III. 5. RELATION OF IRON TO SULFHYDRYL GROUPS IN FERRITIN

Abraham Mazur and Saul Green

Department of Medicine, Cornell University Medical College
and The New York Hospital, New York City

I. Chemical Properties of Ferritin

Before presenting our data, which suggest a relationship between sulfhydryl groups and some of the iron in ferritin, it would be helpful to review briefly some of the important chemical properties of this unusual protein.

1. In solution, ferritin can be heated to 80° without denaturation; it retains its solubility and can still be crystallized by the addition of $CdSO_4$ (*1*).

2. In the ultracentrifuge, ferritin appears as a mixture of molecules of varying size and color. Some 20% of this mixture is a colorless protein, containing no iron, and has a molecular weight of 465,000. The remainder consists of a series of molecules increasing in total iron content as well as apparent molecular size (*2*).

3. The iron in ferritin can be removed by treatment with hydrosulfite at pH 4.6 and dialysis in the presence of α,α'-dipyridyl. The colorless protein can be crystallized with $CdSO_4$ and corresponds to the colorless protein fraction, called apoferritin, observed in the ultracentrifuge (*1*).

4. Ferritin, despite its inhomogeneity with respect to iron content, moves as a single component during electrophoresis and has mobilities identical with that of apoferritin over a range of pH from 4 to about 8. Ferritin and apoferritin also behave in an identical manner when examined for viscosity and for the manner in which they react quantitatively with specific antibody prepared against either protein. On the basis of

these similarities, in experiments which essentially test surface properties, we concluded that the bulk of the iron in ferritin is situated within the protein molecule (*3*). This has been confirmed by electron microscopic studies reported by Farrant (*4*).

II. Presence of Noncolloidal Iron in Ferritin

Although the bulk of ferritin iron is tightly bound and unavailable for reaction with iron-binding compounds, a small portion of its iron appears to be present in a state which makes it available for chemical reaction at neutral pH. It can catalyze the oxidation of adrenaline (*5*), it can react with α,α'-dipyridyl thus indicating the presence of some ferrous iron, and it can be reduced to the ferrous state by treatment with mild reducing agents at neutral pH. The ability of this small quantity of ferrous iron to dissociate from the protein is seen by the increasing quantity of ferrous-dipyridyl complex formed as the ferritin solution is diluted or as the pH is made more acid. It appears, therefore, that in addition to the colloidal micelles of iron hydroxide—iron phosphate which make up the bulk of its iron, ferritin also contains some iron in the ionizable state, at or near the surface of the protein.

One can see some of the relationships of this ionizable iron by analysis of ferritin fractions obtained by high-speed centrifugation or by serial precipitation with increasing concentrations of ammonium sulfate, followed by extensive dialysis to remove the salt. The centrifugal method, if carried out for a sufficient time period (2 hours at 30,000 r.p.m.) yields a brown pellet which has an extremely high content of iron and which does not completely redissolve. The top fraction is the lightest in color and has the least iron. During the ammonium sulfate fractionation, the earliest precipitate contains the most iron. When such fractions are dialyzed and adjusted to equal concentrations of total nitrogen one obtains the results shown in Table I. It can be seen that the ratio of ferrous iron to total iron

TABLE I

RELATIONSHIP OF TOTAL IRON AND FERROUS IRON TO FERRITIN PROTEIN

Fraction	Total Iron: Total N (μmoles/mmole)	Ferrous Iron: Total N (μmoles/mmole)	Ferrous Iron: Total Iron (μmoles/mmole)
Original	454	0.8	1.7
A(0-27)	549	0.7	1.2
B(27-31)	454	0.6	1.4
C(31-34)	361	0.6	1.6
D(34-50)	251	0.6	3.2

varies considerably; that fraction containing the most total iron contains the least ferrous iron. However, the ratio of ferrous iron to total nitrogen is relatively constant. This suggests that the ferrous iron is associated with specific chemical groupings at or near the surface of the protein molecule, in sharp contrast to the bulk of iron which varies in a manner independent of the protein.

