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Role of Phosphate in Fe^{2+} Binding to Horse Spleen Holoferritin†

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ABSTRACT: In order to identify the function and location of phosphate associated with the iron core of horse spleen ferritin (HoSF), the phosphate content of native HoSF was altered by two procedures. Adjustment of pH from 7.0 to 10.0 *irreversibly* released 53% of the phosphate and 10% of the iron, while lowering the pH to 5.0 *reversibly* released 43% of the phosphate and 35% of the iron. Reversible release of 85% of the initial phosphate (but little iron release) also occurs upon reduction with methyl viologen (MV) or dithionite. Most of the phosphate is released in the early stages of reduction of the iron core, suggesting that the phosphate resides primarily on the mineral core surface. Reduction followed by chelation altered both the iron and phosphate content of the HoSF mineral cores. HoSF iron cores first reconstituted in the absence of phosphate and then incubated with added phosphate did *not* bind phosphate. However, when HoSF was first reconstituted in the absence of phosphate and then equilibrated anaerobically with both Fe^{2+} and phosphate, then phosphate was incorporated in amounts similar to native HoSF. Fe^{2+} binding to native, phosphate altered, and reconstituted HoSF in the presence and absence of phosphate clearly showed that Fe^{2+} binding to the mineral core depends on the presence of core-bound phosphate. Fe^{2+} binding to phosphate-depleted mineral cores or to cores reconstituted with 621, 2158, and 3013 Fe/HoSF core in the absence of phosphate bound only eight Fe^{2+} per entire ferritin molecule, clearly showing that Fe^{2+} has no measurable affinity for the phosphate-free, reconstituted mineral core. Because this same Fe^{2+}/HoSF binding stoichiometry was observed in apo-HoSF, we conclude that the eight Fe^{2+} bound to HoSF containing phosphate-free mineral cores only bind to the eight protein sites present in apo-HoSF.

Horse spleen ferritin (HoSF) is a highly symmetrical protein structure of 120 Å in overall diameter (Ford et al., 1984) composed of 24 subunits arranged to form a protein shell enclosing a central cavity of 70-Å diameter. Up to 4500 iron atoms can be contained within the core cavity in the form of an FeOOH mineral particle bound by the inner surface of the protein shell, although as typically isolated only 2000–2500 iron atoms are present. In addition to iron, the mineral core of various ferritins also contains phosphate in variable amounts (Treffry et al., 1987), the chemical role, structure, and nature of which have remained obscure, although summarized evidence suggests that the majority of the phosphate is surface bound (Treffry & Harrison, 1978). In contrast to HoSF and other ferritins with their low and variable phosphate content are the bacterial ferritins (Watt et al., 1986; Rohrer et al., 1990; Moore et al., 1986) with much higher phosphate contents and with the phosphate uniformly distributed (Rohrer et al., 1990) within the mineral cores. Thus, the presence of naturally occurring phosphate in the various isolated ferritin types suggests a possibly important function for this constituent of the ferritin mineral cores, but just what the role is remains an ongoing question in understanding ferritin behavior.

An important step in understanding the role for phosphate in early core formation was recently described by Cheng and Chasteen (1991). These authors have shown that the presence of phosphate greatly accelerates the rate of Fe^{2+} oxidation and is involved in the translocation of the resulting Fe^{3+} within the apoferritin interior initiating core formation. Their results suggest that at least three phosphate groups are involved in the translocation process but that the phosphate is incorporated only at low levels into the developing core.

A recent report of Fe^{2+} binding to both apo- and holo-HoSF under anaerobic conditions (Jacobs et al., 1989) has established that both forms strongly bind Fe^{2+} in a pH-dependent manner, and an initial suggestion was made that OH or phosphate groups present in or on the mineral core were serving as binding sites for the incoming Fe^{2+}. The dramatically higher level of Fe^{2+} binding (Watt et al., 1992a) to holo- *Azotobacter vinelandii* bacterial ferritin (AVBF) compared to holo-HoSF and the detailed comparison of Fe^{2+} binding to AVBF mineral cores with chemically altered phosphate levels clearly established a relationship of Fe^{2+} binding ability with phosphate content in AVBF and suggested that each Fe^{2+} was bound at a site consisting of about two phosphate ions, presumably attached to the mineral core. In both holo-AVBF and -HoSF under anaerobic conditions, Mössbauer measurements showed that the incoming Fe^{2+} ions

