967, Kimura, 1968, and San Pietro, 1968). These proteins generally show characteristic absorption bands (or shoulders) in the regions of 315–335, 410–420, and 450–460 mμ and, upon reduction, a unique electron paramagnetic resonance signal is seen at $g = 1.94$ (reviewed by Beinert, 1966). These properties have been attributed to a complex in which the iron is bonded to sulfhydryl groups on the protein and to inorganic sulfur. In several cases, more detailed structures have been envisioned for these complexes (Blomstrom *et al.*, 1964; Tanaka *et al.*, 1965; Brintzinger *et al.*, 1966; Bayer *et al.*, 1969). However, the exact structures of the iron–sulfur chromophore of these nonheme iron proteins have not yet been established.

Searching for a possible model for the above proteins, Beinert *et al.* (1965) showed that pentacyanonitrosyl ferrate gives an electron paramagnetic resonance signal in which the g value is <2. On the other hand, the typical absorption spectrum of the nonheme iron protein is not duplicated in the above compound or in other simple iron complexes

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[1] In this paper, "nonheme iron proteins" refer to those that also contain "labile sulfide." This category includes both smaller molecular weight ferredoxins and larger nonheme iron flavoproteins.

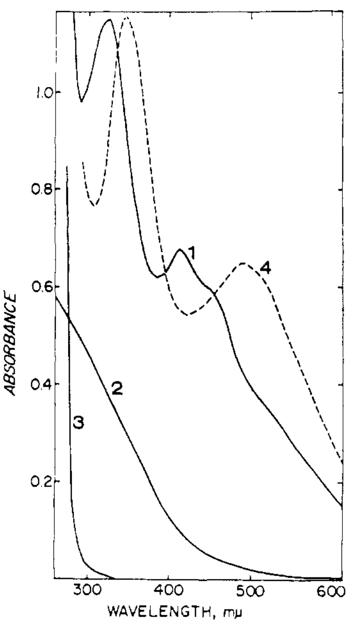

FIGURE 1: Absorption spectrum of the model iron–sulfur complex. Curve 1, $FeCl_3$ (0.16 mM), mercaptoethanol (44 mM), and Na_2S (0.2 mM) at pH 9; curve 2, $FeCl_3$ (0.16 mM), immediately after adjusting to pH 9; curve 3, same as curve 1 except for the omission of $FeCl_3$; and curve 4, same as curve 1 except for the omission of Na_2S. Curves 1 and 4 were obtained by extrapolating the time-dependent spectra to zero time.

(Vallee and Williams, 1968). Recently, we have shown that a complex prepared from $FeCl_3$, mercaptoethanol, and Na_2S closely resembles the nonheme iron proteins in its absorption characteristics and in its equimolar content of iron and inorganic sulfide (Yang, 1969; Yang and Huennekens, 1969). The present communication presents additional information about this "model complex" and discusses its possible relevance to the structure of nonheme iron proteins.

Experimental Section

Materials. Chemicals were obtained from the following sources: ferric chloride and ferrous chloride from Mallinckrodt Chemical Works; ferric ammonium sulfate and ferrous ammonium sulfate from Fisher Scientific Co.; 2-mercaptoethanol, 2-hydroxyethyl disulfide, 1-propanethiol, 2-thiophenemethanol, and 2,2′-thiodiethanol from Aldrich Chemical Co.; L-cysteine·HCl and dithiothreitol from Calbiochem Co.; 2-mercaptoethylamine from Sigma Chemical Co.; 2-aminothiazole from Matheson Co.; sodum sulfide from Allied Chemical Co.; and thioglycolic acid from Eastman Co.

Solutions of ferric and ferrous salts were freshly prepared with glass-distilled water; if necessary, HCl was added to prevent the formation of the hydroxides. Na_2S solutions (0.01 M in 0.01 M NaOH) were prepared under oxygen-free conditions; the amount of HS^- present was determined by I_2 titration. Solutions of other reagents used in anaerobic experiments were also prepared under oxygen-free conditions.

