Pro-(carboxypeptidases A) from whole pig pancreas

Their mass, size, shape and solvation

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Sedimentation analysis and light-scattering measurements were made with the two forms of pig pancreas pro-(carboxypeptidase A), in order to determine some of their physical properties. The following values were found (the first value applies to the binary complex and the second one to the monomer). The $A_{280,1\text{cm}}^{1\%}$ values were 19.9 ± 0.3 and 16.3 ± 0.3 . The partial specific volumes $\bar{v}^0$ were $0.707 \pm 0.016 \text{ cm}^3/\text{g}$ and $0.714 \pm 0.015 \text{ cm}^3/\text{g}$. The sedimentation coefficients $s_{20,w}^0$ were $4.90 \pm 0.15 \text{ S}$ and $3.75 \pm 0.15 \text{ S}$. The diffusion coefficients $D_{20,w}^0$ were $(5.8 \pm 0.1) \times 10^{-7} \text{ cm}^2/\text{s}$ and $(6.95 \pm 0.15) \times 10^{-7} \text{ cm}^2/\text{s}$. From these data the following values were calculated. Relative molecular masses M_r were $71\,000 \pm 4\,000$ and $46\,000 \pm 3\,000$. The frictional ratios f/f_{min} were 1.37 ± 0.06 and 1.31 ± 0.07 ; assuming a value for the solvation of the molecules ($\delta = 0.5 \text{ g/g}$) the asymmetry values range from 3 to 5 for the binary complex and from 2 to 4 for the monomer. The M_r values found in the present work coincide with those found by means of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate [Martínez, Avilés, SanSegundo & Cuchillo (1981) *Biochem. J.* **197**, 141–147]. Therefore the low values obtained by those authors when using gel-filtration chromatography must be the result of the interaction of the zymogens with the gel matrix, as the asymmetry is too small to justify the large discrepancies found.

In the pro-(carboxypeptidase A) system from pig pancreas the proenzyme appears in two different forms, one as a binary complex with pro-(proteinas E) and the other as a monomer (Kobayashi *et al.*, 1978; Martínez *et al.*, 1981). Some of their properties have been studied, and a discrepancy has been noticed between the M_r values found by means of gel-filtration chromatography and those found by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate.

It is known that the two methods used for the determination of M_r are subject to important errors (e.g. see Ackers, 1970; Andrews, 1970; Jacobson *et al.*, 1973). Therefore other methods such as sedimentation analysis and light-scattering were

chosen in order to gather more information both about the actual M_r values of the two proteins and about their hydrodynamic properties. In this way it was possible to decide which of the two sets of M_r values was more accurate. Moreover, knowledge about the shape of the protein could give some hints about possible causes for the anomalous behaviour found on gel filtration.

In order to obtain reliable data it was necessary to determine also the absorption coefficients and the partial specific volumes of the proteins.

Materials and methods

Preparation of the proteins

Pure and homogeneous solutions of the monomeric pro-(carboxypeptidase A) and pro-(carboxypeptidase A)–pro-(proteinase E) binary complex were prepared by a procedure developed in our laboratory (Martínez *et al.*, 1981).

The purity of the samples was checked by sodium

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dodecyl sulphate/polyacrylamide-gel electrophoresis (Davis, 1964) and by analytical boundary sedimentation (see below).

The partial specific volume $\bar{v}$, the diffusion coefficient D and the sedimentation coefficient s were determined in 0.1 M-Tris/HCl buffer, pH 7.5, containing 0.05 M-NaCl.

Sedimentation analysis

The sedimentation coefficients were measured by boundary sedimentation, with an MSE analytical ultracentrifuge with double-sector cells and absorption optics at 280 nm in combination with an automatic photoelectric scanner device. Rotor speeds ranged between 45 000 and 55 000 rev./min. A four-hole or six-hole rotor allowed simultaneous measurements on different samples.

