The Quaternary Structure of Yeast Aminopeptidase I

1. Molecular Forms and Subunit Size

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Aminopeptidase, Yeast, Structure, Subunit Composition

The smallest active form of aminopeptidase I (EC 3.4.11.1) from yeast has a molecular weight of 6.4×10^5. At neutral pH the active enzyme is in equilibrium with two inactive subfragments ($M_r=3.2\times10^5$ and 1.1×10^5) as well as with higher aggregates ($M_r \geq 1.2\times10^6$). All of these species may be dissociated to give a single type of subunits with a molecular weight of 5.3×10^4. It is concluded that the active enzyme is a dodecamer whereas the subfragments correspond to dimeric and hexameric forms.

Introduction

Aminopeptidases (L-aminoacyl L-peptide hydrolases EC 3.4.11.1) catalyze the stepwise removal of single amino acid residues from the NH_2-terminus of peptide chains. Aminopeptidases from a variety of sources have been isolated; most of them where shown to be zinc metalloenzymes (*cf* refs 1–4).

In yeast at least 4 different aminopeptidases are found; their relative amounts vary within wide limits depending on the state of growth of the cells and on the composition of the culture medium [5, 6]. One of these enzymes which is not encountered in significant quantities before the yeast culture approaches the stationary state has been purified and characterized in our laboratory [7]. Aminopeptidase I – as we have named it – seems to be identical with the 'aminopeptidase III' of Matile *et al.* [5] and with an aminopeptidase described by Johnson in 1941 [8]. Our enzyme may be clearly distinguished, however, from the homonymous aminopeptidase I isolated from yeast autolysates by Masuda *et al.* [9] by its uncommonly high molecular weight which by far exceeds the molecular weights of the other yeast peptidases. This feature and the marked molecular polymorphism observed at physiological pH [7] have prompted further studies on the quaternary structure of aminopeptidase I.

The present report deals mainly with experiments on the size and subunit composition of the enzyme. In an accompanying paper [10] the results of electron microscopic studies with aminopeptidase preparations are presented. Based on the evidence provided by either approach a model for the symmetry of the aminopeptidase molecule and the mode of assembly of its subfragments is proposed.

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Materials

Aminopeptidase I from brewer's yeast was purified as described previously [7]. In contrast to former preparations 1×10^{-5} M EDTA was added to all buffers during the isolation and storage of the enzyme. This prevents autolysis of enriched aminopeptidase preparations thus improving yields. The enzyme samples applied in the sedimentation experiments had specific activities between 400 and 800 U/mg (measured with 20 mM Leu-Gly-Gly as described below). They were derived from the pooled active fractions of Sepharose 6B eluates concentrated to 3–15 mg protein/ml by ultrafiltration and stored frozen until use.

If not specified otherwise, the chemicals used were products of Merck, Darmstadt – in general of 'p.a.'-grade. Dimethyl suberimidate·HCl and dansyl chloride were purchased from Serva, Heidelberg. Cyanogen bromide was a product of Fluka, Buchs. The proteins applied as molecular weight standards were supplied by Boehringer Mannheim (β-galactosidase from *E. coli*, phosphorylase A from rabbit muscle and alkaline phosphatase from calf intestine) or by Serva, Heidelberg (bovine catalase, bovine serum albumin and lysozyme). L-asparaginase from *E. coli* was a gift of Bayer, Wuppertal.

Methods

Routinely, aminopeptidase activities were determined spectrophotometrically at 40 °C in 0.1 M phosphate/borate buffer, pH 7.8, containing 100 mM

Cl^- and 50 μM Zn^{2+}. In most cases 10 mM or 20 mM L-Leu-Gly-Gly was used as substrate the hydrolysis of which was followed at 235 nm. Occasionally, leucine-*p*-nitroanilide in a concentration of 2 mM was employed, the splitting of this substrate was measured at 405 nm.

Aminopeptidase concentrations were determined spectrophotometrically based on an $A_{280}^{0.1\%}=0.85$. Amino acid analyses and sodium dodecyl sulfate gel electrophoreses were carried out as described elsewhere [7].

Crosslinking of the enzyme with dimethylsuberimidate was achieved by incubation of enzyme with reagent (1 mg/ml each) in triethanol amine buffer, pH 8.5 for 16 h at room temperature. The resulting solution was then incubated with 0.1% SDS for another 3 h at 40 °C and subjected to SDS gel electrophoresis as usual.

