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NEUTRON SMALL ANGLE SCATTERING OF THE Mo-Fe PROTEIN (NITROGENASE) FROM CLOSTRIDIUM PASTEURIANUM

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ABSTRACT: Neutron small angle scattering measurements of solutions of the Mo-Fe protein from C. pasteurianum have yielded the following results. The molecular weight of the protein is 208,000 ± 10,000, in agreement with figures obtained by other methods. The radius of gyration is 39.8 ± 0.7 Å in H_2O, and 37.6 ± 0.3 Å in D_2O. The experimental scattering curves have been compared with the calculated scattering curves of simple homogeneous bodies. It is concluded that the MoFe protein from C. pasteurianum is a non spherical particle having an axial ratio of 2:1, and that it probably has little, if any, solvent containing cavities.

INTRODUCTION

The nitrogenase enzyme system consists of two proteins and catalyzes the reduction of N_2 to NH_3 in a Mg-ATP dependent reaction. The larger of these proteins (Mo-Fe protein) contains molybdenum, non heme iron, sulfide, and carries the substrate reducing site. The smaller protein (Fe protein) contains non heme iron, sulfide, and serves as a specific reductant for the Mo-Fe protein (1, 2). Nitrogenases extracted and purified from a number of bacteria display remarkable structural and functional similarities. Mo-Fe proteins have $\alpha_2\beta_2$ oligomeric structures and molecular weights of 200,000-250,000 (α and β = 50,000-60,000). Fe proteins have γ_2 oligomeric structures and molecular weights of 55,000-70,000 (1). One of the key steps in the catalytic turnover of nitrogenase is the electron transfer from the Fe protein to the Mo-Fe protein (2), during which the two proteins bind reversibly to each other (3).

Our present understanding of the interaction of the two nitrogenase components at the molecular level would be greatly improved by data on the three dimensional structure of these proteins in solution, and on the con-

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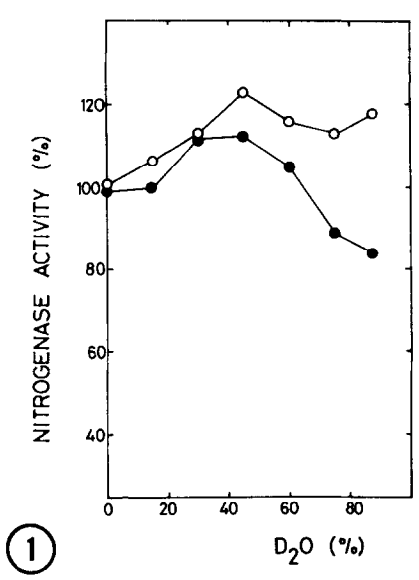
formational changes that may occur upon binding of ATP, of ADP, or of the two proteins to each other.

Neutron and X-ray small angle scattering have been developping into important methods for structural investigations of biological macromolecules in solution. These techniques provide a very low resolution structural information which is, nevertheless, valuable : The size of the particles can be determined, and useful indications of their shapes can be obtained. In addition, under appropriate conditions, conformational changes can be detected. The specific advantages of neutron scattering have been discussed elsewhere (4, 5, 6). In the present paper we describe neutron small angle scattering experiments conducted on the Mo-Fe protein from Clostridium pasteurianum. The molecular weight and radius of gyration have been determined, and the possible shapes of the protein will be discussed.

MATERIALS AND METHODS

C. pasteurianum W5 (ATCC 6013) cells were grown as described by Rabinowitz (7) and stored in liquid nitrogen. The two components of nitrogenase were purified as described by Tsö et al. (8). Nitrogenase activity was assayed in 8 ml flasks flushed with argon, stoppered with rubber septa and containing 100 mM MOPS (morpholinopropane sulfonic acid) pH 7.0, 20 mM creatine phosphate, 5 mM Mg ATP, 5 units of creatine phosphokinase, 15 mM dithionite, and nitrogenase in a final volume of 2 ml. 10 % C_2H_2 was added to the gas phase. Ethylene was assayed as described previously (9). Under these conditions the specific activities of the Mo-Fe and Fe proteins were 2000 and 2300 nmoles $C_2H_4 \times min^{-1} \times mg^{-1}$, respectively. H_2 evolution activity was assayed under the same conditions, except that C_2H_2 was omitted from the gas phase. H_2 was measured on a Intersmat IGC 120 gas chromatograph (Intersmat, 93320 Pavillons-sous-Bois, France) having a thermal conductivity detector and provided with a Porapak Q column (80-100 mesh, 2 m x 3.2 mm). Proteins were assayed by the method of Bradford (10), with crystalline bovine serum albumin as a standard. This assay was found to yield lower figures than the biuret (11). Appropriate corrective coefficients were calculated and consequently the protein concentrations obtained from the Bradford assay were increased by 20 % for the Mo-Fe protein and 40 % for the Fe protein.