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were converted to Fe^{3+} (Frankel et al., 1987; Jacobs et al., 1989) upon binding to phosphate groups attached to the surface of the mineral particle, releasing the electron into the mineral core. Although the added Fe^{2+} was clearly separated into Fe^{3+} and an electron, the process in both HoSF and AVBF is reversible as was shown by adding bipyridine and recovering the initial Fe^{2+} as the bipyridine complex in greater than 90% yield. These results suggest a possibly important role for the phosphate groups on the mineral core surface in not only participating in the mineral core structure formation but also in facilitating the oxidation and stabilization of the incoming Fe^{2+} as Fe^{3+}. If the smaller amount of phosphate present in HoSF compared to AVBF is surface bound, as has been suggested (Treffry & Harrison, 1978), and is responsible for Fe^{2+} binding, as is the case in AVBF, then altering the phosphate content of HoSF should dramatically change its Fe^{2+} binding ability. If iron binding to the phosphate layer is important in iron deposition, then the rate of iron deposition should respond to the phosphate levels on the mineral surface, a prediction consistent with the results of Cheng and Chasteen (1991).

We report here the preparation of holo-HoSF with decreased core phosphate levels, but with only small to moderate changes in the iron content. These modified ferritin species were produced either by partial reduction of the mineral core or by pH adjustment of native holoferritin. We have also prepared reconstituted HoSF in the absence of phosphate in order to evaluate the Fe^{2+} binding ability of HoSF having no phosphate present in the core. Using all of these phosphate-altered ferritins, we show that the phosphate content is essential in the binding of Fe^{2+} to the ferritin mineral core.

MATERIALS AND METHODS

Type I horse spleen ferritin at 100 mg/mL obtained from Sigma and similar quality preparations (cadmium-free) of 50 mg/mL obtained from Boehringer-Mannheim were used in the experiments to be described. These ferritin preparations had iron (Lovenberg et al., 1963) and phosphate contents (Cooper, 1971) ranging from 2165 to 2463 iron per HoSF and 0.63 to 0.91 μmol of P_i/mg of HoSF (284–410 Pi/HoSF), respectively. Reduced methyl viologen (MV) was prepared by the method of Corbin and Watt (1990). The redox reactions involving the reduction of HoSF with MV or sodium dithionite were conducted in a Vacuum Atmospheres glove box under purified nitrogen or argon (O_2 < 1.0 ppm).

Three procedures were used to vary the phosphate content of native HoSF. The first consisted of reducing 0.20-mL samples of holoferritin to varying degrees with dithionite or reduced methyl viologen followed by anaerobic chromatography on a G-25 Sephadex column. This procedure produced HoSF with up to 80% of the phosphate removed with corresponding iron losses from the core ranging from 10 to 30%, depending upon the degree of reduction and the reductant used.

The second method consisted of first reducing the HoSF to the desired degree and removing the Fe^{2+} by bipyridine chelation, thereby releasing both the reduced iron and its attached phosphate. This procedure modified both the phosphate and iron content, depending upon the degree of reduction carried out. The details of these first two methods followed the procedure previously described for AVBF (Watt et al., 1992a), using either dithionite ion or reduced MV as reductants. Excess Fe^{2+} was also examined as a possible reductant under anaerobic conditions by reacting 0.2–0.5 mL of HoSF at 10–25 mg/mL with a 100–350-fold excess of Fe^{2+}, allowing the mixture to incubate anaerobically for 30 min and removing the excess, unbound Fe^{2+} by G-25 Sephadex chromatography. The emerging protein was then analyzed for phosphate and iron by the procedures described above.

The third procedure consisted of adjusting the pH of 0.2–1.0 mL of HoSF solution to either acidic (pH 4.5) or basic (pH 10) values with 1 M HCl or NaOH, allowing the mixture to equilibrate for 30 min, and then passing the ferritin solution through a 1.0 × 15 cm G-25 Sephadex column equilibrated with 0.05 M Tes (*N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid) adjusted to the appropriate pH. The emerging protein was analyzed for phosphate and iron by the procedures described above. The phosphate and iron losses were determined by difference. In some cases, the solution trailing behind the protein band from the column was collected and analyzed to directly determine the iron and phosphate released by the pH treatment.

The reversibility of the phosphate and iron loss accompanying a high or low pH change was examined by first adjusting the pH of the native HoSF solution to pH values between 7.0 and 4.5 or between 7.0 and 10.5, allowing the solution to stand for 30 min at the altered pH, readjusting the pH to 7.0, and then passing it through a G-25 column to remove any small molecules or ions released by the pH treatment. The iron and phosphate content of the emerging protein was determined and compared to the native HoSF prior to pH treatment.

