THE ORIGIN OF INORGANIC SULFIDE IN ARTIFICIAL NON-HEME IRON PROTEIN FORMED FROM BOVINE SERUM ALBUMIN

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The origin of the inorganic sulfide present in many non-heme iron proteins has been the subject of extensive investigation. Clostridial ferredoxins have been used most widely for these studies since they are small proteins (molecular weight about 6000) which contain 7 moles of inorganic sulfide. It was originally thought (Lovenberg et al, 1963) that this sulfide arose from a source other than polypeptide-bound cysteines since the reaction of ferredoxin with mercurials indicated a three fold larger mercaptide formation than could be accounted for by the cysteine content. This view was questioned by Bayer et al (1964) and Bayer and Parr (1966) who suggested that inorganic sulfide from non-heme iron proteins arose entirely via β elimination of sulfide from polypeptide bound cysteine. Although the evidence presented by Bayer and his co-workers was not very convincing, Malkin and Rabinowitz (1966a, 1966b) and Keresztes-Nagy and Margoliash (1966) clearly demonstrated that this was not so and that the inorganic sulfide in the case of bacterial or plant ferredoxin arose from a source other than the polypeptide bound cysteine.

Of considerable interest was the recent observation (Suzuki and Kimura, 1967) that an artificial non-heme iron protein is formed by the interaction of iron and bovine serum albumin in the presence of 2-mercaptoethanol. Al-

though no inorganic sulfide was present in the reaction mixture, the protein obtained contained equivalent amounts of iron and inorganic sulfide leading these workers to conclude that the inorganic sulfide was arising by β elimination of the polypeptide-bound cysteine. The current work indicates that inorganic sulfide must be added to the reaction mixture to obtain an artificial non-heme iron protein, and that the inorganic sulfide of this non-heme iron protein does not arise from the cysteine residues.

MATERIALS AND METHODS

Sodium sulfide, ^{35}S 11.7 μc/mole was purchased from Nuclear Chicago Corp. and Pentex crystalline bovine serum albumin was obtained from Calbiochem. Inc.; 2-Mercaptoethanol (B.P. 153-157)was obtained from Eastman Organic Chemical Co. Amino acids were analyzed on a Beckman 120C amino acid analyzer equipped for high sensitivity analysis, after hydrolysis of the protein *in vacuo* in 6 N HCl for 17 hrs. at 110°. Iron, inorganic sulfide, and protein were determined by methods described previously (Lovenberg *et al*, 1963). Performic acid oxidation of the proteins was performed by the method of Hirs (1956) at 0°.

RESULTS

Using the reaction conditions described previously (Suzuki and Kimura, 1967), we have been unable to form significant amounts of the artificial non-heme iron protein. However, the product described by these workers could be formed if Na_2S was included in the reaction mixture. The following conditions gave the most consistent results: Twenty milligrams of bovine serum albumin is dissolved in 2 ml of 0.15 M PO_4 buffer pH 6.5 containing 4 mM ferrous ammonium sulfate. Next 100 λ of 2-mercaptoethanol is added followed by the addition of Na_2S (final concentration is 4 mM). This mixture is incubated at 37° for 10 minutes followed by 5 minutes at room temperature. The protein is separated by gel filtration on a Sephadex G-50 column (1.1 x 15 cm). The absorption spectrum of the final product is shown in Figure 1 and is very similar to that reported previously (Suzuki and