III. Sulfhydryl Groups in Ferritin

During our early studies concerned with several biological properties of ferritin, we were able to establish that such activities were associated with the presence of sulfhydryl groups as well as with ferrous iron. Chemical treatment or treatment with liver slices which destroyed these biological activities resulted in a decrease in SH as well as ferrous iron content. Table II gives some data for the SH content of ferritin making use of the

TABLE II

SULFHYDRYL CONTENT OF FERRITIN (IODOACETAMIDE METHOD)

	SH Content[a]		SH Content[a]
Ferritin	27	Ferritin	31
Denatured with urea and alkali	55	Treated with liver slices in N_2	36
Treated with GSH and dialyzed	35	Treated with liver slices in O_2	17
Denatured after GSH treatment	64	Last prep. treated with liver slices in N_2	36

[a] SH = μmoles per 100 mg. total N.

reaction between ferritin and iodoacetamide, precipitation of ferritin and oxidation of the HI formed by H_2O_2 to free iodine (*6*). It can be seen that the SH content of ferritin is increased by denaturation. Treatment with reduced glutathione followed by extensive dialysis also appears to increase the SH content of ferritin. The results obtained with rat liver slices are self-explanatory, although they do not explain the nature of the oxidizing or reducing agents concerned.

When the SH groups of ferritin are blocked or oxidized, one notes a decrease in ferrous iron content. It should be noted that this apparent oxidation of ferrous iron takes place more readily than does the spontaneous oxidation which occurs when untreated ferritin solutions are stored. The results can be seen in Table III. They suggest that the ionizable surface ferric iron is bound to sulfhydryl groups in a tight ferric mercaptide linkage, making the SH groups less available for reaction with sulfhydryl

TABLE III

RELATIONSHIP OF SH GROUPS AND FERROUS IRON IN FERRITIN

	SH Content[a]	Ferrous Iron[b]
Ferritin	17	3
Treated with PCMB	0	1
Treated with iodoacetamide	0	1
Treated with iodosobenzoate	0	1
Ferritin	25	1.7
Treated with liver slices in N_2	34	6.5
Last prep. treated with liver slices in O_2	15	2.3

[a] SH = μmoles per 100 mg. total N.
[b] Fe = μmoles ferrous iron per mmole total Fe.

reagents. Reduction of ferric to ferrous iron would result in a liberation of SH groups.

Additional evidence for such a relationship is seen in Table IV which shows analytical data obtained by the ammonium sulfate fractionation method and measurement of SH content by reaction with iodoacetamide. The data in the second group were obtained with fractions separated by high-speed centrifugation. In this case a new method for measuring ferritin sulfhydryl groups was used. The ferritin was treated with *p*-chloromercuribenzoate (PCMB), the mixture subjected to centrifugation for 2 hours at 30,000 r.p.m. and the PCMB content of the clear, colorless supernatant solution determined spectrophotometrically. By comparing this value with that obtained with an equal quantity of PCMB in the absence of added ferritin one obtains a measure of SH groups in the protein.

The results show that the relationship between SH groups and protein nitrogen varies in a manner which suggests that the apoferritin-rich frac-

TABLE IV

RELATIONSHIP OF SH GROUPS AND FERROUS IRON IN FERRITIN FRACTIONS

	High-Speed Centrifugation		Ammonium Sulfate Precipitation	
	Total Iron[a]	SH[b]	Total Iron[a]	SH[b]
Original	0.46	4.2	0.46	4.1
Bottom	0.70	3.8	0.55	3.2
Middle	0.39	4.2	0.45	3.6
Top	0.04	5.2	0.25	5.1

[a] Total Fe = moles per mole total N.
[b] SH = μmoles per 100 mg. total N.

tion contains a somewhat greater quantity of SH groups. The possibility therefore exists that SH groups on the apoferritin surface are associated with the binding of iron.

The results of experiments in which Fe^{59} was injected intravenously into rats and the liver ferritin fractionated by high-speed centrifugation make it seem probable that apoferritin, the fraction of ferritin which contains little bound iron, is the fraction which is concerned with maximum binding of iron *in vivo*. The results, shown in Table V, indicate that the

TABLE V

INCORPORATION OF PLASMA Fe^{59} INTO FERRITIN FRACTIONS[a]

Fraction	1 hour	4 hours	1 day	8 days	15 days
Original	2.0	6.9	11.5	8.0	6.8
Bottom	1.5	6.7	10.8	8.6	6.4
Intermed.	3.0	14.0	14.0	8.1	6.6
Intermed.	4.5	25.3	14.5	7.4	7.7
Top	7.4	31.2	14.8	6.4	6.0

[a] Values are recorded in terms of specific activity (counts per minute per μg. ferritin iron).

earliest incorporation of iron into ferritin takes place to the greatest extent in the apoferritin-rich fraction. They also show that what was at first apoferritin is converted to an iron-rich fraction of the protein. Experimental data obtained by Fineberg and Greenberg (7) using C^{14}-labeled amino acids prove also that apoferritin is first synthesized, and then iron is inserted.

