Bovine Serum Albumin Coordinated Iron-Sulfur Cluster as a Hydrogenase Model†

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The bovine serum albumin coordinated Fe_4S_4-cluster, which had hydrogenase activity, was synthesized. Its physical and chemical characteristics were then compared with those of natural hydrogenase. Though the affinities of hydrogenase and the albumin-coordinated Fe_4S_4-cluster for the substrate, methyl viologen, were similar, the hydrogenase activity for hydrogen evolution was 10^5 times greater than the cluster. Two types of Fe_4S_4-clusters, coordinated by the fragments of the cleaved albumin obtained by treatment with cyanogen bromide, were synthesized, and their hydrogenase activities were compared. The activity with the iron–sulfur cluster incorporated in the fragment with MW 40000 was much higher than that of the one incorporated in the fragment with MW 20000. The amino acid arrangement to form the active site was discussed on the analogy of the other iron-sulfur proteins.

The core structures of iron-sulfur proteins have been studied extensively, and the physicochemical investigations have conclusively established that iron–sulfur proteins have at least three fundamental types of central cores: $Fe(S\text{–}Cys)_4$, $Fe_2S_2(S\text{–}Cys)_4$, and $Fe_4S_4(S\text{–}Cys)_4$.[2]

Holm *et al.*[3–11] have reported the synthesis of the tetranuclear cluster complexes $[Fe_4S_4(SR)_4]^{2-}$ and binuclear cluster complexes $[Fe_2S_2(SR)_4]^{2-}$ (R=alkyl or aryl), whose structure and properties demonstrate them to be a close representation of the central cores of iron–sulfur proteins, and have developed the core-extrusion method for the identification of active sites in native iron-sulfur proteins. Rabinowitz *et al.*[12–14] have also applied the method to the preparation and isolation of apoprotein of ferredoxin from *Clostridium pasteurianum*, and have reconstituted ferredoxin by the addition of iron(II) ions, sodium sulfide, and 2-mercaptoethanol. This procedure, which has considerable utility, is now being applied to other iron–sulfur proteins.[15–17]

Recently we found,[18–21] by the extrusion and reconstitution techniques mentioned above, that the hydrogenase from *Desulfovibrio vulgaris* contained two Fe_4S_4-type clusters per enzyme molecule; we also found[18,20] two active sites per enzyme molecule by kinetics and an inhibition method with mercury(II) chloride. The presence of three Fe_4S_4-type clusters has been established in the case of the hydrogenases from *Desulfovibrio gigas*[22] and *Clostridium pasteurianum*,[11] which contain 12 iron atoms and 12 labile sulfur atoms per molecule. Generally, hydrogenase is regarded as an iron–sulfur protein with Fe_4S_4-type clusters. Although the Fe_4S_4-cluster alone does not have the hydrogenase activity,[23] the Fe_4S_4-clusters synthesized in the presence of apo-hydrogenase has the catalytic activity.[21] Therefore, the Fe_4S_4-cluster and also the environment of the Fe_4S_4-cluster within the protein, such as apo-hydrogenae, play important roles in the hydrogenae activity.

In this paper we wish to describe the development of a hydrogen-evolution system which combines the Fe_4S_4-cluster and some protein, and to compare the characteristics of the artificial and natural hydrogenase.

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Experimental

Materials. The methyl viologen (1,1′-dimethyl-4,4′-bipyridinium dichloride) and trypsin were purchased from the Tokyo Kasei Kogyo Co. and ICN Pharmaceuticals Inc. respectively. The bovine serum albumin, α-chymotrypsinogen-A, and lysozyme were products of the Sigma Chemicals Co. The other chemicals, obtained from the Wako Pure Chemicals Co., were the best commercial grades available. All solutions were degassed and stored under nitrogen. The nitrogen gas was purified by passage through a copper column at 200 °C.

