The Ultrastructure of Ferritin Macromolecules. The Lattice Structure of the Core Crystallites*

(ultrahigh resolution electron microscopy/dark-field imaging/ferric crystallites/iron)

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ABSTRACT The ultrastructure of the crystalline ferric mineral that forms the central core of ferritin macromolecules has been examined by means of ultrahigh resolution electron microscopy at 100 kV. Very high magnification dark-field images reveal the presence of either a single large crystal or several smaller crystallites within many of the cores. When the highly crystalline core contents are suitably oriented to transmit their Bragg reflections through the objective aperture, regular fringes separated by 2–9.5 Å have been visualized. The geometrical relations of lattice fringes and of periodically organized point details in these individual crystallites largely confirm the structural model proposed by Towe and Bradley (1967). The highly variable occupancy of ferric ions in certain planes of the lattice suggests that 20–33% of the iron content of fully saturated ferritin should undergo more rapid physiological release than does the remainder, and that iron uptake will have kinetics that depend upon more than only the maximal rate of crystallization.

The variable iron content of the iron storage protein, ferritin, is present in a ferric mineral located within the central cavity of its apoprotein complex, apoferritin (5, 6). X-ray and electron diffraction of ferritin (9, 10, 23) has elicited polycrystalline ring patterns, thus indicating that some of the mineral forming the core is organized into the crystalline state. Diffraction patterns from ferritin cores do not correspond to the crystal structures of the well-known iron oxides in the natural world (9). Two models, based upon the diffraction data, have been proposed for the lattice structure of ferritin core crystallites. Harrison *et al.* (10) have concluded that these have a hexagonal unit cell with $a = 2.94$ Å and $c = 9.40$ Å. The best agreement of their postulated atomic positions with the actual diffraction patterns was achieved by proposing that the iron atoms are equally and randomly distributed among all of the available sites; both tetrahedral and octahedral coordination of the iron atoms with the oxygen groups was postulated. Towe and Bradley (23) have concluded that the ferritin core crystallites have a hexagonal unit cell with $a = 5.08$ Å and $c = 9.4$ Å. Their model proposes that occupancy by the iron atoms is optional in one of every four layers between the close-packed oxygen groups; in this model, only octahedral coordination was postulated. In addition to these two studies, Brady *et al.* (1) have concluded that ferritin core analogs prepared by the hydrolysis of ferric nitrate have only tetrahedrally coordinated ferric atoms placed in a folded polymeric structure.

Recent biochemical and biophysical studies have not been able to establish the validity of any of these postulated model

structures. A more accurate definition of the lattice structure of the ferritin core crystallites could allow more meaningful correlations to be made between this atomic organization and the physiological uptake and release of iron by these macromolecular complexes. The present study has used very high resolution dark-field electron microscopy at 100 kV to image the lattice planes in *individual* ferritin cores. Direct visualization of the spacings and interrelations between the lattice planes has confirmed the model structure proposed by Towe and Bradley (23) from their study of *populations* of ferritin cores.

MATERIALS AND METHODS

Specimen Preparation. Specimen supports consisted of an ultrathin amorphous carbon film (24) overlaying a fenestrated thick film that had been lightly coated with gold by evaporation (16). A sonicated aqueous suspension of graphitized carbon particles was applied to the ultrathin supporting film and was air dried; the 3.44-Å lattice spacings in this material served as an internal calibration of the magnification (11). After glow discharge treatment to render all surfaces hydrophilic, freshly diluted unfractionated horse-spleen ferritin (Calbiochem, Inc., La Jolla, Calif. 92037) was deposited upon the specimen supports; after about 30 sec, excess liquid was blotted off and the grid was allowed to dry rapidly.

Ultrahigh Resolution Electron Microscopy. A JEM-100B electron microscope [JEOL (U.S.A.), Inc., Medford, Mass. 02155], fitted with a pointed filament and an additional anti-contamination device (12), was operated at 100 kV. Condenser apertures with a diameter of 200 μm and objective apertures with a diameter of 40 μm were used routinely. Bright-field and dark-field electron micrographs were recorded at nominal magnifications of 200,000–500,000 times; exact magnifications for *each* region studied were determined by photographing in bright-field the graphitized carbon particles on the same or a closely adjacent fenestration (15). All images were recorded upon Electron Image Plates (Eastman Kodak Co., Inc., Rochester, N.Y. 14650); exposure times were less than 6 sec for bright-field images, and from 15 to 45 sec for dark-field images.

