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On the molecular size of thymosins

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The immunoregulatory polypeptide prothymosin α and its biologically active N-terminal fragment thymosin α_1m, with relative molecular masses of 12 500 and 3108 respectively, were found to behave as oligomers (trimers to hexamers) in gel-filtration measurements. This phenomenon of an apparent association of polypeptides has been reported for other thymosins – parathymosin α, thymosin β_4 and thymosin β_{10}. In contrast, sedimentation equilibrium ultracentrifugation shows that thymosin α_1 is a monomer with a relative molecular mass of 3000±200. Measurement of the diffusion coefficient as 221 $\mu m^2/s$ suggests that the molecule is approximately spherical. The implications for the molecular species of prothymosin α, parathymosin α, and β-thymosins are discussed.

Thymosin α_1; Prothymosin α; Sedimentation equilibrium; Gel filtration

1. INTRODUCTION

Thymosins are peptides affecting the differentiation and maturation of T-cells (review [1]). The first members of α- and β-thymosins isolated from thymosin fraction 5 (f5) – a complex extract of calf thymus [2] – were the 28-residue thymosin α_1 [3] and the 43-residue thymosin β_4 [4].

Later, new members were added to the α- and β-thymosin families (fig.1), namely thymosins des(25-28)-α_1 and α_{11}, consisting of the thymosin α_1 sequence minus 4 and plus 7 amino acid residues respectively at the C-terminus [5]; prothymosin α, approx. 113 residues in length and the natural precursor, from which thymosin α_1 and its related short peptides are derived by proteolytic degradation during the preparation of f5 [6,7]; parathymosin α, a polypeptide of approx. 105 residues and partly homologous with prothymosin α [8,9]; thymosins β_9 and β_{10}, the 41- and 42-residue peptides found to accompany thymosin β_4 in mammalian tissues [10,11]; thymosin β_8, apparently another artifact from f5, as it is identical to thymosin β_9 with two residues removed [10]; and thymosin β_{11} isolated from trout tissues [12].

Thymosin α_1 has been reported to: modulate the levels of terminal deoxynucleotidyl transferase activity in lymphocytes [13]; stimulate the production of macrophage inhibition factor [14] and interferon [15]; and increase the percentage of glucocorticoid-resistant lymphocytes [16]. Thymosin α_1 was also found to increase the expression of interleukin-2 receptors in normal human lymphocytes [17]. In vivo, thymosin α_1 has been reported to protect sensitive or immunosuppressed mice against opportunistic infections [5,18,19]; to cooperate with interferon in boosting natural killer activity in immunosuppressed animals [20]; and in lung cancer patients, immunosuppressed by radiotherapy, to raise percentages of helper T-cells and improve relapse-free and general survival [21]. Thymosin α_{11} was found to be equally potent to thymosin α_1 in enhancing the resistance of susceptible mice to challenge with *Candida albicans* [5],

