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SMALL ANGLE NEUTRON SCATTERING OF THE MITOCHONDRIAL ADP/ATP CARRIER PROTEIN IN DETERGENT

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ABSTRACT: Small angle neutron scattering measurements have been performed on the mitochondrial ADP/ATP carrier protein in micelles of the detergent, laurylamidodimethylpropylaminoxide (LAPAO). The carrier protein was stabilized in the forms of the carboxyatractyloside- and bongkrekic acid- carrier protein complexes. Using a D_2O/H_2O ratio of 10%, which annuls the contribution of LAPAO to the scattering, the calculated molecular weight of the carrier protein was 61000, assuming no exchangeable H in the protein, and 56000, assuming 65% exchangeable H. Based on a minimal molecular weight close to 32000 calculated from the amino acid sequence, the neutron scattering data indicate that both the carboxyatractyloside- carrier and bongkrekic acid-carrier complexes solubilized in LAPAO are in a dimeric state.

The mitochondrial ADP/ATP carrier is an intrinsic protein spanning the inner mitochondrial membrane in an asymmetric way, the outer and inner sides recognizing two different classes of highly specific inhibitors, namely the atractylosides and bongkrekic acids respectively (for review see (1)). This protein is easily purified in a one step procedure, using hydroxyapatite chromatography (2), and the transport activity has been successfully reconstituted by incorporation of the purified carrier protein into liposomes filled with ADP (3,4). Recently, the complete primary structure has been obtained (5). Physicochemical studies on the ADP/ATP carrier protein have been performed using the intrinsic fluorescence of the protein (6) and the extrinsic fluorescence of substrate analogues (7, 8, 9). Specific labeling of a peptide segment located in the middle of the polypeptide chain of the carrier by photoactivable derivatives of atractyloside has been demonstrated (10). Nevertheless, little has been published on the three dimensional structure of this protein. Hydrodynamic studies of the purified carboxyatractyloside-carrier complex in Triton X-100 (11) have shown that the molecular weight of the protein in the complex corresponds to a dimer ; a shape of the complex in detergent was also proposed. Both these measurements rely on independent assumptions regarding the protein/detergent ratio, protein partial specific volume, and preferential interactions.

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The latter could be important because Triton X-100 is poly-disperse, with molecules of different chain length.

The present work describes a small angle neutron scattering (SANS) study of the ADP/ATP carrier protein in LAPAO (laurylamido N-N' dimethylpropylaminoxide), a homogeneous detergent obtained by chemical synthesis (4). Using contrast variation, it has been possible to analyse directly the contribution of the protein alone to the scattering and to obtain its molecular weight, independent of assumptions regarding protein-detergent ratio, protein partial specific volume and interactions.

MATERIALS AND METHODS

Carboxyatractyloside was purchased from Boehringer. Bongkrekic acid was prepared as described in (12). LAPAO was synthetised as described in (4). (^{14}C) labeled LAPAO was from CEA Saclay. The ADP/ATP carrier protein from beef heart mitochondria was prepared as a carboxyatractyloside- or bongkrekic acid-carrier complex by chromatography on hydroxyapatite (Biorad) as described in (2), except that the concentration of detergent was constant (1%) during lysis and purification. Desalting and D_2O equilibration of the purified protein was performed by gel filtration on Ultrogel AcA 202 (Réactifs IBF). The purified protein was concentrated 4 to 5 fold by ultrafiltration through PM 10 membrane (Amicon) ; the final concentrations of protein and detergent were determined by the Lowry method and estimation of the radioactivity of the (^{14}C)LAPAO respectively.

