Kinetics of the urea-induced dissociation of human plasma α_2 -macroglobulin as measured by small-angle neutron scattering

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The kinetics of the urea-induced dissociation of human plasma α_2 -macroglobulin into two half-molecular fragments was investigated at 21.0 °C by using small-angle neutron scattering. The relative change in molecular mass that occurs upon dissociation was monitored by recording the forward scattering of neutrons as a function of time. All these kinetic data can be explained by a reaction that is first-order with respect to the concentration of undissociated α_2 -macroglobulin. The velocity constant is a function of urea concentration and it varies within wide limits. For instance, the half-life of the reaction at the lowest concentration of [^3H]urea studied (2.70 M) is 328 h, whereas the same value at the highest concentration of [^3H]urea (6.24 M) is only 8 min. Measurements were made both with [^1H]urea in $^1\text{H}_2\text{O}$ and with [^3H]urea in 99% $^2\text{H}_2\text{O}$, and it was found that there is a pronounced kinetic isotope effect, i.e. the dissociation is 4 times faster in the ^1H -containing medium as compared with the ^2H -containing medium at the same molar concentration of urea. From the angular dependence of the neutron scattering it can be concluded that the dissociation is associated with a drastic change in structure. This is directly shown by the radius of gyration, which increases from about 7.4 nm immediately after the addition of urea up to about 9.4 nm when the protein is fully dissociated. A structural analysis shows that the scattering curve of urea-dissociated α_2 -macroglobulin can best be explained by that of a Gaussian coil with a radius of gyration equal to 9.44 nm. These data indicate that the so-called non-covalent interaction of α_2 -macroglobulin probably is more complicated than just a pure hydrophobic interaction. Finally, it is also shown that the dissociation is accompanied by a loss in trypsin-binding activity, which is directly related to the fraction of dissociated protein.

INTRODUCTION

Human α_2 -macroglobulin ($\alpha_2\text{M}$) is one of the major proteinase inhibitors of blood plasma. It is unique among plasma proteinase inhibitors in its broad spectrum of reactivity for proteinases from all four classes. For recent reviews see refs. [1] and [2]. $\alpha_2\text{M}$ consists of four, probably identical, polypeptide chains, each of molecular mass about 180 kDa [3]. The native molecule has a highly organized quaternary structure, which is necessary for the proteinase elimination reaction [4].

The four subunits of $\alpha_2\text{M}$ are linked in pairs by disulphide bonds, and two such disulphide-bonded dimers are associated by so-called non-covalent interactions to form the native tetramer [5]. There is, however, only a limited knowledge about the nature of the contact areas of the subunits, as well as about the forces that hold the functional subunit assemblies together. The strategy in many of the investigations of $\alpha_2\text{M}$ has therefore been simply to dissociate the molecule by using different types of agents. As a result of such investigations it is possible to obtain a deeper insight concerning the forces that keep the subunits together, as well as information regarding the organization of the subunits in the total molecule.

The disulphide intersubunit interactions seem to be rather well defined in the sense that $\alpha_2\text{M}$ easily dissociates into half-molecules upon reaction with dithiothreitol [6,7]. The non-covalent interactions, on the other hand, are more complicated because a large excess of dodecyl sulphate [8] or low pH [9] is needed for dissociation, a process that is accompanied by a change in the structure of the protein.

A number of investigators have used urea as dissociation agent, and it has been shown that half-molecular fragments are

formed [5,10–14]. Such fragments have been used in several investigations in order to study the properties of the disulphide-bonded half-molecule of $\alpha_2\text{M}$ [11–14]. There are, however, conflicting reports concerning the concentrations of urea and the exposure times that are necessary for dissociation, and no quantitative investigation of the kinetics of dissociation has been made. Some authors incubate $\alpha_2\text{M}$ with 3 M-urea for 48 h [11], whereas others claim that 30 min in 3 M-urea is enough in order to dissociate more than 90% of the population of $\alpha_2\text{M}$ [13]. There are also conflicting reports on the effect of urea upon the proteinase-binding activity of $\alpha_2\text{M}$ [5,11,13,14].