Reconstituted cores were prepared by stirring 1-mL portions of apoferritin in 0.1 M Tes, pH 7.0–8.0, in air while adding Fe^{2+} in increments of 100 Fe^{2+}/HoSF and adjusting the pH if necessary. Ferritin reconstituted with 621, 2158, and 3013 Fe^{3+}/HoSF ratios were prepared. When the proper amount of iron had been incorporated, the reconstituted ferritin was filtered through a 0.45-μm Millipore filter to remove any $Fe(OH)_3$ that might have formed. Total protein was determined by the Lowry method, and the iron content was measured colorimetrically by dithionite reduction and bipyridyl chelation using 8400 M^{-1} cm^{-1} at 520 nm. Phosphate was added to phosphate-free reconstituted HoSF samples by first making them anaerobic, adding sodium phosphate adjusted to pH 7.5 to make the solution 1 mM, and then adding Fe^{2+} to the desired levels. The solution was then allowed to react anaerobically for 30 min. The excess Fe^{2+} and phosphate were separated by G-25 chromatography and the Fe^{2+}/HoSF stoichiometry and phosphate-incoporated was determined.

Fe^{2+} binding to the modified, native HoSF proteins and the reconstituted ferritins was carried out anaerobically by the G-25 Sephadex column method previously described (Jacobs et al., 1989).

RESULTS

Phosphate Loss by Reduction. Figure 1 demonstrates that phosphate but little iron is released from the mineral core by sequential reduction of the iron in the HoSF mineral core. With dithionite as reductant slightly more Fe^{2+} is lost than with MV, presumably because the sulfite ion which is formed as a redox byproduct acts as a weak chelator, whereas, with MV as a reductant, little Fe^{2+} is lost because MV does not have any complexing ability.

Figure 1 clearly shows that a small degree of reduction of the iron in the core mobilizes large amounts of phosphate. For example, a 5% and 10% reduction of the iron in the HoSF core releases 41% and 59% of the phosphate initially present in the native HoSF mineral core, which in absolute terms corresponds to the reduction of 110 and 220 Fe^{3+} to Fe^{2+} and

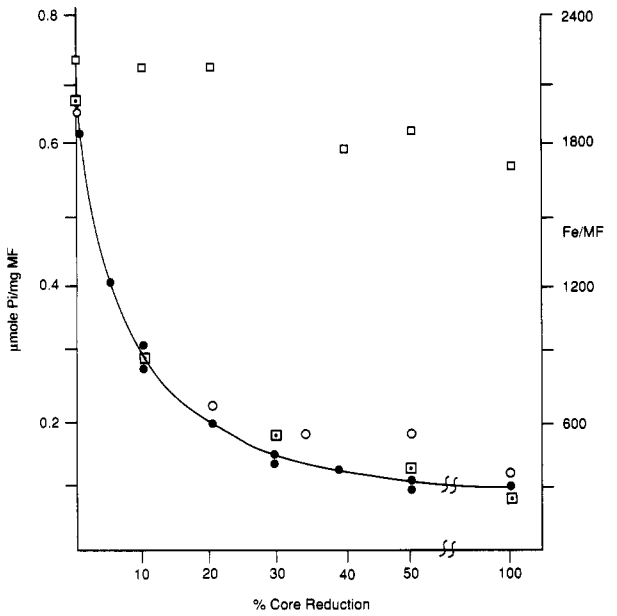

FIGURE 1: Phosphate release from the iron core of horse spleen holoferritin during reduction. The ferritin core initially containing 2250 Fe/molecule and 0.68 μmol of phosphate/mg of protein was reduced anaerobically with MV and on an independent measurement with dithionite (○), and the protein was isolated by anaerobic G-25 Sephadex chromatography. Phosphate (left axis) (●) and iron content (□) (right axis) of the resulting protein were determined as a function of iron core reduction. After reduction and phosphate removal, the phosphate-altered ferritins were air oxidized and made anaerobic, and Fe^{2+}-binding was measured (⊡). To determine the number of Fe^{2+} bound to holoferritin, multiply the left axis by 100. The solid line is an exponential fit of the phosphate content with degree of reduction for the MV-reduced horse spleen ferritin.

the release of 125 and 180 phosphate ions, respectively. At this early stage of reduction, it appears that each Fe^{3+} that undergoes reduction has approximately one phosphate ion associated with it which is released upon reduction. As the percent of iron reduction increases, phosphate loss continues to occur but the amount lost per iron reduced declines until a limiting value of 85% phosphate loss occurs at 100% reduction. This corresponds to mobilization of 260 of the 306 phosphates initially present. From these experiments alone it is not clear whether the remaining 15% (46 phosphates/HoSF) of the original phosphate is thermodynamically stable and bound by the reduced mineral core or it it is just physically trapped. The solid line in Figure 1 is an exponential fit of phosphate loss with increasing extent of reduction and is consistent with the phosphate being localized and associated with a small fraction of the iron present which releases the phosphate upon reduction. These results are consistent with the phosphate being bound to iron atoms at the mineral surface and also show that the major portion of the mineral core is stable in the fully reduced Fe(II) form.