A number of protein-detergent complexes have been examined by SANS (13, 14). Molecular weight determination by SANS from solution has been described in (15). The relationship between forward scattered intensity, I(0), and the molecular weight of a protein-detergent complex is given by equation 1 :

$$\frac{I(0)}{I_{inc}(0)} = \left(f \frac{4\pi\ T_s e \times 10^{-3}}{1 - T_{H_2O}}\right)\left(\frac{C\ Mr}{N_A}\right)\left[N_A\left[R_p b_p + R_d b_d - \frac{\rho^\circ}{N_A}\left(R_p \bar{V}_p + R_d \bar{V}_d\right)\right]\right]^2$$

where $I_{inc}(0)$ is the forward incoherent scattering of H_2O and T_{H_2O} its transmission factor (used for calibration) ; f is a correction factor for the wavelength used ; Ts is the sample transmission ; e (cm) is the pathlength of the sample ; C (mg/ml) is the concentration of the complex in the solution ; Mr is the molecular weight of the complex ; N_A is Avogadro's number ; b_p (cm), $\bar{V}_p$ (cm3/g) R_p are neutron scattering length per unit of molecular weight, partial specific volume, and molecular weight ratio of protein in the complex, respectively ; subscript d denotes the same values for detergent ; ρ° (cm^{-2}) is the scattering length density of the solvent.

Equation 1 is similar to those used in X-ray, light scattering, and sedimentation equilibrium studies (16). The value in brackets which is squared is the neutron scattering density increment. For X-rays, it would be the electron density increment, and for sedimentation equilibrium studies, it would be the mass density increment. An important advantage of neutron scattering is that ρ° can be varied over a large range by D_2O/H_2O exchange (17, 18, 19). By choosing ρ° such that $b_d = (\rho^\circ/N_A)\bar{V}_d$ which annuls the contribution of the detergent to I(0), it is possible to observe the protein alone. In that case equation 1 reduces to :

$$\frac{I(0)}{I_{inc}(0)} = f\left(\frac{4\pi\ T_s e \times 10^{-3}}{1 - T_{H_2O}}\right)\left(\frac{C_p M_p}{N_A}\right)\left[N_A\left(b_p - \frac{\rho^\circ \bar{V}_p}{N_A}\right)\right]^2 \qquad \text{(Equation 2)}$$

where C_p is the concentration of the protein in the solution and M_p its molecular weight within the protein-detergent complex. Another advantage of this approach is that the value of ρ°/N_A which matches the detergent was found to be close to zero (see Table I) so that the density increment is not very sensitive to the value attributed to $\bar{V}_p$. An uncertainty arises, however, because of the exchange of labile H atoms on the protein so that b_p is a function of D_2O in the solvent (see Table I) ; b_p in H_2O and b_p in D_2O were calculated from the amino acid composition of the protein (5). Experiments were performed on D11 at the Institut Laüe-Langevin Grenoble, France (20). The scattering curve I(Q) for dilute protein solution was analysed in the Guinier approximation (21).

$$I(Q) = I(0) \exp\left(\frac{-1}{3} R_G^2 Q^2\right) \qquad \text{(Equation 3)}$$

where $Q = \frac{4\pi \sin\theta}{\lambda}$, 2θ is the scattering angle and λ the neutron wavelength, (7Å). R_G is an experimental parameter that, in dilute solutions, is the radius of gyration of scattering density in the particle. Values of I(0) and R_G^2 are obtained from plots of $Ln[I(Q)]$ versus Q^2.

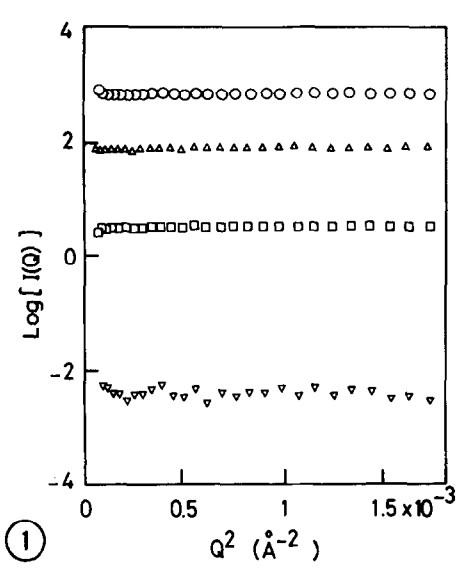
At relatively high concentrations of particles in solution,

