Ferritin: Iron Incorporation and Iron Release

Apoferritin, a hollow protein sphere with a diameter of 120 Å and a molecular weight of 450,000, takes up Fe^{2+} under oxidizing conditions in vitro[1]. Ferritin, on the other hand, releases iron into the surrounding medium under reducing conditions[2]. Based on electron microscopic studies, PAPE[3] has proposed the following hypothesis of ferritin synthesis: The complete apoferritin molecule is in equilibrium with apoferritin subunits; the iron micelles forming independently of the apoferritin moiety are trapped as the apoferritin subunits assemble to form a shell (Figure 1a). According to this hypothesis, only 2 steps in the formation of ferritin should exist: iron-free apoferritin and ferritin with the full complement of iron. Actually, however, native ferritin always contains a spectrum of molecules with intermediate iron loads. To rescue PAPE's hypothesis, one has therefore to assume that the liver ferritin pool contains molecules at various stages of iron core *degradation*.

Doubts have been cast on the validity of PAPE's model by DRYSDALE's experiments[4]. He isolated rat liver ferritin after an injection of ^{14}C-leucin. Radioactivity appeared first in iron-poor ferritin. He concluded that newly synthetized apoferritin was *gradually* filled up with iron. However, an alternative explanation could again rescue PAPE's model: If we assume that iron stabilizes the ferritin molecule so that iron-rich ferritin dissociates only slowly into subunits in comparison to iron-poor ferritin or apoferritin, then it is clear that newly synthetized subunits, when interacting with the pre-existing liver ferritin pool, is faster incorporated into iron-poor ferritin than into iron-rich ferritin.

Other arguments against PAPE's theory[5,6] are less convincing. I therefore studied the incorporation of iron into apoferritin in vitro, thus avoiding the liver ferritin pool which had complicated the interpretation of DRYSDALE's results. 1 ml of a 1% apoferritin solution and 40 mg ferrous ammonium-sulphate were mixed in the presence of 5% sodium thiosulphate and 0.25% potassium iodate (according to a personal communication by P. HARRISON). Samples were analyzed at various time intervals with respect to iron content per molecule by isopycnic centrifugation in an urografin gradient (Figure 2). PAPE's theory predicts a mixture of completely filled ferritin and apoferritin. However, a *gradual uptake* of iron by apoferritin was found. It therefore seems that PAPE's idea has to be given up.

As an alternative, I wish to suggest the 'penetration hypothesis': The hollow apoferritin sphere is riddled with holes; at the *inner* surface of the protein shell, oxidation of the iron is strongly catalyzed by certain amino-acid groups; the nascent ferric ions readily form an intramolecular precipitate and very soon the growing FeOOH-micelle becomes too big to escape (Figure 1b). Circumstantial evidence for the existence of gaps in the apoferritin shell is given by negative contrast electron microscopy, where phosphotungstate can be seen to have entered the apoferritin molecule[3]. If this hypothesis were true, it should be possible to demonstrate a catalytic iron oxidation at sites located on the inside of the apoferritin molecule. Figure 3 shows indeed that apoferritin stimulates iron oxidation.

To identify amino acids in or near the active site, the effects of alkylation and competitive inhibition were studied. Formaldehyde and β-propiolactone, both reacting with free amino groups and the imidazole ring[7,8], and bromoacetate, a weak but rather specific histidine alkylating agent[9], inhibited iron uptake (Figure 4). Alkylations of SH-groups with iodoacetamide had no significant influence on iron incorporation (iodoacetamide concentration 0.5% at various pH from 4.5–8.0 in acetate, imidazole or glycine buffers). The strong inhibition by Zn^{2+}, known to form complexes preferentially with histidine in pep-

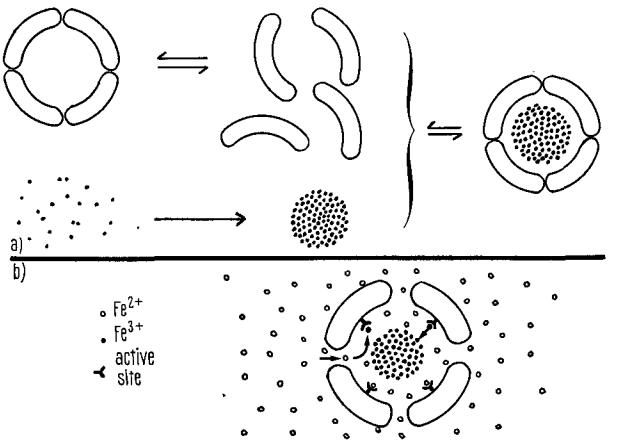

Fig. 1.

