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The Catalytic Activity of Horse Spleen Apoferritin

PRELIMINARY KINETIC STUDIES AND THE EFFECT OF CHEMICAL MODIFICATION

By CHARLES F. A. BRYCE and ROBERT R. CRICHTON*
Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 63, Berlin-Dahlem, Germany, and Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, U.K.

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1. Horse spleen apoferritin catalyses the oxidation of Fe^{2+} to Fe^{3+} with molecular O_2 as electron acceptor under conditions where a number of other proteins have no such effect. The product is similar to ferritin by a number of criteria. 2. The progress curve is hyperbolic and the increase in initial velocity is linear with increasing apoferritin concentration. With respect to Fe^{2+} the reaction follows Michaelis–Menten kinetics. The pH-dependence of the reaction was determined between pH 4.3 and 6.0. 3. Modification of both tryptophan residues/apoferritin subunit with 2-nitrophenylsulphenyl chloride does not affect either $k_{cat.}$ or K_m for the oxidation. Neither does the guanidination of seven out of nine lysine residues/subunit, the modification of nine out of ten arginine residues/subunit with cyclohexanedione, or the nitration of one out of five tyrosine residues/subunit with tetranitromethane. 4. The carboxymethylation of two out of three cysteine residues/subunit and of one out of six histidine residues/subunit can be achieved with iodoacetic acid. This carboxymethylated apoferritin is completely inactive in Fe^{2+} oxidation. 5. Apoferritin does not take up Fe^{3+}. It appears from these results that Fe^{2+} is the form in which iron is taken up by ferritin in a reaction where the protein acts as an enzyme which traps the product in the interior of the protein shell.

Ferritin as isolated from horse spleen varies in its iron content from zero up to a maximum of 4500 atoms (Rothen, 1944; Fischbach & Anderegg, 1965). The iron is present as a ferric hydroxyphosphate micelle and occupies the interior of the molecule, surrounded by a shell of apoferritin protein subunits. Apoferritin has a molecular weight of 440000 and consists of 24 identical subunits each of molecular weight 18500 (Crichton, 1972, 1973).

The mechanism by which iron is taken up by ferritin is not fully understood. Bielig & Bayer (1955) reported the preparation of 'synthetic' ferritin from the incubation of iron-free horse spleen apoferritin in bicarbonate buffer at pH 7.4–7.6 with solid ferrous ammonium sulphate, air oxidation at 4–6°C and subsequent crystallization from $CdSO_4$. The product had an Fe/N ratio of up to 2.4 (about the same as in native ferritin) depending on the amount of ferrous salt added. Loewus & Fineberg (1957) confirmed these observations with rat and horse apoferritins and showed that ferric ammonium citrate could serve as a source of iron for ferritin in the presence of rat liver extract or of ascorbate and molecular O_2. However, the amounts of iron incorporated from Fe^{3+} amounted maximally to only 15% of the original iron content of the ferritin from which the apoferritin had been prepared, whereas uptake from Fe^{2+} was found to be 80% of the original iron content of the ferritin. Uptake of Fe^{2+} by apoferritin was also found in the presence of suitable oxidizing agents or molecular O_2 (Harrison *et al.*, 1967), and apoferritin was reported to influence the rate of oxidation of Fe^{2+} as well as the structure of the micelle produced. Niederer (1970) showed, by following the loss of Fe^{2+}, that apoferritin catalysed the oxidation of Fe^{2+} to Fe^{3+} in the presence of suitable oxidizing agents, in marked contrast with a number of other proteins. He also noted a slight decrease in iron uptake after treatment of apoferritin with formaldehyde, β-propiolactone or bromoacetate, and a strong inhibition of the catalysis by Zn^{2+}. From these observations he postulated that ferritin formation took place by penetration of the apoferritin shell by Fe^{2+} ions, which were oxidized at the inner surface of the protein by a catalytic active site: the Fe^{3+} ions formed an intramolecular precipitate which very soon became too large to escape from the apoferritin shell. Further, he suggested that histidine residues in the protein were involved in the catalytic site. Macara *et al.* (1972) subsequently extended the previous studies of Harrison *et al.* (1967) and found that progress curves for iron uptake by apoferritin were sigmoidal whereas

* To whom reprint requests and enquiries should be directed at the Max-Planck-Institut in Berlin.

those for ferritins of low iron content were hyperbolic. They proposed a crystal growth model in which an initial slow nucleation step was followed by a rapid 'growth' phase. Apoferritin was postulated to have binding sites which are saturated with respect to Fe^{2+}; the same sites are the point of oxidation of Fe^{2+} to Fe^{3+}. A number of models for the growth phase were advanced.

A quite radically different model has been advanced by Pape *et al.* (1967), who proposed that ferritin formation takes place by the hydrolysis and polymerization of low-molecular-weight ferric chelates to form micelles of about 7nm diameter followed by the dissociation of apoferritin molecules into subunits and their reassociation around the micelles to form ferritin. This model has been criticized on a number of grounds, the most cogent of which are the fact that apoferritin does not spontaneously dissociate into subunits under physiological conditions and that when ferritin synthesis is induced by iron administration the first molecules to be formed are poor in iron, which does not agree with the basic tenet of the hypothesis (Drysdale *et al.*, 1968; Niederer, 1970; Macara *et al.*, 1972).

We report here the development of an assay procedure for the study of Fe^{2+} oxidation by apoferritin under conditions in which analysis is not complicated by autoxidation. A preliminary account of the kinetics is presented together with the results of chemical-modification experiments directed towards the identification of amino acid residues involved in the catalytic activity. A preliminary report of this work has been published (Crichton & Bryce, 1972).

