ELECTRON MICROSCOPY OF THE MO-FE PROTEIN
FROM *AZOTOBACTER* NITROGENASE

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INTRODUCTION

Biological N_2 fixation, the reduction of N_2 to
NH_3, is catalyzed by the enzyme nitrogenase.
Fractionation studies of nitrogenases from a variety of organisms have shown that all are composed
of two similar proteins, one contains Mo and Fe
and is called the Mo-Fe protein and the other
contains only Fe and is called the Fe protein.
Recently homogeneous preparations of these
proteins from *Azotobacter vinelandii*, *Clostridium
pasteurianum*, and *Klebsiella pneumoniae* have been

described (1). In the case of *A. vinelandii* a crystalline preparation of the Mo-Fe protein has
been obtained and characterized (2). The present
work describes the quaternary structure of this
resolubilized, crystalline Mo-Fe protein as determined by transmission electron microscopy.

MATERIALS AND METHODS

Isolation and Purification of Nitrogenase

Nitrogenase from *A. vinelandii* (strain OP) was
purified and fractionated and Mo-Fe protein was

crystallized as previously reported (3). Specific N$_2$-fixing activity of the Mo-Fe and Fe protein was determined by the H$_2$ evolution assay (2).

Electron Microscopy

Samples of Mo-Fe protein for negative staining were diluted in 0.1 or 0.25 M ammonium formate at pH 7.1 (purged with N$_2$ for 10 min before use) to a final protein concentration of 70–100 μg/ml. Protein was determined by the biuret method (4). The dilute protein solution was placed onto the surface of a bare carbon or holey carbon film on a 400-mesh copper grid. Excess liquid on the carbon surface was removed with a capillary pipette. The protein was negatively stained by dropwise addition of 1.5% uranyl acetate to the grid and removed after 2 min by blotting with filter paper.

For shadow-casting, the Mo-Fe protein was deposited onto bare carbon films (5) and heated in vacuo for 1 h at 80°C, to insure complete removal of volatile salts. The sample was shadowed with a fused platinum carbon source (Ladd Research Industries, Inc., Burlington, Vt.) which was evaporated through an aperture at a specimen to source, height to length ratio of 6.0:6.0 cm. The vacuum apparatus was operated at a pressure of 1 × 10^{-5} torr during evaporation, while employing liquid N$_2$ trapping of the pumping system.

Electron micrographs were recorded using a Philips EM 200 operated at 80 kV with double condenser illumination at an electron-optical magnification of × 93,000. Both negatively stained beef liver catalase and a diffraction grating having 54,864 lines per inch were used as calibration standards. A liquid N$_2$-cooled anticontamination device, surrounding the area of the specimen, was employed throughout this study. Electron images of negatively stained specimen were printed from the negative, while the image from the shadow-cast specimen was printed from the positive. The marker bar as shown in frame b is the same for frames a–c (inclusive) of Fig. 1 and indicates a distance of 0.1 μm while the marker in frame g represents 0.01 μm and is the same for frames d–g. In Fig. 3 the marker bar represents a distance of 0.01 μm.

Preparation and Use of Ferritin-Conjugated (FC) Anti-Mo-Fe Protein

The ferritin-conjugated immunoglobulin (IgG) fraction of goat anti-Mo-Fe protein (FC anti-Mo-Fe protein) was obtained from Cappel Laboratories, Downingtown, Pa. The total protein content of the conjugate was 8.35 mg/ml as determined by Kjeldahl analysis (6). For the electron microscope identification of the Mo-Fe protein by its specific immune association with FC anti-Mo-Fe protein, Mo-Fe protein (50 μg/ml in 0.25 M ammonium formate)

was mixed 1:1 with FC anti-Mo-Fe protein (diluted 1:100 in phosphate buffer) and incubated at 26°C for 1 h before negative staining.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis of Mo-Fe Protein

Purified Mo-Fe protein was examined in 7.5% SDS gels, using the method of Summers et al. (7). Optionally, samples were made 0.05 M in iodoacetamide before electrophoresis. Gels were stained using the method of Weber and Osborn (8). Marker proteins were purchased from the Worthington Biochemical Corp., Freehold, N. J., and used without further purification, for estimating the molecular weight of the Mo-Fe protein.

