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SEDIMENTATION EQUILIBRIUM STUDIES OF SOME TURTLE CHYMOTRYPSINS

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SUMMARY

Solutions of three turtle pancreatic chymotrypsinogens and their corresponding free enzymes were each examined by a sedimentation equilibrium method to determine the molecular weights of the protein species present, as a function of protein concentration. Two of these zymogens are very susceptible to apparent auto-activation, and specific inhibition of the chymotrypsin product was found to be essential for such measurements.

All six proteins have a monomeric molecular weight in the region of 25 000, but the accuracy of this determination varied with the protein, due to reversible dimerizations which were observed both in the enzymes and in their zymogens. For the chymotrypsin II from *Chelydra serpentina* and for the chymotrypsin I from *Pseudemys elegans*, the data were consistent with the inference drawn from other studies, that very little or no material is released from the zymogen in the activation process. For the chymotrypsin II from *Pseudemys elegans*, these and gel-filtration data are compatible with the previously deduced liberation, in the activation, of fragments containing 18 amino acid residues.

INTRODUCTION

Studies in this laboratory on reptilian chymotrypsins have included the isolation of their zymogens[1,2] from the pancreas of two species of turtle. The reactivity and specificity properties of their chymotrypsins indicated[1-4] that the active centers are similar to, but not identical with, those of mammalian chymotrypsins A and C. The activation behavior of the two chymotrypsinogens from the turtle *Pseudemys elegans* was also studied[2] and showed in one case evidence for the release of fragments (molecular weight about 2000) distinctly larger than those released in the activation of

Abbreviations: TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; M_{app}, apparent molecular weight; $M_{app\,(c)}$, M_{app} as a function of the protein concentration across the ultracentrifuge cell at equilibrium; M_w, weight-average molecular weight.

bovine chymotrypsin A. Pursuing these evolutionary comparisons, information on the molecular weights of the zymogens and enzymes in these cases is needed; such studies are reported here.

MATERIALS AND METHODS

Materials and standard methods

The chymotrypsinogens I and II were isolated in homogeneous form from the pancreas of *P. elegans* as described previously[2], as was the major chymotrypsinogen[1] from *C. serpentina*. Portions of the pure zymogens were activated and at once inactivated with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) or DFP as described previously[2,4]. The excess TPCK was then removed by gel filtration[4]. Other materials and methods not specified were as noted[1,2].

Sedimentation equilibrium

All sedimentation equilibrium experiments were made in a Spinco model E ultracentrifuge modified as described elsewhere, the solution column being always 3 mm high. Depending on the initial protein concentration used, speeds varied between 12 590 and 17 980 rev./min; the cells were 12- or 30-mm optical path. All protein solutions were dialyzed before every run against the solvent chosen. The initial protein concentration was determined in a Hilger model M 154 refractometer at 20 $\pm$ 0.01°, and was expressed in terms of fringes in the ultracentrifuge from a previous calibration (1.0 g/100 ml protein = 42.65 fringes). For the refractometric measurements, a specific refractive index increment of 0.190 ml $\cdot$ g^{-1} at 20° was assumed for the protein. Densities of solution were determined by pycnometry in a bath at 20 $\pm$ 0.01°. For the partial specific volume of chymotrypsinogens and chymotrypsins a value of 0.729 was used, this being the mean of reported values[6,7] for the mammalian A and C proteins.

Usually experiments were run for 36 h. After equilibrium was reached, the interferograms (Fig. 1) were analysed[5] and the values of ln c (where c is the protein concentration in fringes) throughout the cell were plotted as a function of the square of the radial distance (r). In addition, ln c (r) was analysed by the least squares method and expressed as a polynomial of the form

$$P(r) = \ln c(r) = a + b\beta + c\beta^2 + \ldots$$

where a, b, c ... are constants and $\beta = r^2$. Thus, the derivative of this polynomial

$$P'(r) = \mathrm{d} \ln c(r)/\mathrm{d}(r^2)$$

could be used (using an appropriate program on the CDC 6400 computer) to calculate the apparent molecular weight at the concentration, c, at any radial position r in the cell, $M_{\mathrm{app}\,(c)}$, by means of the equations (with the usual symbolism)

$$M_{\mathrm{app}\,(c)} = A\,P'(r), \text{ where } A = RT/(1 - \bar{\nu}\varrho)\omega^2.$$