Experimental

Materials

Ferritin was prepared from horse spleen as described previously (Crichton *et al.*, 1973*a*) or was purchased from Schwarz/Mann, Orangeburg, N. Y., U.S.A. Apoferritin was prepared by dialysis against 1% (v/v) thioglycollic acid (Crichton, 1973). All glassware and cuvettes were soaked in 6M-HCl for 2–3h to remove metal contaminants and were then thoroughly washed in twice-glass-distilled water. All solutions were prepared with twice-glass-distilled water. Ferrous iron was determined as the 2,2′-bipyridyl complex at 520nm by using an experimentally derived molar extinction coefficient of 8158 litre·mol^{-1}·cm^{-1} (Bryce, 1972). Apoferritin concentrations were determined by the absorption of the protein at 280nm ($E^{1\%}_{1cm} = 9.82$), or by the method of Lowry *et al.* (1951), with bovine serum albumin as protein standard.

Thioglycollic acid, 2-nitrophenylsulphenyl chloride and iodoacetic acid were from British Drug Houses, Poole, Dorset, U.K.; *O*-methylisourea was from Serva, Heidelberg, Germany; tetranitromethane was from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.; cyclohexane-1,2-dione and 2,2′-bipyridyl were from Fluka A.G., Buchs, Switzerland. Iodo-[2-^{14}C]acetic acid of specific radioactivity 154μCi/mmol was from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Preliminary assay conditions. To establish the most suitable conditions for studying ferritin formation we examined a number of buffer systems, including those of Bielig & Bayer (1955), Loewus & Fineberg (1957) and Harrison *et al.* (1967), as well as carbonate, Tris–maleate and cacodylate–borate. After having established that in terms of our *a priori* conditions 0.1M-sodium borate–cacodylate buffer was the most suitable, we examined the amount of Fe^{2+} remaining in solution after dissolving ferrous ammonium sulphate (5mM) in this buffer at various pH values and established a time-course for this process at a number of pH values. To be able to follow the reaction directly we determined the molar extinction coefficient of the iron micelle of ferritin at 420nm. The value was $E^{1\%}_{1cm} = 61.4 \pm 1.5$ (Bryce, 1972).

The final assay conditions established after our preliminary studies were as follows: apoferritin or other protein solutions at the appropriate concentration were dialysed for 24h against several changes of 0.1M-borate–cacodylate buffer at the required pH value and 22°C. Ferrous ammonium sulphate was dissolved in the same buffer at an appropriate concentration and two 1ml samples were transferred to two matched 1ml cuvettes. These were placed in a Cary 16 spectrophotometer (Cary Instruments, Monrovia, Calif., U.S.A.) equipped with a Cary 1626 recorder interface and a Honeywell Elektronik 194 recorder (Honeywell Controls, Newhouse, Lanarkshire, U.K.) and the baseline at 420nm was allowed to stabilize. The cuvettes were removed and 50μl or 100μl of the protein solution was added to the sample cell and the same volume of buffer was added to the reference cell. The cell contents were quickly mixed by inverting, the cells replaced and the absorbance at 420nm was monitored continuously for 20–120min. Initial velocities were determined by drawing tangents.

Identification of product. Samples obtained from experiments such as those described above were analysed by a number of techniques. Before determination of iron and protein content unbound iron was removed by gel filtration on columns (1.5cm×25cm) of Sephadex G-25 eluted with 0.1M-sodium borate–cacodylate buffer, pH6.0. A flow rate of 20ml/h was used and 2ml fractions were collected in a Radirac fraction collector (LKB

Produkter, Stockholm, Sweden). Iron was determined after reduction to Fe^{2+} with thioglycollic acid (1%, v/v) by the bipyridyl reaction, and protein concentration by the method of Lowry *et al.* (1951). Sedimentation-velocity experiments were performed as described in the preceding paper (Crichton & Bryce, 1973). Reconstituted ferritin was also analysed by polyacrylamide-gel electrophoresis, by using 6.5% gels in buffer at pH 8.6 as described previously (Crichton *et al.*, 1973*a*). Gels were stained for protein with Amido Black (Crichton *et al.*, 1973*a*) and for iron with potassium ferrocyanide [2g/l, dissolved in 2% (v/v) HCl].

Electron microscopy of reconstituted ferritins was kindly done by Mr. W. Wabl, with a Siemens Elmiskop I apparatus. Samples were negatively stained with uranyl acetate. A magnification of 16250 was used.

Kinetic experiments. The effect of protein concentration, Fe^{2+} concentration and pH was analysed by using the assay system described above. In a number of cases the loss of Fe^{2+} was followed by using a dialysis cell (Bryce, 1972) in which the apoferritin solution was separated from the ferrous ammonium sulphate solution by a semi-permeable membrane, and the Fe^{2+} solution was circulated continuously. Samples were removed at 30–60s intervals and transferred directly to a solution of 2,2′-bipyridyl.

Fractionation of ferritin. Ferritin preparations were fractionated according to their iron content by density-gradient centrifugation in isopycnic gradients of CsCl (Meselson *et al.*, 1957). Native apoferritin was also isolated from ferritin preparations by differential centrifugation (2h at 95000*g*).

Chemical modification of apoferritin. The modification of cysteine residues was by the method of Canfield & Anfinsen (1963). Cysteine modification was also carried out on a sample that had been dissociated into subunits with 1% sodium dodecyl sulphate (Crichton & Bryce, 1970). Modification with iodo[^{14}C]acetic acid was performed in the same way. The extent of modification was determined for radioactive samples by acid hydrolysis and amino acid analysis in the presence of 10nmol of norleucine and radioactivity counting as described previously (Crichton *et al.*, 1973*b*). *S*-Carboxymethylcysteine and 3-monocarboxymethylhistidine were determined by amino acid analysis after acid hydrolysis by using colour factors determined for authentic samples of these two amino acids.