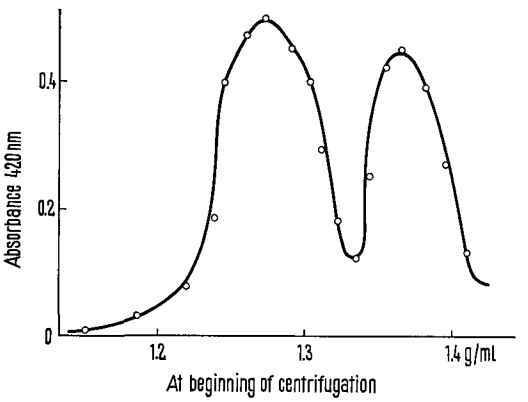

Fig. 2. After 2 or 20 min respectively, iron incorporation was stopped by adding an equal volume of saturated ammonium sulphate to the reaction mixture; both precipitated ferritin samples were redissolved in water, mixed together and centrifuged in a Beckman SW 39 rotor at 39,000 rpm and 20 °C for 18 h on a urografin gradient (0–76% in 20% sucrose). After that, absorbance in the centrifuge tube (at 420 nm indicating ferritin iron) was determined as a function of the density (calculated for the beginning of centrifugation).

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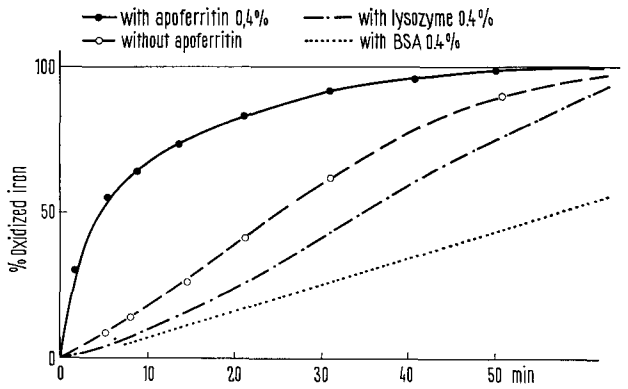

Fig. 3. Reaction mixtures contained 100 mM imidazole buffer, pH 7.45, 1.28 mM ferrous ammonium sulphate, 20 mM potassium jodate and 200 mM sodium thiosulphate at room temperature. Reaction was started by adding the ferrous ions last. At different time intervals samples of 0.1 ml were added to 2 ml of 0.5% α,α-bipyridyl solution (in 0.1 M imidazole buffer, pH 7.45, and 10% ethanol), which stopped further oxidation by forming a red complex with Fe^{2+}; this remaining ferrous iron was determined for each sample by measuring absorbance at 580 nm. Thus percent oxidized iron could easily be calculated. ——, with apoferritin 0.4%; — —, without apoferritin; — · —, with lysozyme 0.4%; – – – – – –, with bovine serum albumine 0.4%.

