Comparative Studies on the Dodecameric and Hexameric Forms of Yeast Aminopeptidase I

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Yeast aminopeptidase I, when purified from autolysates of brewer's yeast, is obtained in two molecular forms a) the enzymatically active dodecameric complex ($M_r = 640\,000$, $s_{20,w} = 22$ S) and b) inactive hexamers ($M_r = 320\,000$, $s_{20,w} = 12$ S). Although the amino acid composition of the 12 S protein is very similar to that of the active enzyme, the hexamers behave differently in ionic exchange chromatography and during electrophoresis on polyacrylamide gels. Moreover, the antigenic properties of 12 S and 22 S aminopeptidase forms suggest a considerable degree of structural diversity. Several strains of *Saccharomyces cerevisiae* did not contain hexameric forms although their 22 S aminopeptidase was immunologically indistinguishable from brewer's yeast aminopeptidase. It is proposed that the hexameric protein is the result of "unproductive" aggregation of aminopeptidase subunits.

Introduction

Yeast aminopeptidase I is a Zn^{2+}-dependent glycoprotein with a molecular weight of 640 000 [1, 2]. When the enzyme is isolated from autolysates of brewer's yeast, its active form is always accompanied by an inactive protein with half of its molecular weight and a sedimentation coefficient of about 12 S. Both forms may be dissociated to give subunits with a molecular weight close to 53 000 [2]. From this findings it was concluded that the 12 S protein is a hexameric fragment of the dodecameric active aminopeptidase. The appearance of negatively contrasted 12 S and 22 S enzyme preparations on electron micrographs was in agreement with this assumption [3]. Fluorescence studies and modification experiments showed that, in the active enzyme, most of the tryptophane residues are masked while about 2/3 of the tryptophanes are exposed to the medium in the 12 S form. As a consequence, the active enzyme is rather resistant to oxidation by N-bromosuccinimide whereas the hexamers are quantitatively broken down to smaller fragments upon incubation with N-bromosuccinimide [4].

However, it remained unclear in which way hexameric aminopeptidase I is formed. Therefore, further comparative studies on the chemical composition and immunological properties of aminopeptidase forms were undertaken.

Materials and Methods

Chemicals were of reagent grade from Merck (Darmstadt, FRG) unless specified otherwise. Freund's adjuvant and the reagents needed for micro complement fixation (amboceptor, sheep blood and guinea pig complement) were obtained from Behringwerke (Marburg, FRG), proteinase inhibitors were from Serva (Heidelberg, FRG). *Aminopeptidase I* was purified from autolysates of commercial brewer's yeast as described elsewhere [1]. In addition, the enzyme was isolated from these cells and a stationary culture of *Saccharomyces cerevisiae* NCYC 366 with the following modifications: Instead of autolysis, homogenization with glass beads was employed for breaking the cells. The procedure was the same as described previously [5] but proteinase inhibitors (1 mM phenylmethylsulfonylfluoride and 1 μM pepstatin) were added to the homogenization medium. Subsequently the enzyme was precipitated from the homogenate by addition of ammonium sulfate to 75% saturation. The pellet, dissolved in buffer containing proteinase inhibitors, was then fractionated on Sephadex G-150. The final steps of the purification were as usual. The method outlined above was chosen to minimize the risk of proteolytic modification of aminopeptidase I.

The assay of aminopeptidase activity [1] and the separation of its main molecular forms by sucrose density gradient centrifugation [2] have been described earlier. Amino acid analyses were performed by standard procedures on a Beckman Multichrom C liquid chromatograph.

Immunological methods: Antisera against 12 S and 22 S were raised in rabbits by repeated subcutaneous injection of 25 – 30 μg aminopeptidase antigen per kg body weight. Injections were administered weekly; the first three contained the antigen in an emulsion with Freund's complete adjuvant, in further injections incomplete adjuvant was used. Although rather low amounts of antigen were given to avoid the inadvertent elicitation of antibodies against trace impurities, precipitating antibodies directed against aminopeptidase appeared as early as three weeks after the first injection.

