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The Formation of Ferritin from Apoferritin

INHIBITION AND METAL ION-BINDING STUDIES

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Inhibition by Zn^{2+} of iron uptake by apoferritin at very low substrate concentrations is shown to be competitive. It is proposed that Zn^{2+} competes with Fe^{2+} for sites on the protein at which the oxidation of Fe^{2+} is catalysed. Interpretation of titration data suggests there are two independent classes of binding site for Zn^{2+} and several other cations. Sites in one such class are probably on the external surface of the apoferritin molecule. The catalytic binding sites are presumed to be internal and may involve histidine or possibly cysteine as ligands.

Ferritin is a large multi-subunit protein which functions as an iron store in all but the most primitive forms of living matter (Granick, 1946; Harrison, 1964; Harrison & Hoy, 1973*a*,*b*). Its hollow protein shell can accommodate up to about 4500 iron atoms as a microcrystalline ferric oxide hydrate (Fischbach & Anderegg, 1965; Haggis, 1965; Harrison *et al.*, 1967). Its iron can be removed by the action of reducing and chelating agents (Granick & Michaelis, 1943) leaving apoferritin. Conversely, ferritin can be reconstituted from apoferritin by the addition of ferrous iron and an oxidizing agent (Bielig & Bayer, 1955; Harrison *et al.*, 1967) and apoferritin has been shown to catalyse the formation of the hydrous ferric oxide of its iron core from Fe^{2+} (Niederer, 1970; Macara *et al.*, 1972, 1973*a*; Crichton & Bryce, 1972; Bryce & Crichton, 1973).

In studies on the kinetics of ferritin formation *in vitro* it was observed that at low iron:protein ratios hyperbolic progress curves were obtained, but the curves became sigmoidal when larger amounts of iron were added (Macara *et al.*, 1972, 1973*b*). Apoferritin was found to catalyse the initial hyperbolic phase of ferritin formation during which nuclei of the microcrystalline ferric oxide hydrate particles are forming in the cavity inside the protein shell. The kinetic data suggest that the first stage in this process is the binding of ferrous iron at the specific sites on the apoferritin molecule at which one or more steps in the subsequent oxidation and hydrolysis is catalysed. Niederer (1970) observed that the cations Zn^{2+}, Ni^{2+}, Hg^{2+}, Cd^{2+}, Co^{2+} and Mg^{2+} all caused inhibition of iron uptake by apoferritin to an extent decreasing in the order listed. He suggested that this order represented the order of affinity of the active site for these ions. Zn^{2+} was found to inhibit both the formation of ferritin from Fe^{2+} under conditions in which sigmoidal progress curves were obtained and also the formation of FeOOH in the absence of apoferritin (Macara *et al.*, 1973*a*). These results were interpreted by the hypothesis that Zn^{2+} competes both for Fe^{2+}-binding sites on the protein and for sites on the hydrous ferric oxide particles, thereby interfering with their growth. The evidence for binding sites was, however, indirect.

In the present studies the nature of inhibition by Zn^{2+} of ferritin formation has been investigated under conditions of very low ferrous iron concentrations where crystal growth is negligible and hyperbolic progress curves are obtained. The binding of Zn^{2+} and other cations has also been followed in separate experiments by measuring their displacement of protons from apoferritin in the pH-stat.

Materials and Methods

Horse spleen ferritin (twice-crystallized, cadmium-free) was purchased from Pentex Co. (Kankakee, Ill., U.S.A.) and was reduced to apoferritin by treatment with sodium dithionite. Some of the ferritin was further purified before reduction by gel filtration on Sephadex G-200. All other reagents were of AnalaR grade (BDH Chemicals Ltd., Poole, Dorset, U.K.).

Apoferritin concentrations were determined by measurement of absorbance at 280nm ($E^{1\%}_{1cm} = 9.0$).

Inhibition studies

The uptake of iron by apoferritin was followed by measuring the change on oxidation in absorbancy at 310nm ($E^{1\%}_{1cm} = 450.0$). Very low concentrations of ferrous iron (48–154μM as ferrous ammonium sulphate) were added to solutions containing 0.58mg of apoferritin/ml and 1.9mM-KIO_3–7.6mM-$Na_2S_2O_3$ as oxidant in 19mM-imidazole buffer, adjusted with 0.1M-HCl to pH7.45. The effect of

adding Zn^{2+} (as $ZnSO_4$) on the initial rates of the hyperbolic progress curves was studied in the range 48–77.0 μM-$ZnSO_4$. Initial rates, v, were corrected to constant ionic strength ($\mu = 0.1$) and to a standard apoferritin concentration (1.0mg/ml) as described previously (Macara *et al.*, 1973*b*).