The copolymer of styrene and *p*-vinylphenylmethanethiol was synthesized according to the procedure of Okawara *et al.*[24]

Hydrogenase from the *Desulfovibrio vulgaris* (Miyazaki type, which has kindly been provided by Professor T. Yagi of Shizuoka Univ.) was purified according to the procedure of Yagi *et al.*[25,26]

Measurement of Hydrogenase Activity. The hydrogenase activity was measured under anaerobic conditions. The hydrogenase activities of the iron-sulfur proteins were determined by the rate of hydrogen evolution from reduced methyl viologen at 30 °C and pH 7.0. The evolved hydrogen was analyzed by gas chromatography on a 2 m×0.5 mm active carbon column (Gasukuro Kogyo Co.) with a nitrogen carrier. The reaction mixture (8.5 ml) consisted of 4.0 ml of the iron–sulfur protein solution with the 7.73×10^{-7} M Fe_4S_4-cluster described in "Synthesis of Iron–Sulfur Clusters," 1.96 μmol of methyl viologen, and 5 mg of $Na_2S_2O_4$ in 4.5 ml of a 0.02 M phosphate buffer (pH 7.0).

Synthesis of Iron–Sulfur Clusters. The $[Fe_4S_4(SR)_4]^{2-}$ complexes (R=phenyl or *t*-butyl) were prepared by the procedure of Holm *et al.*[4] The synthesis of other iron-sulfur clusters was carried out under a nitrogen atmosphere as follows. To a stirred solution of thiol or protein with mercapto ligands (4×10^{-5} mol) in 4 ml of H_2O containing 0.01 M iron(II) chloride, 2 ml of aq Na_2S (0.02 M) were added; the mixture thereupon turned dark brown in each case and was incubated at 30 °C for 30 min. The solution was then centrifuged (20000 g×30 min) to remove fine black precipitate, which may be iron(II) sulfate.

Cleavage of Bovine Serum Albumin. Albumin was cleaved according to the method of King and Spencer.[27] To 12.2 ml of a formic acid (75%) solution containing 26.4 μmol of albumin, we added a solution of cyanogen bromide (2.31 mmol) in 36 ml of formic acid, after which the solution was stirred for 20 h at room temperature. Then the solution was

placed in a Sephadex G-75 column (2.2 cm × 140 cm) and eluted with the 0.02 M Tris–HCl buffer (pH 7.0; to compare the hydrogenase activity, this pH value was employed, for this value was suitable for the hydrogenase). The eluted solution was then concentrated by ultrafiltration with an UM-2 Diaflo membrane.

Results and Discussion

Preparation and Properties of Bovine Serum Albumin Coordinated Iron–Sulfur Cluster. The method used for the preparation of the albumin cluster was a modification of those of Suzuki and Kimura[28] and Lovenberg and McCarthy.[29] The preparation was carried out under a nitrogen atmosphere as follows: eighty milligrams of albumin was dissolved in 6 ml of water containing 6.7 mM iron(II) chloride or ammonium iron(II) sulfate. 2-Mercaptoethanol (0.4 ml) was then added, followed by 2 ml of aq Na_2S (0.02 M).