IV. Biological Reduction of Ferritin Iron

Although there are many compounds of biochemical importance which can reduce ferritin iron, we have established that the system responsible for ferritin iron reduction in the liver involves xanthine oxidase. I shall not go into the experimental observations which led us to this conclusion (*8*), but shall present data pertinent to our discussion.

Purified xanthine oxidase from milk or calf liver can reduce as much as 6% of ferritin iron to the ferrous state during the oxidation of hypoxanthine, xanthine, or purine by the enzyme. The conversion of ferric iron to ferrous iron releases the iron for combination with such iron-binding agents as α,α'-dipyridyl or the plasma iron-binding globulin. The reduction of ferritin ferric iron by the reduced enzyme takes place in the complete absence of oxygen, and somewhat less extensively in the presence of

oxygen. Iron reduction is completely inhibited by 2-amino-4-hydroxy-6-pteridine aldehyde, which also inhibits xanthine oxidase activity when measured in the presence of acceptors other than ferritin iron. That the reduction of ferritin iron by xanthine oxidase serves as a mechanism for the release of iron to the plasma is shown by the hyperferremia produced in animals after the intravenous administration of hypoxanthine, xanthine, or purine compounds, which act as substrates for xanthine oxidase.

V. Mechanism of Reduction of Ferritin Iron by Xanthine Oxidase

The possible sites on xanthine oxidase which may be involved in electron or hydrogen transfer are flavine adenine dinucleotide (FAD), nonheme iron, and molybdenum, all of which have been shown to be present in the crystalline enzyme (*9*). In addition, it should be noted that xanthine oxidase is inhibited by reagents which react with sulfhydryl groups and by cyanide.

Since xanthine oxidase is capable of reducing a variety of acceptors, we attempted a determination of the sites on the enzyme concerned with reduction of such acceptors. These experiments grew out of a discussion with Dr. Irwin Fridovich, who has carried out a study of such sites together with Dr. Philip Handler at Duke University, and who kindly showed us a copy of their paper in advance of publication (*10*). Table VI

TABLE VI

EFFECT OF SUBSTRATE CONCENTRATION ON XANTHINE OXIDASE ACTIVITY FOR VARIOUS ACCEPTORS

	Relative reaction velocity in presence of			
Hypoxanthine concentration (μmoles per ml.)	Oxygen (μmoles per hour of uric acid)	Methylene blue ($1/t \times 10^3$ seconds)	Cytochrome c (μmoles per hour)	Ferritin iron (μmoles Fe^{++} per hour)
0.05	3.2	21	0.06	0.2
0.10	3.8	21	0.07	2.1
0.20	3.4	14	0.07	3.2
0.50	1.4	9	0.07	3.2
1.00	0.8	7	0.06	3.0

shows the activity of xanthine oxidase, measured with a variety of acceptors, including oxygen, methylene blue, ferricytochrome c, and ferritin. One can see that increasing quantities of substrate result in a marked decrease in enzyme activity in the presence of oxygen or methylene blue, in contrast to a lack of inhibition in the presence of ferricytochrome c or

ferritin iron. These results suggest at least two different sites on xanthine oxidase for reaction with its acceptors.

Fridovich and Handler, as a result of their studies, have suggested that the purine substrate is bound by one FAD molecule, resulting in a reduction of this FAD. An electron transfer takes place to enzyme-bound ferric iron, which is present in the form of a ferric-mercaptide linkage, resulting in a reduction of this iron to the ferrous state and a release of SH groups. At this point, the enzyme ferrous iron may transfer its electron in one of two ways. In the presence of molecular oxygen or methylene blue, the transfer of an electron would be to the second FAD molecule which is involved directly with reduction of oxygen or the dye. Excess substrate would result in binding by this FAD group and consequent inhibition of its reaction with oxygen or dye. On the other hand, we feel that another pathway of electron transfer from enzyme-bound ferrous iron can take place, thus bypassing the second FAD group. This would take place by electron transfer by enzyme-bound ferrous iron to ferritin-bound or cytochrome c-bound ferric iron, resulting in reoxidation of the enzyme and the appearance of ferrous iron in ferritin and cytochrome c.