Binding studies

The binding of various cations by apoferritin was studied in the pH-stat by following the displacement of H^+ from their ligands.

A titrator (type TTTIa, plus a Titrigraph type SBR 2b; Radiometer, Copenhagen, Denmark) was used in conjunction with a motor-driven syringe burette (1.0ml). About 6mg of apoferritin [0.3 μmol of subunit, assuming a mol.wt. of 18500/subunit (Bryce & Crichton, 1971)] together with 200–300 μmol of NaCl (to maintain a constant ionic strength) was added to the reaction vessel and made up to a total volume of 1.25ml with distilled water. The solution was mixed by magnetic stirrer. The syringe burette was loaded with 2.5mM-NaOH, and the metal salt solution (0.1M in some experiments, 5mM in others) was added to the reaction mixture in accurate amounts from a 1.0ml Agla glass syringe by means of a calibrated micrometer. The pH of the metal salt solution was first adjusted by small additions of 0.1M-NaOH or 0.1M-HCl, and the protein solution was brought to the same pH by titration in the pH-stat. The metal salt was then added in discrete 0.01ml volumes, and the volume of 2.5mM-NaOH required to maintain the initial pH was recorded by the Titrigraph. Further volumes of metal salt were added and the same procedure was followed until little further reaction occurred, and the number of H^+ displaced was insignificant.

From the recorded volumes for each addition, the concentrations of free metal ion, bound metal ion and protein concentration were computed.

In the first series of experiments $TbCl_3$ and $ZnSO_4$, $CdSO_4$, $CuSO_4$ and $MnSO_4$ were used, all at about pH5.5. In the second series, only $ZnSO_4$ was used, over the range pH4.7–6.3. (The lower limit to this range was determined by the rapid decrease in release of H^+ to insignificant numbers below pH4.7, and the upper limit by the hydrolysis of Zn^{2+} at alkaline pH.)

Results

Inhibition of iron uptake

The effect of Zn^{2+} on the initial rate of iron uptake, studied at several concentrations of Fe^{2+} and of Zn^{2+}, is shown in Fig. 1 as a plot of Zn^{2+} concentration versus the reciprocal of the initial velocity. Even allowing for the high degree of error inherent in measuring the initial slope of the hyperbolic progress curves, the plots are approximately linear, and their point of intersection above the abscissa suggests that Zn^{2+} is acting as a competitive inhibitor, with an apparent inhibition constant of the order of 70–80 μM under the conditions used (1.0mg of apoferritin/ml, $\mu = 0.1$, 18.5mM-imidazole buffer, pH7.45, 25°C).

Binding studies

When small amounts of metal ions were added (as 5mM salt solution) to give from 0.2 to 1.0 metal ions per apoferritin subunit, an almost stoicheiometric displacement of protons was found. The results indicated that Zn^{2+}, Mn^{2+}, Tb^{3+} and Cd^{2+} each displace one H^+, whereas Cu^{2+} displaces two H^+. These values were assumed in all subsequent calculations. The binding data obtained from the addition of these metal ions at high molar excess are presented for various cations in Fig. 2 as a Scatchard plot (Scatchard *et al.*, 1954). Fig. 3 shows the effect of varying pH on the binding of Zn^{2+} only. It was observed that Zn^{2+}, Tb^{3+} and Cd^{2+} ions, when in a molar excess over apoferritin subunit of greater than 30-fold, caused aggregation of the apoferritin to an increasing extent, whereas Cu^{2+} produced a flocculence almost immediately, even at relatively low concentrations (metal ion/protein subunit ratio

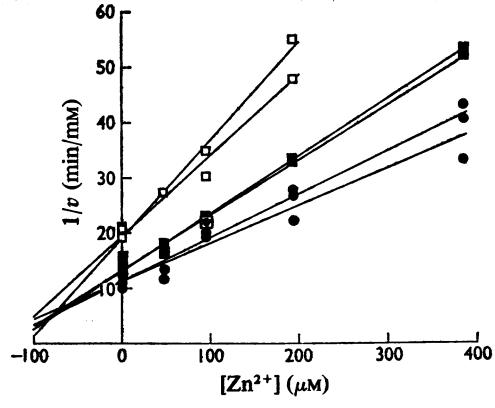

Fig. 1. *Inhibition by Zn^{2+} of apoferritin iron uptake at low Fe^{2+} concentrations*