For neutron scattering measurements, Mo-Fe protein solutions in MOPS 50 mM (pH 7.2), NaCl 100 mM, and dithionite 2 mM were transferred into quartz cells of 1 mm (for H_2O) or 2 mm (for D_2O) pathlength that had been stoppered with rubber septa and flushed with argon. No decrease of the enzymatic activity could be detected after 24 hours. Neutron scattering experiments were carried out less than 6 hours after sample preparation. Buffer solutions in D_2O were prepared by dissolving weighed amounts of MOPS and NaCl in 99.8 % D_2O, and adjusting the pH with a concentrated solution of NaOH in D_2O. The pH of heavy water containing solutions was determined by adding (0.3314 n + 0.0766 n^2) pH units to the pHmeter reading, n being the D_2O fraction (6). The D_2O buffer was then flushed with argon and transferred with a syringe into a argon flushed flask containing solid dithionite (final concentration 2 mM). Solutions of the Mo-Fe protein in D_2O buffer were prepared by anaerobic filtration on a Sephadex G-50 (fine) column equilibrated in D_2O buffer.



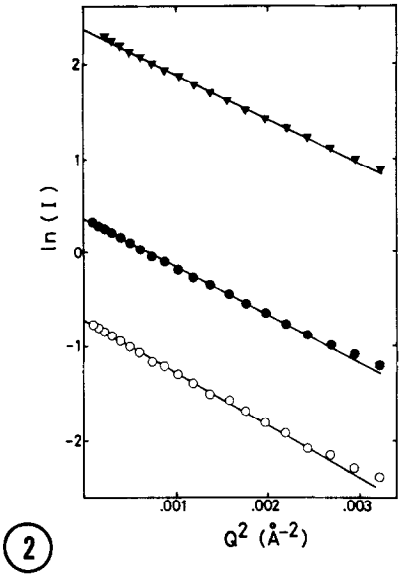


Figure 1. Effect of D_2O on nitrogenase catalyzed C_2H_2 reduction (o) and H_2 evolution (●). Assays were conducted as described in Methods. 98 µg of Fe protein and 210 µg of Mo-Fe protein were present in each assay. In 100 % H_2O the activities were 170 nmoles x min^{-1} and 155 nmoles x min^{-1} for C_2H_2 reduction and H_2 evolution, respectively.

Figure 2. Guinier plots of scattering curves of C. pasteurianum Mo-Fe proteins. Concentrations : 12.2 mg/ml in D_2O (▼), 13.5 mg/ml in H_2O (●), and 4.5 mg/ml in H_2O (o).

Neutron scattering experiments were done at the Institut Laüe-Langevin on the high-flux reactor using the small-angle scattering camera D11 (12). Data were collected for two ranges of the scattering vector, Q ($Q = 4\pi \sin\theta/\lambda$; θ is half the scattering angle and λ is the wavelength). The first range corresponded to $0.013 < Q < 0.09 Å^{-1}$, and was measured with $\lambda = 10Å$ (Figure 2). The second corresponded to $0.02 < Q < 0.18 Å^{-1}$ and was measured with $\lambda = 5Å$ (Figure 3).

RESULTS AND DISCUSSION

The effect of D_2O on H_2 production (under Ar) and on C_2H_2 reduction (under Ar : C_2H_2, 9:1) by nitrogenase is shown in Figure 1. Nitrogenase activity displays little variation (less than ± 25 %) over a wide range of D_2O concentrations (from 0 % to 90 % D_2O). This is in agreement with other studies that have shown that the nitrogenase catalyzed reactions display little primary kinetic isotope effect when D_2O is used as solvent (13, and references therein). It can thus be safely assumed that the use of various H_2O:D_2O mixtures for neutron scattering studies does not alter the three-dimensional structure of the enzyme in any significant way.

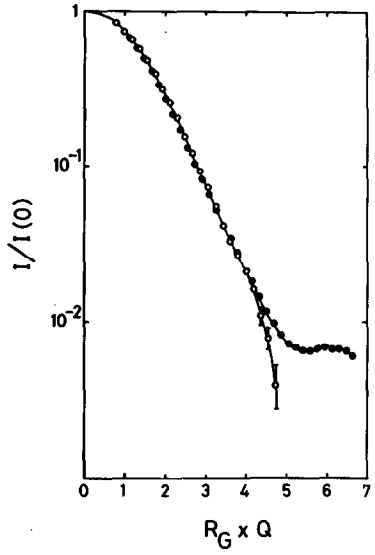


Figure 3. Neutron scattering profiles of C. pasteurianum Mo-Fe protein at 13.5 mg/ml in H_2O (o) and at 12.2 mg/ml in D_2O (●).