Methods. In general, formation of the model complex was initiated by mixing 2-mercaptoethanol, Na_2S, and the Fe^{3+} or Fe^{2+} salt in final concentrations of 42, 0.2, and 0.16 mM, respectively, at pH 9; the pH was obtained by the addition of 1 N NaOH. Anaerobic experiments were carried out in Thunberg-type cuvets in which 2-mercaptoethanol and Na_2S were placed in the cell and the iron solution was present in the side arm. The cuvet was evacuated and flushed with argon for at least six cycles before the solutions were mixed. Absorption spectra were measured with a Cary recording spectrophotometer, Model 14. Oxygen uptakes were measured at 30° with a GME Oxygraph, Model KM, equipped with a Clark electrode. Electron paramagnetic resonance experiments were performed with a Varian electron paramagnetic resonance spectrometer, Model E3.

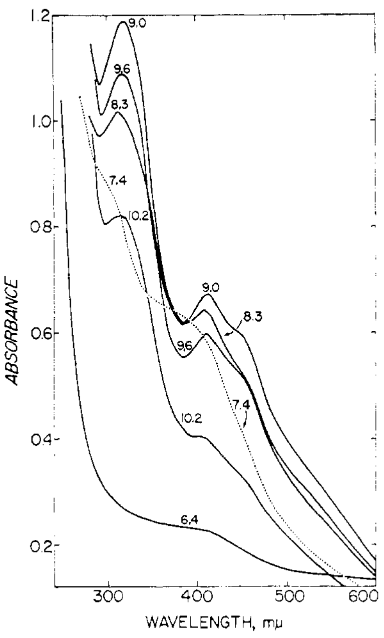

FIGURE 2: pH dependence of model complex formation. Conditions as in curve 1 of Figure 1 with NaOH used to adjust the pH to the values indicated by each curve. For convenience, the spectrum at pH 6.4 was moved upward by 0.1 absorbance unit.

Results

Formation of the Model Complex. When $FeCl_3$ is admixed with an equal (or slightly higher) concentration of Na_2S and an excess of mercaptoethanol at pH 9, a brownish-yellow, soluble complex is formed. The absorption spectrum of this complex (curve 1, Figure 1) has distinct bands at 325 and 412 mμ and a shoulder at 450 mμ. On the assumption (to be discussed subsequently) that all of the iron has been converted into the complex, extinction coefficients for the latter at 325 and 412 mμ have been calculated to be 7.2×10^3 and 4.2×10^3 cm^{-1}, respectively, per g-atom of iron (Yang and Huennekens, 1969).

Complex formation is indicated by complete solubility of the system at an alkaline pH where iron salts would ordinarily precipitate, and by the fact that curve 1 in Figure 1 cannot be accounted for by the sum of the absorbances of the individual components (curves 2 and 3). Curve 4, shown for comparison, is the spectrum of the spectrum of the iron–mercaptoethanol complex formed in the *absence* of Na_2S. The latter complex has its absorption bands at 345 and 480 mμ with extinction coefficients approximately 7×10^3 and

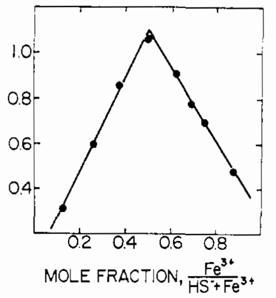

FIGURE 3: Dependence of model complex formation on the mole fractions of iron and inorganic sulfide. The sum of the $FeCl_3$ and Na_2S concentrations was fixed at 0.5 mM and the solution also contained 44 mM mercaptoethanol at pH 9.1.

4×10^3 cm^{-1} per g-atom of iron. The extreme instability of the iron-mercaptoethanol complex ($t_{1/2} \leqq 1$ min) further distinguishes it from the model complex.