Tryptophan was modified with 2-nitrophenylsulphenyl chloride as described by Bryce & Crichton (1971) by the method of Boccu *et al.* (1970) and the degree of modification estimated spectrophotometrically after reversal of cysteine modification.

The procedures for modification of tyrosine, lysine and arginine with tetranitromethane, *O*-methylisourea and cyclohexanedione are described in the preceding paper together with methods for determination of the degree of modification (Crichton & Bryce, 1973).

Results

Establishment of an assay procedure

We selected ferrous ammonium sulphate instead of other ferrous salts on account of its relatively slow autoxidation and solubility. The selection of a suitable buffer was not so easy: the requirements here were for a buffer in which the solubility product of ferrous and ferric salts was not exceeded, in which autoxidation was minimal (thereby allowing any catalytic effect of apoferritin to be conveniently followed), which did not chelate or complex the ferrous salts in such a way that the iron could not be taken up from the complex, and which had a sufficient buffering capacity to cope with the protons generated resulting from hydration of Fe^{3+} (Speakman, 1962).

Increasing the pH of a solution of Fe^{2+} results in an ever more rapid autoxidation to Fe^{3+}, so that above about pH 9.0, no Fe^{2+} is found in solution (Fig. 1).

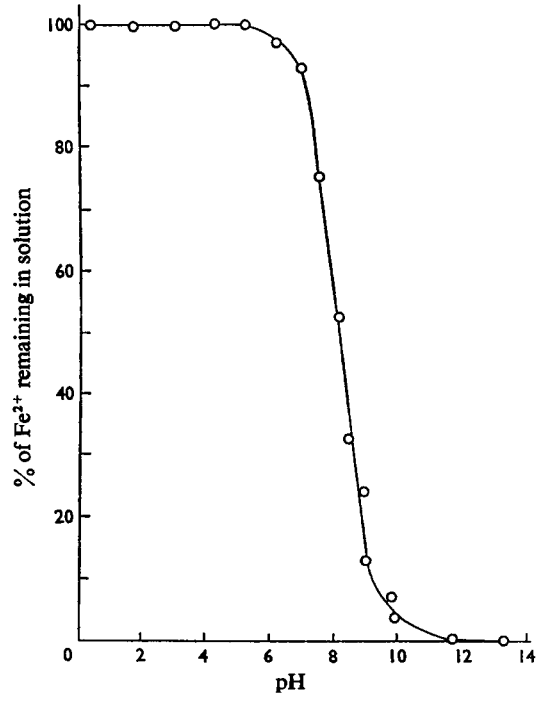


Fig. 1. *Solubility of Fe^{2+} as a function of pH*

Ferrous ammonium sulphate (of 5mM) was dissolved in water and the pH adjusted to the appropriate value with HCl or NaOH. A sample was removed and the content of Fe^{2+} determined as the complex with bipyridyl.

This agrees well with previous results of Conrad (1970) for $FeCl_2$. In Na_2CO_3 buffers a heavy precipitate formed when ferrous ammonium sulphate was added; the same was found with $NaHCO_3$ buffers, pH6.8–8.0. With imidazole buffers (20mM, pH6.7–7.5) a precipitate of ferric oxyhydroxide was formed both in the presence of O_2 and with KIO_3 and $Na_2S_2O_3$ (Macara *et al.*, 1972). Tris–maleate–NaOH buffers were also unsatisfactory; although no precipitate formed, the Fe^{2+} was still rapidly oxidized to Fe^{3+}. Sodium borate–cacodylate buffer was finally chosen and with this system we examined the effect of buffer pH and ionic strength on the oxidation of Fe^{2+}, as well as the extent of changes in the buffer pH caused by hydration of Fe^{2+} and Fe^{3+}. Fig. 2 shows the amount of ferrous ammonium sulphate remaining in solution at a variety of pH values in 100mM-borate–cacodylate buffer as a function of time. Oxidation of Fe^{2+} is extremely rapid at pH7.4, whereas at pH values lower than about 5.5 virtually zero oxidation takes place. By using 100mM-cacodylate–borate buffers of pH3.0–7.0 we could detect virtually no change in buffer pH within 1h of dissolving ferrous ammonium sulphate. Above pH7 the change was quite rapid, falling for example from 7.4 to 7.2 in 10min (Fig. 3). For comparison we have included 20mM-imidazole buffer, pH7.38, and with this buffer the pH change on dissolving 5mM-ferrous ammonium sulphate is extremely rapid: within 1min the pH decreased to 7.13.

We decided on the basis of these preliminary experiments to use as our assay buffer for most purposes (except effect of pH) 100mM-borate–cacodylate buffer, pH5.5. A typical progress curve is shown in Fig. 4. The effect of bovine serum albumin and of lysozyme on the reaction are also shown in Fig. 4.