Ouchterlony double-diffusion experiments and quantitation of antigen amounts by micro complement fixation were performed according to published procedures [6].

Results

Separation of aminopeptidase forms

In a previous paper [2] the separation of 12 S and 22 S aminopeptidase forms by gradient centrifugation was described in detail. For analytical purposes, electrophoresis on 7.5% polyacrylamide gels is equally useful (Fig. 1). While the active enzyme migrates as an apparently single band, the 12 S form is split up in several peaks. Usually, three main bands were seen; occasionally, as in Fig. 1, up to 7 bands could be discerned. All of these species are glycoproteins as demonstrated by staining the gels for carbohydrate [7].

Partial separation of aminopeptidase hexamers and dodecamers was also achieved by ionic exchange chromatography on DEAE-cellulose columns. Aminopeptidase activity was eluted within 5 – 7 neighbouring peaks; all of them contained both 12 S and 22 S material. Analysis of pools A – C (see Fig. 2) on density gradients showed that the fractions eluted first held much more 12 S protein than those appearing at higher ionic strength.

Interconvertibility

As reported previously [2] spontaneous interconversion takes place during storage of isolated aminopeptidase forms. More detailed studies on this point, however, indicated that, under non-denaturing conditions, the extent of interconversion is rather limited. In all cases only trace amounts of 12 S material were formed from active 22 S enzyme even after prolonged standing at 4 °C. On the other hand, after recentrifugation of 12 S preparations, 22 S enzyme was regularly encountered in amounts
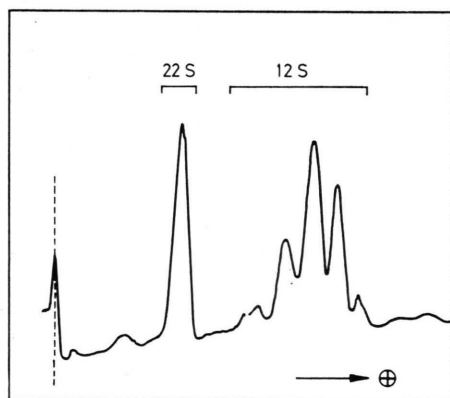

Fig. 1. Separation of aminopeptidase I forms by polyacrylamide gel electrophoresis. An enzyme preparation consisting mainly of 12 S protein was analyzed on a 7.5% polyacrylamide gel at pH 8.9. A densitometric scan obtained after staining with Coomassie Blue is shown. The direction of migration was from the left to the right. Peaks corresponding to 22 S and 12 S protein are marked by bars.

Fig. 2. Fractionation of aminopeptidase I on DEAE-cellulose. 90 mg purified aminopeptidase I were applied to a 2×25 cm column of DEAE-cellulose equilibrated with 10 mM Tris-succinate buffer/pH 7.3. The enzyme was eluted with a linear gradient of 0 – 250 mM NaCl. The protein distribution (———) and activities against leucine-*p*-nitroanilide (—○—) are shown (bottom). Pools A – C were then analyzed by density gradient centrifugation. Relative amounts of 12 S and 22 S protein in each pool are indicated by columns (top).

a)   b)

Fig. 3. Reaction of antisera with various aminopeptidase I preparations. Immuno-precipitates (they are stained with Coomassie Blue) were obtained by double-diffusion of antisera (A 12, A 22) against antigens (12, 22, EC) in 1.5% agar/pH 7.3. For details see text.