Formation of the model complex is markedly dependent upon the pH at which the components are mixed (Figure 2). Complex formation was maximal at pH 9.0; at higher or lower pH values, the curves were characterized by decreased absorbances.

Composition of the Model Complex. The ratio of inorganic sulfide to iron in the complex was determined by the method of continuous variations (Job, 1928). Complex formation (based upon absorbance at 412 mμ) was measured under conditions in which the mole fractions of these two components were varied (Figure 3). Maximal complex formation occurred when the mole fraction of each component was 0.5, indicating that the ratio of iron to sulfide in the complex is 1:1.

The following sulfur-containing compounds were examined for their ability to replace mercaptoethanol as a component of the model complex: 1-propanethiol, 2-mercaptoethylamine, cysteine, thioglycolic acid, dithiothreitol, 2,2′-dithioethanol, 2-thiophenemethanol, and 2-aminothiazole. When admixed with Na_2S and $FeCl_3$ at pH 9, some of these compounds formed colored complexes, but in no case was the desired spectrum obtained (*i.e.*, λ_{max} at 315–335, 410–420, and 450–460 mμ).

Stability of the Model Complex. Although under appropriate conditions it can be maintained at a high steady-state level, the model complex is inherently unstable. This is especially noticeable in the *absence* of oxygen. Spectral changes observed when the complex was allowed to stand under anaerobic conditions are shown in Figure 4. Although the decrease in absorbance at 412 mμ followed first-order kinetics, the complete absorption spectrum changed in a more complicated manner. The initial maximum at 325 mμ[2] shifted to 305 mμ, accompanied by a decrease in absorbance. Some indication of the nature of this transformation is provided by the fact that a complex characterized by an absorption maximum at 305 mμ can be produced by admixing Fe^{2+} with mercaptoethanol under anaerobic conditions; addition of Na_2S does not alter the spectrum of this latter complex. Further substantiating this point, it was found that, upon admitting air to the sample that had stood for 700 min under anaerobic conditions, the spectrum of the original model complex was regenerated. This cycle could be repeated until all the mercaptoethanol had become oxidized. A similar pattern of formation and decay of the model complex was observed under aerobic conditions, except for differences in the time sequence caused by the amount of dissolved oxygen present in the solution.

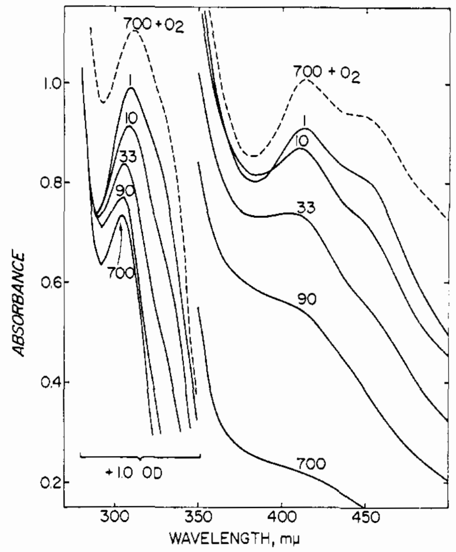

FIGURE 4: Stability of the model complex under anaerobic conditions. The solution contained mercaptoethanol (87 mM), Na_2S (0.3 mM), and $FeCl_3$ (0.29 mM) at pH 9.1. The numbers on each spectrum indicate minutes after mixing the reagents. The dotted line is the spectrum obtained after aeration of the 700-min solution. For convenience, spectra below 350 mμ have been increased by 1.0 absorbance unit.