RESULTS AND DISCUSSION

Electron microscope images of negatively stained crystalline and resolubilized Mo-Fe protein are shown in Fig. 1 a and b, respectively. In these ultracentrifugationally homogeneous preparations of Mo-Fe protein (high specific activity of 1,535 μmol H$_2$/min per mg protein), the particle type was homogeneous, consisting exclusively of structural units whose shapes are approximately square and measure 90 × 90 Å.

Because of the absence of edge-on views of the negatively stained Mo-Fe protein, it was necessary to shadow-cast this protein in order to reveal particle heights for a reasonable estimation of its molecular weight. The resulting particle heights (Fig. 1 c) were determined by measurement of the shadow lengths, employing the expression $H = \tan \theta . L$. At the shadow angle of 45° used for this study, $\tan \theta = 1$, and the length (L) of the shadow is therefore equal to the height of the particle, which in this case was approximately 40 Å. Side to side measurements of the shadow-cast Mo-Fe protein revealed a particle diameter of 112 Å. Correction for a calculated enlargement of particle widths of 10 Å on either side due to lateral growth of the particle during shadowing (9), gives a particle diameter of about 92 Å. Thus, when the lyophilized structural units of Mo-Fe protein are prepared for electron microscopy by metal shadowing, we observe particles with heights of approximately 40 Å and widths that correspond quite well with the 90 Å dimension obtained by negative staining. Since the molecular height to diameter ratio is about 1:2, the protein tends to orient with a 90 × 90 Å surface juxtaposed to the carbon film. Therefore, the
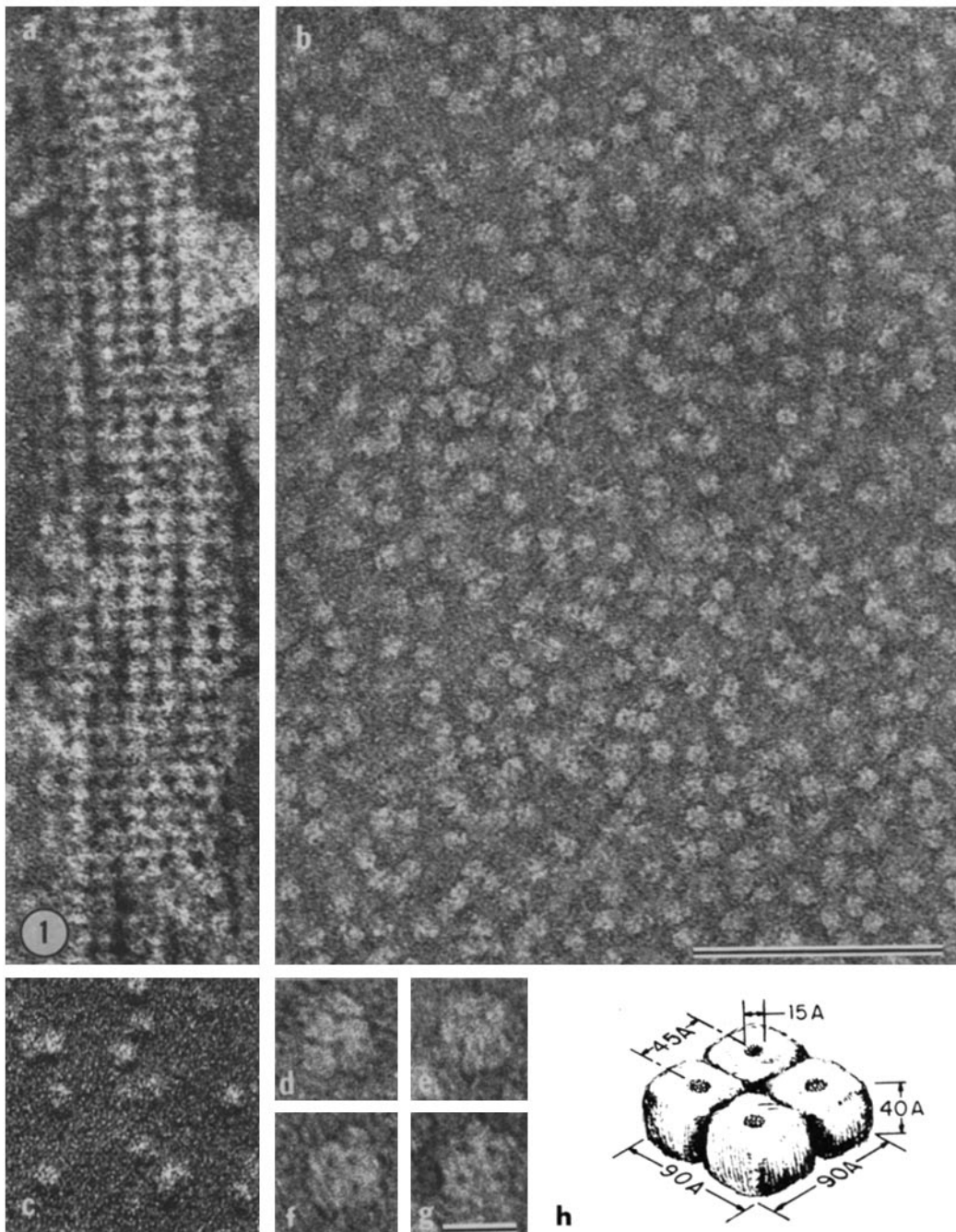
FIGURE 1 *a–c*   Mo-Fe protein (*a*) crystallized twice and (*b*) resolubilized. The particle population is homogeneous with respect to type and consists exclusively of 90×90 Å square structural units considered to be the monomeric form of the protein. Specimen was negatively stained with 1.5% uranyl acetate. The marker bar represents a distance of 0.1 μm and is the same for frames *a–c*, inclusively. (*c*) Shadowed with fused platinum carbon (Pt-C) and demonstrating particle heights of approximately 40 Å. $\times$ 375,200. (*d–g*) Enlargements of single particles of Mo-Fe protein showing characteristic shape of the basic 90×90 Å structural unit and revealing the presence of discrete areas of electron density. Although the total number of dense areas can vary from 0 to 4, their location is regularly positioned at the corners of the monomeric structural unit of the Mo-Fe protein indicating a possible tetrad arrangement of subunits for this protein. Negatively stained with 1.5% uranyl acetate. $\times$ 1,080,000 (bar in *g* is 0.01 μm). (*h*) Drawing of the proposed structure of the Mo-Fe protein from the nitrogenase of *A. vinelandii*. Each subunit of the tetrad measures $45 \times 45 \times 40$ Å and has an estimated molecular weight of 70,000 daltons. On this basis the total estimated molecular weight of the Mo-Fe protein tetrad is about 280,000 daltons.