The upper set of points in Figure 1 represents how the iron content of HoSF changes upon reduction. In contrast to the phosphate content, iron loss is negligible at low levels of reduction but as reduction approaches 100%, up to 23% of the initial iron is lost even in the absence of externally added complexing agents. It is possible that the released phosphate which increases with the extent of reduction, and can accumulate to about 15 mM at 100% reduction under the conditions used in Figure 1, is sufficiently concentrated to act as a weak Fe^{2+} chelating agent and removes some of the reduced iron. Thus, a possible role for phosphate in the HoSF mineral core is to facilitate the release of the iron with which it is closely associated.

To further elucidate the role of phosphate in the mineral core and to gain information regarding its mode of attachment to the iron, we first examined whether the phosphate which is released upon reduction is reattached to the core upon reoxidation. Anaerobic reduction of holoHoSF with MV for 30 min was first carried out, followed by stirring in air for 30 min. The protein was then passed through a G-25 column to remove any free phosphate or iron. This experiment showed that at least 90% of the original phosphate was retained and that all of the original iron was retained. This result indicates that the phosphate either remains weakly attached to the reduced core or that if it is actually released from the HoSF interior during reduction, as is evident by phosphate released upon reduction followed by chromatography, it returns upon oxidation and binds to its original sites of attachment. Thus, the phosphate appears to be able to traverse the protein shell according to redox conditions imposed upon the protein and the iron core.

We next addressed the question of whether phosphate is reattached to the oxidized mineral core. The experimental protocol was to first reduce the HoSF core, remove the released phosphate by anaerobic G-25 chromatography as described above, allow the protein to oxidize in air, and then determine if phosphate binds to the phosphate-free, oxidized core. When this protocol was followed, no phosphate incorporation occurred. This result indicates that once the phosphate binding sites are lost, presumably by replacement with oxo or hydroxo groups, binding of phosphate does not occur to the Fe^{3+} sites which initially bound the original phosphate groups. As will be discussed later, this result demonstrates that Fe^{2+} and PO_4^{3-} must be present for PO_4^{3-} to be incorporated.

The final experiment of this series determined the phosphate binding ability of the iron core in native HoSF. Several independent, native holo-HoSF samples were incubated, both anaerobically and aerobically, with externally added phosphate, and the resulting solution was passed through a G-25 Sephadex column. Following this treatment, we were *not* able to demonstrate any additional phosphate binding to the mineral core, in contrast to AVBF where up to 10% additional phosphate was incorporated (Watt et al., 1992a). This result suggests that the coordination environment of Fe^{3+} in the HoSF core is essentially complete, presumably with phosphate, oxo, or hydroxo groups around the iron, a result which differs from that found with native bacterial ferritin which is able to accommodate additional phosphate.

Mössbauer, measurements have previously shown (Jacobs et al., 1989; Watt et al., 1992a) that when $^{57}Fe^{2+}$ binds anaerobically to native or holo-HoSF or holo-AVBF, the bound $^{57}Fe^{2+}$ undergoes a redox reaction and forms $^{57}Fe^{3+}$ with the electron presumably transferred to the mineral core. In view of the phosphate release by external reductants discussed above (see Figure 1), we reacted HoSF with up to a 350-fold excess of Fe^{2+}/HoSF (which could behave as a reducing agent) under anaerobic conditions for 30 min and then isolated the HoSF fraction. Analysis for phosphate showed that there was no change or only a small decrease in the phosphate content. This result demonstrates that the internal redox reactions involving Fe^{2+} binding are fundamentally different from those involving externally added reductants such as MV or dithionite ion.

Phosphate Loss by Reduction and Chelation. Reduction of HoSF followed by chelation of the resulting Fe^{2+} results in destruction and removal of the reduced iron portion of the mineral core and releases any bound phosphate associated with it. The unreduced portion of the mineral core apparently