Reaction kinetics

The effect of apoferritin concentration on the initial velocity of the reaction determined in the range 0.2–4mg/ml and is shown in Fig. 5. The relationship is linear, i.e. the initial velocity of the reaction shows

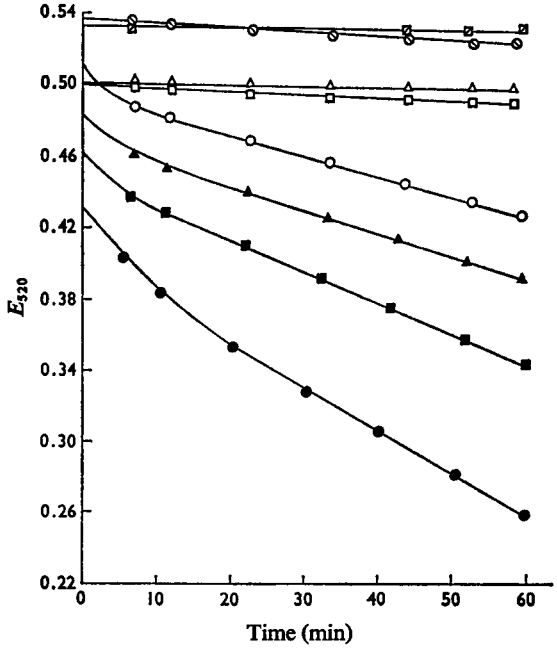


Fig. 2. *Effect of pH on the solubility of Fe^{2+} (at a concentration of 5mM) in 100mM-borate–cacodylate buffer of different pH values as a function of time*

Fe^{2+} was determined as the complex with bipyridyl at 520nm. ⊖, pH4.66; ▨, pH5.22; △, pH5.38; □, pH5.56; ○, pH5.92; ▲, pH6.39; ■, pH6.89; ●, pH7.40.

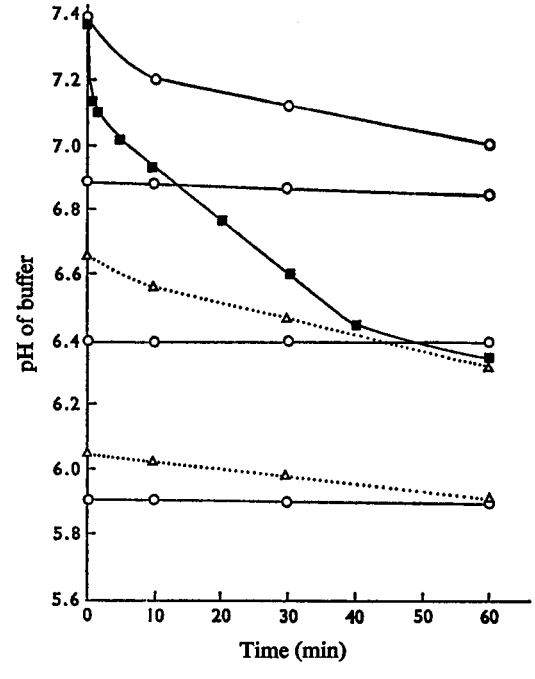


Fig. 3. *Effect of addition of ferrous ammonium sulphate (to a concentration of 5mM) on the pH of various buffers*

For details see the text. ○, 100mM-borate–cacodylate buffers. The initial pH of the buffers was 7.40, 6.89, 6.39 and 5.92. △, 20mM-borate–cacodylate buffers of initial pH6.66 and 6.06. ■, 20mM-imidazole buffer, pH7.38.

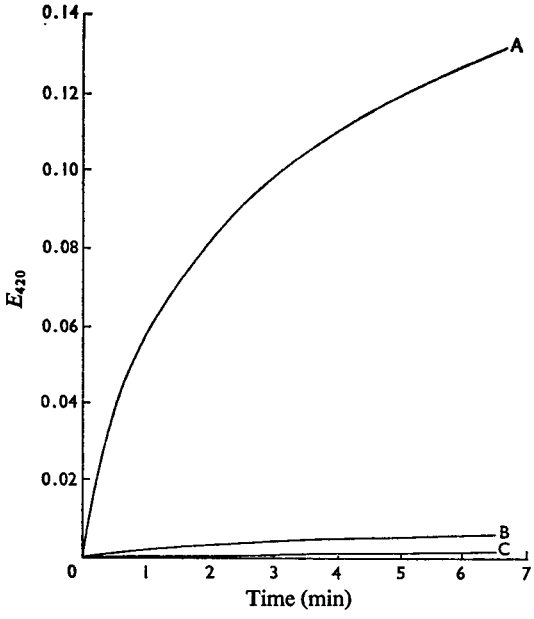


Fig. 4. *Effect of the addition of several proteins on the oxidation of Fe^{2+} in 100 mM-borate–cacodylate buffer, pH 5.50*

For details see the text. Curve A, horse spleen apoferritin (2.27 μM); curve B, bovine serum albumin (13.8 μM); curve C, hen's egg-white lysozyme (26.6 μM).

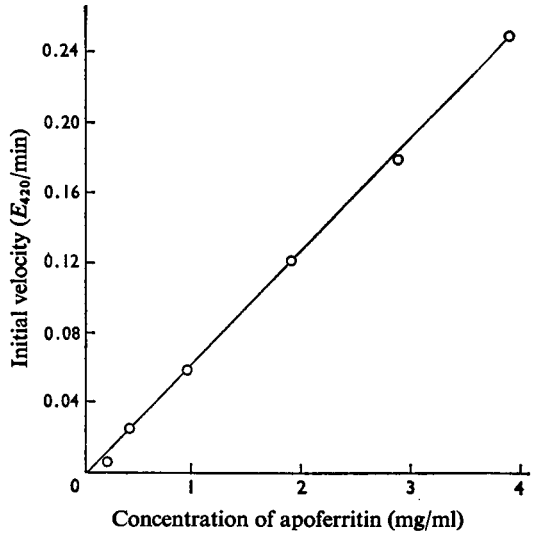


Fig. 5. *Effect of apoferritin concentration on the initial velocity of Fe^{2+} oxidation*

Apoferritin concentrations of 0.1–4 mg/ml were used and the initial velocities calculated as described in the text.