structural unit of Mo-Fe protein is represented by a rectangular parallelepiped particle having dimensions of 90 × 90 × 40 Å. The estimated molecular weight, based on these dimensions and an assumed $\bar{v}$ = 0.73 g/cm³, is 268,000 daltons and is in good agreement with the values of 270,000–300,000 daltons calculated previously from sedimentation data and Mo content (2, 3).

Further examination of negatively stained preparations of Mo-Fe protein revealed the location of discrete areas whose densities approach that of the negative stain and are shown enlarged in Fig. 1 d–g. These areas of increased densities number from 0 to 4 per 90 Å particle, they are 10–15 Å in diameter, and are separated by a distance (center to center) of 45 Å. Although it is possible that these dense areas could be random stain artifacts, the regularity of their location in the Mo-Fe protein as well as their distribution with respect to one another makes this interpretation unlikely. It is also possible that these dense areas represent centers of localization of the Mo and Fe which are the only known metal

components of this protein. Staining with uranyl acetate may enhance the density of these areas by formation of a metallic complex, resulting in the observed positive stain effect. The particle in Fig. 1 d appears as a tetrad but shows no dense staining areas, while those particles in Fig. 1 e–g, show two, three and four dense spots, respectively, located at the corners of the particle. This evidence suggests a tetrad structure for the Mo-Fe protein as shown diagrammatically in Fig. 1 h. Each subunit of the tetrad would have dimensions 45 × 45 × 40 Å and a molecular weight of ca. 67,000 daltons.