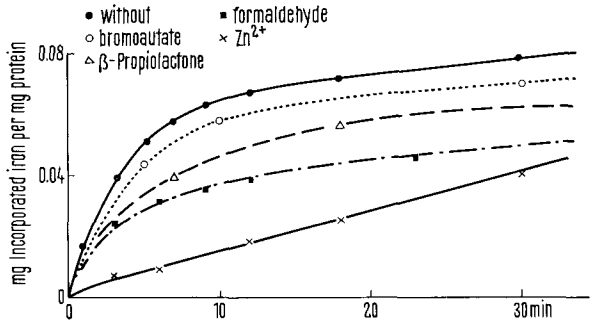

Fig. 4. —, Iron uptake by native apoferritin without further treatment. · · · · ·, Apoferritin alkylated with bromoacetate according to Banaszak[9] (reaction time 7 days, pH 7), followed by ammonium sulphate precipitation and dialyzed against distilled water. –·–·–, Apoferritin alkylated with formaldehyde 0.66% in 0.1 M acetate buffer, pH 5.2. – – –, Apoferritin alkylated with β-propiolactone 0.1% in 0.1 M acetate buffer, pH 5.2. –×–, Apoferritin with 16 mM Zn^{2+} present. All reaction mixtures contained 0.5% apoferritin, 40 mM ferrous ammonium sulphate, 7 mM potassium iodate, 110 mM sodium thiosulphate (room temperature). Iron incorporation was stopped by adding samples of 0.1 ml to 4 ml ammonium sulphate solution (50% saturated, 0 °C). The precipitated ferritin was redissolved in water and the iron content per mg protein determined by measuring ferritin iron directly in the spectrophotometer (absorbance of 1 mM ferritin iron at 480 nm = 0.263) or by a modified method according to Borei[11] with bipyridyl. The protein concentrations were determined according to Lowry[12].

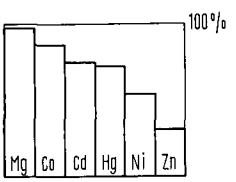

Fig. 5. Iron incorporation with 16 mM divalent metal ions after 10 min. Results given in percent of uninhibited sample. Reaction conditions as in Figure 4.

tides, and much less with cysteine[10] also suggests that the active site contains histidine (Figure 5).

Somewhat different results were obtained by Mazur[13], who was able to suppress iron incorporation with iodoacetamide. He concluded that cysteine might be the iron acceptor in ferritin. But his system, consisting of liver slices supplied with ferric citrate as iron source, was too complex for any conclusion to be drawn, since transferrin, xanthine oxidase and probably other biological substances were involved in the iron-uptake reaction.

It is a surprising finding that the histidine alkylating agent diazonium-H-tetrazole (DHT)[14] does not affect iron uptake. If the gaps in the protein shell postulated by the 'penetration hypothesis' were smaller than the DHT molecule, the reagent could not penetrate to alkylate the internal histidine residues. In fact, only 10% of histidine residues present in apoferritin had reacted with DHT. (I measured the extent of histidine alkylation as described by Horinishi[14] and took 4.8% for histidine content of apoferritin[15].) However, after apoferritin had been disintegrated with acetic acid into subunits (followed by dialysis against 0.01 M glycine buffer, pH 3, as described by Harrison[6]) more than 90% of histidine residues were accessible to alkylation. It seems, therefore, that those histidine residues which are involved in iron uptake are *inside* the apoferritin molecule, as required by the 'penetration hypothesis'. Unfortunately the catalytic iron oxidation could not be demonstrated with subunits and thus also no inhibitory effect by DHT-alkylation of subunits.

Formaldehyde, β-propiolactone and several divalent ions (Zn^{2+}, Cd^{2+}, Mg^{2+}, Fe^{2+}) delayed also iron *release* which I observed with the method described earlier[16]. Thus reduction of ferritin iron seems to involve the same

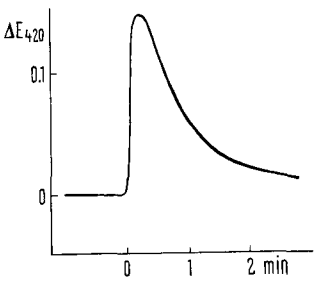

Fig. 6. Absorbance difference between sample cuvette and reference cuvette was written as a function of time. Both cuvettes contained 2 ml of a ferritin solution (0.15 mg/ml in 0.1 M acetate buffer, pH 5.2); the sample cuvette contained 50 mM Zn^{2+}, the reference cuvette 50 mM Fe^{2+}. pH was adjusted with NaOH and then simultaneously (at time 0) 0.15 ml of a 4% sodium dithionite solution was added.