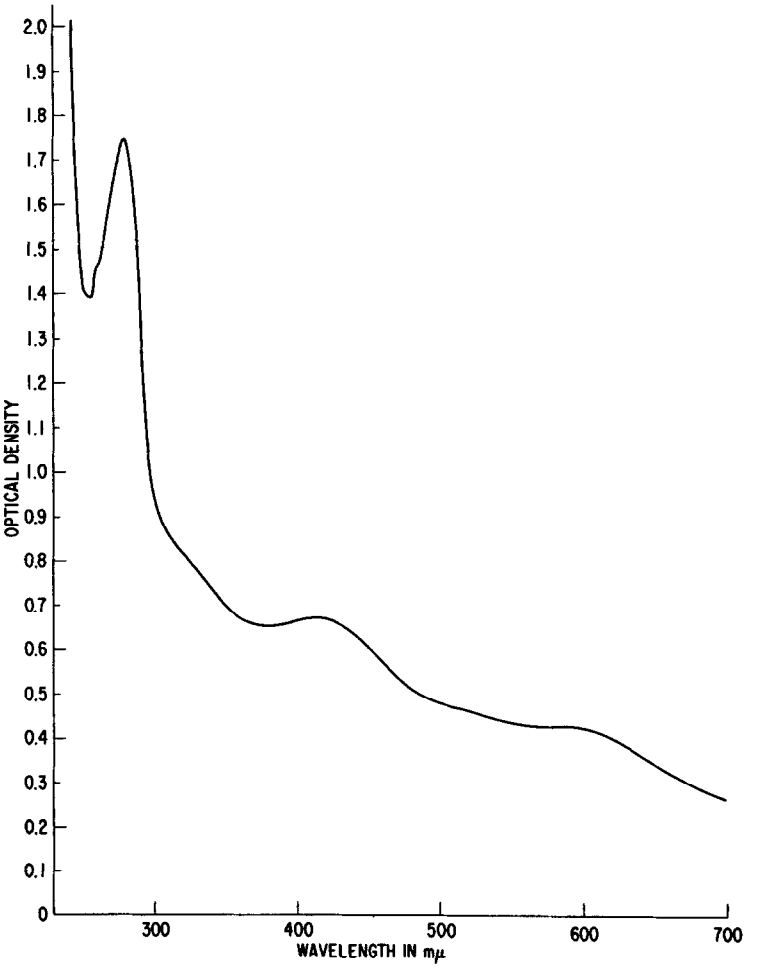

Fig. 1. Absorption spectrum of the artificial non-heme iron protein. The protein concentration was 1.7 mg/ml in 0.15 M phosphate buffer, pH 6.5.

Kimura, 1967). The molar extinction coefficient for the absorption maximum at 410 mμ of this preparation is about 3500 per atom of bound iron. This non-heme iron protein contains from 8 to 10 moles of iron per mole of protein (Table I). The amount of inorganic sulfide found was approximately equivalent to iron when assays were done colorimetrically.

TABLE I

The Iron and Inorganic Sulfide Content of the Artificial Non-Heme Iron Protein

Exp. #	Mole Fe^{++}/mole Protein	Mole $S^{=}$/mole Protein
1	8.7	6.8
2	8.0	6.5
3	8.3	11.0
4	10.0	13.2

Iron was determined as described in text as was the inorganic sulfide in experiments 1 and 2. In experiments 3 and 4 the amount of inorganic sulfide incorporated was determined by measuring the radioactivity incorporated. This radioactive artificial non-heme protein was prepared as described in Figure 2.

When ^{35}S sodium sulfide is used in the reaction mixture, the isotope is incorporated into the protein. Figure 2 shows the separation of this protein from the reactants on a column of Sephadex G-50. The amount of radioactive sulfide bound to the protein considerably exceeds the amount released as

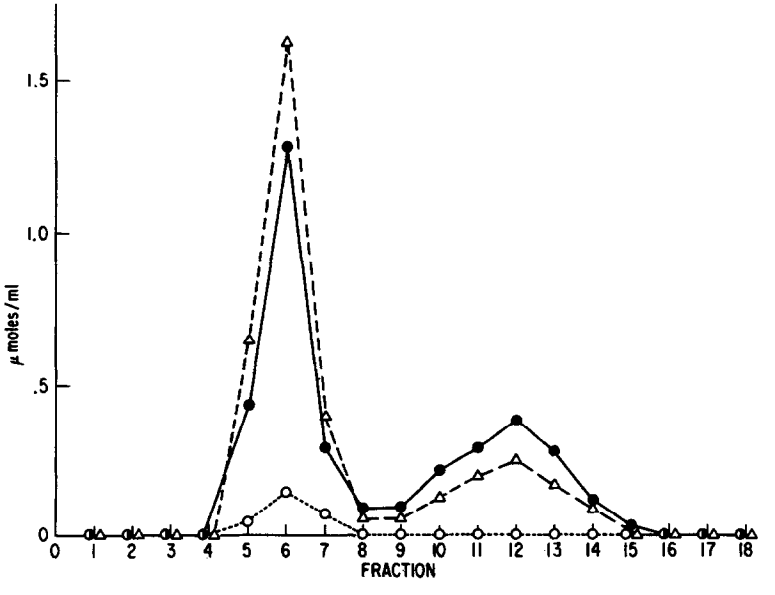

Fig. 2. Isolation of the artificial non-heme iron protein by gel filtration on Sephadex G-50. The protein was prepared and chromatographed as described in the text with the exception that radioactive Na_2S (153,000 cpm/μ mole) was used. Fractions (1.5 ml) were assayed for protein (o--o), sulfide (Δ---Δ) and iron (o—o). The amount of sulfide equivalents present in each fraction was determined by the radioactivity.

inorganic sulfide (Table I). Although the mechanism of this extra sulfide binding is not apparent, it was found (Figure 3) that when a sample of the artificial non-heme iron protein containing about 8 moles of iron per mole of protein is treated with sodium mersalyl, and chromatographed on Sephadex G-50, about 8 moles of $^{35}S=$ are removed from the protein as the mercaptide while 3 to 4 moles of sulfide remain bound to the protein.