Oxygen Uptake Measurements. In the above experiments, decomposition of the model complex and its reformation upon aeration suggested that the overall reaction involved oxidation of mercaptoethanol by molecular oxygen. This would be consistent with the well-known ability of metal ions to catalyze the oxidation of thiols (Cecil and McPhee, 1959). In order to investigate this reaction further, oxygen uptake was measured at pH 9 in systems containing mercaptoethanol in the presence of Fe^{3+} alone, or Fe^{3+} plus Na_2S. In the presence of excess mercaptoethanol, both reactions followed first-order kinetics with respect to oxygen concentration (Figure 5). However, in the reaction catalyzed by Fe^{3+} alone (curve 1), there was a brief, rapid uptake of oxygen followed by the establishment of a slower rate. The initial phase of this curve was even more distinct with higher concentrations of Fe^{3+}. In contrast, the system containing both Fe^{3+} and Na_2S consumed oxygen at a much faster rate and did not show the biphasic phenomenon.

The effect of concentration of each of the two catalysts upon the rate of O_2 uptake is shown in Figure 6. The rate of O_2 consumption increased gradually with increasing concentration of Fe^{3+}, while in the Fe^{3+}–Na_2S system the curve is concave upward. It is apparent from these data that, under the conditions used for the experiments described in

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[2] In these experiments, the recording spectrophotometer required 2 to 3 min to scan from 600 to 300 mμ. For this reason, the maximum of this peak is generally recorded slightly below 325 mμ [*e.g.*, a value of 318 mμ was reported in our previous communication (Yang and Huennekens, 1969)].

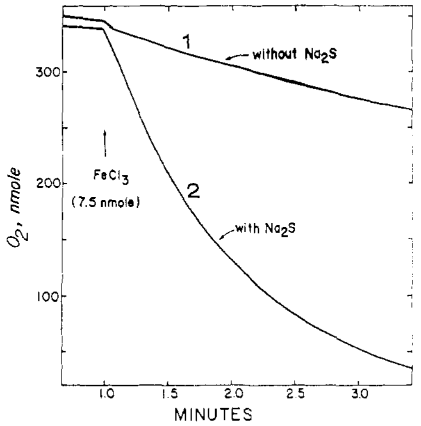


FIGURE 5: O_2 uptake of the iron complexes. Reactions were started by adding 7.5 nmoles (in 10 ml) of $FeCl_3$ into reaction mixtures (1.6 ml) containing 30 μmoles of mercaptoethanol and 12 nmoles of Na_2S (in curve 2 only). Initial amount of O_2 was approximately 350 nmoles. The reaction was carried out at 30° and pH 9.

previous sections (0.16 to 0.3 mM of Fe^{3+} + Na_2S), most of the O_2 in solution would have been consumed instantaneously. Subsequent diffusion of O_2 into the system is rather slow, unless the vessel is shaken vigorously in air.

Electron Paramagnetic Resonance Studies. As shown in Figure 7, a solution of the model complex revealed a strong signal at $g = 4.1$, which is attributed to the complex itself, and weaker signals in the region of $g = 2.0$, due probably to some minor constituent. Because of their possible relevance to the electron spin resonance spectra of nonheme iron proteins, the latter signals were studied further with respect to microwave power saturation and temperature changes. As shown in Figure 8, both signals ($g = 2.01$ and 1.96) increased in a parallel manner with increasing microwave power and power saturation was not apparent under the conditions used. Likewise, both signals responded similarly (in terms of decreased amplitude and resolution) to increases in temperature (Figure 9).

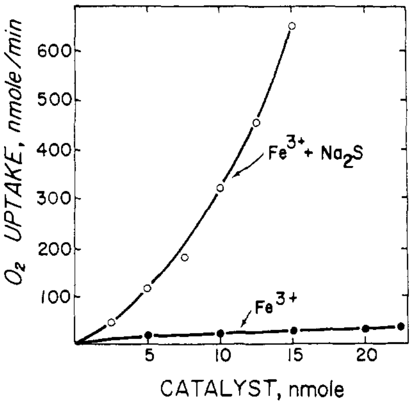


FIGURE 6: Dependence of the rate of O_2 uptake on the concentration of the iron complexes. Conditions are the same as in Figure 5, except that amounts of $FeCl_3$ and Na_2S were varied as indicated. Initial rates were used for both systems, neglecting the initial phase of the Fe^{3+} system.