The concentrations of the protein solutions ranged from 0.2 to 0.5 mg/ml; no concentration effects were observed. Reduction of the sedimentation coefficient to standard conditions of water and 20°C yielded $s_{20,w}^0$ (Marshall, 1978).

Photon correlation spectroscopy of scattered light

The diffusion coefficients have been determined by analysing the intensity fluctuations of the light scattered by proteins in Brownian motions (Foord *et al.*, 1970; Pusey *et al.*, 1974; Bloomfield, 1981).

In particular, single-clipped photon-count autocorrelation spectroscopy was used for the efficient and accurate analysis of the intensity fluctuations of the laser light scattered by a solution of macromolecules. In the case of a dilute and monodisperse solution of macromolecules, which are small compared with the wavelength of the light, the measured correlation function $G(iT)$ decays exponentially with a decay time proportional to the desired diffusion coefficient D :

$$G(iT) = A + B \cdot \exp(-2 D \cdot K^2 \cdot iT)$$

where i is the channel number, T is the chosen sample time, K is the known modulus of the scattering vector and A and B are constants. We have previously described our set-up and experimental procedures (Nieuwenhuysen & Clauwaert, 1977, 1978) and methods of data analysis (Nieuwenhuysen, 1978).

Density and concentration measurements

The density increment at zero concentration and constant chemical potential of all diffusible components $(\delta\rho/\delta c)_\mu^0$ (Eisenberg, 1976), was obtained from a plot of density versus concentration. The solutions were brought to thermodynamic equilibrium of diffusible components by means of exhaustive dialysis against buffer.

The densities were determined with an Anton Paar DMA O2C digital precision density meter

(Kratky *et al.*, 1973) in a thermostatically controlled room. The temperature was kept constant within 0.005°C at 25.0°C by using a Tronac model 40 precision temperature controller and two thermostats in series.

The density of a solution is given by:

$$\rho = A(T^2 - B)$$

where T is the oscillation period and A and B are instrumental constants, which can be obtained from calibration measurements with two samples of known density. Dry air and twice-distilled water that had been made dust-free by filtration through a 0.1 µm Millipore filter and subsequent centrifugation were used for the calibration. The protein solutions were centrifuged before measurement.

Protein concentrations were determined by measuring the absorbance of the solutions at 280 nm with a Zeiss PMQ 3 spectrophotometer. The precision of the $(\delta\rho/\delta c)_\mu^0$ determination is limited by the accuracy of the concentration measurements; therefore special attention was paid to the accurate determination of the $A_{280,1\text{cm}}^{1\%}$ values. For that purpose, the protein content of solutions with known absorbance at 280 nm was determined. Several samples of protein solutions were hydrolysed with 6 M-HCl under N_2 at 110°C for 24 h, followed by amino acid analysis on a Beckman 120 C amino acid analyser, in order to determine the amino acid content quantitatively and, in this way, the protein content.

Results and discussion

Sedimentation coefficient

Analytical boundary sedimentation of monomeric pro-(carboxypeptidase A) and binary complex pro-(carboxypeptidase A)-pro-(proteinase E) showed a symmetric boundary with all the material moving within a single boundary, so that we can say that the homogeneity is better than 90%.

Values for $s_{20,w}^0$ of $4.90 \pm 0.15S$ for the binary complex and of $3.75 \pm 0.15S$ for the monomer were obtained. These agree very well with literature values for the same proteins (Martínez *et al.*, 1981) and, in the case of the binary form, for the analogous protein from bovine pancreas (Brown *et al.*, 1963).

Diffusion coefficient

Fig. 1 shows a typical pattern of either monomer or binary-form pro-(carboxypeptidase A) from the final Sephadex G-200 column. In order to ensure the homogeneity of the protein solutions only the top fractions were used for the measurements.