This value for the subunit molecular weight is substantiated by electrophoretic data. A typical polypeptide profile of purified Mo-Fe protein, after electrophoresis in an SDS-containing polyacrylamide gel, is shown in Fig. 2 a. Treatment of the Mo-Fe protein with iodoacetamide (Fig. 2 b) under reducing conditions (8), failed to produce a faster moving component. By comparison with the electrophoretic migration of bovine serum albumin (BSA) and ovalbumin, the mono-
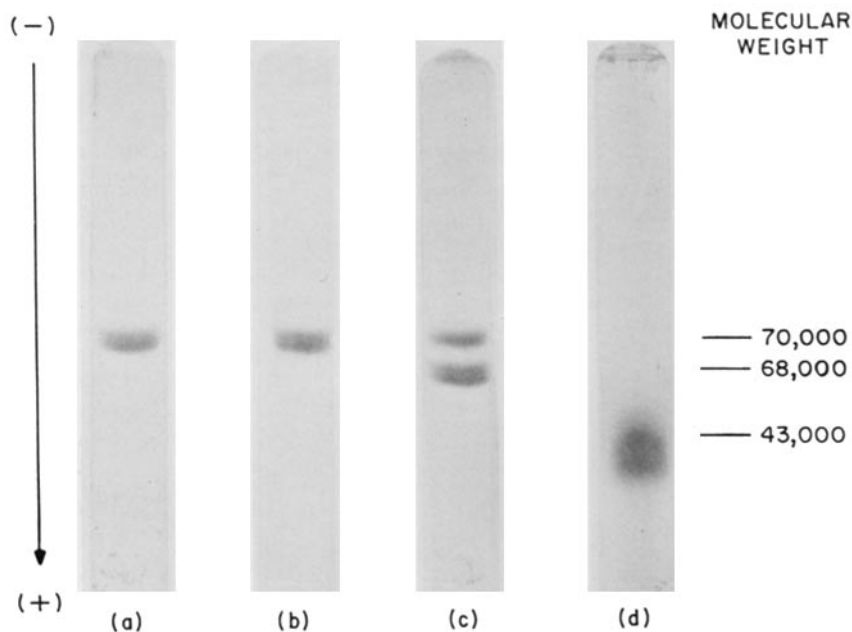