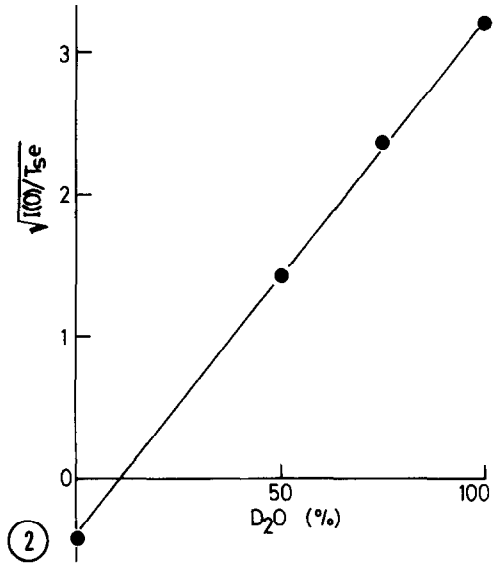
$$I(Q) = S(Q) [F(Q)]^2 \qquad \text{(Equation 4)}$$

where $[F(Q)]^2$ is a form factor which is equal to the Guinier approximation of equation 3 and S(Q) a structure factor arising from interparticle interference (22).

RESULTS AND DISCUSSION

Equation 4 applies to the scattering by the detergent micelle solutions at 4 to 5% (w/v) ; because of compensation between the functions S(Q) and F(Q), it was possible to observe flat profiles in a broad range of Q^2 in the Guinier analysis (Fig.1). Using different D_2O/H_2O ratios for solutions of pure LAPAO at the constant concentration of 4%, the plot of $\sqrt{I(0)/T_se}$ against D_2O/H_2O ratio yielded a straight line (Fig.2), and the contribution of LAPAO to I(0) was found to be null at 10% D_2O. Thus, the protein-detergent SANS studies were performed at 10% D_2O where the protein alone contributes to I(0). In this highly hydrogenated solvent, the detergent alone gave an intense background due to incoherent scattering. Furthermore, it was difficult to obtain a protein solution more concentrated than 2.5 mg/ml. The I(0) due to the protein at 10% D_2O was small compared to the incoherent scattering intensity of the detergent solution. Nevertheless, because the coherent scattering of the detergent was flat and very close to zero in 10% D_2O over the Q range, it was possible to measure the scattering intensity of the detergent-protein complex alone with reasonable precision. A typical protein-detergent complex scattering curve at 10% D_2O is shown in Fig.3. The forward scattered intensity I(0) was measured by extrapolation of the Guinier plot to $Q^2 = 0$ by linear regression. The molecular weight was then calculated from equation 2. This method appears to be a general one for determination on an absolute scale of the molecular weight of intrinsic protein solubilized in detergent. The bongkrekic acid-carrier protein complex is not very stable (23) ; it could not be analysed by hydrodynamic studies. Because free LAPAO does not





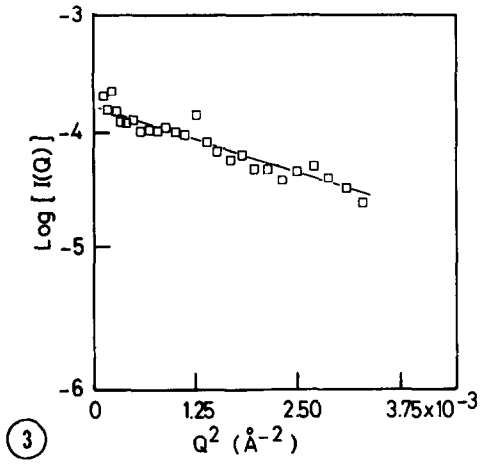


Figure 1 Guinier plots of scattering curves of pure LAPAO micelles. The detergent concentration was 4% (w/v). The pathlength was 0.200 cm for the three upper profiles and 0.100 cm for the lower one. The solvent composition was : 100% D_2O (O-), 75% D_2O (Δ-), 50% D_2O (□-) and H_2O (▽-).

Figure 2 Variation of zero angle scattering from LAPAO micelles with solvent deuteration.
The intersection with the horizontal axis (I(0) = 0) gives the solvent composition whose scattering density is the same as the average scattering density of the micelle i.e. 10% D_2O. Detergent concentration : 4%.