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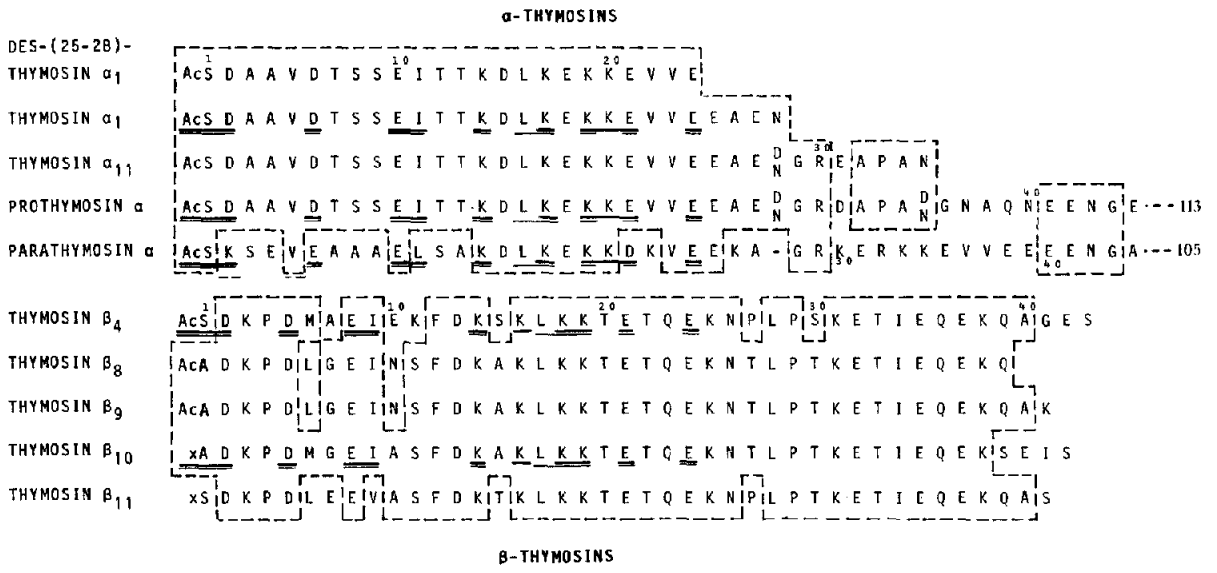

Fig.1. The primary structures of α- and β-thymosins. Amino acid sequences are shown using the single-letter code. Homologous regions are enclosed in dashed boxes. A deletion has been assumed between residues 27 and 28 of parathymosin α for maximum homology. The sequences of prothymosin α and parathymosin α beyond residue 44 are not shown. Similar residues (identical, or conservative substitutions) for the α- and β-thymosins given in table 1 are underlined.

while prothymosin α was found to be active at doses significantly lower (1/20 or 1/30 on a molar basis) than those required for the shorter peptides [8]. In the same test, parathymosin α exhibited much weaker activity, but when administered together with prothymosin α it appeared to block the immunoenhancing effect of the latter [8].

Thymosin β_4 has been reported to: induce terminal deoxynucleotidyl transferase activity in bone marrow cells from normal and athymic mice in vivo [4] and in vitro [22]; inhibit the migration of guinea pig peritoneal macrophages [14], possibly acting directly on these cells [23]; and stimulate the hypothalamic secretion of luteinizing-hormone releasing factor from superfused medial basal hypothalami of female rats [24].

Recently, antisera raised against thymosin α_1 were found to inhibit replication, in cultured cells, of the AIDS-associated virus, HIV [25]. Cross-reactivity of the immune response, which may provide a basis for preparing a vaccine against HIV, is supported by homology between thymosin α_1 and a section of p17, a protein product of the *gag* gene of the virus [25].

In view of the various biological properties invoked for thymosins, it is of interest to establish the molecular entities that exist in solution as their functional units. Gel filtration under denaturing conditions (6 M guanidinium chloride on Sephadex G-75) confirmed the sizes of the polypeptides of thymosins α_1 and β_4 [26]. However, gel filtration without denaturants, on Sephacryl S-200, gave a relative molecular mass near 11 000 rather than 3100 for thymosin α_1, and 32 000 rather than 12 600 for prothymosin α [6]. Parathymosin α and thymosins β_4 and β_{10} also gave molecular sizes several fold higher than those calculated for single polypeptides [8]. These results suggested the formation in solution of oligomers of thymosins. The present investigation was undertaken to establish the size of the molecular species of thymosin in solution by sedimentation equilibrium analysis.

Thymosin α_1 was used because the synthetic peptide was the only thymosin available in sufficient quantity for these experiments. Furthermore, it is the smallest thymosin for which there was an indication of oligomer formation; its sequence is common with other α-thymosins and corresponds to the N-terminal segment of prothymosin α, and is that segment which has the highest degree of

homology with parathymosin α. Therefore, it should be anticipated that the physical properties found for thymosin α_1 would also be shown by these related polypeptides.