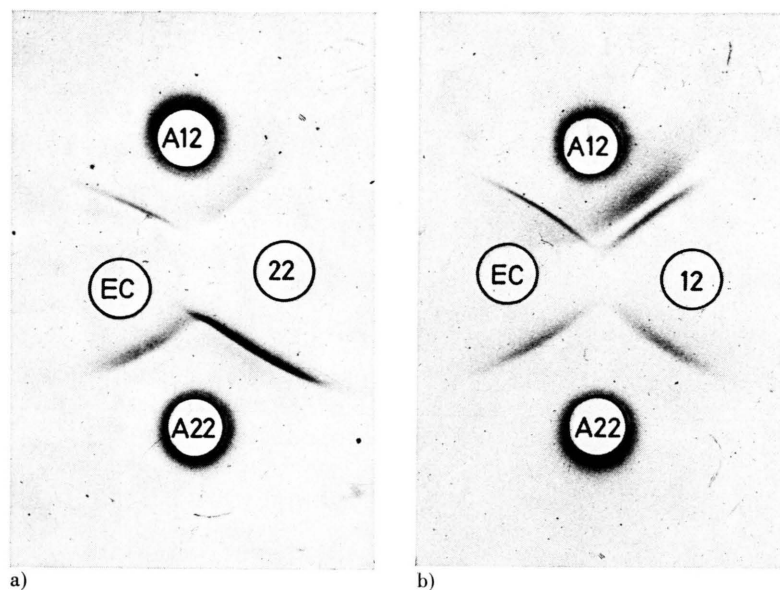of up to 15% of total protein (see Fig. 3). Zn^{2+} and Cl^- ions, both effective activators of aminopeptidase I, did not shift the ratio between 12 S and 22 S material. EDTA and other metal chelating agents were without effect, too.

Amino acid composition

Since earlier amino acid analyses were performed with a mixture of dodecamers and hexamers we reinvestigated the amino acid composition of isolated aminopeptidase forms. The results are summarized in Table I.

Although a close similarity between both sets of data is obvious, the contents of some amino acids (Glu/Gln, His) differ to an extent that exceeds experimental error. We, therefore, analyzed the data by the method of Cornish-Bowden [8] which allows an estimation of sequence homologies between protein chains solely from their amino acid composition. The degree of homology is obtained in quantitative terms by calculating $S \Delta n$, a parameter defined by $S \Delta n = \frac{1}{2} \Sigma (n_{ia} - n_{ib})^2$, where n_{ia} and n_{ib} are the frequencies of amino acid i in the sequences A and B, respectively, which both are assumed to contain a total of N residues. If there is no relationship between the primary structures of A and B beyond that caused by chance, $S \Delta n$ will amount to 0.42 N. With increasing sequence homology $S \Delta n$ decreases, reaching zero with identical chains. The data of Table I yield $S \Delta n = 0.10$ N for the comparison of

Table I. Amino acid analyses of isolated 12 S and 22 S aminopeptidase forms. If not specified otherwise the data are means ± S. D. of three analyses, performed with samples hydrolyzed for 15, 20 and 25 h (6 N HCl, 110 °C). When the yield of an amino acid strongly varied with the duration of hydrolysis, its amount was estimated by extrapolation to zero hydrolysis time. Values of $(n_{ia} - n_{ib})^2$ are used for the calculation of $S \Delta n$ (see text).

Amino acid	Residues per subunit		$(n_{ia} - n_{ib})^2$
	22 S	12 S	
Asp	48.9 ± 6.6	51.2 ± 0.7	5.29
Thr	30 [a]	32 [a]	4
Ser	31.6 ± 1.1	31.3 ± 0.4	0.09
Glu	36.8 ± 1.2	42.7 ± 0.4	34.8
Pro	19.1 ± 0.2	19.9 ± 0.6	0.64
Gly	44.0 ± 2.3	41.3 [b]	7.29
Ala	28.9 ± 0.8	30.5 ± 0.8	2.56
Val	26 [c]	26 [c]	—
Ile	21.3 ± 0.2	19.2 ± 0.1	4.41
Leu	40.8 ± 0.08	40.0 ± 0.7	0.64
Tyr	21 [a]	20 [a]	1
Phe	23.1 ± 0.5	21.1 ± 0.7	4.0
His	10.6 ± 0.1	7.0 ± 0.1	12.96
Lys	30.2 ± 0.9	29.2 ± 0.8	1.0
Arg	16.7 ± 0.5	15.5 ± 0.3	1.44
Trp	2.4 ± 0.2 [d]	3.1 ± 1.4 [d]	0.49
Cys	(5) [e]		
Met	(6) [e]		
	Sum: 442	Sum: 441	Sum: 80.6
			$S \Delta n = 0.10$ N

[a] Extrapolated to zero hydrolysis time.