first-order dependence on the concentration of protein. Since we were carrying out our experiments with dissolved molecular O_2 as electron accepter, we could not easily vary this parameter. However, the concentration of dissolved O_2 was constant in all experiments, so that we could effectively analyse the effect of Fe^{2+} concentration. The curve so obtained has the form of a rectangular hyperbola, typical of an enzyme-catalysed reaction defined by the Michaelis–Menten equation (Dixon & Webb, 1964) and the parameters $k_{cat.}$ ($V_{max.}/[E]_0$; where $[E]_0$ is the initial concentration of enzyme) and K_m were determined from a plot of $1/v$ versus $1/[S]$ (Lineweaver–Burk plot; see Dixon & Webb, 1964) (Fig. 6). The values obtained for $k_{cat.}$ and K_m are 61.5 min^{-1} and 2.82 mM. Native apoferritin was found to have very similar kinetic parameters to those found for apoferritin prepared by chemical reduction (Fig. 6): $k_{cat.}$ was 67 min^{-1} and K_m was 2.84 mM.

The effect of pH on initial velocity was examined over the pH range 4.3–6.0 (Fig. 7), from which it is clear that we are operating well below the optimum pH of the reaction.

All of these kinetic studies were performed by following the formation of ferric oxide hydrate. Typical results obtained with the dialysis cell described above, in which samples were removed for determination of Fe^{2+}, are shown in Fig. 8, and are similar to those found by the direct method.

Chemical modification

The extent of chemical modification of tryptophan, tyrosine, lysine and arginine residues has been determined previously (Bryce & Crichton, 1971; Crichton & Bryce, 1973), and we know that both tryptophan residues/subunit are converted into the nitrophenylsulphenyl derivative, that one out of five tyrosine residues/subunit is nitrated, that seven out of nine lysine residues/subunit are guanidinated and that nine out of ten arginine residues are modified with cyclohexanedione. The ability of these modified apoferritins to catalyse the oxidation of Fe^{2+} is not at all impaired. The kinetic parameters $k_{cat.}$ and K_m are given in Table 1. Carboxymethylation of apoferritin with iodo[2-^{14}C]acetic acid led to the introduction of 3.05±0.01 carboxymethyl groups per 18500 daltons of apoferritin (i.e. per subunit). Amino acid analysis of carboxymethylated apoferritin gave the result shown in Table 2, from which we conclude that treatment with iodoacetic acid leads to the carboxymethylation of two cysteine residues and one histidine residue/subunit. The carboxymethylated protein had

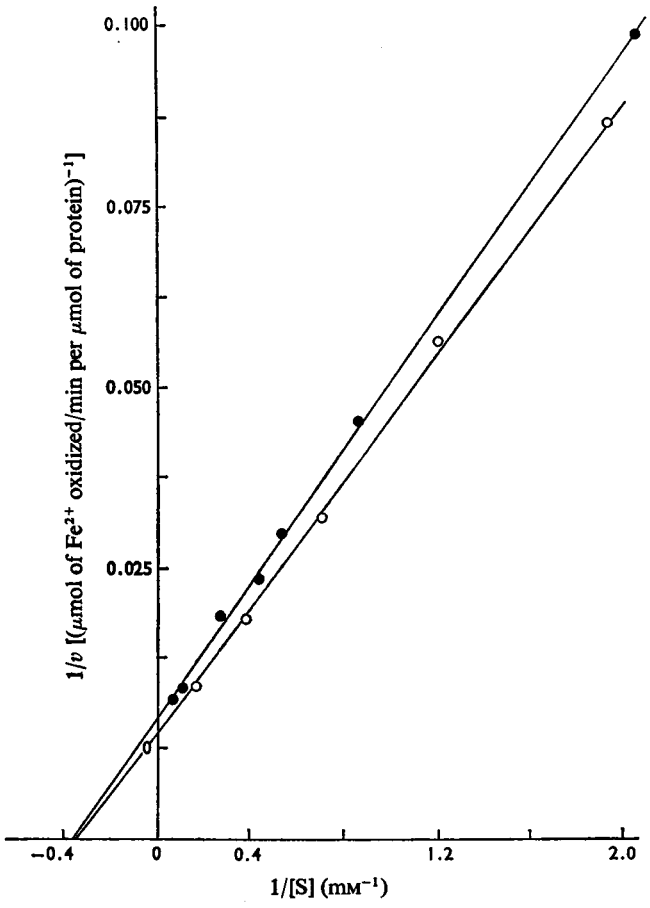


Fig. 6. *Lineweaver–Burk plot for catalysis of Fe^{2+} oxidation by apoferritin prepared by chemical reduction* (●) *and native apoferritin* (○)

Initial velocities were determined at concentrations of Fe^{2+} from 0.5–20mM for both apoferritins and double-reciprocal plots constructed as described in the text.

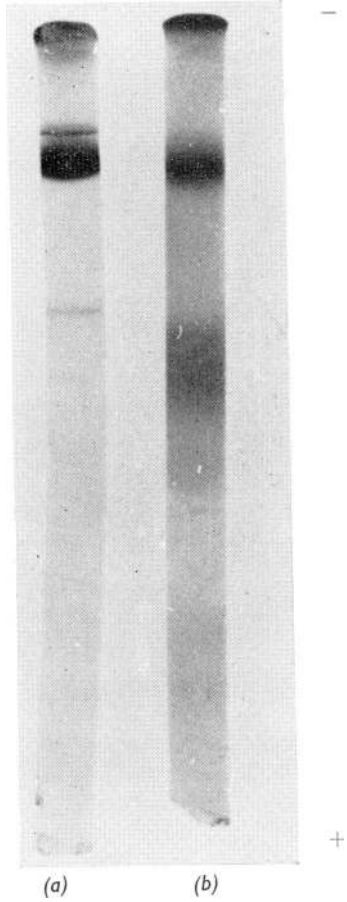
no catalytic activity whatever (Fig. 8), whereas apoferritin that had been treated in the same way, except that no iodoacetate was added, retained its catalytic activity. We also established that the protein was not dissociated into subunits as a result of carboxymethylation.