In order to separate the albumin-cluster from the excess reagents, the solution containing the albumin-cluster was placed in a Sephadex G-50 column (5 cm × 23 cm) with 0.02 M Tris–HCl (pH 7.0) containing 0.08 M NaCl as an eluting buffer. The eluting solution was monitored by means of the absorption bands for proteins and iron–sulfur clusters; the results are shown in Fig. 1. Albumin came out at the elution position peak A. As the eluting solution has the absorption band at 420 nm which is attributable to the iron–sulfur clusters, and also a molecular weight corresponding to albumin, the product is not a crude mixture of albumin and the iron–sulfur cluster, but the iron–sulfur clusters incorporated in albumin. The solution was dark brown, and iron(II) sulfide as a by-product may also be incorporated in the protein. Although the eluting solution corresponding to Peak A was centrifuged at 20000 g for 60 min to remove the black precipitate, it was not separated from the protein completely. The iron–sulfur clusters incorporated in albumin were not synthesized by the reaction of the preformed iron–sulfur cluster and albumin. Therefore, the iron(II) sulfide concomitant in the iron-sulfur incorporated in albumin was not avoided by this method. The specific absorption coefficients, 35600 $M^{-1}\,cm^{-1}$ at 280 nm and 14000 $M^{-1}\,cm^{-1}$ at 420 nm,[28] were used to estimate the concentrations of protein and iron–sulfur clusters respectively. The B peak also has ultraviolet and visible absorption bands which seem to be due to an excess of the 2-mercaptoethanol reagent and the synthesized $Fe_4S_4(S\text{–}CH_2CH_2OH)_4$ dianion respectively.

When the solution of the iron–sulfur cluster incorporated in albumin (albumin-cluster; the spectrum is shown in Fig. 2, Curve a) was incubated under a hydrogen atmosphere at 30 °C, the spectrum of the solution changed as is shown in Fig. 2, Curve b. When exposed in air, the spectrum returned to its original shape. A similar spectrum change has been observed[26] in the reduction of hydrogenase from *Desulfovibrio vulgaris* by hydrogen. In this case, however, cytochrome C_3 is needed as an electron-acceptor. When excess 2-mercaptoethanol is added to the albumin-cluster solution for the identification of the core types, the obtained absorption spectrum (λ_{max}=410 nm) of the resulting solution resembled that of the synthesized $Fe_4S_4(S\text{–}CH_2CH_2OH)_4$ dianion, as is shown in Fig. 2, Curve c. From the above results, the iron–sulfur clusters incorporated in albumin seem to be Fe_4S_4-type clusters, but another possibility still remains that the corresponding Fe_2S_2-clusters were rapidly converted to the Fe_4S_4-clusters.

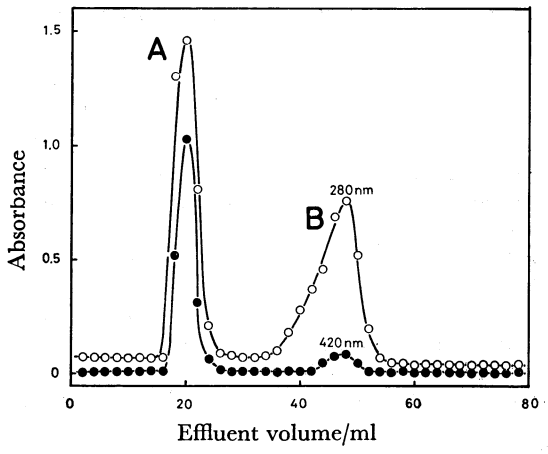

Fig. 1. Elution patterns of the albumin-cluster from a Sephadex G-50 column.

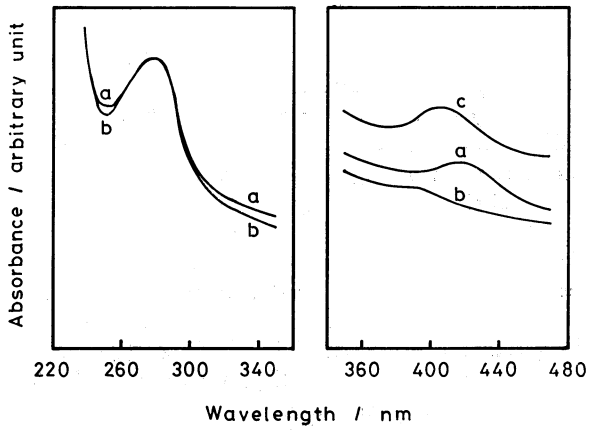

Fig. 2. Absorption spectra of the albumin-cluster.
a: Albumin-cluster, b: Albumin-cluster is incubated under hydrogen for 24 h, c: Mixture of albumin-cluster and 2-mercaptoethanol.