[b] Glycine yields strongly increased with hydrolysis time, the value after a 25 h hydrolysis is shown.

[c] Data were calculated assuming 26 valine residues per subunit.

[d] Determined after a 15 h hydrolysis in the presence of 0.5% tryptamine, mean of two determinations.

[e] Data from ref. [1], not used for estimation of $S \Delta n$.

aminopeptidase I dodecamers and hexamers. This value is by far lower than is expected for non-related polypeptides, especially, if one takes into account, that the experimental error alone (estimated by comparing different analyses of the same protein sample) led to $S \Delta n$ values of $0.03 - 0.05$. In agreement with the theory, the comparison of 22 S aminopeptidase with 5 randomly selected yeast proteins led to $S \Delta n = 0.42 \pm 0.15$. Thus, statistically, more than 90% of the primary structures of 12 S and 22 S aminopeptidase are identical.

Immunological properties

Frequently, valuable information on a structural relationship between proteins is contributed by the investigation of their antigenic properties. The reaction of antisera raised against 12 S and 22 S aminopeptidase preparations with various antigens is illustrated by the double-diffusion experiments shown in Fig. 3. The wells at the top and bottom of each

Fig. 4. Reaction of aminopeptidase forms with anti-22 S-serum as detected by complement fixation. Isolated 12 S protein was recentrifuged in a density gradient (direction of sedimentation from the right to the left). The resulting fractions were analyzed for protein (———) and aminopeptidase activity against leucine-*p*-nitroanilide ($- \times -$). In addition, the ability to fix complement after incubation with an antiserum against 22 S aminopeptidase was tested with 5 μl-samples from each tube. Under the conditions chosen, the percentage of complement fixation ($- \bullet -$) and the amount of aminopeptidase antigen were related in a roughly linear fashion. Between fractions 18 and 26 the fixation values were slightly negative. Therefore, in this range the abscissa was displaced to the 10% level.

plate were filled with antisera (A 12, A 22), the outer wells contained purified aminopeptidase forms (12, 22) or an aminopeptidase I preparation isolated from *Saccharomyces cerevisiae* NCYC 366 (EC).

Serum A 22 turned out to be monospecific with respect to 22 S aminopeptidase. A single precipitation line was formed during diffusion of A 22 against 22 S antigen (Fig. 3 a, lower right). The 22 S material inevitably present in 12 S aminopeptidase preparations was also detected by A 22 (Fig. 3 b, lower right). Serum A 12 formed a single precipitate with homogeneous 22 S antigen (Fig. 3 a, upper right) and a couple of lines with 12 S preparations (Fig. 3 b, upper right). In the latter case, the diffuse band located near the antiserum well corresponds to the A 12-12 S precipitate while the sharper line near the antigen well resulted from the interaction of A 12 with 22 S molecules (in each case precipitates contaning 22 S material are recognized by their fusion with the single precipitate of both A 12 and A 22 with antigen EC).

Thus, antigens 12 and 22 do not seem to cross-react with respect to serum A 22. The most probable explanation for the reaction of A 12 with both 12 S and 22 S aminopeptidase is, to assume that A 12 contains two different types of antibodies reacting independently with either antigen. This was experimentally verified as follows: Samples of serum A 12 were mixed with different amounts of homogeneous 22 S antigen. After removal of precipitates by centrifugation, the resulting supernatant was allowed to diffuse against 12 S and 22 S aminopeptidase as usual. It was found that with increasing excess of 22 S antigen initially added, the serum lost its ability to form precipitates with 22 S protein in the subsequent double-diffusion, whereas its reaction with 12 S antigen was unaffected.