2. MATERIALS AND METHODS

Thymosin α_1 was chemically synthesized [27], and rat prothymosin α [6] and thymosin β_4 [28] were isolated from thymus tissue. Sephacryl S-200 was purchased from Pharmacia, Sweden.

A phosphate buffer, pH 7.0, $I = 0.1 + 0.2$ M KCl (2.79 g KH_2PO_4, 4.61 g K_2HPO_4 and 14.9 g KCl in 1 l solution) was used for gel-filtration and analytical ultracentrifugation experiments. Gel-filtration experiments were also performed in 1 M HCOOH-0.2 M pyridine buffer, pH 2.8, $I = 0.2$ (38 ml formic acid and 14.4 ml pyridine per l solution).

Columns of Sephacryl S-200 (1.5 × 90 cm) equilibrated with buffer were calibrated using 0.8 ml of 1.5% glycerol solution in phosphate buffer containing 5 mg of each of the following marker proteins: bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), ribonuclease (13.7 kDa) and aprotinin (6.5 kDa). Samples containing 6 μg thymosin α_1 and 20 μg prothymosin α were applied to the same column, and the peptides were detected by radioimmunoassay [29]. The flow rate was 5.7 ml/h and 1.14 ml fractions were collected.

The sedimentation equilibrium experiments were carried out using a Beckman model E analytical ultracentrifuge, fitted with an RTIC unit. The temperature was maintained at 4°C, and the initial protein concentration was approx. 7 mg/ml. Before gel-filtration and ultracentrifugation experiments the lyophilized peptide was dissolved in phosphate buffer, pH 7.0, $I = 0.1 + 0.2$ M KCl. A value of 0.722 ml/g was used for the partial specific volume of thymosin α_1 calculated [31] from the amino acid composition. Data on the densities of the salt solutions were taken from International Critical Tables.

Weight-average and z-average molecular weights, M_w and M_z, were determined from sedimentation equilibrium experiments [30], using schlieren optics and solution columns of 3 mm. The initial concentration was determined in a separate synthetic boundary experiment and, assuming conservation of mass for a sector-shaped cell, numerical integration was used to give the distribution of concentration for the calculation of M_w [30]. Heterogeneity of preparations was detected by plots of log c vs r^2 and of $(1/r \cdot dc/dr)$ vs $(c_r - c_a)$, where c_r and c_a are the concentrations at radial distance, r, and at the meniscus, respectively.

The diffusion coefficient was obtained by measuring the spreading of a synthetic boundary in a low centrifugal field [32], these experiments also being done at 4°C.

3. RESULTS AND DISCUSSION

Formate-pyridine buffer at pH 2.8 was used initially for gel filtration because, after separation, the samples were either directly subjected to reversed-phase HPLC, or else the volatile buffer components could be readily removed by lyophilization. Here, a pH near the physiological value was used. Gel filtration, on Sephacryl S-200, in phosphate buffer at pH 7 gave a relative molecular mass of 17000 for thymosin α_1 and 56000 for prothymosin α (fig.2). These values are approx. 5-fold, rather than the 3-fold increases obtained in pyridine-formate buffers at pH 2.8 (table 1), and apparently confirm the self-association of thymosins.

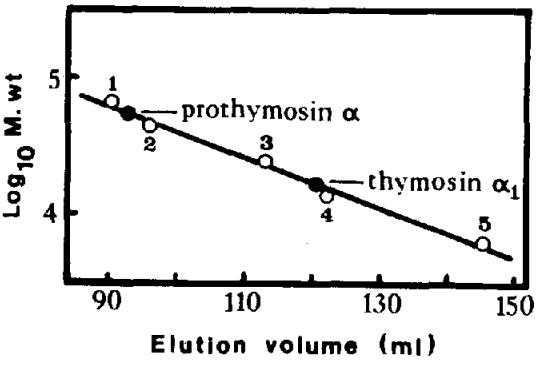

Fig.2. Gel filtration on Sephacryl S-200. The graph shows the elution volumes of synthetic thymosin α_1 and rat prothymosin α in relation to the marker proteins: bovine serum albumin (1); ovalbumin (2); chymotrypsinogen A (3); ribonuclease (4); and aprotinin (5). The buffer was phosphate pH 7.0, $I = 0.1 + 0.2$ M KCl, and the temperature was 4°C.