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active site as iron uptake. I found that Zn^{2+} has apparently a stronger affinity to the active site than Fe^{2+} (Figure 6). This is of special interest because it is known that in vivo Zn^{2+} stops iron incorporation into ferritin[17].

Zusammenfassung. Es wurde eine neue Hypothese für die Eisenaufnahme durch Ferritin experimentell geprüft. Es scheint, dass die zweiwertigen Eisenionen in die Apoferritinhohlkugel eindringen können und im Innern an histidinhaltigen aktiven Stellen katalytisch oxydiert werden; das entstehende Fe^{3+} bildet sofort ein (FeOOH)-Mikropräzipitat, welches bald so gross ist, dass es nicht mehr durch die Lücken der Apoferritinhohlkugel entweichen kann.

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PRO EXPERIMENTIS

Bromination of Nucleosides

Recently there has been considerable interest in the bromination of purine nucleosides[1-6] and nucleotides[7]. The reagents that have been employed for such brominations are bromine in dioxane, N-bromoacetamide and bromine water. We have now found that N-bromosuccinimide (NBS) in DMF solution is capable of brominating pyrimidine and purine nucleosides. It is known that NBS effects aromatic bromination when both reagent and substrate are in solution. DMF was chosen as the reaction medium since it dissolves NBS as well as the nucleosides (except guanosine) on slight warming.

The nucleosides in this study which were brominated are uridine, cytidine, adenosine, 2′,3′-*O*-isopropylidene-adenosine and guanosine[8]. The general procedure followed in these reactions is illustrated by the preparation of 8-bromoguanosine and 5-bromouridine. The progress of these reactions was followed by change in UV-absorptions and paper chromatography[9]. The structure of the bromonucleosides was confirmed by hydrolysis with *N* HCl to the corresponding brominated bases.

8-Bromoguanosine. Guanosine (283 mg, 1.0 m*M*) was suspended in anhydrous DMF (8 ml), NBS (200 mg, 1.14 m*M*) added and the suspension stirred overnight at room temperature. By this time all the guanosine had dissolved to a clear yellow solution. Solvent was removed under reduced pressure (40–50 °), water added to the residue and the separated solid filtered and recrystallized from hot water. Yield 290 mg (80%), Rf 0.60.

5-Bromouridine. Uridine (244 mg, 1.0 m*M*) was dissolved in DMF (2 ml), NBS (200 mg, 1.14 m*M*) added and the clear light-yellow solution allowed to stand at room temperature for 16 h. The solution, which had turned red, was evaporated in vacuo (40–50 °C). After thorough removal of DMF, the residue was crystallized from acetone to give 202 mg (62%) of the product, mp 175° (ref.[10]), 181°. Rf 0.63[9].

5-bromocytidine, 8-bromoadenosine and 8-bromo-2′,3′-*O*-isopropylidene adenosine were similarly obtained in 83, 40 and 50% yields, respectively. However, when this reaction was applied to triacetylinosine or to inosine, which is insoluble in DMF, no reaction was observed during 16 h at room temperature and hypoxanthine was obtained when the reaction mixture was heated at 70–80 °C for 6 h[11].

Zusammenfassung. Mit N-Bromsuccinimid können Nukleoside in Dimethylformamid mit guter Ausbeute zu den in 5-Stellung bromierten Derivaten umgewandelt werden.

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Collagen Substrate Films for Localizing Collagenolytic Activity Histologically

Collagenolytic activity has been reported in animal and human tissues under both physiologic and pathologic conditions[1,2]. Such collagenolytic activity has been demonstrated employing the methods or various modifications of GROSS et al.[3]. This procedure involves the use of collagen gels, obtained by extraction of mammalian skin, as substrates. The properties of extracted collagenases have been studied by viscometry and electrophoresis[4].

Substrate films on microscope slides have been employed to demonstrate proteolytic activity and to localize deoxyribonuclease, ribonuclease, amylase and hyaluronidase[5]. Although such substrate film techniques