Ferrous ammonium sulphate (48–158 μM) was added to a solution containing 0.58mg of apoferritin/ml, 1.4mM-KIO_3 and 7.6mM-$Na_2S_2O_3$ as oxidant, 19mM-imidazole, pH7.45, and 4.8–77 μM-$ZnSO_4$. The uptake of iron was followed by measuring the absorption of the ferric oxide hydrate product at 310nm ($E^{1\%Fe}_{1cm} = 450$). ●, 158 μM-Fe^{2+}; ■, 96 μM-Fe^{2+}; □, 48 μM-Fe^{2+}. The lines represent the computed best fit to the unweighted data, ±1 S.D. for the slope and intercept.

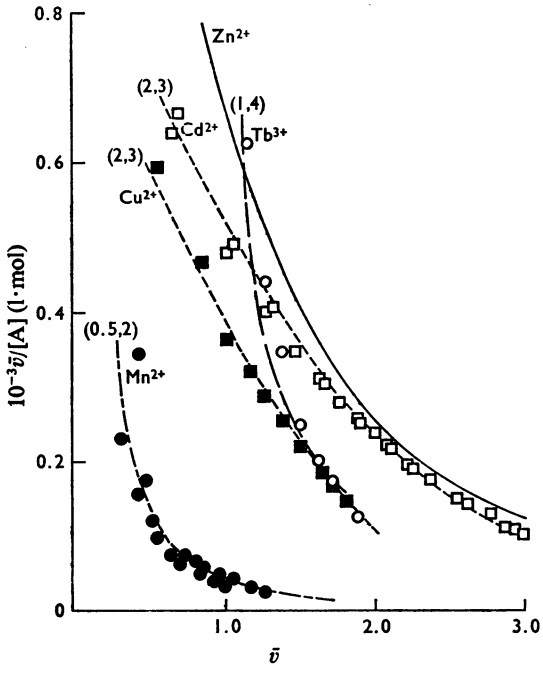

Fig. 2. *Binding of metal ions by apoferritin*

Binding was estimated by measurements in the pH-stat of the displacement of H^+ from the protein. Apoferritin (6mg) was added to 0.2ml of 1M-NaCl plus water to a volume of 1.25ml, and the mixture was adjusted to the exact pH of the 0.1M-metal sulphate solution. The metal salt was added to the protein solution in discrete volumes of 0.01ml. The displacement of H^+ by the metal was measured by recording the amount of 2.5mM-NaOH required to maintain the initial pH of the solution. In preparing the Scatchard plot no correction was made for electrostatic parameters. $\bar{v}$, Number of mol of metal ion bound/mol of apoferritin subunit; [A], concentration of free metal ion. Curves shown as broken lines were computed by assuming independent binding of the ions to two classes of site. The numbers on the curves refer to the number of sites in each class and the apparent binding constants are given in Table 1. The continuous line represents the experimental curve for Zn^{2+} binding, the data for which are given in Fig. 3. ●, Mn^{2+}, pH5.55; ■, Cu^{2+}, pH4.7; □, Cd^{2+}, pH5.5; ○, Tb^{3+}, pH5.55.

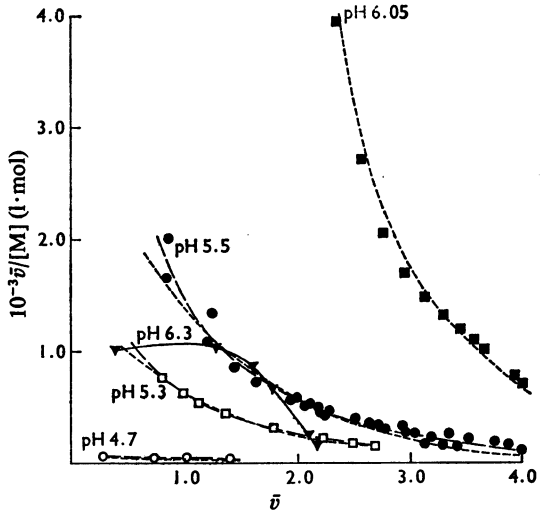

Fig. 3. *Effect of varying pH on the binding of Zn^{2+} by apoferritin*

Conditions and method were as described in Fig. 2. The pH was adjusted by the use of 0.1M-HCl or 0.1M-NaOH. ———, Simulated curves, assuming two classes of one and four sites respectively; ----, simulated curves assuming two classes of two and three sites respectively. ▼, pH6.3; ■, pH6.05; ●, pH5.5; □, pH5.3; ○, pH4.7. The anomolous nature of the curve at pH6.3 (▼) can be explained either by the loss of a stoicheiometric relationship between Zn^{2+} bound and protons released or by the buffering action of certain groups on the protein.

approx. 3.0). Dialysis of the precipitate against distilled water resolved the protein, showing that the binding and aggregation are reversible.