An interesting feature of these two studies is that we have arrived at a configuration for ferritin ferric-mercaptide groups similar to those in xanthine oxidase. This would suggest an electron transfer across similar types of iron-sulfur linkages, from one protein to another. Table VII

TABLE VII

ACTION OF XANTHINE OXIDASE-HYPOXANTHINE ON FERRITIN IRON AND SULFHYDRYL GROUPS

Ferritin treatment	SH content[a] of ferritin	Ferritin Fe^{+++} reduction by xanthine oxidase (% of control ferritin)	X.O. activity in presence of ferritin (% of X.O. activity in presence of untreated ferritin)
Control	100	100	100
+ PCMB	0	0	0
+ Iodoacetamide	12	95	
+ Iodosobenzoate	17	82	

[a] Treatment of ferritin with xanthine oxidase-hypoxanthine under anerobic conditions led to an increase in ferritin sulfhydryl content from 11.3 to 18.4 μmoles SH per 100 mg. ferritin nitrogen.

shows some preliminary evidence for a liberation of ferritin sulfhydryl groups after xanthine oxidase action. As is the case pictured in xanthine oxidase, reduction of ferritin ferric iron would result in a release of its SH

groups for reaction with sulfhydryl reagents. This table also shows what happens to the ability of xanthine oxidase to reduce ferritin ferric iron after the ferritin has been treated with a variety of SH reagents and the excess of such reagents removed by dialysis. In the case of iodoacetamide and iodosobenzoate, although the SH sites of the ferritin have been reduced by some 85%, its ferric iron can still be reduced by the enzyme to a considerable extent. However, in the case of ferritin treated with PCMB, the ability of xanthine oxidase to reduce ferritin iron is completely inhibited. Because of the reversibility of the PCMB-sulfhydryl complex, this ferritin specimen was added to a mixture of xanthine oxidase and hypoxanthine and its effect on oxygen uptake was measured. Complete inhibition took place. This would suggest a transfer of PCMB from ferritin sulfhydryl groups to xanthine oxidase sulfhydryl groups with a consequent inhibition of its aerobic activity.

In summary, our data may be interpreted to mean that the mechanism of ferritin iron reduction involves a transfer of electrons from the iron atoms of one protein molecule, xanthine oxidase, to the iron atoms of another, ferritin, each iron atom in association with sulfhydryl groups. The consequent reduction of ferritin iron results in a weakened bond to the protein, allowing iron-binding agents such as the iron-binding globulin of the plasma to remove this iron to the general circulation, from which it can be removed by the bone marrow for purposes of hemoglobin synthesis.

References

1. S. Granick, *Chem. Revs.* **38**, 379 (1946).
2. A. Rothen, *J. Biol. Chem.* **152**, 679 (1944).
3. A. Mazur, I. Litt, and E. Shorr, *J. Biol. Chem.* **187**, 473 (1950).
4. J. L. Farrant, *Biochim. et Biophys. Acta* **13**, 569 (1954).
5. S. Green, A. Mazur, and E. Shorr, *J. Biol. Chem.* **220**, 237 (1956).
6. A. Mazur, I. Litt, and E. Shorr, *J. Biol. Chem.* **187**, 485 (1950).
7. R. A. Fineberg and D. M. Greenberg, *J. Biol. Chem.* **214**, 107 (1955).
8. S. Green and A. Mazur, *J. Biol. Chem.* **227**, 653 (1957).
9. P. G. Avis, F. Bergel, and R. C. Bray, *J. Chem. Soc.* p. 1100 (1955).
10. I. Fridovich and P. Handler, *J. Biol. Chem.* **231**, 899 (1958).

Discussion

JENSEN: In regard to Dr. Mazur's postulate that the PCMB mercaptide is dissociated, we found the silver was bound to the sulfhydryl of serum albumin much less strongly than we had anticipated even though Hg is bound tighter than Ag. It is not unreasonable to suppose that there is a certain amount of dissociation, especially if you have a potential acceptor there for it.