Table 1

Relative molecular masses for thymosins according to gel filtration

Thymosin	M_r (calculated)	M_r (observed)	Buffer[a]	Ref.
Thymosin α_1	3108	10500	HCOOH/pyridine	[6]
Thymosin α_1	3108	17000	phosphate	fig.2
Prothymosin α	12500	32000	HCOOH/pyridine	[6]
Prothymosin α	12500	56000	phosphate	fig.2
Parathymosin α	11800	32000	HCOOH/pyridine	[8]
Thymosin β_4	4963	17000	HCOOH/pyridine	[28]
Thymosin β_{10}	4737	17000	HCOOH/pyridine	[28]

[a] The HCOOH/pyridine buffer had a pH of 2.8, I 0.2, and was used at 22°C, while the phosphate buffer had a pH of 7.0, I 0.3, and was used at 4°C

In contrast, according to sedimentation equilibrium ultracentrifugation at pH 7.0 and $I = 0.3$, the M_w for thymosin α_1 was 2800 and the M_z was 3200, close to the value of 3108 calculated from the amino acid sequence. The graph of log c vs r^2, used for the calculation of M_w, was linear (fig.3), as was the graph of $(1/r \cdot dc/dr)$ vs $(c_r - c_a)$ used for the calculation of M_z (fig.4). Therefore, under the above conditions, a discrete molecular species was found corresponding to the monomer. Although the experiments were carried out at a pH far removed from the isoelectric point of thymosin α_1, the problems of non-ideality that could arise from charge interactions were avoided by using a high ionic strength in the buffer. Thus, under the present experimental conditions with a net charge of -6 on the peptide (fig.1), the ion imbalance

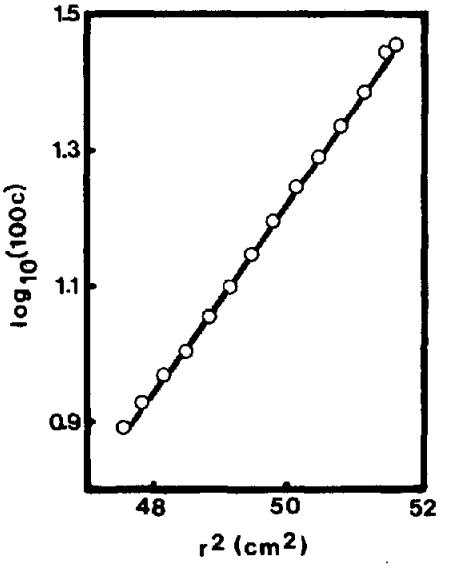

Fig.3. Sedimentation equilibrium giving M_w for thymosin α_1. The graph shows the logarithm of concentration plotted vs the radial distance. The slope corresponds to an M_w value of 2840. The buffer was phosphate, pH 7.0, $I = 0.1 + 0.2$ M KCl, at 4°C; rotor speed was 42040 rpm, and the duration of the experiment was 18 h. The concentration is in arbitrary units.