Figure 3 Typical Guinier profile of LAPAO-carrier protein complex (protein liganded to carboxylatractyloside) in 10% D_2O. For this solvent composition, the contribution of detergent to I(0) was null. The protein concentration was 2.19 mg/ml and the LAPAO concentration 4.94%.

Table I : Parameters for equation 2

Parameter	value	error or uncertainty	Effect of uncertainty on M_p determination
$I(0)/I_{inc}(0)xC$	0.20	≃ 10%	≃ 10%
f	0.86 for λ = 7Å	< 1%	< 1%
T_s	0.531	< 1%	< 1%
T_{H_2O}	0.512	< 1%	< 1%
e	0.100 cm	——	——
$\bar{V}_p$	0.73 cm^3/g **	2% (estimated)	< 0.3%
ρ°	1.34 . 10^9 cm^{-2} (in 10% D_2O)	——	
b_p*	n = 0 2,275 x 10^{-14} cm/MW unit		M_p 53000 to 61000
	n = 0.65 2.385 x 10^{-14} cm/MW unit		
	n = 1 2.444 x 10^{-14} cm/MW unit		

*b_p = b_p (H_2O) + nX [b_p (D_2O) - b_p (H_2O)], where b_p (H_2O) and b_p (D_2O) are the scattering lengths per molecular weight unit of the protein in H_2O and D_2O respectively, X the D_2O/H_2O ratio, and n in the proportion of labile hydrogen within the protein that could be exchanged by deuterium (no exchange n=0, complete exchange n=1 ; n=0.65 is a reasonable value for protein in detergent).

**The value of $\bar{V}_p$ = 0.73 cm^3/g is that calculated from the amino acid composition (11).

interfere with SANS measurements at 10% D_2O, excess of detergent does not have to be eliminated from the purified protein. This allowed SANS studies to be performed within five hours after the solubilization of the ADP/ATP carrier from mitochondria. Therefore, molecular weight determination of both carboxyatractyloside- and bongkrekic acid- complexes were performed.

The values of the parameters used in equation 2 are summarized in Table I. From this Table and Fig.3, it is clear that uncertainties in our determination arise mainly from the unknown number of labile hydrogen within the protein core that could be exchanged with deuterium, and from the error of the I(0) determination. Calculations were performed in two extreme cases : for no exchangeable hydrogen (complete protection of the protein by the detergent micelle), or for complete exchange of labile hydrogen with deuterium. Another calculation was performed with a 65% hydrogen to deuterium exchange, a reasonable value for protein in detergent solutions. Different protein concentrations were used, namely 2.85 to 1.51 mg/ml and 2.55 to 1.55 mg/ml for the carboxyatractyloside- and bongkrekic acid- carrier complexes respectively. Compatible molecular weights were found for all concentrations and for both liganded forms i.e. 61000 in the case of no exchangeable hydrogen within the protein, 56000 in that of 65% of exchangeable

hydrogen with deuterium, and 53000 for 100% hydrogen deuterium exchange. The maximum relative error was estimated to be 15%. These values are compatible with those found by hydrodynamic studies for the carboxyatractyloside-carrier complex (11). Because of the minimum molecular weight of the ADP/ATP carrier protein, close to 32000 calculated from the primary structure (5), our findings indicate that the solubilized protein in LAPAO is in a dimeric state whatever the liganded form of the carrier, carboxyatractyloside-carrier or bongkrekic acid-carrier.

For this measurement, no assumption regarding the protein-detergent ratio, no accurate $\bar{V}_p$ determination and no high protein concentrations were needed. This is in contrast with hydrodynamic and X-ray small angle scattering determinations of protein molecular weight for which an accurate determination of $\bar{V}_p$ is required. The SANS method appears therefore to be very suitable for rapid and direct determination of the molecular weight of labile intrinsic protein in detergent solutions and to complement the other techniques. The radius of gyration of the protein-detergent complex in 10% D_2O solvent was about 30Å. Whereas it is true that the contribution of detergent in the complex to I(0) is negligible, this is not the case for the radius of gyration, because of inhomogeneity in the scattering density of the detergent molecules (24, 25). This study will be continued to accurately determine the protein radius of gyration using partially deuterated detergent with homogeneous scattering density.

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