MODEL SYSTEMS FOR THE IRON-SULFUR CHROMOPHORE OF NONHEME IRON PROTEINS

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Received April 21, 1969

Although a number of nonheme iron proteins* have been isolated and studied extensively (reviewed by Beinert, 1966 and Kimura, 1968), there is still considerable uncertainty regarding the structure of the iron-sulfur complexes responsible for their absorption spectra and oxidation-reduction properties. Acidification of these proteins destroys the iron-sulfur complex although, in the case of ferredoxin and putidaredoxin, treatment of the resulting apoprotein with 2-mercaptoethanol, Na_2S and Fe(II) restores both the chemical composition and the enzymatic activity (Hong and Rabinowitz, 1967; Tsibris, et al., 1968). Similarly, an artificial nonheme iron protein having a ferredoxin-like absorption spectrum has been prepared by treating bovine serum albumin with the above chemical agents (Suzuki and Kimura, 1967; Lovenberg and McCarthy, 1968).

The present communication provides further information about the nature of the iron-sulfur chromophore in the nonheme iron proteins that has been obtained from: (a) models prepared from Fe(III), inorganic sulfide and proteins having lower molecular weights and fewer cysteine residues than

*In this paper, "nonheme iron proteins" refer to those that also contain "labile sulfide."

bovine serum albumin; and (b) a "minimal model" in which the protein has been replaced by mercaptoethanol. Additional information about the latter complex will be reported elsewhere (Yang and Huennekens, in preparation).

Soybean trypsin inhibitor, which consists of a single polypeptide chain (MW = 21,500) containing only two disulfide linkages, was found to be one of the better proteins for model studies. The complex was prepared as follows: 20 mg of the protein was incubated for 15 min at 37° with 45 μmoles of dithiothreitol (or 700 μmoles of mercaptoethanol) and 4 μmoles each of Na_2S and $FeCl_3$ in 1.5 ml of 0.1 M Tris-HCl buffer, pH 7.5. The resulting artificial nonheme iron protein was then separated from the other reagents by filtration through a Sephadex G-25 column using 0.01 M Tris-HCl buffer, pH 7.5, as the eluant. Similar nonheme iron proteins were also prepared by the above procedure using ribonuclease, lysozyme or chymotrypsinogen, except that in those instances, 4 M urea had to be added to prevent precipitation of the reduced proteins.

Absorption spectra of the nonheme iron proteins prepared from soybean trypsin inhibitor and ribonuclease are shown in Fig. 1. These spectra are similar to those of the bovine serum albumin model and to the naturally-occurring nonheme iron proteins with respect to the characteristic absorbances in the 315-335, the 410-420 and the 450-460 mμ regions. Extinction coefficients at 318 and 415 mμ for the artificial nonheme iron proteins (summarized in Table I) are similar in magnitude to those of spinach ferredoxin (ϵ_{mM} = 6.0 and 3.9 at 325 and 420 mμ; Tagawa and Arnon, 1968).

The soybean trypsin inhibitor complexes had half-lives of 10 to 30 minutes at room temperature, as judged by the decrease in absorbance at 415 mμ. Despite this instability, the data in Table I clearly show the iron to inorganic sulfide ratio in these model systems was always near unity, even

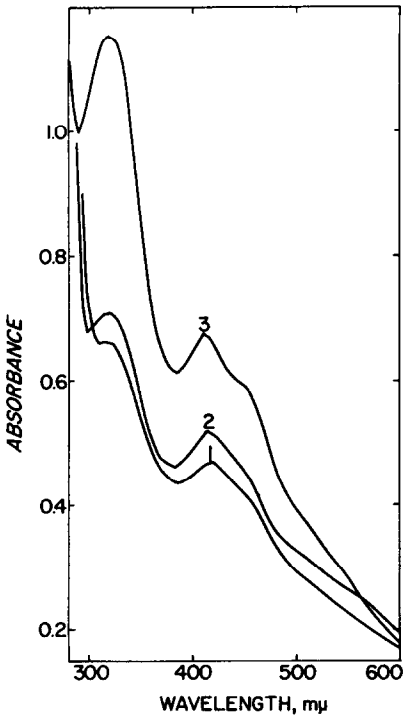

Fig. 1. Absorption spectra of the model complexes. Curve 1, soybean trypsin inhibitor (51 μM); Curve 2, ribonuclease (173 μM); Curve 3, mercaptoethanol. The iron-sulfur concentrations in these models were 112, 114 and 160 μM, respectively.