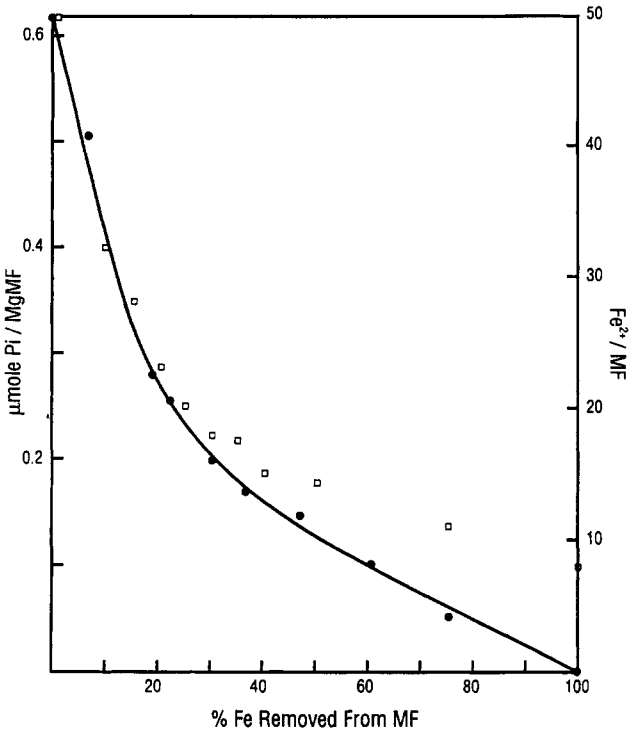

FIGURE 2: Phosphate loss from reduced horse spleen holoferritin after chelation of reduced iron. The same procedure was followed as in Figure 1 except the reduced iron was chelated by bipyridine prior to G-25 Sephadex chromatography. The left axis is the phosphate content (●) of the horse spleen ferritin following the reduction and chelation steps. The right axis is the number of Fe^{2+} ions that bind (□) to the core-altered ferritin under anaerobic conditions.

remains undisturbed. This treatment not only removes associated surface-bound phosphate but also alters the iron content and size of the mineral core. The phosphate loss by this reduction process as a function of percent reduction is shown in Figure 2 and is similar to Figure 1 up to about 60% reduction, where 15% or less of the original phosphate still remains. However, in contrast to Figure 1, where only a small amount of the Fe^{2+} is lost upon reduction, the iron content of HoSF shown in Figure 2 decreases linearly with the degree of reduction because the resulting Fe^{2+} is removed by chelation. Above 60% reduction, the remaining phosphate and iron decrease together with increasing reduction and corresponding iron loss, indicating that the remaining mineral core (containing ~1000 iron atoms) is essentially uniform with a Fe/phosphate ratio > 18. These results are also consistent with the view that the majority of the phosphate present in the HoSF core is surface bound and that the iron which is first reduced and chelated also resides on the mineral surface (Harrison & Treffry, 1978).

pH-Induced Iron and Phosphate Loss. Figure 3 shows that the phosphate present in holo-HoSF is most stable near pH 7.0 but that both iron and phosphate are mobilized and easily separated by G-25 chromatography following either lowering or raising of the pH. Figure 3a demonstrates that raising the pH to 10.0 removes 53% of the phosphate while lowering the pH to 5.0 releases 43%. The loss of phosphate by dilute alkali was initially observed by Granick and Hahn (1944), but this reaction was not studied in detail. Quite the opposite effect is seen for iron loss as shown in Figure 3b. The iron present in HoSF is quite stable in the pH range 7.0–9.0, but up to 35% of the iron is lost at pH 5.0 and about 10% is lost at pH 10.0.

These results suggest that two quite different processes are involved in removing phosphate and iron from the HoSF core

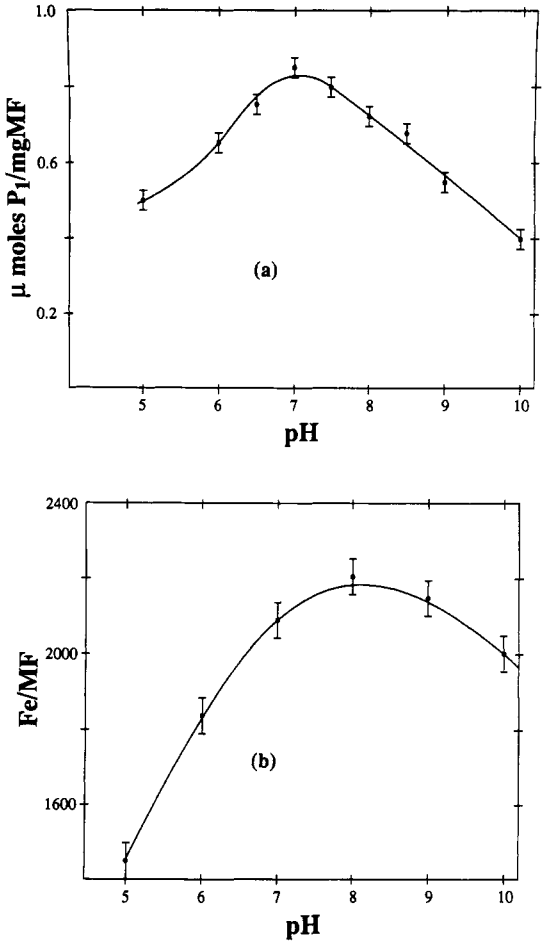

FIGURE 3: Phosphate and iron release from horse spleen ferritin by pH adjustment. The pH of horse spleen ferritin initially at pH 7.5 in 0.025 M Tes, 0.10 M NaCl, was adjusted to the pH indicated on the *x*-axis, held for 15 min, and then passed through a G-25 Sephadex column equilibrated with 0.025 M Tes, 0.10 M NaCl, adjusted to the same pH. The emerging protein was then analyzed for phosphate, (curve a) and iron (curve b). The error bars represent the uncertainty from duplicate and some triplicate measurements.