Since a weak cross-reactivity is easily overlooked in immunoprecipitation experiments we, in addition, compared the reaction of antigens 12 and 22 with A 22 by micro complement fixation, a technique much more sensitive in revealing structural homologies in proteins [9]. A 12 S aminopeptidase preparation was recentrifuged in a sucrose gradient and the resulting fractions were assayed for aminopeptidase activity, protein and their effectivity in complement fixation after incubation with serum A 22 (Fig. 4). While 22 S aminopeptidase (fractions $5 - 13$) was readily detected by its enzymic
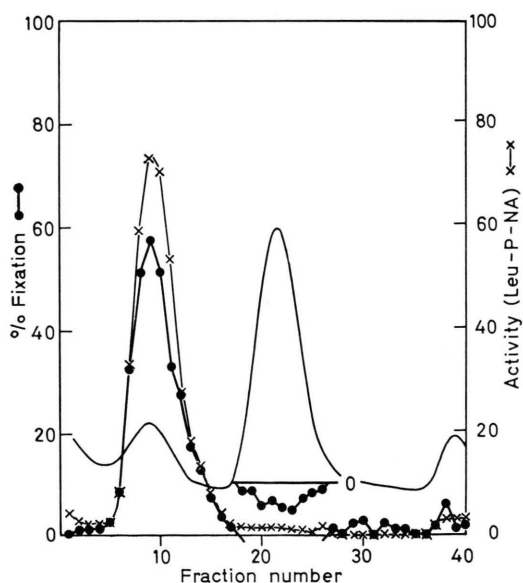
activity and a positive complement fixation reaction, the 12 S form, although present in much higher amounts (fractions 18 – 28), did not interact with A 22 as indicated by its failure to inhibit complement action. Thus, according to generally accepted immunological criteria, the hexamers bear only a remote, if any, structural resemblance to the active enzyme.

Aminopeptidase forms in other yeasts

The availability of antisera against the main aminopeptidase I species considerably simplified their detection in cell homogenates. Serum A 22 readily reacted with enzymes present in some strains of *Saccharomyces cerevisiae* kept in our laboratory. Fig. 3 shows that the precipitation lines formed by the 22 S enzyme from brewer's yeast (22) and aminopeptidase I from *S. cerevisiae* NCYC 366 (EC) are largely fused, indicating a considerable degree of structural relationship (Fig. 3 a, bottom). However, a small spur visible at the point of fusion, shows that slight differences in the antigenic properties of either protein exist. Surprisingly, as judged by immunodiffusion, aminopeptidase I purified from strain 366, did not contain 12 S forms (Figs 3 a and b, upper left). Likewise, by micro complement fixation hexamers were not detected in homogenates of *S. cerevisiae* S 288 C, although cross-reactive 22 S enzyme was present. The absence of 12 S material from strain 366 was confirmed by analysis of antigen EC in density gradients which failed to demonstrate the presence of a protein sedimenting at 12 S. On the other hand, an enzyme preparation isolated from brewer's yeast in the same way as EC (*i. e.* by homogenization in the presence of proteinase inhibitors and rapid separation of the enzyme from low-molecular weight proteins) contained the usual amount of 12 S forms. Thus, it appears that the occurrence of hexameric aminopeptidase I is not a regular property of yeasts.

