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Crystallization of the *Azotobacter vinelandii* Nitrogenase Iron Protein*

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The iron protein from *Azotobacter vinelandii* nitrogenase has been crystallized in the reduced form. The needle-shaped crystals are in space group $P2_12_12$ (a = 94.6 Å, b = 179.9 Å, c = 74.1 Å) and diffract to at least 3.5-Å resolution. Five or six Fe-protein monomers are present in the asymmetric unit.

Only a limited number of procaryotes have the ability to reduce dinitrogen to ammonia, yet this activity accounts for all the enzymatically fixed nitrogen which enters the biosphere (1). Biological nitrogen fixation is catalyzed by the nitrogenase enzyme complex which consists of two proteins, a molybdenum-iron (MoFe-) protein and an iron (Fe-) protein (2). The MoFe-protein from *Azotobacter vinelandii* is an $\alpha_2\beta_2$ tetramer ($M_r \approx$ 220,000–240,000) containing 30–32 Fe atoms, two Mo atoms, and 24–32 inorganic sulfurs (2–4). The Mo atoms are arranged in two centers, each associated with 6–8 Fe atoms while the remaining Fe appear to be arranged as four atypical 4Fe:4S clusters (5–7). The Fe-protein from *Azotobacter vinelandii* is a dimer (M_r = 63,184) of identical subunits (8, 9) and contains a single 4Fe:4S cluster (8, 10). Recently, we have provided direct chemical evidence that the center may be symmetrically coordinated between subunits and that the ligands are cysteinyl residues 97 and 132 (11).

Electrons generated from metabolic reactions are passed to the Fe-protein via the electron carriers flavodoxin and ferredoxin. Subsequent transfer of electrons to the MoFe-protein is coupled to ATP hydrolysis (12). The MoFe-protein is apparently the site of substrate reduction, although it has not been established whether the Fe-protein is complexed to the MoFe-protein during the substrate reduction steps. The Fe-protein is the only known reductant of the MoFe-protein which results in catalytic activity. At least 2 ATP molecules are hydrolyzed per electron transferred, or 12 ATP per reduced nitrogen (13, 14). The ATP requirements of nitrogen fixation can consume over 40% of the ATP generated by nitrogen-fixing cells (15). Two tight binding sites for MgATP exist on the Fe-protein (K_d = 17 μM (16)). Binding of MgATP to these sites apparently results in significant conformational changes in the Fe-protein, as indicated by a decrease in reduction potential by over 100 mV (17), alterations in EPR spectrum (18), changes in cysteine thiol reactivity (11, 19), increased iron accessibility (20, 21), and increased oxygen sensitivity (22).

To understand the structural basis for these observations, we have initiated work on the three-dimensional structure of the Fe-protein by x-ray diffraction methods. The first stage in this investigation is the preparation of diffraction quality crystals, which are described in this paper for *A. vinelandii* Fe-protein. In addition, crystals of MoFe-protein from *A. vinelandii* and *Clostridium pasteurianum* have recently been described (23). Comparison of the completed Fe- and MoFe-protein structures will facilitate understanding of the interaction between these two proteins and the mechanism of dinitrogen reduction.

RESULTS AND DISCUSSION

The Fe-protein from *Azotobacter vinelandii* (ATCC 13705) was purified by a modification of the procedure of Burgess *et al.* (24). The specific activity of Fe-protein from different preparations ranged from 1600–2000 nmol of C_2H_2 reduced/min/mg of Fe-protein, with an iron content of 3.5–4.4 Fe/dimer. Due to the extreme oxygen sensitivity of the protein, strict anaerobic conditions were required for the purification and crystallization steps. Large needle-shaped crystals were prepared by dialysis of the Fe-protein in 2 mM dithionite at pH values between 7.0 and 8.0, using 2-methyl-2,4-pentanediol as the precipitant. Brown crystals appeared after several days and reached maximum size (dimension 0.4 × 0.4 × 3.0 mm) in approximately 1 week.

From oscillation and precession photographs, the Laue symmetry of the Fe-protein crystals is *mmm*, corresponding to an orthorhombic space group. Systematic absences identify the space group as $P2_12_12$. Cell parameters (a = 94.6 Å, b = 179.9 Å, c = 74.1 Å, with a unit cell volume of 1.26×10^6 Å^3) were determined by least squares refinement against the setting angles observed for reflections on a Syntex $P2_1$ diffractometer. A precession photograph of the $0kl$ zone is shown in Fig. 1. The diffraction pattern extends to at least a 3.5-Å resolution on oscillation and still photographs.