by pH alteration. At low pH, the increased proton concentration begins to compete favorably with the Fe^{3+} for the phosphate bound to the mineral surface forming protonated phosphate which detaches from the Fe^{3+} of the core surface and is then free to move from the HoSF interior. The low pH is probably effective in mobilizing some iron by protonating the oxy–hydroxy matrix in addition to the phosphate. This reactivity results in the release of some of the Fe^{3+}, thereby explaining the accompanying iron loss shown in Figure 3b. From differences in acid–base titrations of both apo- and holoferritin, Breslow and Silk (1977) suggested that the phosphate groups on the mineral core surface were being reversibly protonted, thereby accounting for the observed difference in their titrations of apo- and holoferritin. This prediction is consistent with the results reported here.

Phosphate loss at high pH probably occurs by a quite different process involving hydroxide ions instead of protons. As the pH increases, the hydroxide ion concentration increases and, because it interacts more strongly with Fe^{3+} than phosphate, hydroxide begins to compete with the Fe^{3+}-bound phosphate on the surface forming $Fe(OH)_3$ and releasing the bound phosphate. Because the iron is strongly coordinated in the mineral core particle, little iron is released by hydroxide ions, explaining why only small amounts of iron are released at the high pH values.

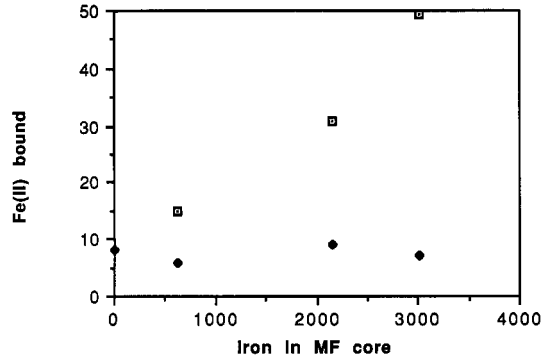

FIGURE 4: Ferrous ion binding to reconstituted horse spleen ferritin. Ferritin was reconstituted to the indicated core sizes shown on the x-axis, as described in the Materials and Methods Section. These reconstituted ferritins were made anaerobic, reacted with excess Fe^{2+} for 30 min, and separated by G-25 Sephadex chromatography, and the number of Fe^{2+} bound to the ferritin was determined (◆). The same anaerobic protocol was followed except that 1 mM phosphate was present (⊡).

These two pH-induced processes have quite different chemical effects on the mineral core surface structure and composition, some of which are easily demonstrated to be reversible while others are irreversible. For example, if the pH is adjusted initially from 7.0 to 4.5, held for 30 min, and then returned to 7.0 (without separating the mobilized iron and phosphate), then at least 90% of the phosphate and iron are reattached to the mineral core and the initial HoSF composition results. This may demonstrate that iron and phosphate are lost by either alteration of the protein shell or changes to the mineral core at this low pH. The removal of phosphate by protonation and its reattachment by deprotonization appear to be quite reversible. However, when phosphate and iron are released by pH adjustment from 7.0 to 10.0 and then back to 7.0, only about 40% of the released phosphate is reattached, demonstrating considerable irreversibility. The irreversibility likely results from the inability of phosphate to replace the more strongly bound hydroxide groups on the newly formed $Fe(OH)_3$.

Fe^{2+} Binding to Phosphate-Modified HoSF Cores. Fe^{2+} binding to native and phosphate-altered HoSF prepared as described above was examined under anaerobic conditions to evaluate the role of phosphate in the Fe^{2+} binding process. Figure 1 shows a strong correlation between the amount of Fe^{2+} which binds to modified HoSF cores and the phosphate content remaining within the core. A similar result is shown in Figure 2 where the Fe^{2+} binding ability of modified HoSF closely follows the phosphate content of the cores which have been modified by reduction and chelation of the reduced iron. Figure 2 further shows that as the core size, and consequently the core phosphate content, decreases to zero, the number of Fe^{2+} bound approaches eight, the value measured previously for Fe^{2+} binding to apo-HoSF (Wardeska & Chasteen, 1986; Jacobs et al., 1989). Although not shown, the same Fe^{2+} binding behavior was demonstrated for HoSF samples whose phosphate content was altered by pH adjustment. These results clearly show that Fe^{2+} binding to the holo-HoSF core requires the presence of phosphate, which as discussed above and elsewhere (Treffry & Harrison, 1978) is likely to be surface bound.