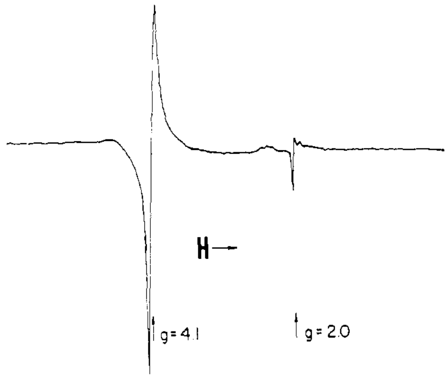


FIGURE 7: Electron paramagnetic resonance spectrum of the model complex system at 108°K. Concentration of the complex, 5 mM; microwave power, 160 mW; modulation amplitude, 12.5 G; and time constant, 1 sec.

Discussion

The present model complex, prepared from Fe^{3+}, mercaptoethanol, and Na_2S, has an absorption spectrum (λ_{max} at 325 and 412 mμ) closely resembling those of a number of nonheme iron proteins (San Pietro, 1965; Beinert, 1966; Rajagopalan and Handler, 1968; Kimura, 1968). Extinction coefficients (expressed per gram atom of iron) for peaks in these regions of the spectrum are in the range of 9×10^3 to 10×10^3 and 5×10^3 to 6×10^3, respectively, for most nonheme iron proteins (Beinert, 1966), although values as low as 6.0×10^3 and 3.9×10^3 have been reported for spinach ferredoxin (Tagawa and Arnon, 1968). The corresponding extinction coefficients of 7.2×10^3 and 4.2×10^3 for the model complex (Yang and Huennekens, 1969) are comparable with the above values. In the region above 430 mμ, the complex is also spectrally similar to some of the nonheme iron proteins although, with the latter, the bands at 450–460 mμ and the shoulders near 550 mμ are generally more sharply defined.

Although the possibility of electron delocalization makes the assignment of a valence state for iron alone somewhat arbitrary, several lines of evidence suggest that in the model complex the iron may be considered to be in the Fe^{3+} state: (a) the similarity of its spectrum to those of the above nonheme iron proteins which are believed to be in the oxidized state; (b) its electron paramagnetic resonance signal at $g = 4.1$; (c) inability of Fe^{2+} to form the complex under anaerobic conditions; (d) transformation of the complex into a presumed Fe^{2+} complex upon standing; and (e) regeneration of the model complex upon aeration of the Fe^{2+} complex.

Because of its general instability, most of the structural features of the complex have had to be deduced indirectly. A *1:1 ratio* of inorganic sulfide to iron in the complex was established by the method of continuous variations (Figure 3). The fact that inorganic sulfide competes so successfully with much greater concentrations of mercaptoethanol suggests that it has a high affinity for iron. At pH 9, inorganic sulfide is present as HS^-, but it is possible that the proton may be displaced when binding to the iron occurs.

Studies with analogs of mercaptoethanol have suggested that *both* the thiol and hydroxyl groups are involved in

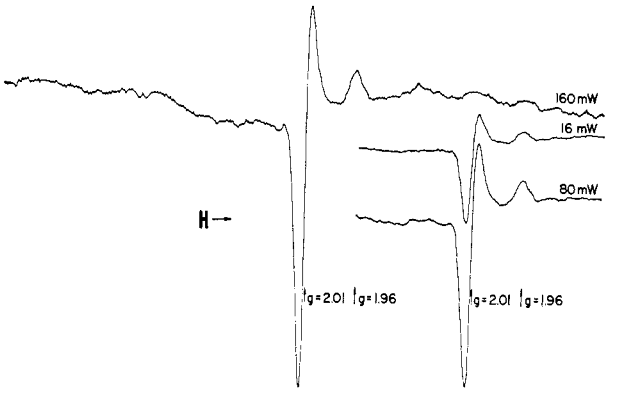


FIGURE 8: Dependence of electron paramagnetic resonance signals on microwave power. Conditions as in Figure 7, except for variations in microwave power as indicated. For convenience, two of the spectra have been offset.