An estimate of the number of molecules per unit cell was obtained by measuring the crystal density in a bromobenzene-xylene density gradient (25). From the observed crystal density, 1.24 ± 0.01 g/cm^3, the number of Fe-protein monomers per asymmetric unit may be calculated (26) to be 5.6, assuming a partial specific volume (v_p) for the protein of 0.738 cm^3/g. This value of v_p was obtained from the amino acid composition (8), using the tables of Cohn and Edsall (27). For the iron protein of *Klebsiella pneumoniae*, however, the measured v_p was found to be 0.04 cm^3/g lower than the value calculated from the amino acid composition (28). If a similar phenomenon occurs for the *A. vinelandii* Fe-protein, the number of

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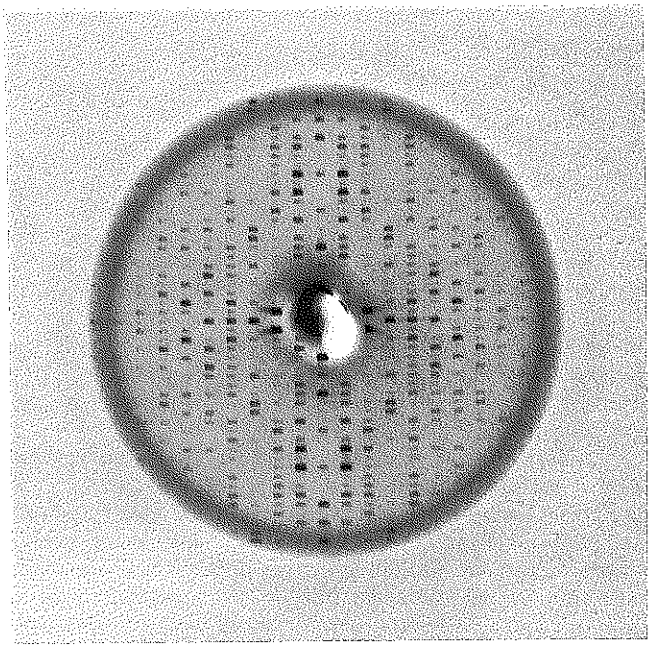

FIG. 1. **0*kl* diffraction photograph of Fe-protein crystal from *A. vinelandii*.** The precession angle equals 6° and the *c* axis is horizontal. The crystal was exposed for 12 h on a Elliott GX6 rotating anode operating at 40 kV and 40 mA, with a 200-μ focusing cup.

monomers per asymmetric unit would be 4.8. Consequently, the density measurements cannot distinguish between 5 or 6 monomers in the asymmetric unit. The ratios of the total protein mass to volume in the asymmetric unit for both 5 monomers (2.02 Å^3/dalton) and 6 monomers (1.7 Å^3/dalton) in the asymmetric unit are within the range observed for crystalline globular proteins (29), although the latter value is on the low end of the range.

Occasionally, we observed diffraction patterns from Fe-protein crystals which have "extra" rows of reflections perpendicular to the *c* axis, corresponding to a doubling of the *c* axis. Since these diffraction patterns are most commonly observed from older crystals, the doubled lattice may have arisen as a consequence of oxidation of Fe-protein. To test this hypothesis, Fe-protein crystals were soaked for 3 days in a solution containing 0.05 mM indigo carmine. This dye has been used to reversibly oxidize Fe-protein.[1] The diffraction patterns from these crystals were essentially identical with those from the reduced Fe-protein crystals. However, the doubled lattice may have resulted from an irreversibly oxidized form of Fe-protein, which occurs during oxygen inactivation. Regardless of the origin of the doubled lattice, it is possible to reproducibly obtain crystals of Fe-protein with the shorter *c* axis in both the reduced and indigo carmine-oxidized states, and these are the crystal forms which are being used in the structure determination.

Addition of 1 mM MgATP to Fe-protein crystals leads to disruption and dissolution of the crystals. This behavior is consistent with previous observations suggesting that ATP binding to Fe-protein is accompanied by significant conformational changes. In contrast, the crystals are stable in 1 mM MgADP for several days. The diffraction pattern from these crystals is isomorphous with the native patterns, but distinct differences in the intensities of reflections are present. Because MgADP is a competitive inhibitor toward MgATP (16), it should be possible to identify the MgATP binding site in the completed Fe-protein structure by difference Fourier studies of MgADP binding to these crystals. Details of the conformational change accompanying MgATP binding to Fe-protein will likely require independent structure determination of a MgATP-Fe-protein complex.

[1] G. L. Anderson and J. B. Howard, unpublished results.

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