though the quantity of the iron-sulfur complex per mole of protein varied with the reducing agent used (cf. preparations A and C from soybean trypsin inhibitor). Involvement of protein sulfhydryl groups in the binding of the iron was suggested by the observation that carboxymethylation of the reduced protein prevented complex formation. Since there are 4 cysteine residues per molecule of reduced soybean trypsin inhibitor, it is of interest that preparation B in Table I contained, per mole of protein, 4 gram atoms of nonheme iron and 4 moles of inorganic sulfide. Thus, inorganic sulfide and a sulfhydryl group on the protein provide two of the ligands to the iron while the other ligands, which may vary from model to model, appear to have little influence upon the absorption spectrum of the iron-sulfur chromophore.

The facile formation of these complexes suggested that the protein might be replaced by a simple organic molecule containing a sulfhydryl group

Table I

Properties of the Model Complexes

Component of the Complex*	Nonheme Iron	Inorganic Sulfide	ϵ mM	
	g atoms/mole protein	moles/mole protein	318 mμ	415 mμ
Soybean trypsin inhibitor‡, Prep. A	2.3	2.0	5.9	4.2
Ditto, Prep. B	4.1	3.9	5.8	4.1
Ditto, Prep. C	1.5	1.4	6.0	3.9
Ribonuclease	0.69	0.63	6.2	4.6
Mercaptoethanol	1:1 ratio		7.2	4.2 (at 412 mμ)

*In addition to $FeCl_3$ and Na_2S.

‡In preparations A and B, the protein was reduced with dithiothreitol before treatment with $FeCl_3$ and Na_2S; in preparation C, mercaptoethanol was used. Preparations A and C were obtained after filtration of the mixture through Sephadex G-25. Preparation C was one of the fractions obtained when the nonheme iron protein complex was resolved by passage through Sephadex G-100.

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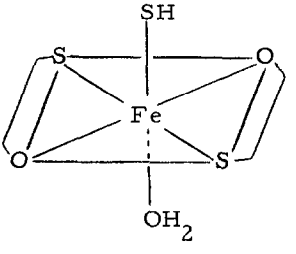
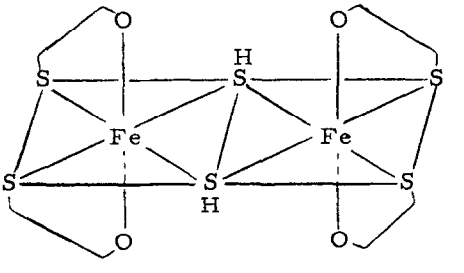
and perhaps other potential ligands to the iron. Mercaptoethanol proved to be ideal for this purpose since, when it was admixed with $FeCl_3$ and Na_2S (final concentrations: 42, 0.16 and 0.16 mM, respectively) at pH 9, a soluble complex was formed whose spectrum (cf. Fig. 1) was similar to those of the nonheme iron proteins. Assuming complete complex formation, the extinction coefficients of this model at 318 and 412 mμ are calculated to be 7.2 and 4.2 x 10^3, respectively, per gram atom of iron.