The neutron scattering curves of C. pasteurianum Mo-Fe protein in solution in H_2O and in D_2O are shown in figure 2 as Guinier plots (14). Straight lines were fitted to $0.0002 < Q^2 < 0.002 Å^{-2}$ and the radius of gyration (R_G) was calculated from the slopes of these lines (6, 14). In H_2O R_G was found to be 39.8 ± 0.7Å. R_G is not dependent on protein concentration over the range investigated. In 99.8 % D_2O, R_G is 37.6 ± 0.3Å, thus significantly smaller than in H_2O. The smaller value of R_G in D_2O can be interpreted in the following way (15). In D_2O the contrast (difference between the scattering length densities of the solvent and of the solute) for hydrophobic groups, which are closer to the center of the protein, is higher than for hydrophilic groups, which are closer to the outside of the protein. Thus, the particle appears to be "denser" inside. In H_2O the relative contrasts for hydrophilic and hydrophobic groups are reversed, and the particle appears to be "denser" outside, thus having a larger R_G than in D_2O. If the Mo-Fe protein were a homogeneous particle, R_G would not depend on the contrast $\bar{\rho}$, and would be intermediate between the R_G values in H_2O and in D_2O. This theoretical value of R_G can be calculated by plotting R_G^2 as a function of $\bar{\rho}^{-1}$ and taking R_G for $\bar{\rho}^{-1} = 0$, i.e. infinite contrast (15). We have found R_G ($\rho = \infty$) = 38.4Å. This figure can be used as follows to obtain information concerning the shape of the Mo-Fe protein. The volume V of the protein can be calculated by taking a molecular weight of 220 000 and a hydration of 0.4 ± 0.1 g of water per g of protein (16) : $V = (3.75 \pm 0.27) \times 10^5 Å^3$. The radii of gyration of simple

homogeneous bodies having the same volume as the Mo-Fe protein can be calculated by using known formulas (17) : A sphere having the same volume as the Mo-Fe protein would have a R_G of 34.7Å. An ellipsoid of revolution with an axial ratio of 3:1, again with the same volume, would have a R_G of 46Å. Thus, the R_G value of the Mo-Fe protein from C. pasteurianum would suggest that this protein is a non spherical particle having an axial ratio of 2 ± 0.5.

From the intensities extrapolated to zero angle, I (o), in H_2O and D_2O, we find a match point ($\bar{\rho}$ = 0) of 41 % D_2O for the protein, which is a similar value as for other soluble proteins (4).

The molecular weight M can be calculated from the Guinier plots by using the following relation (5) :

$$\frac{I(o)}{C} = 4\pi \frac{T}{1-T} \times 10^{-3} \times t \times N_A \left[\frac{\Sigma b'}{M'} - \rho_S \times \frac{\bar{\nu} \times 10^{24}}{N_A}\right]^2 \times M$$

I(o) is the scattered intensity ae zero angle divided by the incoherent scattering of water in the same solid angle. I(o) is obtained by extrapolation of the Guinier plot to Q = 0. C is the protein concentration in mg/ml. T, the transmission of the neutron beam through the solvent. t, the pathlength through the sample in cm. N_A, Avogadro's number. ρ_S = -.00562 . 10^{-12}cm x Å^{-3}, the neutron scattering density of H_2O. $\Sigma b'/M'$ is the sum $\Sigma b'$ of the scattering length of the amino acid residues for a given mass of protein M'. $\Sigma b'/M'$ = 2.20 x 10^{-14}cm/dalton was calculated for the C. pasteurianum Mo-Fe protein by using known scattering lengths for amino acids (4) and the amino acid composition given by Chen et al. (18). $\bar{\nu}$ = .735 cm^3/g, the partial specific volume of the protein, was calculated from the amino acid composition (18) and from the specific volumes of amino acid residues (19). Mo-Fe-S and Fe-S clusters present in the Mo-Fe protein (20) have smaller specific volumes ($\bar{\nu}$) and specific scattering lengths ($\Sigma b'/M'$) than the polypeptide chain. However, their mass being only 1.3 % of the protein mass, their contribution to $\bar{\nu}$ and $\Sigma b'/M'$ has been neglected. The resulting error on the molecular weight determination is less than 1 %. The total error on the molecular weight is estimated to about 5 %. When calculated as described above, the molecular weight was found to be 208,000 ± 10.000 for protein concentrations of 4.5 mg/ml and 13.5 mg/ml (Figure 2). This figure is in good agreement with molecular weights obtained by other techniques : 200,000 by gel filtration (21), 221,800 from the amino acid composition (18), and 220,000 from the subunit composition (21). It confirms a tetrameric structure $\alpha_2\beta_2$ for the Mo-Fe protein in solution. No aggregation is apparent at the highest protein concentration used (13.5 mg/ml). Partial dissociation of the tetramer into