Anastigmatic dark-field images were obtained by deflecting the incident beam about 1° with reference to the axially centered objective aperture (Fig. 3). Spatial frequencies corresponding to real lattice periodicities of around 1–5 Å were transmitted to the image plane (Fig. 3); the maximum arc of the diffraction rings being transmitted was near 80°. The

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FIGS. 1 and 2. Bright-field (Fig. 1) and dark-field (Fig. 2) images of the same field of unfractionated ferritin upon an ultrathin carbon supporting film. In the dark-field image, most cores appear to be without ordered substructure; scattering points less than $5 \text{ \AA}$ in diameter are present in some of these cores (arrowheads). Two cores consist of a single large crystal (arrows). Bar denotes $100 \text{ \AA}$.

"best" dark-field focus usually did *not* correspond to the precisely determinable bright-field focus, probably due to hysteresis effects and to stray fields from the beam-deflecting coils. For ferritin, critical dark-field focus was determined by the criteria presented by Cowley *et al.* (4) or by directly observing the $9.5\text{-\AA}$ lattice fringes that frequently were visible at the high magnifications used.

RESULTS

The same ferritin cores that reveal considerable substructure in their dark-field images only very rarely show substructure in their corresponding bright-field images (Figs. 1 and 2), even when these latter are recorded near the optimal defocus for thin phase-objects (e.g., ref. 3). In high-resolution dark-field images, many of the randomly oriented ferritin cores in these unfractionated samples appear to have unstructured contents with relatively low scattering powers (Fig. 2). Intensely scattering points less than $5 \text{ \AA}$ in diameter are present in some of these cores (Fig. 2). Other cores show one or more regions with high scattering intensities, representing crystalline materials in a Bragg orientation (Figs. 2, 4, and 5). Certain cores consist of a single large crystal (Figs. 2 and 6), while many cores have several smaller crystallites (Fig. 5), which sometimes are situated among substances appearing to have lower scattering intensities within the same central cavity (Fig. 4).

In all preparations where critical dark-field focussing has been achieved, some ferritin cores show contents with periodic arrays of scattering material (Figs. 2, and 4–14). The periodic substructure is of three types: (1) two or more parallel fringes of varied lengths (Figs. 4, 5, 7, 9–11, and 14); (2) sets of crossed parallel fringes (Figs. 6, 12, and 13); and, (3) periodically arrayed points (Fig. 8), sometimes appearing to be extensions of the linear systems. Regular periodic spacings of $2\text{--}9.5 \text{ \AA}$ have been observed. Many of the scattering regions visualized as fringes represent lattice planes that are being seen edge-on

(see *Discussion*). Observed crystallites with visible fringe systems have ranged in size from about $14 \text{ \AA}$ up to the entire diameter of a core, which can be as great as $98 \text{ \AA}$. As few as one or two lines of intensely scattering material have been observed. Some of the fringes do not have uniform scattering densities, and appear to be discontinuous (Fig. 11) or much thinner near their ends (Figs. 4, 9, and 14). In certain cases, the lattice fringes are not strictly parallel or straight, and can appear to show other structural defects such as those resembling stacking faults (Fig. 14).

All observations of the fringes in ferritin core crystallites can be interpreted in terms of variable iron occupancy in a lattice having a hexagonal unit cell with $a = 5.1 \text{ \AA}$ and $c = 9.5 \text{ \AA}$ (see *Discussion*). With reference to the polycrystalline diffraction data (refs 9, 10, and 23, and unpublished results), the fringes having $9.5\text{-\AA}$ periodicities must represent the (0001) planes. To date, up to 9 of the $9.5\text{-\AA}$ fringes have been observed within a single core. The $9.5\text{-\AA}$ fringes can have either one or two layers of scattering material situated between them (Figs. 10 and 11); these often have a weaker intensity than do the main fringes. At times, pairs of fringes, each about $2\text{--}3 \text{ \AA}$ wide, are observed separated from other pairs by about $9.5 \text{ \AA}$ (Fig. 11); at other times, wider layers of scattering materials, each over $5 \text{ \AA}$ in width, are visible and are also $9.5 \text{ \AA}$ apart (Figs. 4, 9, and 12–14). When images have been consecutively recorded, some of the single $9.5\text{-\AA}$ fringes have changed into pairs of fringes (Figs. 9 and 10). A set of fringes with a spacing of about $2.5 \text{ \AA}$ is frequently seen intersecting the (0001) system at angles of about 90° (Fig. 12); these probably represent the $(11\bar{2}0)$ planes. In one instance, where a core has evidently rotated between two consecutive exposures, these fringes change into a system running at an angle of about 55° to the same $9.5\text{-\AA}$ system (Figs. 12 and 13); such observations emphasize the full three-dimensional crystallinity of the core crystallites.