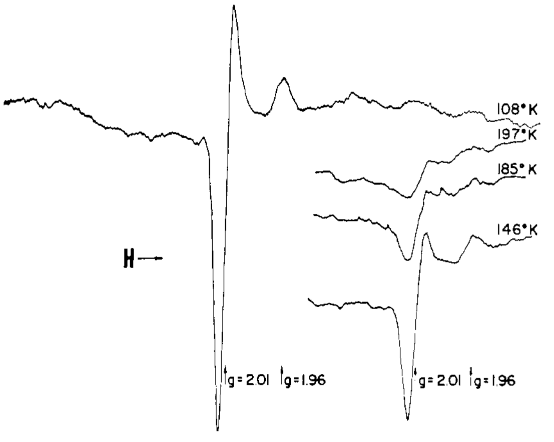
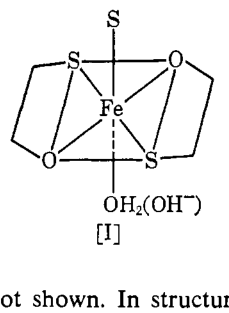


FIGURE 9: Temperature dependence of electron paramagnetic resonance signals. Conditions as in Figure 7, except for variations in temperature as indicated. For convenience, three of the spectra have been offset.

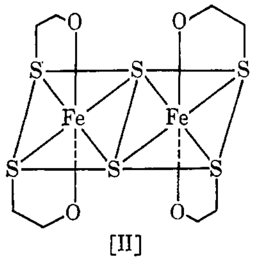
chelation of the iron. This would lead to the formation of a 5-membered chelate ring or, since a large excess of mercaptoethanol is present, possibly two rings in an octahedral complex. The mercaptide ion is known to form strong ligands to iron (Cecil and McPhee, 1959) and the involvement of an aliphatic hydroxyl group has been suggested in the chelation of Fe^{3+} by *N,N*-dihydroxyethylglycine (Chaberek *et al.*, 1953; Toren and Kolthoff, 1955). It is not known, however, whether the proton of the ethanolic group is displaced by iron in the present model complex. Attempts to establish this point (and also whether a proton is released from HS^-) by direct titration have been hampered by the necessity of maintaining a high concentration of mercaptoethanol in the system.

Inorganic sulfide and the thiol group of mercaptoethanol are believed to be independent ligands, rather than existing as the persulfide structure suggested by Miller and Massey (1965) for the "labile sulfide" in nonheme iron proteins. A persulfide structure for the model complex would involve a 6-membered ring which is less favorable thermodynamically than a 5-membered one. Moreover, the mercaptide ion would certainly be expected to have a higher affinity for the trivalent iron than for the negatively charged sulfur atom of inorganic sulfide.

Possible mononuclear and binuclear octahedral structures for the model complex are shown below. For clarity, and since they may possibly have been displaced during complex formation, the H atoms of HS^- and the hydroxyl groups are not shown. In structure [I] the bottom ligand could be H_2O or OH^-. Binuclear structures similar to [II] (except for hydroxyl bridges) have been postulated for Fe^{3+} complexes in aqueous solution (Schugar *et al.*, 1967, 1969) and for iron thioglycolate (Michaelis and Schubert, 1930; Martell and Calvin, 1952). By analogy, HS^- (or S^{2-}) could also serve as the bridging ligands and, indeed, ethyl mercaptide bridges have recently been shown by X-ray diffraction to occur in a Fe^{3+}–thioxanthate complex (Coucouvanis *et al.*, 1969). Dimeric or polymeric forms of [I] *via* S–S (inorganic sulfur) bridges are also possible.