We lack the extinction coefficient data for the spectrum required for a quantitative analysis of the $Fe_4S_4(S\text{–}CH_2CH_2OH)_4$ dianion. However, it is clear that the Fe_4S_4-cluster is the dominant product.

Synthesis of Various Iron–Sulfur Clusters. The method used for the preparation of the iron–sulfur clusters with various mercapto ligands was the same as that used for the albumin-clusters. Though a number of Fe_4S_4 cores are synthesized in the presence of some thiols, SH-containing polymer, and natural proteins containing cysteinyl residues instead of apo-hydrogenase, an estimation has not been made whether each product is a crude mixture of protein and the iron–sulfur cluster or the iron–sulfur cluster incorporated in a compound as a ligand. As is evident from Table 1, no cluster complex but the albumin-coordinated Fe_4S_4-cluster can catalyze the hydrogen-evolution reaction from reduced methyl

Table 1. Catalytic activities of synthesized iron–sulfur clusters

Ligand	Solvent[a)]	Activity (mol-H_2/min/mol-Catal.)
2-Methyl-2-propanethiol	DMF/H_2O	0
Thiophenol	HMPA/H_2O	0
2-Mercaptoethanol	H_2O	0
Dithiothreitol	H_2O	0
Glutathione	H_2O	0
L-Cysteine	H_2O	0
Copolymer of styrene and *p*-vinylphenylmethanethiol	DMSO/H_2O	0
Bovine serum albumin	H_2O	9.62×10^{-3}
Lysozyme	H_2O	Trace
α-Chymotrypsinogen	H_2O	0
Papain	H_2O	0

a) DMF: *N*,*N*-Dimethylformamide; HMPA: hexamethylphosphoric triamide; DMSO: dimethyl sulfoxide.

viologen, although its activity is lower than that of the actual enzyme, hydrogenase.

Specificity for Electron Carriers and Kinetics. When the albumin-cluster was added to a solution of dithionite-reduced methyl viologen, hydrogen evolved under the same conditions of hydrogenase-activity measurement. Various redox carriers were tested to see if they would replace methyl viologen and mediate the electron transfer to the albumin-cluster from dithionite. The electron-carrier specificities for albumin-cluster and hydrogenases are compared in Table 2. The albumin-cluster was specific for methyl viologen and nicotinamide–adenine dinucleotide.

The time course showed that the amount of hydrogen evolved from reduced methyl viologen increased linearly with the time. It is reasonable to assume that the iron–sulfur cluster in the albumin-cluster (which has not been separated from iron(II) sulfide) has a central role in hydrogen evolution, because it has been confirmed experimentally that iron(II) sulfide has no activity for hydrogen evolution. With a 44-h reaction time, the molar ratio (the turnover number) of the evolved hydrogen for the iron–sulfur cluster was 18.[1)] This means that the albumin-cluster behaves as a catalyst. Though the stability of the albumin cluster was not examined, it seems fairly stable in the long term because it was still intact after a 44-h reaction.

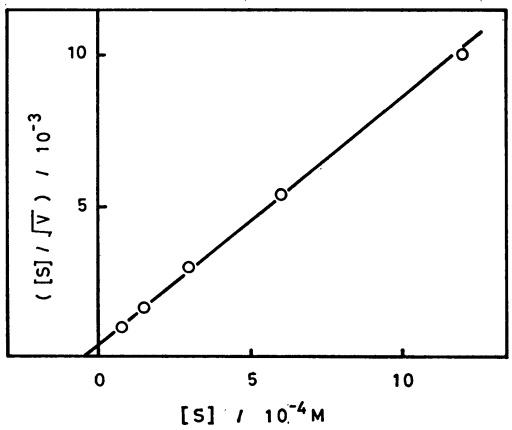

Fig. 3. Relation between $[S]/\sqrt{V}$ and [S].