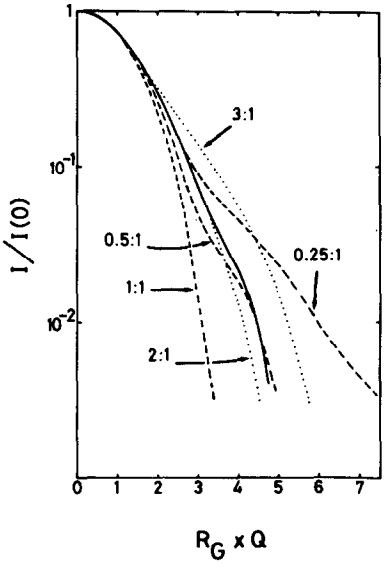


Figure 4. Comparison of the experimental scattering profile of C. pasteurianum Mo-Fe protein (—) with calculated scattering profiles of right angle prisms of various shapes : (...) elongated prisms, (---) flattened prisms. The first figure is the height of the prism, the second is the side of its base. The scattering profile of the cube (1:1) displays a subsidiary maximum (at $R_G xQ = 4.5$) which has been omitted for the sake of clarity.

a dimer has been evidenced by sedimentation equilibrium (21). We have not used concentrations lower than 4.5 mg/ml, and have detected no such dissociation.

Neutron scattering curves of C. pasteurianum Mo-Fe protein in H_2O and in 99.8 % D_2O are shown in Figure 3. The scattering intensities were plotted as log I/I(o), where I(o) is the intensity at zero angle, as a function of $R_G xQ$. The scattering curves in H_2O and D_2O are identical up to $R_G xQ$ values of 4.5. At larger angles, a subsidiary maximum at $R_G xQ = 6$ can be observed on the scattering curve in D_2O. Due to low signal to background ratio, the data in H_2O are not accurate enough to show a subsidiary maximum. In any case, if such a maximum indeed exists, it will have a lower intensity than the one observed in D_2O. Theoretical scattering curves have been calculated for a number of shapes (17). In Figure 4, the experimental scattering curve of the Mo-Fe protein in H_2O is compared to calculated scattering curves of right angle prisms having a square section. The best fit is obtained with prisms having a side : height ratio of about 2:1 or 0.5:1. Comparison with scattering curves of ellipsoids of revolution (not shown) lead to similar conclusions. Better fits to the experimental scattering curve would require

the use of more complicated models. Additional, although more uncertain, information can be obtained from the intensity of the subsidiary maxima (22). Indeed, it has been shown that, for a given shape, the scattering curve of a hollow particle shows a more intense subsidiary maximum than that of a solid particle (17). This parameter has not yet been extensively investigated. However, the low intensity subsidiary maxima of the scattering curves of the Mo-Fe protein suggest that this macromolecule has small, if any, solvent containing cavities.

Our results may be interestingly compared to electron microscopical observations of the Mo-Fe protein from Azotobacter vinelandii (23). The latter protein has the same tetrameric structure $\alpha_2\beta_2$ as the corresponding protein from C. pasteurianum, but is slightly larger : the molecular weights of the subunits are 61,000 and 59,000 for A. vinelandii (24), 60,000 and 50,000 for C. pasteurianum (21). On the electron micrographs, the four subunits of the A. vinelandii Mo-Fe protein are at the corners of a square, and the molecule is seen as a right angle prism having a square base (side = 90 Å) and a height of 40 Å (23). The height : side ratio (.44) is in very good agreement with our R_G values and with the shape of the neutron scattering curve.

Another comparison can be made with the Mo-Fe protein from Klebsiella pneumoniae : The latter protein has been reported to have a frictional ratio of 1.08 (25), which corresponds to an axial ratio of 1.5 to 2.

Further neutron scattering studies of nitrogenase will include characterization of the Fe protein, and attempts to detect conformational changes of one or both proteins under a variety of conditions.

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