RESULTS

C. serpentina chymotrypsinogen and chymotrypsin

The chymotrypsinogen fraction obtained from the pancreas of the snapping turtle *C. serpentina*, was previously shown to contain two of those zymogens, which we shall now designate as I and II. The chymotrypsinogen II is the major and more

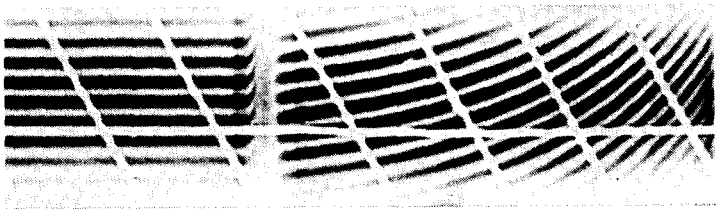


Fig. 1. Liquid column section of an interferogram at equilibrium (with the reference frame still in position) of a sample of *P. elegans* chymotrypsin II. Initial protein concentration, 0.48 mg/ml; speed, 17 980 rev./min.

cationic species, emerging later in CM-cellulose chromatography. This chymotrypsinogen II was isolated here in pure form after chromatography and re-chromatography[1]. This material was dialysed at pH 5.5 and 4°, in preparation for ultracentrifugation, but it was observed that most of it became dialysable during the 48-h period employed, due to auto-activation. Further samples were, therefore, treated with DFP (at a final concentration of 1 mM) in, and dialysed at 0° for 2 days against, acetate–NaCl buffer (I = 0.10, pH 5.5), to which further DFP was added to 0.1 mM concentration, with two or three changes daily of this medium. Sedimentation equilibrium analysis at 3° then led to a plot of ln c *versus* r^2 which showed an appreciable concavity upwards, indicating polydispersity. A computation was, therefore, made in this and further cases to exhibit the molecular weight as a function of protein concentration across the cell, $M_{app\,(c)}$. This test showed a range in apparent molecular weight from 21 000 to 31 000.

Another sample of the purified chymotrypsinogen was, immediately after the final isolation, activated and then inhibited by TPCK treatment[3]. After a 48-h dialysis against acetate buffer (I = 0.023, pH 4.0), similar analyses were made. The plot of ln c *versus* r^2 here gave what is normally considered in such plots to be reasonable linearity. The least squares analysis, assuming linearity, gave M_w = 25 500. However, the $M_{app\,(c)}$ plot showed that the range of M_{app} values is from 24 500 to 28 000, although the inhomogeneity is definitely small in this case.

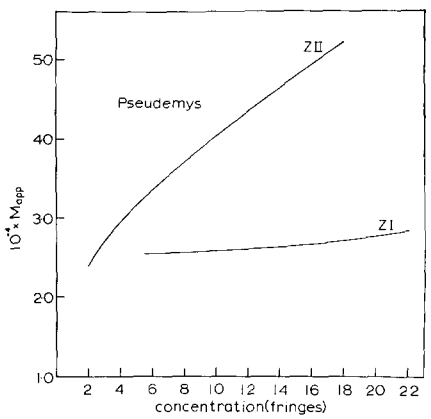
P. elegans chymotrypsinogens

The two chymotrypsinogens, I and II, from the turtle *P. elegans* were similarly isolated[2] in pure form. DFP treatment was applied before and during dialysis as in the case described above (in the pH 5.5 buffer). In the subsequent equilibrium analysis of the zymogen I, the plot of ln c *versus* r^2 showed only the very slightest departure from linearity, giving M_w = 26 000, and M_{app} varied from 25 500 to 28 500 (Fig. 2).

In the case of the chymotrypsinogen II, in contrast, the plot of *ln c versus* r^2 showed a pronounced concavity upwards, and M_{app} varied from 24 000 to 52 000 (Fig. 2).