The initial rate of hydrogen evolution increased with the methyl viologen concentration until it finally reached a constant value. In the case of the hydrogenase, the rate of hydrogen evolution from reduced methyl viologen was expressed as follows:[20,30)]

$$V=\frac{kK^2[S]^2}{(1+K[S])^2},$$

or

$$\frac{[S]}{\sqrt{V}}=\frac{[S]}{\sqrt{k}}+\frac{1}{\sqrt{k}K},$$

where k and K are constants and where [S] is the concentration of methyl viologen. In the case of the albumin-cluster, a good linear relation was also obtained between $[S]/\sqrt{V}$ and [S], as is shown in Fig. 3. Although the $[S]/V$ *vs.* [S] plot on the basis of the first-order kinetic equation:

$$V=\frac{kK[S]}{1+K[S]}$$

was also tried, there were some deviations. The albumin-cluster may also catalyze hydrogen evolution by the same mechanism as that of the hydrogenase. From the slope and the intercept, the equilibrium constant, K, and the rate constant, k, for hydrogen evolution from

Table 2. Electron-carrier specificity in H_2 evolution by the hydrogenase from *Desulfovibrio vulgaris* and the albumin-cluster

Electron carrier	Concentration M	Activity (mol-H_2/min/mol-Catal.)	
		Hydrogenase	Albumin-cluster
Cytochrome C_3	5.94×10^{-7}	245	0
Flavin monoculeotide	2.45×10^{-4}	Trace	0
Nicotinamide–adenine dinucleotide	1.61×10^{-4}	13	4.54×10^{-3}
Coenzyme II	1.72×10^{-4}	0	0
Methyl viologen	2.48×10^{-4}	1770	9.62×10^{-3}
Methylene Blue	1.96×10^{-4}	0	0
Neutral Red	2.03×10^{-4}	47	0
Safranine T	2.07×10^{-4}	40	0
Phenosafranine	1.91×10^{-4}	34	Trace
Potassium hexacyanoferrate(III)	2.01×10^{-4}	0	0

reduced methyl viologen at 30 °C were calculated as follows:

Catalyst	K / M^{-1}	k / min^{-1}
Hydrogenase	8.8×10^{3}	2.3×10^{3}
Albumin-cluster	1.9×10^{4}	1.5×10^{-2}

Judging from the K values, the affinity of the albumin-cluster for methyl viologen seems to be similar to that of the hydrogenase. The k value of hydrogenase, however, was 10^5 times greater than that of the albumin-cluster.

Effect of Mercury(II) Chloride. As has been reported earlier[15,20] some heavy metals, mercury, copper, and silver, strongly inhibit the activity of the hydrogenase, even though their concentrations are low in comparison with that of the hydrogenase. Although the albumin-cluster was inhibited in the presence of mercury(II) chloride, as is shown in Table 3, it was less inhibited than the hydrogenase at the same concentration. Mercury atoms are exchangeable with the iron atoms in the hydrogenase,[20] and the inhibition of the hydrogenase by mercury(II) chloride is a result of the exchange between mercury and iron. Although the inhibition by metal ions is explained by the binding of these ions to the thiolate sulfurs, the inhibition of the albumin-cluster by mercury(II) chloride may be caused by the exchange between mercury and iron, just as in the case of the hydrogenase.

Cleaved Bovine Serum Albumin Coordinated Iron–Sulfur Clusters. Though the Fe_4S_4-cluster of the albumin-cluster combines cysteine residues of albumin, none of the cysteine residues (there are 35 cysteine residues per albumin molecule[31]) participate in the cluster formation, for the concentration of the synthesized clusters is low compared to that of the cysteine residues. To determine the situation needed for the hydrogenase model, the hydrogenase activities were measured with Fe_4S_4-clusters with cleaved albumin as ligands.