FIG. 3. Superimposed images of diffraction patterns from polycrystalline gold, placed in the positions for bright-field (BF) and dark-field (DF) imaging. The objective aperture (arrow) always remains in its axially centered position.

FIGS. 4-14. Selected dark-field images showing ferritin core ultrastructure. Bar in Fig. 4 denotes 50 Å; a similar magnification is used for all of these figures. Fig. 4: Periodically ordered material is present among apparently unordered substances in this core. Fig. 5: Two distinct crystallites are present in this core; the one at the right shows periodicities of 2.4 Å. Fig. 6: At least three sets of lattice fringes are visible in the single large crystal comprising this core; horizontal periodicities are 2.2 Å. Fig. 7: The single large crystal filling this core has periodicities of 2.6 Å with somewhat uniform intensities. Fig. 8: Orthogonally ordered small points of scattering material form much of this core; horizontal periodicities are 3.8 Å. Figs. 9 and 10: Two consecutively recorded images of the same core, showing individual 9.5-Å fringes changing into pairs or triplets of fringes. Fig. 11: Fringes with lesser intensity are present midway between the main 9.5-Å fringes in this core; note the nonuniform intensity and discontinuities within some of these fringes. Figs. 12 and 13: Two consecutively recorded images of the same core, showing 2.6-Å fringes (aligned with the arrows) crossing the 9.5-Å fringes in two different orientations. Fig. 14: The set of 9.5-Å fringes in this core is interrupted by a pair separated by 4.4 Å (arrows), probably due to a stacking fault.

In the dot patterns, intersecting rows of dots have been observed from orthogonal orientations (Fig. 8) to near-hexagonal arrays. Each of the dots appears to be around 2 Å in diameter, and thus could represent a projection looking down a row of iron atoms.

DISCUSSION

The ultrahigh resolution imaging of crystal lattice planes within individual ferritin core crystallites permits an evaluation of the several lattice models derived from the diffraction studies of populations of these same materials. In addition, establishment of the crystalline ultrastructure of the cores allows refinement of biochemically and biophysically formulated concepts of core structure, formation, and dissolution. Before considering these topics in detail, it is necessary to

critically examine the present dark-field observations in terms of electron optics.

High-Resolution Dark-Field Imaging of Thin Crystals: Relation of Image Details to Object Structure. In the present case, dark-field images of small crystalline regions are formed by interference in the image plane of those diffracted beams which are transmitted through the objective aperture that is placed asymmetrically with reference to their diffraction pattern. The particular beams that are transmitted and their relative amplitudes and phases are strongly dependent on the orientation of the individual crystallites. It can *not* be assumed that the high-resolution details in an image produced by interference of selected transmitted beams has any well-defined relationship with those in an ideal dark-field image formed by all of the diffracted beams, or with the elements

of the actual crystal structure (2). However, the present circumstances do allow some conclusions as to the image geometry and some reasonable deductions regarding intensities. Since the array of diffraction spots transmitted through the aperture from any crystallite must correspond to part of a near-planar section of its reciprocal lattice, the periodicities of the image must be those of the corresponding projection of the crystal structure if the aperture is large enough. Hence, from a sufficient number of images, the geometry of the unit cell may be deduced unequivocally.