The non-linearity of the Scatchard plots shown in Figs. 2 and 3 could be a consequence either of independent binding at multiple classes of site or of negative co-operativity, or a mixture of both. Negative co-operativity seems in this case to be an unlikely hypothesis and it was decided to analyse the results on the assumption that there are several independent classes of independent and equivalent sites. Too few parameters were known to apply electrostatic correction factors to the data, and this might also in part be responsible for the non-linearity, although the effect is likely to be small.

Two techniques were used to determine first the number of classes of site and then the number and binding constant of the sites in each class. The first technique involves the superposition of a graph of $\log y = \log(x/1+x)$ over the data plotted as $\log\bar{v}$ versus log[A], where $\bar{v}$ = μmol of bound metal ion per subunit of protein and [A] = concentration (mM) of free metal ion (Scatchard *et al.*, 1957). This indicated the presence of two classes of site for Zn^{2+} and Cd^{2+}. The second method was the use of a non-linear

Table 1. *Binding of metal ions to apoferritin*

The binding data were analysed on the assumption that there are two independent classes of site per apoferritin subunit (assuming a subunit mol.wt. of 18500), which bind according to the equation:

$$\bar{v} = \frac{n_1 K_1 [A]}{1 + K_1 [A]} + \frac{n_2 K_2 [A]}{1 + K_2 [A]}$$

where $\bar{v}$ = number of ions bound/subunit and [A] = concentration of free ion; n_1 and n_2 are the numbers of sites within the two classes and K_1 and K_2 are the apparent binding constants. Calculated values are given below. R, in the last column of the table, is the normalized root mean square of the difference between the observed and calculated data. A perfect fit with no experimental error would give a value of zero, and random data within the range of the experimental results give a value for R of >1. R was calculated from the equation:

$$R = \frac{\sqrt{n\Sigma(\bar{v}_0 - \bar{v}_c)^2}}{\Sigma\bar{v}_0}$$

where $\bar{v}_0$ = observed number of ions bound/subunit, $\bar{v}_c$ = calculated number of ions bound/subunit and n = number of data points.

Cation	pH	No. of H^+ displaced	n_1	$\log K_1$	n_2	$\log K_2$	R
Zn^{2+}	4.70	1	2	1.48	3	0.48	0.032
			1	1.78	4	0.65	0.033
Zn^{2+}	5.30	1	2	2.70	3	1.30	0.026
			1	3.22	4	1.65	0.028
Zn^{2+}	5.50	1	2	2.98	3	1.48	0.058
			1	3.50	4	1.84	0.084
Zn^{2+}	6.05	1	2	~4.7	3	2.53	0.023
			1	~5.0	4	2.74	0.065
Cd^{2+}	5.50	1	2	2.88	3	1.48	0.017
			1	3.43	4	1.74	0.065
Cu^{2+}	4.70	2	2	2.87	3	0.00	0.035
			1	3.26	4	1.60	0.037
Mn^{2+}	5.55	1	0.5	3.30	2	1.30	0.060
Tb^{3+}	5.55	1	2	3.08	3	0.30	0.081
			1	4.3	4	1.62	0.047

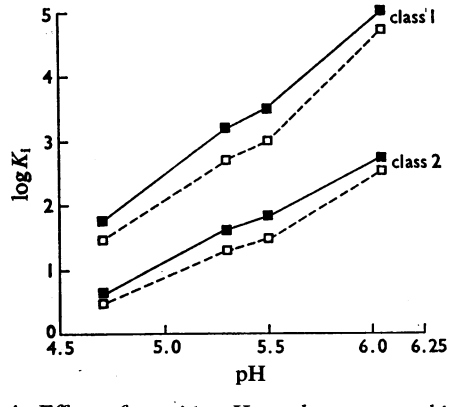

Fig. 4. *Effect of varying pH on the apparent binding constants for the binding of Zn^{2+} to apoferritin*