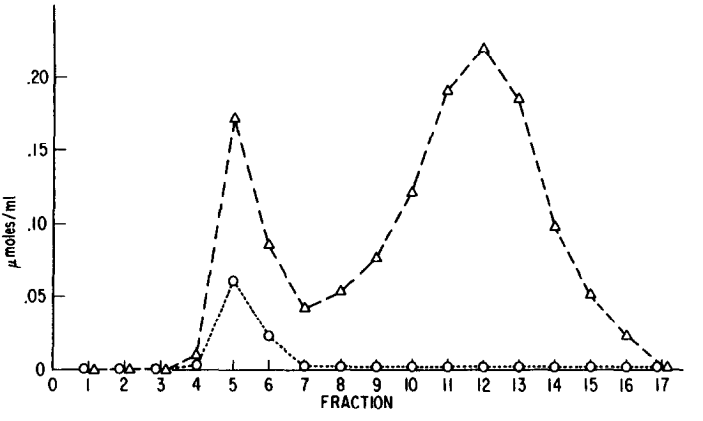

Fig. 3. Gel filtration of the artificial non-heme iron protein after reaction with sodium mersalyl. One ml of 0.15 M phosphate buffer containing 0.12 μ moles of the artificial non-heme iron protein prepared as described in Fig. 2 was treated with 40 μ moles of sodium mersalyl for 2 minutes at room temperature. The bleached reaction mixture was chromatographed on a G-50 Sephadex column equilibrated with 0.15 M phosphate buffer. Protein (o--o) is expressed as μ moles per ml and radioactivity (△--△) as μ mole of sulfide equivalent per ml.

Finally, the cysteic acid content of a sample of acidified artificial non-heme iron protein was compared with that of serum albumin after each protein had been oxidized with performic acid. The amino acid content of the two proteins appeared to be very similar. The apparent cysteic acid residues per mole of oxidized protein were 31 for serum albumin and 32 for the artificial non-heme iron protein from which the inorganic sulfide had been released. This demonstrates that the inorganic sulfide is not arising from the cysteine residues.

COMMENT AND CONCLUSIONS

It is apparent from the current observations that formation of the artificial non-heme iron protein from serum albumin requires the presence of both iron and inorganic sulfide. This is in contrast to the original observations by Syzuki and Kimura (1967) that no addition of inorganic sulfide was necessary to obtain a protein which would release inorganic sulfide upon acidification. It is also obvious that in the case of the artificial non-heme iron protein the inorganic sulfide is not arising from the cysteine residues. This is in complete agreement with the findings of Malkin and Rabinowitz (1966a, 1966b) and Hong and Rabinowitz (1967) concerning clostridial ferredoxins. The similarity of the conditions required for reconstitution of ferredoxin and the conversion of serum albumin into an artificial non-heme iron protein establishes a relationship between these two proteins.

REFERENCES

1. Bayer, E., Parr, W. and Kazmaier, B,, Arch. Pharm., 298, 196 (1964).
2. Bayer, E. and Parr, W., Agnew. Chemie., 78, 824 (1966).
3. Hirs, C.H.W., J. Biol. Chem., 219, 611 (1956).
4. Hong, J. and Rabinowitz, J.C., Biochem. Biophys. Res. Comm., 29, 246 (1967).
5. Keresztes-Nagy, S., and Margoliash, E., J. Biol. Chem., 241, 5955 (1966).
6. Lovenberg, W., Buchanan, B.B., and Rabinowitz, J.C., J. Biol. Chem. 238, 3899 (1963).
7. Malkin, R., and Rabinowitz, J.C., Biochemistry, 5, 1262 (1966a).
8. Malkin, R., and Rabinowitz, J.C., Biochem. Biophys. Res. Commun., 23, 822 (1966b).
9. Suzuki, K., and Kimura, T., Biochem. Biophys. Res. Commun. 28, 514 (1967).