Ferritin core crystallites are sufficiently small to allow the image intensities to be treated using the phase-object approximation (2, 3). This considers that the incident electron waves undergo a phase change proportional to $\Phi(xy)$, which is the projection of the potential distribution in the direction of the incident beam. Applying this approximation to the formation of dark-field images, Cowley has shown (2) that in ideal cases the image intensity depends on $|\Phi(xy) - \Phi_0|^2$, where Φ_0 is the value of the projected potential averaged over a suitably defined small region. Thus, maxima of intensity in a dark-field image of ferritin could come from either positive or negative deviations from the average, i.e., from regions having either a high or a low concentration of iron atoms. Dark-field images obtained with large displaced apertures, as in the present case, are expected to retain something of this character, but also may show intensities proportional to the square of the rate of change of the potential in the direction of the aperture displacement. This latter effect would give contrast where the specimen changes thickness, and may account for some cases of the lines of weak intensity that are seen in cores without much other substructure. Most of the lines of strong intensity in the present dark-field images of ferritin core crystallites with Bragg diffracting orientations can be interpreted mainly in terms of planes of iron atoms seen edge-on. It must be borne in mind that some fringes could also represent planes containing fewer iron atoms than the average of the projected structure. On this basis, the present observations can be used to distinguish with reasonable certainty the relative merits of the several models proposed for the structure of these crystallites.

Ono *et al.* (20) recently have described observations of parallel lines in ferritin cores deposited upon thin single-crystal graphite layers. These lines were interpreted as being Moiré fringes formed by the unresolved periodicities in the ferritin crystallites and the resolved lattice planes in the graphite. The observations made in the present study by the use of ultrathin supporting films of amorphous carbon indicate that this Moiré explanation is unlikely, and that actual lattice planes in the ferritin cores are being imaged.

Lattice Structure of Ferritin Core Crystallites. The model of Towe and Bradley (23) differs from that of Harrison *et al.* (10) chiefly in postulating a variable occupancy of the iron atoms in certain planes between the close-packed layers of oxygen groups, and in postulating that the ferric atoms have only an octahedral coordination. With the proposals of the latter model (10), statistical fluctuations in density would be present, but otherwise any set of lattice planes being imaged should show equal and uniform intensities, and only planes 2.35 Å apart should be seen when the electron beam is perpendicular to the *c*-axis.

In fact, when the incident beam is parallel to the (0001)-planes of the oxygen network, the most prominent and fre-

quently observed spacing is 9.5 Å, which corresponds to a periodicity of four close-packed oxygen layers. There is often a gap of several Ångströms between these fringes. The observed width of the 9.5-Å fringes is usually too great to correspond to a single planar layer of iron atoms seen exactly edge-on. Slight tilting of the angle of incidence certainly would tend to broaden the projection of any single plane so that its image would be several Ångströms wide. However, there is sometimes a substructure with pairs or triplets of lines, spaced about 2–3 Å apart, which are separated by the 9.5-Å spacing (Fig. 10); this suggests an image of a structure involving several planes of iron atoms. Such would be consistent with a projection of the Towe and Bradley structure where the planes having the highest density of iron atoms are paralleled by planes having half the number of iron atoms at a distance of 2.4 Å. Less intense fringes have been observed half-way between 9.5-Å fringes, in a position suggested by Towe and Bradley (23) for layers having an occupancy of iron atoms variable from zero upwards. The half-way fringes could indicate concentrations of iron atoms at these positions. However, it is more likely that many such fringes arise from an absence of iron atoms in these planes, i.e., a periodic negative deviation from the average density of iron atoms in the entire crystal lattice. Periodicity within the basal planes shows up in images (unpublished observations) where the 9.5-Å fringes appear to be modulated with maxima about 4.4 Å apart, corresponding to the lattice structure projected approximately along its *a*-axis. The angles of intersection of various fringes with the 9.5-Å and other fringe systems are in every case consistent with the proposed unit cell having $a = 5.1$ Å and $c = 9.5$ Å. The appearance of fringes corresponding to sets of planes with periodicities of 2.4 and 2.5 Å is consistent with any structure based on a hexagonal close-packed oxygen lattice. In the Towe and Bradley model (23), the projection of the structure along the *c*-axis should be hexagonal with a periodicity of 5.1 Å. Analysis of images showing a hexagonal arrangement of spots with spacings of about 5.1 Å (unpublished observations) has indicated a correlation of the spots with regions having a low density of iron atoms.