Photon-count autocorrelation spectroscopy of the light scattered from these essentially monodisperse solutions of both forms of pro-(carboxypeptidase A) yielded their diffusion coefficients. Owing to the

Fig. 1. Typical elution pattern of monomeric pro-(carboxypeptidase A) or binary complex pro-(carboxypeptidase A)-pro-(proteinase E) from Sephadex G-200 chromatography

The column size was 2.6 cm $\times$ 100 cm, and elution buffer was 0.1 M-Tris/HCl, pH 7.5, containing 0.05 M-NaCl. The experiments were performed at 4°C.

Fig. 2. Determination of partial specific volumes for monomeric pro-(carboxypeptidase A) (●) and binary complex pro-(carboxypeptidase A)-pro-(proteinase E) (▲): relationship between densities and concentrations

Measurements were made in an Anton Paar DMA O2C digital precision density meter at $25 \pm 0.005^\circ\text{C}$. For more details see the text.

unfavourable measurement conditions, caused by the limited solubility so that the maximum concentration was 6 mg/ml for both proteins, the quality factor Q of individual spectra ranged from -0.1 to 0.2 . The numerous spectra were all taken into account to allow an optimal estimate of the diffusion coefficients as described previously (Nieuwenhuysen, 1978). No concentration-dependence was found. Thus the values obtained for the standard diffusion coefficient $D_{20,w}^0$ were $(5.8 \pm 0.1) \times 10^{-7} \text{ cm}^2/\text{s}$ for the binary complex and $(6.95 \pm 0.15) \times 10^{-7} \text{ cm}^2/\text{s}$ for the monomer.

Absorbance, density increment and partial specific volume

The concentration of the proteins was derived from the absorbance of the solutions at 280 nm. To allow an accurate conversion of A_{280}/ml values into mg/ml values, the protein content of solutions of known absorbance was calculated by determining

quantitatively the amino acid content after hydrolysis of the protein solutions at 110°C during the appropriate time, followed by amino acid analysis. In that way the following values were obtained: $A_{280,1\text{ cm}}^{1\%} = 19.9 \pm 0.3$ for the binary complex and $A_{280,1\text{ cm}}^{1\%} = 16.3 \pm 0.3$ for the monomer. The higher absorbance of the first one may be due to its higher tryptophan content as compared with the second one (Martinez *et al.*, 1981). Fig. 2 shows the plots of the density ρ of the solutions of binary complex and monomer versus their absorbance at 280 nm; this illustrates the accuracy of the measurements and the linearity of the relation between ρ and concentration. By using the above values for the absorbance, the following results were obtained:

$$\left(\frac{\delta\rho}{\delta c}\right)_\mu^0 = 0.290 \pm 0.008 \text{ for binary complex pro-(carboxypeptidase A)-pro-(proteinase E)}$$

$$\left(\frac{\delta\rho}{\delta c}\right)_\mu^0 = 0.283 \pm 0.008 \text{ for monomeric pro-(carboxypeptidase A)}$$

From the equation:

$$\left(\frac{\delta\rho}{\delta c}\right)_\mu^0 = 1 - \bar{v}^0\rho_0$$

where ρ_0 is the density of the solvent, it was found for the partial specific volume at infinite dilution:

$$\bar{v}^0 = 0.707 \pm 0.015 \text{ cm}^3/\text{g} \text{ for binary complex pro-(carboxypeptidase A)–pro-(proteinase E)}$$

$$\bar{v}^0 = 0.714 \pm 0.015 \text{ cm}^3/\text{g} \text{ for monomeric pro-(carboxypeptidase A)}$$

These values are within the range of 0.69–0.75 reported for proteins containing only amino acids

$$f/f_{\min.} = 1.37 \pm 0.06 \text{ for pro-(carboxypeptidase A)–pro-(proteinase E) binary complex}$$

$$f/f_{\min.} = 1.31 \pm 0.07 \text{ for monomeric pro-(carboxypeptidase A)}$$

(e.g. see Cantor & Schimmel, 1980), and are slightly lower than the values of 0.727 and 0.730 cm³/g that were calculated for binary and monomeric form respectively from their amino acid composition and from estimates of the specific volumes of the individual amino acid residues (Spragg, 1981; Kobayashi *et al.*, 1978).