Figure 2 SDS polyacrylamide gel electrophoresis of Mo-Fe protein. (a) Polypeptide profile of Mo-Fe protein reveals a single major component corresponding to an approximate molecular weight of 70,000 daltons. (b) Same as (a) above, but treated with iodoacetamide before electrophoresis. (c)Polypeptide profile of Mo-Fe protein and BSA monomer employed as an internal calibration standard for estimation of molecular weight. The BSA monomer has a known molecular weight of 68,000 daltons. The combined sample was treated with iodoacetamide before electrophoresis. (d) Polypeptide profile of ovalbumin (43,000 daltons) used as a marker for estimation of molecular weight.
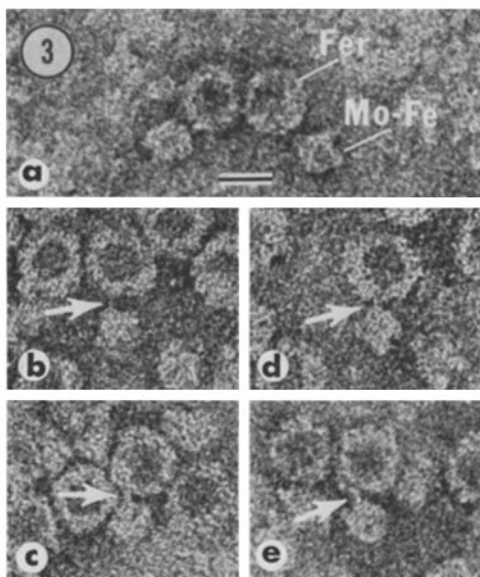
FIGURE 3 a–e Mo-Fe protein after incubation with the FC of goat anti-Mo-Fe protein IgG and subsequent negative staining with 1.5% uranyl acetate. The Mo-Fe protein (Mo-Fe) is shown connected to the antibody (IgG, see arrows) which is conjugated to the ferritin (Fer) marker (frames a–e). The preferential attachment of the FC to the corners of the Mo-Fe protein indicates that the corners are the principal antigenic sites on the molecule and lends supprt for a quadrangular tetrad arrangement of subunits. The tetrameric nature of the Mo-Fe protein was also shown earlier, by direct electron microscope observation (Fig. 1 d and g) and was substantiated by the electrophoretic data (Fig. 2) which revealed that the Mo-Fe protein is divisible into subunits, each with a molecular weight of approximately 70,000 daltons. The indicator bar located in (a) represents a distance of 0.01 μm and is the same for frames a–e, inclusively. × 720,000.

mers of which have known molecular weights of 68,000 (10) and 43,000 (11) daltons, respectively (Fig. 2 c–d), the subunit molecular weight of the Mo-Fe protein is estimated as 70,000 daltons.

Further electron microscope evidence for the tetrad arrangement of subunits within the Mo-Fe protein is the preferential attachment of the FC of anti-Mo-Fe protein to the corners of the Mo-Fe protein antigen, as observed after negative staining of the immune response (Fig. 3 a–e). The ferritin is identified by its characteristic dense, iron core region (70 Å diameter) which is surrounded by a negatively stained protein shells. In most cases, the antibody to Mo-Fe protein can be seen as a bridge, connecting the ferritin to a corner of the Mo-Fe protein. This mode of attachment indicates that the corners are the principal antigenic sites on the molecule and supports a tetrad subunit arrangement for the Mo-Fe protein.

The widths of the antibody (IgG) bridges conjugated to ferritin, and shown attached to the Mo-Fe protein (Fig. 3 a–e), ranged from 20 to 30 Å. Their lengths also varied, and IgG segments longer than 43 Å were not observed. Although it is likely that the native IgG is larger in both dimensions than we observe, such factors as the configuration (12) and flexibility (13) of the IgG, the mode of attachment of IgG to ferritin after conjugation, as well as the meniscus of the negative staining agent can influence the observed dimensions of the IgG bridge.

It has been estimated, however, that the minimum diameter of the combining site of an antibody molecule is 10–20 Å (14), and the dimensions of the combining site at the surface of the Mo-Fe protein (antigen) exceed this minimal size range. The further utility of the immune response between FC anti-Mo-Fe protein IgG and the Mo-Fe protein, for the intracellular localization of Azotobacter Mo-Fe protein, has been described (15) and will be reported more extensively elsewhere.

The results reported in the present paper, which include data from negative staining, shadow-casting, and gel electrophoresis, consistently indicate that the Mo-Fe protein is a rectangular parallelepiped particle of dimensions 90 × 90 × 40 Å and is composed of four subunits (45 × 45 × 40 Å) arranged as quadrants of a square tetramer when viewed face-on. The estimated molecular weight of 70,000 daltons for each subunit and about 280,000 for the Mo-Fe protein is consistent with previously reported values based on Mo content and analytical ultracentrifugation. A previous electron microscope study of Azotobacter Mo-Fe protein has been reported (16), but its value is limited by the use of highly impure Mo-Fe protein (the specific activity was only 15% of that used in the present experiment). Structural data on other Mo-Fe proteins will be of interest, especially in view of the similarity of the number of subunits and Mo atoms per molecule of clostridial Mo-Fe protein (17) to that of Azotobacter Mo-Fe protein.

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