Characterization of product

The products of reconstitution experiments were analysed as described in the Experimental section. In all cases the Fe/N ratio was greater than 0.8, and in several cases values in excess of 2.0 were obtained. The product sedimented as a broad diffuse band with an approximate *s* value of 35–60S, unlike apoferritin (about 17S). Duplicate polyacrylamide gels were stained respectively for iron and for protein after electrophoresis and most of the iron migrated with the protein (Plate 1). The appearance of the product in the electron microscope also differed from apoferritin and closely resembled that of ferritin (Plate 2).

Discussion

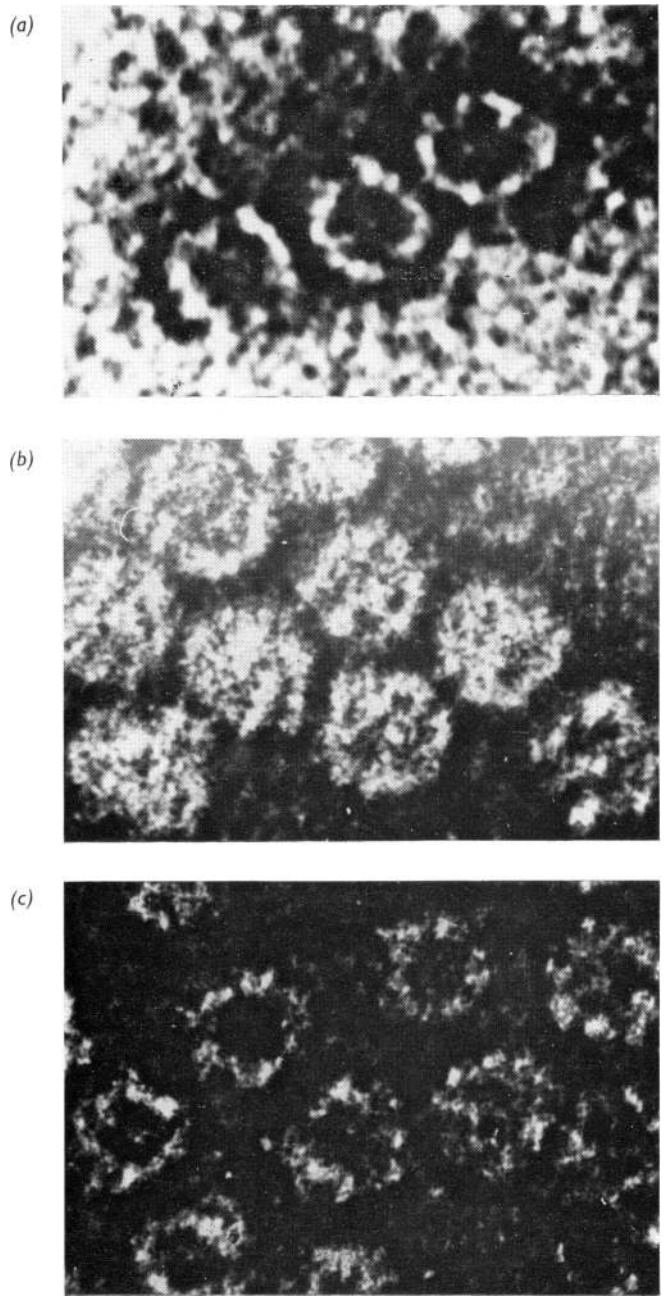
From the results presented here we would conclude that the mechanism of ferritin formation can be simply explained on the basis of the 'penetration' hypothesis advanced by Niederer (1970). However, Macara *et al.* (1972) have proposed a much more detailed and complex model for ferritin formation, based on the experimental results found by them. The primary reason for their considering a crystal growth



EXPLANATION OF PLATE 1

Gel electrophoresis of reconstituted ferritin

A sample (50μg) of protein was applied to two gels which were run in parallel as described in the text and stained for protein (*a*) and iron (*b*).



EXPLANATION OF PLATE 2

Electron micrographs of (*a*) *ferritin,* (*b*) *apoferritin and* (*c*) *reconstituted ferritin*

For details see the text. Magnification ×16250.

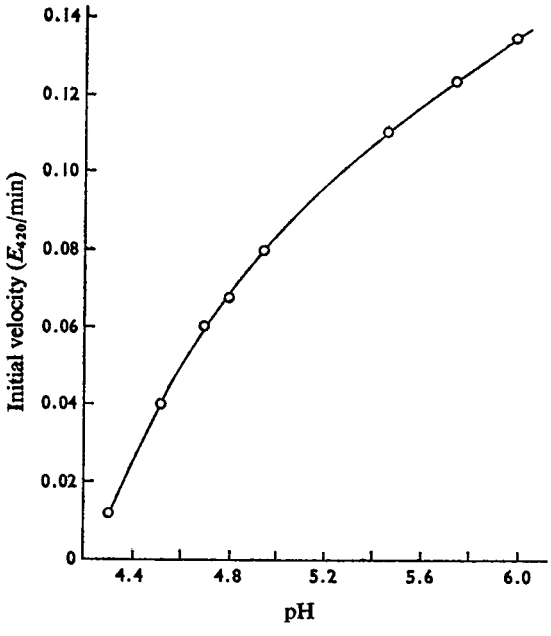


Fig. 7. *Effect of pH on the initial velocity of Fe^{2+} oxidation catalysed by apoferritin*

Conditions are given in the text.