Relative molecular mass, shape and solvation

Substitution of the present experimental values for $s_{20,w}^0$, $D_{20,w}^0$ and $(\delta\rho/\delta c)_\mu^0$ in the relation (e.g. Eisenberg, 1976; Cantor & Schimmel, 1980):

$$M_r = \frac{s_{20,w} \cdot RT}{D_{20,w}^0 \cdot \left(\frac{\delta\rho}{\delta c}\right)_\mu^0}$$

where R is the gas constant and $T = 293 \text{ K}$, yields the following values for the molecular weights: $M_r = 71\,000 \pm 4\,000$ for pro-(carboxypeptidase A)–pro-(proteinase E) binary complex and $M_r = 46\,000 \pm 3\,000$ for monomeric pro-(carboxypeptidase A).

These values are in very good agreement with those obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Martínez *et al.*, 1981; Kobayashi *et al.*, 1978), but not with those determined by gel-filtration chromatography (Martínez *et al.*, 1981). This indicates either an interaction between the proteins and the gel matrix or an asymmetrical structure of the proteins.

From $D_{20,w}^0$, $s_{20,w}^0$ and $\bar{v}^0$ one can obtain, not only M_r , but also information about the asymmetry and the solvation of the particle.

Therefore, the frictional ratio $f/f_{\min.}$ is calculated, where f is the frictional coefficient calculated from the diffusion coefficient:

$$f = \frac{RT}{N \cdot D_{20,w}^0}$$

(where N is the Avogadro number) and $f_{\min.}$ is the

frictional coefficient of an unsolvated sphere with the same molecular mass, so that:

$$f_{\min.} = 6\pi \cdot \eta_{20,w} \cdot \left(\frac{3M_r \cdot \bar{v}^0}{4\pi \cdot N}\right)^{\frac{1}{3}}$$

where $\eta_{20,w}$ is the viscosity of water at 20°C.

From the above measurements, the following values are obtained:

The values of $f/f_{\min.}$ for both molecules are within the range of 1.1–1.4 for globular proteins (Squire & Himmel, 1979).

It is also possible to express explicitly the contribution of the asymmetry and the solvation by partitioning the frictional ratio ($f/f_{\min.}$) into two parts:

$$f/f_{\min.} = \frac{f}{f_0} \cdot \frac{f_0}{f_{\min.}}$$

where f_0 is the frictional coefficient of a sphere with a volume equal to that of the solvated particle. The factor $f_0/f_{\min.}$ depends on the solvation δ . On the other hand, the factor f/f_0 depends on the shape, and has been calculated for geometric forms such as oblate and prolate ellipsoids of revolution (e.g. Cantor & Schimmel, 1980).

As a protein particle is not a perfect sphere and as it contains some solvent, a compromise calculation, accepting a solvation $\delta = 0.5 \text{ g/g}$, should give a reasonable picture of the solvated proteins (Squire & Himmel, 1979); then the asymmetry values range from 2 to 4 for the monomer and from 3 to 5 for the binary complex. These values again suggest globular proteins.

The hydrodynamic data presented in this paper show that the pig pancreas pro-(carboxypeptidases A) are not too asymmetrical, and therefore the explanation for their anomalous behaviour must be related to an interaction of the molecule with the gel.

According to Nozaki *et al.* (1976), chemical affinity for the gel substance may be more common than has been generally realized, and this may be the cause of unexplained anomalies observed for several proteinases (Voordouw *et al.*, 1974). However, in the case of pig pancreas pro-(carboxypeptidases A) this interaction should be of a non-specific nature with respect to the matrix, as the results are very similar whether a dextran-type gel such as Sephadex, an acrylamide-type gel such as Bio-Gel or a mixed gel such as Ultrogel is used (Martínez *et al.*, 1981).

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