In the present investigation we have studied the kinetics of the urea-induced dissociation of $\alpha_2\text{M}$ by using the small-angle neutron-scattering (s.a.n.s.) method. The s.a.n.s. data have been combined with tests of trypsin-binding activity of the urea-produced half-molecule of $\alpha_2\text{M}$.

MATERIALS AND METHODS

Preparation of α_2 -macroglobulin

$\alpha_2\text{M}$ was isolated from fresh blood and freshly prepared plasma by using a slight modification [15] of the method described by Harpel [16]. No impurities could be detected when a sample of $\alpha_2\text{M}$ was subjected to agarose-gel electrophoresis and crossed immunoelectrophoresis [17]. Protein concentration was determined by absorbance measurements at 280 nm by using the absorption coefficient $0.91 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ given by Dunn & Spiro [18].

Small-angle neutron-scattering measurements

The s.a.n.s. method is very well suited for the investigation of

Abbreviations used: $\alpha_2\text{M}$, human plasma α_2 -macroglobulin; s.a.n.s., small-angle neutron scattering.

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dissociation reactions of macromolecules since it offers a rapid and non-destructive determination of the average molecular mass of particles directly in a solution. It also gives direct information about structural changes that might occur upon dissociation. Furthermore, in comparison with many other methods, e.g. ultracentrifugation and gel electrophoresis, s.a.n.s. is an ideal method because neutrons cause only a very small disturbance of the system.

Data were collected with the s.a.n.s. instrument at Risø National Laboratory, Risø, Denmark (K. Mortensen), set at a sample-to-detector distance equal to 386 cm and a neutron wavelength $\lambda = 0.73$ nm. ($\Delta\lambda/\lambda$ in all measurements = 16% full width at half maximum.) For the analysis of the structure of urea-dissociated α_2 M additional data points were collected at sample-to-detector distances equal to 200 and 500 cm. Scattering intensities, normalized to the primary-beam monitor, were measured by a two-dimensional position-sensitive detector that encodes the position of the detected neutrons and sums them into elements of a 128×128 array. The sample containers were ordinary quartz cells with path lengths equal to 0.100 cm ($^1\text{H}_2\text{O}$) and 0.200 cm ($^2\text{H}_2\text{O}$). The data were corrected for background by using the intensity profiles obtained from the quartz cells filled with dialysis buffer containing the corresponding concentration of urea, an empty cell and with the primary beam blocked with boron-containing plastic. Next, the data were corrected for non-linearity in detector response by dividing through with the incoherent scattering from 1 mm of water [19]. Finally, the radially averaged intensity distributions were calculated.

All the data were collected at 21.0 °C. In order to increase the signal-to-noise ratio the main part of s.a.n.s. measurements were made with [^2H]urea in 99% $^2\text{H}_2\text{O}$. In order to check for a possible kinetic isotope effect some measurements were also made with [^1H]urea in $^1\text{H}_2\text{O}$. The protein concentration was varied in the range 1.7–6.7 mg/cm³ in buffers containing 6.5 mM- NaH_2PO_4 and 65.2 mM- Na_2HPO_4 . The introduction of $^2\text{H}_2\text{O}$ into the solutions was made by dialysing the protein for 36 h against buffers made up in $^2\text{H}_2\text{O}$. The protein concentrations were chosen as a compromise between accuracy and the risk of interparticle scattering effects. At the concentration range used interparticle scattering is still negligible. Furthermore, the concentrations used are not very far from the physiological concentration of α_2 M, which is about 1.2 mg/cm³ [20].

The kinetic data were collected simply by mixing solutions containing α_2 M and urea directly into a quartz cell. The time of mixing was defined as time $t = 0$ and the reaction was monitored by collecting consecutive spectra. Since a s.a.n.s. spectrum, with acceptable precision, can be collected in times as short as only a couple of minutes it is possible to study reactions with half-lives down to about 10 min. The urea-produced half-molecule of α_2 M is very stable; there was no noticeable changes in the s.a.n.s. spectra, even if the samples had been standing for several days.