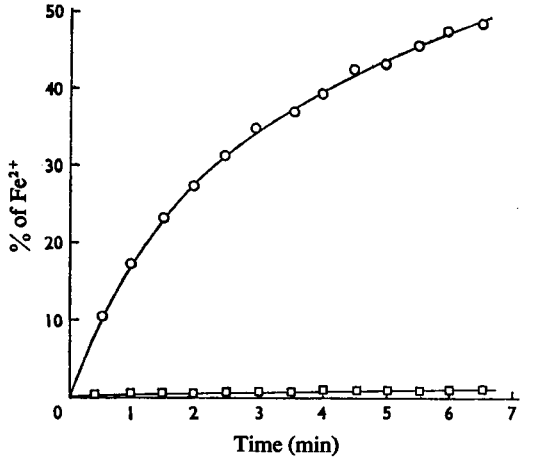


Fig. 8. *Oxidation of Fe^{2+} catalysed by apoferritin as a function of time measured with the dialysis cell described in the text*

Decrease in Fe^{2+} was determined with the bipyridyl reaction. ○, Apoferritin; □, carboxymethylated apoferritin.

Table 1. *Kinetic parameters of modified and native apoferritins*

The values $k_{cat.}$ and K_m were determined as described in the text from Lineweaver–Burk plots in the range 0.5–20mM-ferrous ammonium sulphate.

Protein	K_m (mM)	$k_{cat.}$ (min^{-1})
Apoferritin*	2.82	61.5
Native apoferritin	2.84	67
Apoferritin modified with 2-nitrophenylsulphenyl chloride	2.32	63
Apoferritin modified with tetranitromethane	2.71	58
Apoferritin modified with *O*-methylisourea	2.44	55
Apoferritin modified with cyclohexanedione	2.30	57

* Prepared by chemical reduction as described under 'Methods'.

Table 2. *Amino acid composition of carboxymethylated apoferritin*

The results are presented as residues of each amino acid/subunit (18500 daltons). The colour factors for carboxymethylcysteine and carboxymethylhistidine were determined by using authentic samples of these amino acid derivatives. The results for the modified protein are the mean of ten determinations. N.D., not determined.

Amino acid residue	Content (residues/subunit) Control	Modified
Cysteic acid	N.D.	N.D.
Aspartic acid	17.50	17.39
Threonine	5.21	5.57
Serine	8.99	8.93
Glutamic acid	23.73	24.04
Proline	2.41	3.25
Glycine	9.70	9.88
Alanine	13.88	14.11
Valine	6.91	6.30
Methionine	2.65	2.38
Isoleucine	2.95	3.75
Leucine	24.77	24.85
Tyrosine	4.94	4.80
Phenylalanine	7.11	6.83
Histidine	5.81	5.07
Lysine	8.55	8.21
Arginine	10.11	9.24
S-Carboxymethylcysteine	0.00	2.05
3-Carboxymethylhistidine	0.00	1.02

model instead of a simple penetration model was the finding that progress curves for iron uptake by apoferritin were sigmoidal whereas those for ferritins of low iron content were hyperbolic. The method used by Macara *et al.* (1972) to initiate the reaction, namely addition of ferrous ammonium sulphate to a solution containing apoferritin or bovine serum albumin in 20mM-imidazole buffer, pH7.45, plus oxidants, may be the cause of the 'complex' progress curve. On initiation of the reaction under these conditions, a number of reactions contribute to the appearance of absorbance at 420nm. First, Fe^{2+} ions must be hydrated, with a corresponding small amount of protons being released. Secondly, autoxidation of Fe^{2+} to Fe^{3+} occurs, as shown in Fig. 2. Thirdly, hydration of Fe^{3+} ions generates protons which, as we have demonstrated in Fig. 3, may cause the pH value of this buffer to fall. And finally, superimposed on all of these other processes, apoferritin catalyses the oxidation of those Fe^{2+} ions that have managed to diffuse into the protein shell (whether this oxidation occurs in the channels between subunits or in the interior of the protein shell is not known). To analyse such a complex series of reactions kinetically would be rather difficult. To proceed from such an analysis to the mathematical models advanced by Macara *et al.* (1972) does not seem to be justified.

The assay method used in our study has the advantage that the rate of autoxidation of Fe^{2+} in the absence of apoferritin is slow and can be continuously monitored in the reference cell, so that the course of the protein-catalysed reaction is presented directly on the recorder trace. The buffering capacity of the borate–cacodylate is sufficient to prevent any change in the pH of the reaction mixture during the course of the oxidation. By initiating the reaction with addition of apoferritin we avoid the problems associated with dilution of ferrous ammonium sulphate solutions, namely that the effective increase in O_2 concentration causes a rapid autoxidation of Fe^{2+}. However, we have not been able to examine the reaction at physiological pH values, and by using dissolved O_2 as our electron acceptor we are limited to studying the effect of only one substrate in what is clearly at least a two-substrate reaction. By using the dialysis cell we were able to show that under N_2 in the absence of dissolved O_2 (with buffers that had been thoroughly degassed) no oxidation occurs. We have also established that under our assay conditions ferric iron (as $FeCl_3$) is not taken up by apoferritin, which excludes the possibility that apoferritin is merely accelerating the apparent rate of oxidation of ferrous iron by trapping the product.