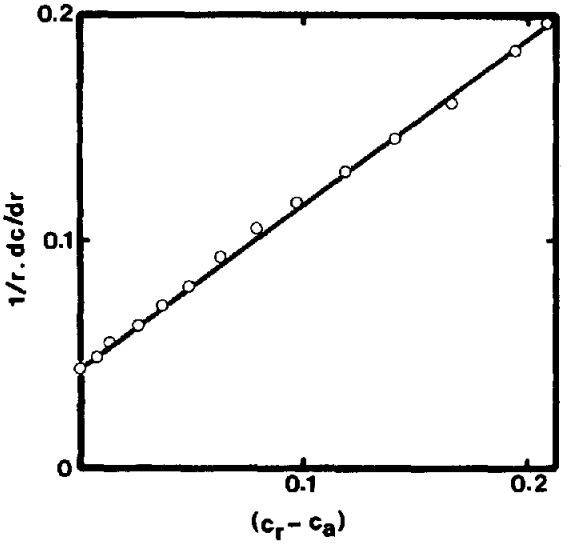

Fig.4. Sedimentation equilibrium giving M_z for thymosin α_1. The graph shows the ratio of refractive index increment to radial position plotted vs increase in concentration from the meniscus. The slope corresponds to an M_z value of 3200. Experimental conditions were as given for fig.3. The concentration is in arbitrary units.

arising from the Donnan effect would be less than a factor of 1.03 [33].

An erroneously high molecular weight could be inferred from gel filtration of a highly asymmetric molecule. Accordingly, the diffusion coefficient of thymosin α_1 was measured in the same buffer, i.e. phosphate, pH 7, 0.3 I, and the $D_{20,w}$ was found to be 221 $\mu m^2/s$. Combining this value with a relative molecular mass of 3100 and partial specific volume of 0.722 ml/g, gives a frictional ratio of 1.01, indicating approximate spherical symmetry for the thymosin molecule. For comparison, ribonuclease with a relative molecular mass of 13 700 has a $D_{20,w}$ value of 122 $\mu m^3/s$ [30] and a diffusion coefficient lower than that of ribonuclease would be required to explain the relative elution volume on gel filtration of thymosin α_1.

In the absence of aggregation or asymmetry, anomalous behaviour on gel filtration may be caused by exclusion of thymosin from the gel by charge repulsion. This could arise from the occasional negative charges on the polysaccharide matrix preventing access to small pores by a high density of negative charges on the peptide. This property could explain the high apparent molecular weight for thymosin α_1 at pH 7, but not the apparent increase in size at pH 2.8 [6], since the carboxyl groups should be extensively protonated at low pH. Correspondence to the monomer of the molecular size in 6 M guanidinium chloride [26] suggests that, besides unfolding the peptide, the denaturant obscures any charge interactions by contributing a very high concentration of ions.

Other proteins that are rich in acidic amino acids also give anomalously high molecular weights on gel filtration. These include calmodulin and troponin-C, which behave like molecules of relative mass 30 000 on gel filtration rather than 17 000–18 000 [34], and the chromosomal 'high-mobility group protein', HMG2, which gave a value of 45 000 rather than 29 000 for its relative molecular mass [35]. The discrepancy in thymosin α_1 is more pronounced, which may arise from the small size of the peptide and its correspondingly higher charge density.

Since the sedimentation equilibrium experiments were carried out at a high concentration – 7 mg/ml or 2.2 mM of thymosin α_1 – the peptide should be monomeric at the low concentrations that occur in vivo; for example, prothymosin α is present at a concentration of 400 $\mu g/g$ or 0.03 mM in the rat thymus [28].

As the apparently high molecular size of thymosin α_1 seems to be an artifact of gel filtration, the same consideration may well apply to the other thymosins that have been subjected to this technique. The high charge density of thymosin α_1 that could be responsible for its anomalous behaviour on gel filtration is present in the N-terminal segment of prothymosin α, and a homologous sequence occurs at the N-terminus of parathymosin α. It is of interest that the region near the N-terminus (and not the C-terminus) is conserved between rat and human prothymosins [36], which suggests that this segment is significant for its biological function. As trout prothymosin α also shows a high degree of aggregation according to gel filtration, with an apparent molecular weight of 55 000 [1], it would be of interest to know whether the sequence near its N-terminus is homologous with the other prothymosins.

Conversely, although the present observations show that thymosin α_1 is monomeric, there remains the possibility that in an extended polypeptide, as in prothymosin α, the surface profile may be modified to allow association. Further investigations of the hydrodynamic properties of prothymosin α, parathymosin α, and the β-thymosins should prove of importance in understanding the functional behaviour of these regulators of T-cell proliferation.

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