Only rarely have images been obtained that could possibly be interpreted in terms of a structure in which the iron atoms occur with almost equal probability between all close-packed oxygen layers (10). Indeed, most of the fringes observed, even in cores consisting of a single large crystal, do not have a uniform intensity along their length. The present observations of individual cores thus are mostly consistent with the model of Towe and Bradley (23), and do not support the equal occupancy model proposed by Harrison *et al.* (10). Additional support for the validity of the Towe and Bradley model comes from the electronic absorption spectroscopy data of Gray (8), which indicates a strong octahedral coordination of iron in the ferritin cores. Gray also has ruled out (8) tetrahedral coordination for iron in the synthetic analogs of the ferritin cores (1); thus, if the structural analogy extends to the atomic level, as the diffraction data suggest (23), then it is likely that the analogs also have the octahedral coordination found in the ferritin core crystallites (23).

Variations of the *c*-axis spacings in some images suggest that deviations from the idealized structure of the Towe and Bradley model (23) do occur. The regular spacings of 9.5 Å between fringes are sometimes substituted by one or more

spacings of 14, 7, or 4.6 Å (e.g., Fig. 14). These correspond to intervals of 6, 3, or 2 close-packed oxygen layers between the planes of highest iron content, as opposed to the 4 layers indicated by the model.

Ferritin Core Substructure. A previous study of ferritin by dark-field high-voltage electron microscopy (16) already has indicated that the cores can have a substructure based upon their polycrystallinity. Crystallite sizes ranging from 40 to 75 Å have been estimated by x-ray and electron diffraction (9, 10); these average figures fall within the even broader range of measurements of individual crystallites obtained in the present study. Fischbach and Anderegg (7), on the basis of their x-ray scattering curves, have postulated that the cores of fully saturated ferritin consist of a uniformly dense spherical particle about 73 Å in diameter. These probably correspond to those cores having a single large crystal that were observed in the present study. While some of the small core crystallites are quite elongated, the present observations do not readily match the postulated (7) particles extending asymmetrically across the diameter of the central cavity in ferritin with intermediate iron contents.

On the basis of the present observations, one can refine the prevailing concepts of core crystallinity. The dark-field images clearly show that some cores are composed of a single large crystal. Many of the other cores that show neither periodic substructure nor strongly scattering regions are probably maloriented such that their crystallites are not directing Bragg reflections through the opening in the objective aperture; indeed, during changes in the orientation of given cores, such as occur when one of the ultrathin supporting films has ruptured and curls up, fleeting lattice images and large changes in scattering intensities have been observed in cores that originally had not shown such features (unpublished observations). These and related observations all emphasize the full three-dimensional crystallinity of these small mineral crystallites. The diffuseness of the x-ray diffraction rings, as noted by Harrison *et al.* (10), now can be seen to be due not only to the very small sizes of some of the crystallites, but also to their variable iron occupancy and to the presence of lattice imperfections.

Structural Correlations with Core Growth and Iron Release. The present establishment of the Towe and Bradley model (23) necessitates recognizing that a ferritin particle which is only partially saturated, with regard to its maximum iron content, could have fully completed the growth of a crystalline lattice that fills the entire volume of its central cavity. Further iron accumulation by such a particle would be independent of core growth *per se*, and could have quite different kinetics than the precedent iron uptake of the crystallization stage. In fact, both *in vivo* (17) and *in vitro* (18) studies have shown that partially saturated populations have greater uptakes than do those with lower saturations. The present direct observation of several very small particles and crystallites in some individual cores confirms suggestions based on biochemical and morphological studies that there may be several potential nucleation sites for crystallite formation (e.g., refs. 14, 19, and 22). These same observations also emphasize the need to include intercrystallite competition

for iron as a factor in the dynamics of core growth, as well as the traditional competition between single cores (e.g., ref. 14).

On the basis of the Towe and Bradley model (23) one can predict that there should be a definite inflection point in a plotted curve giving the rate of iron release from ferritin against the remaining iron content. This inflection should occur at 67–80% saturation, and is caused by the fact that the iron atoms in planes of optional occupancy are much less tenaciously bound than are the iron atoms in other planes; hence, the former should be more easily or rapidly released from the crystalline mineral. A more exact value for the predicted inflection cannot be made at present due to the uncertain equilibrium conditions for iron occupancy at physiological pH. While measurements of iron release from the well-defined homogeneous populations of ferritin that are needed to test this prediction have not yet been reported, two *in vitro* studies of chelation-mediated release from undefined populations have reported that a phase of more rapid release precedes a longer period of slow release (13, 21).

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