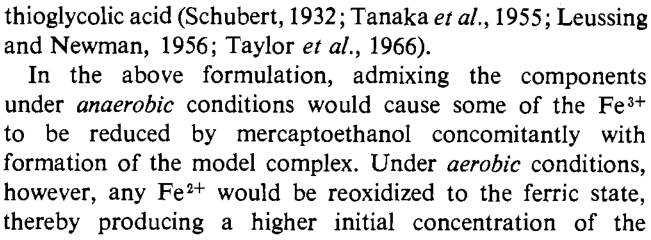


[I]



[II]

Complexes represented by structures [I] or [II] would not be expected to be stable chemically because of the facile reduction of Fe^{3+} by the thiol group. A possible route for the formation, decay, and re-formation of the Fe^{3+} complex is summarized below. Similar sequences have been postulated previously for the iron-catalyzed oxidation of cysteine and thioglycolic acid (Schubert, 1932; Tanaka *et al.*, 1955; Leussing and Newman, 1956; Taylor *et al.*, 1966).



In the above formulation, admixing the components under *anaerobic* conditions would cause some of the Fe^{3+} to be reduced by mercaptoethanol concomitantly with formation of the model complex. Under *aerobic* conditions, however, any Fe^{2+} would be reoxidized to the ferric state, thereby producing a higher initial concentration of the complex. Repeated aeration of the system gradually increases the absorbances in the 412- and 320-mμ regions owing to the accumulation of the oxidized product, 2-hydroxyethyl disulfide. The contribution of the latter to the peak absorbances can be estimated by its effect upon the adjacent troughs at 383 and 290 mμ (*cf.* Figure 4). It should be noted that high concentrations of the disulfide inhibit complex formation.

The spectral changes in Figure 4 can be attributed mainly to three factors: (a) disappearance of the (Fe^{3+}) model complex, which is accompanied by a decrease in the 412- and 325-mμ bands; (b) appearance of the Fe^{2+} complex with its absorption band at 305 mμ; and (c) accumulation of 2-hydroxyethyl disulfide which, in addition to its inherent absorbance, may bind iron and thus create an additional absorbing species. These factors complicate kinetic studies on the formation and disappearance of the model complex and related entities.

The above type of mechanism also appears to be operative even in the absence of HS$^-$. Under such conditions, the Fe^{3+}–mercaptoethanol complex (absorption maxima at 345 and 480 mμ) is less stable than the model complex, having a half-life of about 1 min as compared with about 100 min for the model. This demonstrates the stabilization of the bond linking Fe^{3+} to the sulfur of mercaptoethanol that results when HS$^-$ is also a ligand to the iron.

The pronounced effect of the HS$^-$ ligand on the iron complex is shown further in the O$_2$-uptake experiments (*cf.* Figures 5 and 6). If reduction of O$_2$ occurs only *via* the mechanism shown in the above scheme, the overall rate should be determined by the slowest step, which is most probably transformation of the Fe^{3+} complex to the Fe^{2+} complex (*cf.* Figure 4). An estimate of the rate of O$_2$ uptake of the two systems, based upon the above assumptions, can be made from the first-order decay rates of the corresponding ferric complexes. From spectrophotometric experiments similar to that shown in Figure 4, it can be calculated that 10 nmoles of the model complex or the Fe^{3+} complex should be able to reduce 0.7 and 6 nmoles of O$_2$ per min, respectively. The observed rates, however, are 320 and 24 nmoles per min (*cf.* Figure 6). Similarly, at the concentration (160 μM) of the model complex present in Figure 1, one-half of the O$_2$ in solution should be consumed in about 10 min. However, this event occurred too rapidly even to be followed on the Oxygraph.