The cyanogen bromide cleavage of albumin between two amino acid residues, 183 (Met) and 184 (Arg), gives two peptides with molecular weights of 40000 and 20000.[27] A chromatogram of the formic acid solution containing albumin and cyanogen bromide obtained by means of a Sephadex G-75 column is shown in Fig. 4. Fragment 1 is the original albumin, while Fragments 2 and 3 are examples of cleaved albumin with molecular weights of 40000 and 20000 respectively. The reagents to produce the Fe_4S_4-cluster, iron(II) chloride, 2-mercaptoethanol, and Na_2S, were added to a solution containing the protein corresponding to Fragment 2 or 3, and then the mixture was placed in a Sephadex G-50 column to remove the excess reagents as well as to produce albumin-cluster synthesis. As is shown in Table 4, the activity of the iron-sulfur cluster incorporated in Fragment 2 was much higher than that of the one incorporated in Fragment 3.

Table 3. Inhibition by mercury(II) chloride

Hg^{2+}/Cluster (M/M)	% Activity[a]	
	Hydrogenase	Albumin-cluster
0	100	100
5	82	100
20	42	100
50	0	53
100	—	0

a) Samples were preincubated with $HgCl_2$ for 24 h at 4 °C. Methyl viologen: 2.31×10^{-4} M.

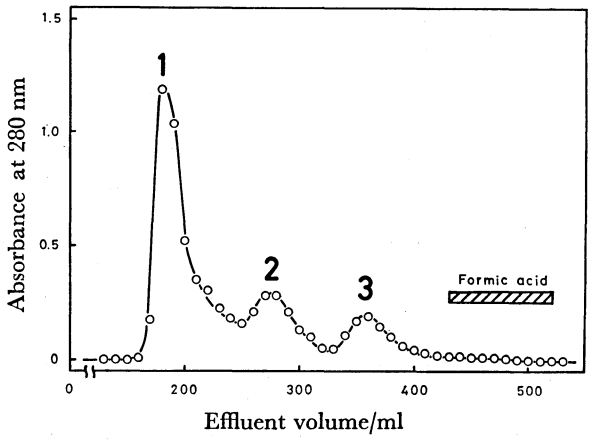

Fig. 4. Separation of cyanogen bromide fragments of albumin on a Sephadex G-75 column.

In albumin, two cysteine residues are adjacent to each other except for the two cysteine residues 276 and 280, where three other amino-acid residues are incorporated between them. This part belongs to Fragment 2 in the cleaved albumin, and there is no other remarkable difference in geometrical arrangement between Fragments 2 and 3. Judging from the chelate structures of other iron–sulfur proteins determined by X-ray diffraction studies,[2] the following two situations in amino acid sequence seem to be required for Fe_4S_4-cluster formation: the existence of four cysteine residues as ligands close to each other, and the presence of two, three, or four other amino acids between the cysteine residues. As Fragment 2 contains the portion of the 263—287 residues which satisfies the situation, the iron–sulfur cluster will be incorporated at this portion of Fragment 2. The concentration of the iron–sulfur cluster is comparable with that of Fragment 2 protein.

Table 4. Hydrogenase activities with some clusters[a]

Protein source	Protein concentration / M	Cluster concentration / M	Activity (mol-H_2/min/mol-cluster)
Albumin whole chain	3.74×10^{-6}	7.73×10^{-7}	7.75×10^{-3}
Fragment 2	1.86×10^{-6}	1.34×10^{-6}	4.84×10^{-3}
Fragment 3	3.42×10^{-6}	2.46×10^{-6}	5.70×10^{-4}

a) See text for reaction conditions.

This means that only one cluster is formed per protein molecule.

After all, not only the proper distance between two cysteine residues which is suitable for the formation of the Fe_4S_4-core in the albumin molecule, but also the proper amino acid residues near the active-site core seem to be required for the hydrogenase activity.

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