As in the case of the protein models, the exact composition of this non-protein complex could not be determined because of its instability. However, certain of its structural features were deduced indirectly. A 1:1 ratio of iron to inorganic sulfide in the complex was established by the method of continuous variations (Job, 1928). The thiol group of mercaptoethanol and the inorganic sulfide are believed to be independent ligands, rather than in the form of a persulfide structure such as suggested by Miller and Massey (1965). Under anaerobic conditions the complex could be prepared only from ferric salts but, in the presence of oxygen, ferrous salts could also be used. The 318 and 412 mμ complex was not formed when mercaptoethanol was replaced by 1-propanethiol, 2,2′-thiodiethanol, cysteine or dithiothreitol. This suggests that both the thiol and hydroxyl groups of mercaptoethanol are involved in iron binding (with the formation of a 5-membered ring). The presence of two mercaptoethanols per atom of iron would be expected to confer added thermodynamic stability to the complex. Possible mononuclear and binuclear octahedral structures for the complex are shown below.

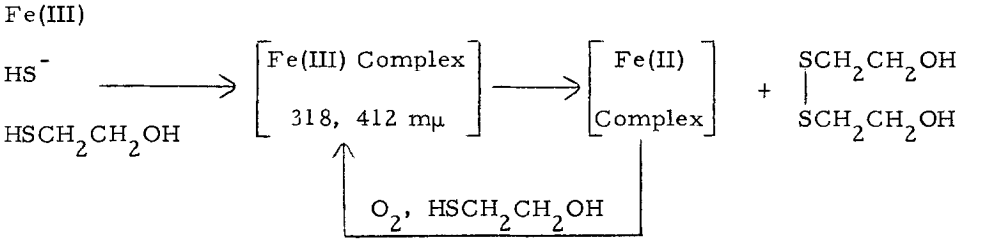




Dimeric structures involving sulfur or oxygen as bridge atoms have been suggested previously for other iron complexes (Schugar, et al., 1967; Coucouvanis, et al., 1968). Polymeric structures seem unlikely, since the complex was stable to dilution.

Attempts to isolate this "minimal model" complex using solvent

precipitation, column chromatography and electrophoresis have not been successful, since removal of the excess mercaptoethanol resulted in destruction of the complex. Even in the presence of excess mercaptoethanol, the absorption bands at 318 and 412 mμ decreased with time, but aeration regenerated the complex; this cycle could be repeated until all of the mercaptoethanol had become oxidized. These observations are consistent with the following scheme:



In the model complex characterized by absorbance bands at 318 and 412 mμ, the iron is believed to be in the Fe(III) state. Loss of this characteristic spectrum is thought to be due to an intramolecular electron transfer from thiol to iron. Additional complexes undoubtedly occur in this cyclic process, but when both mercaptoethanol and oxygen are present in excess, the ferric complex can be maintained at a high steady-state concentration.

When solutions containing the above complex were examined at 110° K, an EPR signal was seen at g = 4.1, probably due to a high-spin ferric complex. There was also a weaker signal in the central field ($g_{\perp}$ = 2.01 and $g_{/\!/}$ = 1.96), which appeared to result from a transient iron-sulfur complex containing a delocalized unpaired electron.

Based upon spectroscopic criteria, a similar type of iron-sulfur complex appears to be involved in both the artificial nonheme iron proteins and in the mercaptoethanol model. In the former case, the proteins provide polydentate ligands to the iron and the resulting complexes are sufficiently

stable to be isolated by gel filtration. However, formation of these artificial nonheme iron proteins is a rather non-specific process with respect to the thiol used to reduce disulfide bonds in the protein and the initial oxidation state of the iron when it is mixed with the protein. The characteristic chromophore with absorption bands in the 315-335 and 410-420 mμ regions is observed when one ligand to the iron is a sulfhydryl group and another is inorganic sulfide. The remaining coordination sites of the iron can be occupied by a variety of other ligands which contribute to the thermodynamic stability and, possibly, to the oxidation-reduction properties.

ACKNOWLEDGMENTS

This work was supported by grants from the American Heart Association (66-796) and the National Cancer Institute, National Institutes of Health. We are indebted to Dr. Alan Bearden for his advice on the ESR measurements.

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