Summarizing the results of Figs. 4–8, we conclude (a) that apoferritin increases the rate of Fe^{2+} oxidation with concomitant formation of a ferritin-like product; (b) that the reaction rate increases linearly with increasing apoferritin concentration; (c) that the kinetics of the reaction at constant O_2 concentration are standard Michaelis–Menten with respect to Fe^{2+} concentration; (d) that the pH optimum of the reaction is (as might be expected) in excess of 6.5, and (e) that although $k_{cat.}$ for the process is low, the value of 61–67min^{-1} compares quite favourably with that reported at pH7.4 in the presence of a large excess of oxidant by Macara *et al.* (1972), namely 270min^{-1}.

The effects of carboxymethylation show that the 'active centre' of apoferritin can be blocked by chemical modification (Fig. 8). Unfortunately, from our analysis of the carboxymethylated protein we cannot at present be sure whether histidine or cysteine residues, or indeed both, are required for oxidation. We have recently analysed a modified apoferritin in which one cysteine residue had been made to react with *N*-ethylmaleimide, and found no effect on the catalytic function of the protein (K. Wetz & R. R. Crichton, unpublished work). The results of Niederer (1970) are indicative though not conclusive on this point. He found a decrease in the rate of oxidation on treatment of apoferritin with a number of reagents that might modify histidine, although the effect with bromoacetate, the most specific of these, decreased the rate of reaction by less than 10%. Niederer (1970) found no effect of iodoacetamide at pH values between 4.5 and 8.0 on iron incorporation, although Mazur *et al.* (1960) reported that iodoacetamide suppressed iron incorporation into ferritin. The effect of Zn^{2+}, on iron incorporation both *in vitro* (Niederer, 1970; Macara *et al.*, 1973) and *in vivo* (Coleman & Matrone, 1969) would suggest that histidine is involved in the catalysis. However, Niederer (1970) himself reported that the histidine-alkylating agent diazonium tetrazole did not affect iron uptake, although from his results we conclude that approximately one histidine residue/subunit was modified. Further speculation seems unjustified, and the detailed analysis of histidine and cysteine reactivity in apoferritin together with functional analysis of such modified proteins must await further investigation.

A number of rather important questions remain to be answered with regard to the 'ferroxidase' activity of apoferritin. We would like to know in what form Fe^{2+} is presented to apoferritin within the cell (certainly not as ferrous ammonium sulphate). The biological electron acceptor may be molecular O_2, but this is not at all certain. And finally the products of the reaction (other than ferritin) remain to be established. We presume that under our conditions the other product is most likely to be H_2O_2. A more detailed analysis must be made before these points can be clarified.

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References

Bielig, H. J. & Bayer, E. (1955) *Naturwissenschaften* **42**, 125–126

Boccu, E., Veronese, F. M., Fontana, A. & Benassi, C. A. (1970) *Eur. J. Biochem.* **13**, 188–192

Bryce, C. F. A. (1972) Ph.D. Thesis, University of Glasgow

Bryce, C. F. A. & Crichton, R. R. (1971) *J. Biol. Chem.* **246**, 4198–4205

Canfield, R. E. & Anfinsen, C. B. (1963) *J. Biol. Chem.* **238**, 2684–2690

Coleman, C. B. & Matrone, G. (1969) *Biochim. Biophys. Acta* **177**, 106–112

Conrad, M. E. (1970) in *Iron Deficiency: Pathogenesis, Chemical Aspects, Therapy* (Hallberg, L., Horweth, H. G. & Vannotti, A., eds.), p. 87, Academic Press, New York and London

Crichton, R. R. (1972) *Biochem. J.* **126**, 761–764

Crichton, R. R. (1973) *Angew. Chem.* **85**, 53–62; *Angew. Chem. (Int. Edn. Engl.)* **12**, 57–65

Crichton, R. R. & Bryce, C. F. A. (1970) *FEBS Lett.* **6**, 121–124

Crichton, R. R. & Bryce, C. F. A. (1972) *Biochem. J.* **130**, 36P

Crichton, R. R. & Bryce, C. F. A. (1973) *Biochem. J.* **133**, 289–299

Crichton, R. R., Millar, J. A., Cumming, R. L. C. & Bryce, C. F. A. (1973*a*) *Biochem. J.* **131**, 51–59

Crichton, R. R., Eason, R., Barclay, A. & Bryce, C. F. A. (1973*b*) *Biochem. J.* **131**, 855–857

Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., pp. 54–116, Longmans, London

Drysdale, J. W., Haggis, G. H. & Harrison, P. M. (1968) *Nature (London)* **219**, 1045–1046

Fischbach, F. A. & Anderegg, J. W. (1965) *J. Mol. Biol.* **14**, 458–473

Harrison, P. M., Fischbach, F. A., Hoy, T. G. & Haggis, G. H. (1967) *Nature (London)* **216**, 1188–1190

Loewus, M. W. & Fineberg, R. A. (1957) *Biochim. Biophys. Acta* **26**, 441–443

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Macara, I. G., Hoy, T. G. & Harrison, P. M. (1972) *Biochem. J.* **126**, 151–162

Macara, I. G., Hoy, T. G. & Harrison, P. M. (1973) *Biochem. Soc. Trans.* **1**, 102–104

Mazur, A., Green, S. & Carleton, A. (1960) *J. Biol. Chem.* **235**, 595–603

Meselson, M. F., Stahl, F. W. & Vinograd, J. (1957) *Proc. Nat. Acad. Sci. U.S.* **43**, 581–588

Niederer, W. (1970) *Experientia* **26**, 218–220

Pape, L., Multani, J. S., Stitt, C. & Saltman, P. (1967) *Biochemistry* **7**, 606–612

Rothen, A. (1944) *J. Biol. Chem.* **152**, 679–693

Speakman, J. C. (1962) *An Introduction to Electronic Theory of Valency*, Arnold Ltd., London