Reconstituted HoSF Cores. Figure 4 shows the Fe^{2+}-binding ability of reconstituted HoSF containing a variety of core sizes. The lower points demonstrate that a nearly constant level of Fe^{2+} binding occurs with reconstituted cores which is independent of the core size as measured by iron content. The amount of Fe^{2+} bound to reconstituted HoSF is about eight Fe^{2+} per ferritin, a value which was previously reported (Wardeska & Chasteen, 1986; Jacobs et al., 1989) for apo-HoSF. Therefore, it appears that Fe^{2+} does not bind to the phosphate-free mineral core surface but binds instead to the 3-fold channels of the protein (Jacobs et al., 1989). This conclusion is also supported by the results in Figures 1 and 2, which show that Fe^{2+} binding occurs on the mineral core surface only if there is phosphate present. Exposure to oxygen of this reconstituted HoSF which contains eight Fe^{2+} results in the oxidation of the bound Fe^{2+} to Fe^{3+} with regeneration of the Fe^{2+}-binding sites. This result suggests that Fe^{2+} oxidation occurs at protein oxidation sites and does not necessarily involve the mineral core surface.

The exposure of reconstituted HoSF to Fe^{2+} under anaerobic conditions in the presence of phosphate produces HoSF with both Fe^{2+} and phosphate bound, presumably on the mineral surface. The upper points of Figure 4 demonstrate that the presence of bound phosphate increases the Fe^{2+} binding ability of reconstituted HoSF. Figure 4 shows the Fe^{2+} which binds to the reconstituted cores in the absence of phosphate is most likely binding in the same sites where Fe^{2+} binds to apoferritin. This figure demonstrates that no Fe^{2+} binds to the iron core unless phosphate is present which may explain the increased rate of binding and oxidation observed by Cheng and Chasteen (1991). Kinetic measurements in our laboratory monitoring the increase in absorbance of 350 nm upon the addition of Fe^{2+} to reconstituted HoSF (1300 Fe/HoSF) in the absence and in the presence of bound phosphate show that iron incorporation into HoSF is greatly enhanced when phosphate is present.

DISCUSSION

The presence of phosphate in the native HoSF mineral core has been recognized as an integral part of the HoSF core since its initial discovery (Michaelis et al., 1943), but an adequate explanation of its purpose and function is still lacking. Treffry and Harrison (1978) and Treffry et al. (1987) have discussed the properties and relative concentrations of bound phosphate in both native and reconstituted ferritins. Their survey of many different naturally occurring and some reconstituted ferritins indicated that phosphate/iron ratio values can range from zero in reconstituted ferritin cores to nearly one, as is found in native bacterial ferritins. These compositional studies and the ability to form iron cores in both mammalian and bacterial ferritin in the absence of phosphate have led Treffry et al. (1987; Mann et al., 1987) to conclude that while phosphate is often present in ferritins, it is not an essential component of the ferritin iron core. Such a conclusion based upon "static" conditions may not be valid under the dynamic conditions of iron release or deposition where phosphate may perform important physical and chemical roles.

A possible dynamic role for phosphate in the early formation of reconstituted iron core has recently been presented by Cheng and Chasteen, (1991). These authors have shown that phosphate accelerates not only the rate of Fe^{2+} oxidation but also the translocation of the resulting monomeric Fe^{3+} into oligomeric ferritin core. Previous anaerobic Fe^{2+}-binding studies have demonstrated that Fe^{2+} binds to the mineral core of both HoSF and AVBF (Jacobs et al., 1989; Watt et al., 1992a) and phosphate groups were proposed to act as Fe^{2+}-binding sites for incoming Fe^{2+}. These same Fe^{2+} binding sites were also shown to provide an environment which thermodynamically favored Fe^{3+} and allowed for the oxidation, under anaerobic conditions, of the surface-bound Fe^{2+} to Fe^{3+}

with the electrons being transferred internally, presumably to the mineral core or to some other protein-bound electron accepting site (Watt et al., 1992b). Phosphate is thus seen to be involved in an active way in various in vitro processes responsible for core formation and such reactions are likely to be important in in vivo processes as well.