These observations, as well as the first-order kinetics shown in Figure 5, suggested (especially for the model complex system) that other mechanisms besides the above scheme might account for O$_2$ reduction. The model complex may be able to activate O$_2$ very efficiently by bonding the O$_2$ directly to the iron (*e.g.*, by displacement of the bottom ligand in structure I or an ethanolic group in structure II). The accelerated rates of oxygen uptake at higher concentrations of the model complex (*cf.*, the concave upward curve in Figure 6) suggest the possible involvement of a dimeric or polymeric species (*e.g.*, a binuclear complex similar to II). Complexes involving Fe^{2+}–O$_2$ and Fe^{3+}–O$_2$ linkages have been postulated by Michaelis (1951) and by Sutin (1965). Activated species such as $\cdot O_2^-$ and $\cdot SR^-$ have also been postulated as intermediates in the iron-catalyzed oxidation of thioglycolate (Lamfrom and Nielsen, 1957). However, no direct evidence for such types of O$_2$ activation was obtained in the present study. The detailed kinetics and mechanism of the model complex-catalyzed reduction of O$_2$ remain to be investigated.

The electron paramagnetic resonance studies shown in Figures 8 and 9 suggest that the g = 2.01 and 1.96 signals are due to a single species. Since it accounts for only a small fraction of the iron present (*cf.* Figure 7), this species is probably a transient intermediate (containing an unpaired electron bound to an iron–sulfur complex) that is not shown in the above scheme. Electron paramagnetic resonance studies at liquid helium temperatures and with isotope substitutions might provide further insight into the nature of this unknown species. The presently observed signals ($g_\perp = 2.01$ and $g_\parallel = 1.96$) differ from those of the reduced nonheme iron proteins in having an average g value above 2 and a higher value for $g_\perp$ than for $g_\parallel$. In this respect, it is interesting to note that an inverse order of the g values ($g_\perp > g_\parallel$) was also observed for the pentacyanonitrosyl ferrate model of Beinert *et al.* (1965). The present model also differs from the iron–sulfur complex of nonheme iron proteins in its stability. Thus, with regard to two criteria (electron paramagnetic resonance signals and stability), it is not a completely adequate model for the naturally occurring entities. In other respects, however, it appears to be the best model complex presently available. It reproduces the light absorption features of the nonheme iron chromophore and provides an experimental basis for interpreting the spectra of these naturally occurring electron carriers. The present model, together with the artificial nonheme iron protein models described earlier (Yang and Huennekens, 1969), delineate the minimal structural requirements for the iron–sulfur chromophore in the naturally occurring nonheme iron proteins. The demonstration that HS$^-$ is bound to the iron in a ratio of 1:1 in the model complex provides further support for the belief that the labile sulfide of nonheme iron proteins does not originate from cysteine residues (reviewed by Kimura, 1968). Information regarding the reactivity of the present model system with O$_2$ may also be applicable to certain flavoproteins that contain nonheme iron. It has been shown previously that treatment of a mitochondrial DPNH dehydrogenase with *o*-phenanthroline (Hatefi *et al.*, 1969) or by incubation at 38° and pH 4.8 (Hatefi, 1968) causes a parallel decline in catalytic activity and labile sulfide content. However, even in the absence of iron chelator and at neutral pH, aging of DPNH dehydrogenase[3] and succinic dehydrogenase (Der Vartanian *et al.*, 1966) decrease the enzymatic activities in a manner roughly parallel to the loss of absorbance due to the iron–sulfur chromophore. The rate of this aging inactivation was reduced significantly under anaerobic conditions. These results suggest that destruction is referable to oxidation of protein sulfhydryl groups bound to iron which, in turn, leads to destruction of the nonheme iron complex. In the DPNH dehydrogenase this type of damage can be repaired (as evidenced[3] by restoration of part of the catalytic activity) when the O$_2$-inactivated enzyme is treated with Fe^{3+}, mercaptoethanol, and Na$_2$S.

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