P. elegans chymotrypsins

The zymogens I and II were activated and treated with DFP as before. It was, at first, intended to study this enzyme as a self-associating system, for which purpose a number of consecutive sedimentations at different total protein concentrations were made. However, it was found that both proteins showed with ageing an increase in



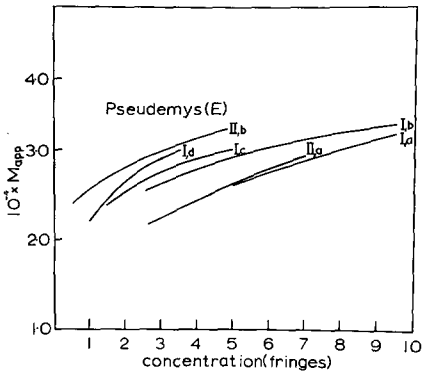


Fig. 2. Plots of M_{app} *versus* c for chymotrypsinogens I and II from *P. elegans*.

Fig. 3. Plots of M_{app} *versus* c. I, a, b, c, d: chymotrypsin I *(P. elegans)* measured at successive intervals of 2–3 days. II, a, b: the same for chymotrypsin II.

the range of $M_{app\,(c)}$ values calculated (Fig. 3). Therefore, only the general behavior of these two proteins in sedimentation can be described. With the fresh material, the chymotrypsin I showed M_{app} ranging from 26 000 to 32 000 with increasing concentration (Fig. 3), indicating slight self-association. Chymotrypsin II showed, correspondingly, a range for $M_{app\,(c)}$ from 22 000 to 29 500, and on ageing both types extended to at least 33 000 (Fig. 3).

Table I summarizes the results obtained with all the proteins studied here.

DISCUSSION

Although the methods used are among the most accurate available for protein molecular weight analysis, they have not yielded precise values for all the species examined here. This is due to the problems of partial auto-activation, auto-digestion and protein aggregation found in this series. Although the exact extent of auto-activation and auto-digestion cannot be precisely determined, we believe that the precautions taken here by the addition of proteolytic inhibitors (see MATERIALS AND METHODS) have minimized these effects. The firmest conclusions that can be drawn

TABLE I

MINIMUM AND MAXIMUM M_{app} AND ESTIMATES OF M_w OF UNDEGRADED MONOMERS

Turtle	*Protein*	M_{app}^{*} *(min)*	M_{app}^{*} *(max)*	M_w
C. serpentina	Zymogen II	21 000	31 000	approx. 25 000
	Enzyme II	24 500	28 000	25 500
P. elegans	Zymogen I	25 500	28 500	approx. 26 000
	Enzyme I	26 000	32 000	26 000
	Zymogen II	24 000	52 000	?
	Enzyme II	22 000	33 000	?

* For protein concentration ranges and further details see text.

are those for *C. serpentina* chymotrypsinogen and for *P. elegans* chymotrypsinogen I. These gave rise to plots of ln *c versus* r^2 that are very close to linear; these plots yielded apparent M_w values (at initial protein concentrations of about 1 mg/ml) of 25 500 and 26 000, respectively. The further computational analysis shows, however, that even these solutions contain a range of protein species, although the contribution of those significantly differing in weight from those M_w values is undoubtedly small.

The other proteins examined show greater poly-dispersity. Bovine chymotrypsin A is known to polymerise reversibly[6,9] but this is said not to be paralleled with its zymogen[10]. The turtle chymotrypsins studied here all show some self-association. (It is unlikely that the behavior of the native chymotrypsins would be different from that of the inactivated enzymes used here, since only a single modifying group has been introduced and since the bovine enzyme shows the same polymerisation behavior in the native and specifically inactivated forms[10]). The very low degree of association observed for the *C. serpentina* chymotrypsin is probably due to the much lower ionic strength (0.02, at pH 4) used for that experiment, these being the conditions where MORIMOTO AND KEGELES[10] showed that bovine α-chymotrypsin A becomes essentially monomeric due to the less-screened intermolecular repulsions. The zymogens from the turtle, too, show some self-association, this being least in the case of the *P. elegans* chymotrypsinogen I. Even at the relatively low total protein concentrations used (1–2 mg/ml), it appears that a monomer–dimer equilibrium is present to a certain degree in each of these cases (and in the case of *P. elegans* chymotrypsinogen II, higher aggregates are also significant).