Trypsin-binding activity

The irreversible loss of trypsin-binding activity of α_2 M after being exposed to urea was measured in $^1\text{H}_2\text{O}$ by using an assay developed by Ganrot [21]. The assay is based on the ability of α_2 M-bound trypsin to hydrolyse the synthetic substrate N^α -benzoyl-DL-arginine p -nitroanilide, while being unaffected by soya-bean trypsin inhibitor.

First, α_2 M was mixed with an urea solution to the desired final urea concentration and a protein concentration equal to 2.5 mg/cm³. Then at regular time intervals samples were taken out from the mixture and the reaction was stopped by diluting with a large volume of buffer. Finally, the trypsin-binding activity was assayed as previously described [9,21].

DATA ANALYSIS AND RESULTS

Kinetics of dissociation

In this work we utilize the forward scattering of neutrons, $I(0)$, to monitor the dissociation of α_2 M. This parameter is directly proportional to the molecular mass and it starts at a value $I_0(0)$ at time $t = 0$ and decreases to $\frac{1}{2}I_0(0)$ when the protein is fully dissociated into half-molecules. It is assumed that there is no other time-dependent change except dissociation that will influence the scattering power of the system. We have reasons to believe that this assumption is fulfilled since the experimental values of $I(0)$ always reached the value $\frac{1}{2}I_0(0)$ at large t .

The determination of $I(0)$ was made by fitting an exponential to the s.a.n.s. data within the Guinier range [22]. The effect of the unsymmetric wavelength distribution was considered by smearing the exponential curve before it was fitted to the experimental data [23]. As a by-product we also obtain the radius of gyration, R , which reflects structural changes and/or dissociation of the molecule.

For a reaction that is first-order with respect to the concentration of undissociated protein $I(0)$ varies according to the equation:

$$I(0) = \frac{1}{2}I_0(0)[1 + \exp(-kt)] \quad (1)$$

where $I_0(0)$ is the forward scattering at $t = 0$ and k is the velocity constant.

The analysis of the kinetic s.a.n.s. data was made simply by fitting the unknown parameters $I_0(0)$ and k directly to the experimental data by minimizing a least-squares sum based on the differences between the theoretical and experimental $I(0)$ values. Minimization was made by using the subroutine VA05A of the Harwell library [24]. Theoretical $I(0)$ values were calculated from eqn. (1) and by averaging over the time interval used for collecting an experimental data point.

It was found that all the experimental data could be excellently explained by the first-order eqn. (1), i.e. the dissociation is first-order with respect to the concentration of undissociated α_2 M. A few representative sets of $I(0)/I_0(0)$ data are shown in Fig. 1. In Fig. 2 we summarize all the experimental values of the velocity constant k obtained at the different urea concentrations.

Fig. 1. Examples of experimental velocity data for the dissociation of α_2 M

The data shown were recorded in 99% $^2\text{H}_2\text{O}$ at 21.0 °C. They are presented as relative forward scattering versus time. Data are shown for the following concentrations of [^2H]urea: sample A, 2.70 M; sample B, 3.40 M; sample C, 3.81 M; sample D, 4.68 M; sample E, 5.63 M. The protein concentration was equal to 3.5 mg/cm³. The continuous curves were calculated by using eqn. (1) together with the values of the first-order velocity constants given in Fig. 2. The measurements at 2.70 M- and 3.40 M-urea were extended up to 90 h and all the data can be well explained by the theoretical model curves (not shown in the Figure).

Fig. 2. Experimental values of the first-order velocity constant

The logarithm to base 10 of the velocity constant k (in h^{-1}) is plotted versus urea concentration. Data are shown both for $[^1\text{H}]\text{urea}$ in $^1\text{H}_2\text{O}$ (Δ) and for $[^2\text{H}]\text{urea}$ in 99% $^2\text{H}_2\text{O}$ (+, $\circ$ and $\square$). The following concentrations of $\alpha_2\text{M}$ were used: +, 1.7 mg/cm^3 ; $\circ$, 3.5 mg/cm^3 ; $\square$ and Δ , 6.7 mg/cm^3 . The continuous curves are arbitrarily chosen least-squares functions that summarize the result and that can be used for interpolating the data. The vertical distance between the two parallel curves is equal to 0.605, which means that the reaction is 4 times faster with $[^1\text{H}]\text{urea}$ in $^1\text{H}_2\text{O}$ as compared with $[^2\text{H}]\text{urea}$ in 99% $^2\text{H}_2\text{O}$. The equation of the lower curve is:

$$\log k = -2420.807 + 4090.907[\text{urea}]^{0.0244823} - 1679.337[\text{urea}]^{0.0549113}$$

The upper curve is obtained just by adding 0.605 to the lower one.