Discussion

Our results show that the relationship between hexameric and dodecameric aminopeptidase I forms is much more complex than could be described by a simple association-dissociation equilibrium. Firstly, both forms are hardly interconvertible *in vitro*; they differ with respect to their chemical composition. Moreover, an unexpectedly low degree of immuno-logical cross-reactivity suggests that the conformation of 12 S aminopeptidase is drastically altered as compared to that of the active enzyme. Up to now, our data are insufficient to establish the process by which aminopeptidase hexamers are formed. Among others the following mechanisms have to be taken into account:

1. The hexamers may be preparation artifacts

The artifactual modification of yeast enzymes in the course of their purification has been repeatedly observed. In some cases limited proteolytic degradation was identified as the underlying process [10, 11]. In our large-scale purification from brewer's yeast the enzyme is liberated by autolysis, proceeding for 3 – 4 days at room temperature [1]. Therefore, the risk of hydrolytic modification of the enzyme is considerable. On the other hand, as a vacuolar enzyme, aminopeptidase I is rather insensitive to proteolytic attack [12]. Moreover, when cells were broken with glass beads instead of autolysis, 12 S material was already present as demonstrated by immunodiffusion. Thus, experimental artifacts seem to play a minor role, if any, in the formation of aminopeptidase hexamers.

2. The hexamers may be precursors or physiological degradation products of the active enzyme

This view implies that the 12 S protein is a regular cell constituent, either to be modified in such a way that active aminopeptidase I can form or, alternatively, derived from its partial degradation. It would be uncommon, however, to find that an enzyme precursor or an intermediate of its breakdown accumulate in amounts comparable to those of the enzyme itself. Moreover, the absence of 12 S material from strains NCYC 366 and S 288 C indicates that the hexamers are not indispensable intermediates of aminopeptidase turnover. Possibly, measurable amounts of 12 S protein are formed only under the rather special conditions to which brewer's yeast is exposed during the brewing process.

3. The hexamers may be the result of "unproductive" aggregation of aminopeptidase subunits

This assumption is suggested by the protein patterns displayed by aminopeptidase dodecamers and hexamers upon polyacrylamide gel electrophoresis. As shown in Fig. 1 the bands attributable to the

12 S fraction, are distributed rather symmetrically with highest amounts in a central peak. Such a pattern will be generated if hexameric complexes are built up from two types of subunits of equal size but with different chemical composition and thus different net charge. If they combine at random, seven electrophoretically discernible species are formed; their relative amounts will be 1 : 6 : 15 : 20 : 15 : 6 : 1 * and the resulting electrophoretic pattern, therefore, not unlike that given by 12 S aminopeptidase. If one assumes, in addition, that only certain types of hexamers are able to form dodecamers by pairwise aggregation, the final products of the aggregation process will consist of a relatively homogeneous group of dodecamers and a still heterogeneous population of hexamers. Moreover, since pools of hexamers and dodecamers contain the subunits in different proportions, their overall chemical compositions will also not be the same.

This explanation for the molecular heterogeneity of aminopeptidase I is not an entirely speculative one. As shown by Zuber and his coworkers [13]

aminopeptidase I from *Bacillus stearothermophilus*, an enzyme resembling yeast aminopeptidase I in many ways, occurs in various molecular forms that are composed of two types of monomers (α and β) in different ratios. In the cell a dodecamer with the composition $\alpha_8 \beta_4$ is the predominant species, in addition $\alpha_{10} \beta_2$ and $\alpha_6 \beta_6$ were found. The interconversion of these hybrids at neutral pH proved to be extremely difficult. Interestingly, under *in-vitro* conditions only α-subunits recombined, yielding active α_{12} enzyme. Hexameric forms were not described.

In view of these findings, it is tempting to speculate that the aminopeptidase hexamers of brewer's yeast are the result of "unproductive" aggregation processes, prematurely terminated at the hexameric level. Such events should be rare in normally growing cells but could be strongly favoured under particular non-physiological conditions. In order to substantiate such a hypothesis it would be necessary to demonstrate the presence of at least two forms of subunits with different net charge in the yeast enzyme. To the present date, however, experiments with this objective remained unsuccessful.

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* Estimated from the probability that a complex containing n subunits of one type will form ($n = 0, 1 \ldots 6$) from a large pool containing equal amounts of each monomer. The amounts of hexamers with $n = 0$ and $n = 6$ were set $= 1$.

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