The present study has now established that the phosphate in the HoSF mineral core is an essential constituent of the Fe^{2+}-binding sites and demonstrates that phosphate must be present for Fe^{2+} to bind to the iron core surface. It is seen in both Figures 1 and 2 that as the phosphate level decreases the Fe^{2+} binding ability decreases in parallel. When only small amounts of phosphate are present in the mineral core, but yet relatively large iron cores are still present (>1000 Fe/HoSF), Fe^{2+} binding approaches that found in apo-HoSF, indicating that the remaining mineral surface lacking phosphate does not bind incoming Fe^{2+}, and the Fe^{2+} which is bound is probably attached to the 3-fold channels as was previously measured (Wardeska & Chasteen, 1986; Jacobs et al., 1989). This result is also confirmed by the Fe^{2+} binding to reconstituted HoSF cores (Figure 4) which shows that only 8 Fe^{2+} bind in the absence of phosphate. Thus, the total Fe^{2+} which is bound to native, holo-HoSF consists of the Fe^{2+} bound to the phosphate sites on the mineral core surface plus the eight Fe^{2+} bound in the 3-fold channels. Another interesting consequence of this discovery is that the increased rate of iron oxidation in the presence of an iron core, previously observed (Macara, et al., 1972; Harrison et al., 1986), may not be due to Fe^{2+} binding and oxidation on the core surface as was proposed but may be due to other causes. Perhaps the presence of the developing iron core shifts the redox potential of the ferroxidase center (Cheng & Chasteen, 1991) or alters the passages to or from the ferroxidase center, enhancing oxidation of bound Fe^{2+}.

The phosphate in the HoSF cores behaves similarly to that in AVBF, except with HoSF the phosphate is localized to a small group of iron atoms (probably the surface iron atoms) whereas in AVBF the phosphate is distributed more or less uniformly throughout the mineral core (Rohrer et al., 1990). A comparison of Fe^{2+} binding to HoSF and AVBF containing phosphate-altered cores prepared by reduction and chelation clearly demonstrates that phosphate is essential for Fe^{2+} binding. As described above for HoSF, once the surface iron and associated phosphate is removed Fe^{2+} binding decreases drastically (Figures 2 and 3) and results from the binding to residual phosphate and protein binding sites. In AVBF, the Fe^{2+}-binding ability is initially greater than in HoSF, and since the phosphate is evenly distributed throughout the core, Fe^{2+} binding remains much higher with decreasing core size (Watt et al., 1992a) than with HoSF. The biological mechanism governing how phosphate is placed just on the core surface in HoSF but yet is distributed throughout the core in AVBF core are questions that are important to understand and important in explaining how the two cores are formed, but these are questions which currently remain unanswered.

Experiments using HoSF with cores reconstituted in the absence of phosphate demonstrate that neither Fe^{2+} nor phosphate bind to these reconstituted cores. The binding of Fe^{2+} to reconstituted cores in the presence of phosphate and the reversible binding of phosphate released from HoSF upon reduction and subsequent oxidation as reported here demonstrate that both Fe^{2+} and phosphate must be present for Fe^{2+} binding to occur and for phosphate incorporation to take place. The Fe^{2+}-binding results previously reported (Jacobs et al., 1989) and those reported here offer some insights into the processes for incorporating phosphate and iron into the mineral core.

One possibility is that phosphate acts as a transport agent to carry the Fe^{2+} across the protein shell to the interior. However, the observation that Fe^{2+} binds to native, holo-HoSF core suggests that free Fe^{2+} may enter the interior of the protein without the aid of phosphate since the phosphate in the native, holo-HoSF core most likely remains bound to the core surface. In order for the phosphate to act as a transport agent, it would have to be released from the native holo-HoSF core, diffuse to the Fe^{2+}-binding site, and then return with with bound Fe^{2+} which then binds on the core surface. This would require significant internal reactivity which has not been demonstrated.

Another possibility is that phosphate binds to or is associated with the protein in some manner which causes a protein conformational change allowing the Fe^{2+} ions entry into the interior. Cheng and Chasteen (1991) have considered a similar possibility with regard to the accelerating effect of phosphate on the rate of Fe^{2+} oxidation and suggested that the phosphate may operate at the ferroxidase site by shifting the redox potential of Fe^{2+} to a more negative value. No firm evidence for phosphate binding to apo-HoSF or to reconstituted HoSF is available except in the presence of Fe^{2+}. Therefore, no evidence exists for phosphate directly associating with the protein to allow Fe^{2+} to enter the interior.

A final possibility, then, is that both Fe^{2+} and phosphate have access to the protein interior, and although neither bind alone, together they interact and are able to bind to the core surface. Ferrous ion appears to have access to the interior as explained above, and phosphate appears also to have access to the interior as demonstrated here by the experiment which showed that phosphate is released upon reduction and is reincorporated upon subsequent oxidation. With the present experimental observations, this third explanation appears to be the best model for Fe^{2+} and phosphate incorporation into the ferritin core.

The observations reported here could have possible in vivo significance since phosphate is usually present at 1 mM in the cell and may always be present inside the ferritin interior. When Fe^{2+} is introduced into the interior of ferritin, phosphate could interact with Fe^{2+} to provide conditions appropriate for the formation of a Fe^{2+}–phosphate layer on the mineral surface. This may be a possible in vivo process which occurs in ferritin during iron incorporation.

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