Fig. 3. Experimental s.a.n.s. intensity values compared with a theoretical model curve

Data are shown on a relative scale and they are plotted versus $Q = (4\pi/\lambda) \cdot \sin \theta$; 2θ is the scattering angle and λ is the neutron wavelength. The samples of $\alpha_2\text{M}$ were treated with 4.68 M- $[^2\text{H}]\text{urea}$ in 99% $^2\text{H}_2\text{O}$ for 15 h (sample D in Fig. 1). The continuous curve was calculated for a Gaussian coil with a radius of gyration equal to 9.44 nm.

It should be noted that the dissociation is a rather slow process and that a relatively large concentration of urea is needed. For instance, the half-life at the lowest $[^2\text{H}]\text{urea}$ concentration studied (2.70 M) is 328 h, whereas the same value at the highest concentration of $[^2\text{H}]\text{urea}$ (6.24 M) is only 8 min.

In the data analysis above we have considered only molecular masses on a relative scale. It should, however, be mentioned that, for every solution, we have also checked the absolute molecular masses. Within $\pm 12\%$ they agreed with the expected values for $\alpha_2\text{M}$ and its half-molecule. In these calculations we assumed that the partial specific volume of $\alpha_2\text{M}$ is independent of urea concentration. Scattering densities of the solvents at the different

concentrations of both $[^1\text{H}]\text{urea}$ and $[^2\text{H}]\text{urea}$ were calculated from the solution densities measured by the mechanical oscillator technique [25].

Kinetic isotope effect

From Fig. 2 we can also see that there is a pronounced kinetic isotope effect, i.e. the velocity is strongly dependent upon whether a ^1H -containing or ^2H -containing medium is used. The vertical distance between the two parallel curves in Fig. 2 is 0.605, which means that the dissociation is 4 times faster with $[^1\text{H}]\text{urea}$ in $^1\text{H}_2\text{O}$ as compared with the same molar concentration of $[^2\text{H}]\text{urea}$ in $^2\text{H}_2\text{O}$.

Structure of urea-dissociated $\alpha_2\text{M}$

In the above discussion we have used only the forward scattering $I(0)$ in order to monitor the relative change in molecular mass upon dissociation. By considering the shape of the scattering profile it is, however, also possible to study structural changes of $\alpha_2\text{M}$ that occur during the dissociation process.

In this case the dissociation is accompanied by an increase in the radius of gyration from about 7.4 nm immediately after mixing the urea/protein solution up to about 9.4 nm when the protein is dissociated into half-molecules. The only way to explain this result is to assume that the dissociation is accompanied by a structural change, otherwise it should lead to a decrease in the radius of gyration.

A more detailed shape analysis was made by using standard procedures involving model fitting directly to the experimental intensity data [23]. The effect of the asymmetric wavelength distribution was considered by smearing the theoretical scattering curves. Fig. 3 shows experimental intensity values obtained after treating $\alpha_2\text{M}$ with 4.68 M- $[^2\text{H}]\text{urea}$ for 15 h. Best fit with these data was obtained for a Gaussian coil with a radius of gyration R equal to 9.44 nm (Fig. 3). In such a model the scattering elements are linked together in the manner of random walk and the distances between the elements follow a Gaussian distribution. The scattering $I(Q)$ for a Gaussian coil can be calculated by using the equation [26]:

$$I(Q) = (2/X^2)[\exp(-X) + X - 1] \quad (2)$$

where $X = (RQ)^2$ and $Q = (4\pi/\lambda) \cdot \sin \theta$; 2θ is the scattering angle and λ is the neutron wavelength.