Another difference seen between the turtle and bovine proteins is the very facile auto-activation of the turtle chymotrypsinogens. The *P. elegans* zymogen I is moderately stable, but the other two zymogens studied were so labile, even at 4° and pH 5.5, that no results could be obtained on them unless extended DFP treatment was used to arrest auto-activation. Even so, some autolysis can proceed slowly over the periods required for the sedimentation equilibrium experiments; thus, the *C. serpentina* chymotrypsinogen solution showed the presence of some material of lower molecular weight than that in its chymotrypsin. The latter appears to be almost entirely stable after its preparation, this being attributed either to the use of TPCK (in optimal conditions) instead of DFP (in sub-optimal conditions) or to the change from pH 5.5 to 4.0: shortage of the turtle material prevented further investigations in the latter conditions. The auto-activation inferred in the *P. elegans* zymogen II is consistent with the finding[2] that the zymogen II was unstable in electrophoresis, giving after short times several bands due to activation products (and finally a single band), whereas the zymogen I was stable. These chymotrypsins I and II showed a slow increase in M_{app} over several days at pH 5.5 and 4°; this ageing is presumably due either to further aggregation secondary to slow denaturation, or to further stages of activation produced by persisting traces of active enzyme, with the final products capable of greater aggregation. The great susceptibility of the *P. elegans* chymotrypsinogen II to activation may be the reason for the unusually large amount of a trypsin inhibitor detected in the pancreas of this species in an earlier study[1].

Information was sought here on decreases in weight occurring in the activation process. For *C. serpentina* chymotrypsinogen II, no evidence was obtained for a significant decrease. M_w for the final activation product is 25 500, and the parent zymogen also has a molecular weight in this range, but its value is, so far, indeterminate

due to the phenomena described above. When a solution of this zymogen, after full activation and TPCK inhibition, was gel filtered to separate activation peptides[2], no released peptides were detected (W. MÖCKEL AND E. A. BARNARD, unpublished results), although the release of single amino acids at a low level was not excluded.

For *P. elegans* chymotrypsin I, the zymogen and enzyme both have M_w near 26 000: the former was determined with reasonable accuracy, while the chymotrypsin associates but the lowest M_{app} measurable was not, in fact, lower than this. This corresponds to the finding[2] that no peptides or amino acids could be detected upon gel filtration of the activation mixture. In contrast, liberated products containing about 18 amino acid residues were isolated[2] from the activation mixture of *P. elegans* zymogen II. The molecular weight results are not inconsistent with this. The zymogen II associates, but the lowest M_{app} calculated was about 24 000. The shape of the curve (Fig. 2) suggests the presence of contaminating peptides. M_w of the monomeric enzymic product could not be determined by ultracentrifugation, due to the complications described above, although it was clearly in the same general range. Gel filtration analysis[8] shows[3] that the predominant species in the preparation of the chymotrypsin II have a size close to that of those from the chymotrypsin I. The combined data are compatible with a reduction of about 2000 in molecular weight occurring in the formation of the chymotrypsin II, but to establish this firmly by physical methods would require further investigation.

The release of as many as 18 residues from chymotrypsinogen II is of interest in a comparative sense. In the formation of bovine α-chymotrypsin A, only 4 residues are released, as is known with certainty from the amino acid sequence[11]. Similarly, 3 residues are liberated in the formation of bovine chymotrypsin B (ref. 12). None were detected in the activation of a dogfish chymotrypsinogen[13]. The only other type of chymotrypsin characterized in this respect is porcine chymotrypsin C (ref. 7). There, a change from 31 800 to 23 800 was estimated. This was based, however, upon use of the values of $D_{20,w}$ and $s_{20,w}$ only, extrapolated linearly to zero protein concentration (with the lowest points studied being at least 10 times higher than the lowest in the present case); complications of the types found here are not excluded by the data presented in that study[7].

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