Cyanogen bromide peptides were prepared by stirring enzyme (5 mg in 2.5 ml of 0.1 N HCl) with a 100-fold excess (with respect to Met) of CNBr for 20 h at room temperature. After lyophilization the reaction products were separated by gel filtration on a 0.9×100 cm column of Sephadex G 25 fine, equilibrated with 1 N acetic acid. The material appearing with the void volume of the column was further analyzed by SDS gel electrophoresis, smaller peptides eluted in later fractions were detected by the ninhydrin reaction and their ultraviolet absorption.

Performic acid oxidations were carried out by incubating enzyme with 3% H_2O_2 in 70% formic acid for 1 – 2 h at 4 °C, followed by lyophilization.

Dansylation of the enzyme was performed as described by Gros *et al.* [11]. After hydrolysis with 6 N HCl (4 – 6 h at 110 °C) the resulting dansyl amino acids were identified by two-dimensional thin layer chromatography on silica gel G [solvents: a) benzene : pyridine : acetic acid, 80 : 20 : 5; b) toluene : 2-chlorethanol : 25% ammonia, 6 : 10 : 4].

Succinyl aminopeptidase was prepared by addition of several small volumes of 0.2 M succinic acid anhydride in acetone to an enzyme solution at pH 8.5 in 30-min intervals. After standing overnight the succinyl derivative was purified by chromatography on Sephadex G 75.

Most of the sedimentation experiments were performed by the sucrose density gradient technique [12]. Linear sucrose gradients (4.5 ml, 5 – 20% sucrose in 0.1 M buffers) were prepared with a LKB Ultrograd gradient mixer and stored for at least 3 h in the cold room before application of the samples (50 – 100 μl per gradient). Usually the gradients were run for 15 – 17 h at 36 000 rpm and 4 °C in a Beckman SW 65 swinging bucked rotor.

Fractionation of the gradients was carried out as follows: The tubes were inserted into a block of clear plastic which could be tightly closed by a screw cap bearing two bores. Through one of these – located above the center of the tubes – a syringe needle connected to 0.4 mm teflon tubing was carefully lowered to the bottom of the tube, through the other bore water was pumped onto the top of the gradient at a rate of about 10 ml/h thus gradually forcing the contents from the bottom of the tube upwards into the central needle. A Labotron LDP-11 precision piston pump was used for this purpose. In this way a constant pulse-free flow and thereby a highly reproducible fractionation of the gradients could be maintained.

The gradient solution was first directed through a micro flow-cell (5 mm path length, about 10 μl of volume) adapted to a Zeiss PMQ II spectrophotometer and then to a Gilson Microcol fraction collector where about 50 fractions (0.1 ml each) were obtained from a tube. The distribution of protein within the gradients was derived from the A_{280} values recorded during the fractionation of the gradients.

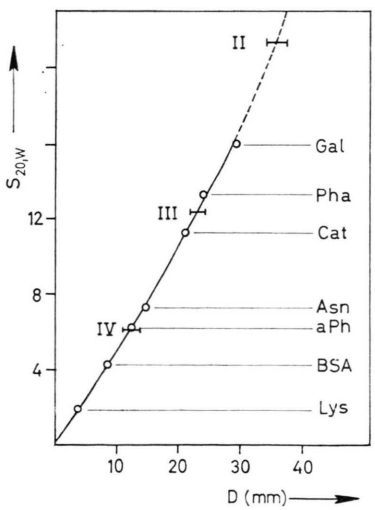

Fig. 1. Standard curve for the estimation of sedimentation coefficients from sucrose density gradient centrifugations. The gradients were run in a SW 65 rotor (k-factor=46 at maximal speed) for 15 h at 36000 rpm and 4 °C. The distance D migrated by the protein bands under these conditions are plotted vs. the respective sedimentation constants. The reference proteins and their $S_{20,w}$-values were: Gal-β-galactosidase from *E. coli* −16.0S; Pha-phosphorylase A from rabbit muscle −13.4S; Cat-catalyse −11.3S; Asn-L-asparaginase from *E. coli* −7.6S; aPh-alkaline phosphatase from calf intestine −6.1S; BSA-bovine serum albumin −4.2S and Lys-lysozyme −2.0S. Roman numerals indicate the positions of aminopeptidase I species (cf. Fig. 2).