It should be mentioned that several other models, for instance solid three-axial bodies, were tested, but they all fail to explain the experimental data. As criteria for the quality of the fits of the different models we used the weighted least-squares sum based on the differences between the experimental and theoretically calculated intensity values (eqn. 5 in reference [23]). For all the models tested the least-squares sum was more than 3 times larger than that obtained for the Gaussian coil. Most of the three-axial bodies could also be rejected because they obtained physically unrealistic dimensions.

Thus the results of the present study indicate that the highly organized structure of native $\alpha_2\text{M}$ is lost after treatment with urea and that the dissociation and structural change are processes that are closely connected with each other.

Trypsin-binding activity

Tests of trypsin binding by $\alpha_2\text{M}$ exposed to urea were performed at 3.80 M-urea. The results show that urea induces a loss in trypsin-binding activity (Fig. 4). The drawn curve in Fig. 4, representing the fraction of undissociated $\alpha_2\text{M}$, was calculated on the basis of the s.a.n.s. kinetic data. It follows that the activity of $\alpha_2\text{M}$ is directly proportional to the fraction of undissociated

Fig. 4. Trypsin-binding activity of α_2 M as a function of time of exposure to 3.80 M-[1 H]urea in 1 H $_2$ O

The concentration of α_2 M was equal to 2.5 mg/cm 3 . The points are the activity data, and the continuous curve, representing the fraction of undissociated α_2 M, was calculated from the first-order velocity constant $k = 0.36$ h $^{-1}$, obtained from the s.a.n.s. data (Fig. 2).

α_2 M. Apparently the trypsin-binding activity of α_2 M is lost after dissociation of the molecule into half-molecules.

DISCUSSION

This study clearly demonstrates that a relative large concentration and/or a long time of exposure to urea is needed for dissociation of α_2 M into half-molecules. Consequently, in studies of the properties of the half-molecule, it is very important to check that the time of exposure to urea is long enough. For instance, in 3 M-[1 H]urea in 1 H $_2$ O the half-life is 24 h. After 30 min 99 % of the population of α_2 M is still undissociated. This might be the reason why there are so many conflicting reports in the literature regarding the proteinase-binding capacity of the urea-produced half-molecule of α_2 M. The result is simply dependent upon whether the protein really is dissociated or not; in this work it was found that the trypsin-binding activity directly follows the concentration of undissociated protein.

The urea-induced dissociation of α_2 M is well-defined in the sense that the reaction is first-order with respect to the concentration of undissociated protein. First-order kinetics have been found in several [27,28], but not all [29], urea/protein systems. The dependence of the rate constant upon the urea concentration is more complicated and cannot be explained by a simple kinetic law. The equations used for calculating the full-drawn curves in Fig. 2 were chosen because they are the simplest equations found that can describe the experimental data.

In this investigation it was shown that the dissociation is 4 times faster when using [1 H]urea in 1 H $_2$ O as compared with the same molar concentration of [2 H]urea in 2 H $_2$ O. There is, however, no indication of a change in reaction mechanism, since the two sets of data fall on parallel curves. Such a kinetic isotope effect has been previously observed in an investigation of urea denaturation of ovalbumin, where it was found that the substitution of 1 H for 2 H leads to a 2.8-times slower reaction [30].

The structural analysis of the s.a.n.s. data clearly shows that the urea-induced dissociation of α_2 M is associated with a

breakdown of its native structure. Thus urea mediates a transition that results simultaneously in dissociation, disorganization of the conformation of the subunits and loss in trypsin-binding activity. Consequently the so-called hydrophobic interaction of α_2 M is probably more complicated than just a pure hydrophobic interaction. This result is in agreement with previous investigations with dodecyl sulphate [8] and H $^+$ [9] as dissociating agents. In these two studies it was found that the dissociation is accompanied by a change in structure and loss of activity. Also, with regard to the reversibility of the dissociation this study is in agreement with the previous ones [8,9]; attempts to re-associate the half-molecules by dilution or by dialysis against a buffer containing no urea gave a product having properties different from that of native α_2 M.

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