It was assumed that there are two independent classes of n_i independent sites. Values for $\log K_i$ were taken from Table 1. ■, $n_1 = 1$, $n_2 = 4$; □, $n_1 = 2$, $n_2 = 3$.

least-squares computer program to fit the basic data to a two-term binding equation:

$$\bar{v} = \frac{n_1 K_1 [A]}{1 + K_1 [A]} + \frac{n_2 K_2 [A]}{1 + K_2 [A]} \quad (1)$$

where n_i = number of sites within the class i, and K_i = the apparent binding constant for the sites within that class. The results from both analyses were put back into eqn. (1) to produce simulated Scatchard plots, which allowed a direct comparison with the experimental results.

The two classes of site to which Zn^{2+}, Cd^{2+} and Tb^{3+} bind appear to possess either one or two and either three or four sites per subunit respectively. Mn^{2+}, however, seems to possess 0.5 and two or three sites in each class, and Cu^{2+} may bind to only a single class of site, if the slight departure from linearity of the Scatchard plot for this ion is due to other factors. Values of K have been calculated, however, on the assumption that two classes of binding site do in fact exist for this cation.

Table 1 gives estimates of the apparent binding constants for all the cations used in the experiments, calculated from eqn. (1), plus a value for the residual mean square, R, which is a measure of the 'goodness of fit' between the simulated curves and the experimental points. The apparent binding constants for Zn^{2+} rise logarithmically with pH (Fig. 4). Above pH6.05, however, a rapid change occurs, as can be seen in Fig. 3, and the two-term binding equation no longer holds.

Discussion

The kinetic data show that Zn^{2+} inhibits Fe^{2+} uptake by apoferritin at very low substrate concentrations. The competitive nature of this inhibition provides supporting evidence for the conclusion (Macara *et al.*, 1973*b*) that the first step in the catalytic action of apoferritin in converting Fe^{2+} into hydrous ferric oxide in its interior is the binding of Fe^{2+} by the protein. It is proposed that oxidation occurs at these binding sites and that Zn^{2+} competes with Fe^{2+} for these sites.

The titration studies provide further evidence that Zn^{2+} (and other cations) are bound by apoferritin. The results suggest that Zn^{2+}, Cd^{2+}, Mn^{2+} and Tb^{3+} bind to two separate classes of site, one class having a much higher binding constant than the other. Zn^{2+}, Cd^{2+} and probably Tb^{3+} appear to bind to the same sites. Mn^{2+} may bind differently, since site 1 apparently binds only one Mn^{2+} for every two subunits, suggesting that it lies on the twofold axes between subunits. Cu^{2+} may also bind at different classes of sites, since, unlike the other cations, it displaces two H^+ on binding (instead of only one) and precipitates the protein at much lower metal ion:protein ratios than do the other cations. It seems likely that this binding site is on the outside of the protein molecule. Aggregation and precipitation of the apoferritin also occur with Zn^{2+} and Cd^{2+} when these ions are in greater than about 30-fold molar excess per subunit, and Cd^{2+} is commonly used for crystallization of ferritin and apoferritin (Michaelis, 1947). The second class of binding sites for these ions is therefore probably also on the external surface of the protein molecule. The first class of sites, which have a higher affinity for these cations, can be presumed to include the catalytic sites. There may be two binding sites in this class and it is also possible that the catalytic site may involve the binding of two Fe^{2+} in close proximity. The binding data cannot, however, be interpreted unambiguously and there may be a single site in this class.

The apparent binding constant of Zn^{2+} at the first class of site rises approximately logarithmically with increasing pH up to pH6.05, but at pH6.3 the binding curve is anomalous. This could be explained if either the group to which Zn^{2+} binds has a pK of about 6.0–7.0, so that in this pH region there is no longer any stoicheiometric relationship between Zn^{2+} bound and protons released, or else other groups on the protein are buffering the solution at this pH. The former hypothesis suggests that the binding group may be histidine or possibly cysteine. It has been reported (Bryce & Crichton, 1973) that modification of two cysteine residues and one histidine residue abolishes the catalytic activity of apoferritin, whereas modification of a single cysteine does not. Since only a single proton is released on binding Zn^{2+} or Cd^{2+} it can be concluded that if other groups are involved in binding they are either uncharged (e.g. the carbonyl oxygen of the peptide group) or are already negatively charged in the pH range under study (e.g. ionized carboxyl groups with pK below 4.7).

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