In addition, after appropriate dilution the enzyme activities and ultraviolet absorption of the collected fractions were measured. Sedimentation coefficients were estimated by comparison of the distances between meniscus and peak maxima with standard curves constructed by running a series of proteins with known $S_{20,w}$-values under identical conditions (see Fig. 1). The variation coefficients of the sedimentation constants determined in this way were better than 0.05.

Some additional sedimentation experiments were performed in a Beckman model E analytical ultracentrifuge equipped with a photoelectric scanner system. Sedimentation coefficients for the main aminopeptidase species were determined by band centrifugation according to Vinograd *et al.*[13]. The sedimentation equilibrium technique of Yphantis[14] was used to estimate the molecular weight of the 12.5S aminopeptidase subfragment. (Registration after a 38 h-run at 3600 rpm at 20 °C in Beckman double-sector cells.)

Results

Molecular heterogeneity

As described previously[7], even with highly purified aminopeptidase preparations the existence of several protein species may be demonstrated by chromatography on Sepharose columns. By sucrose density gradient centrifugation these species may be completely separated. This is shown in Fig. 2 which contains protein patterns and the distribution of aminopeptidase activities in density gradients run at pH 6 and pH 10, respectively. Clearly, at pH 6 aminopeptidase activity is almost exclusively associated with peak II which has a sedimentation coefficient of about 22S (*cf* Fig. 1). Another aminopeptidase species, quantitatively less important but with similar specific activity sediments with 30 – 35S (peak I). Under appropriate conditions the existence of active material with still higher sedimentation coefficients may be demonstrated. In addition, a peak lacking aminopeptidase activity (peak III, sedimentation constant about 12S) and some material sedimenting with about 6S (peak IV) are encountered. At pH 10 one more active peak is found which is completely absent at lower pH and sediments with about 17S (peak IIa Fig. 2 b).

Employing the fractionation procedure described above we were able to isolate the main peaks from density gradients in a pure state. Their comparison strongly suggests that all of them, regardless whether active or not, originate from only one protein chain.

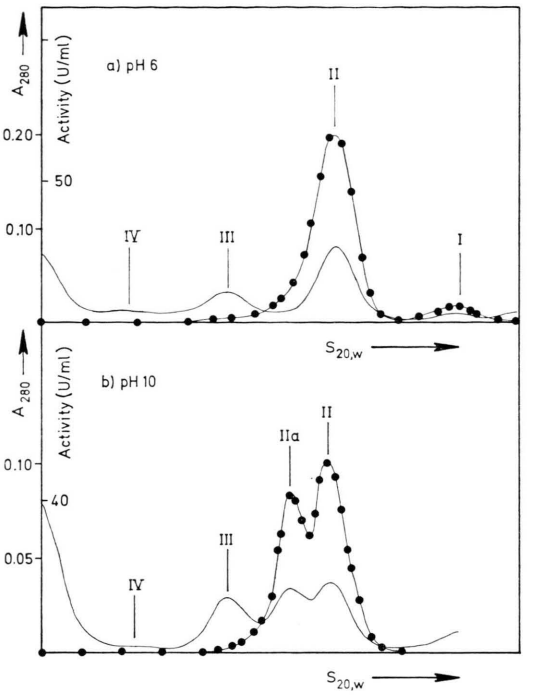

Fig. 2. Separation of aminopeptidase forms by density gradient centrifugation. Centrifugations were performed and evaluated as described in section 'methods'. a) 0.16 mg of aminopeptidase were separated in a gradient buffered with 0.1 M borate/succinate buffer, pH = 6.0. The absorption at 280 nm (measured with a 5 mm cell, solid line) and the activity of the collected fractions (filled circles, measured with 10 mM Leu-Gly-Gly under standard conditions) are shown. b) The same experiment was carried out using a gradient made up with 0.1 M glycine/NaOH buffer, pH = 10.0. The enzyme sample had been preincubated with this buffer for 2 days in the cold room. Again the protein distribution (solid line) and activities against Leu-Gly-Gly (circles) are given.

1. The protein patterns obtained in SDS gel electrophoresis with purified peak I – III proteins are identical (see Fig. 3).
2. All of these species are interconvertible. So at neutral pH purified peak II protein slowly forms peaks III and IV on standing. Similarily, peaks II and IV are formed from peak III material after some time (see Fig. 4). At 4 °C these processes have half-times of a few days.
3. The amino acid compositions of peaks I, II and III are very similar to each other.

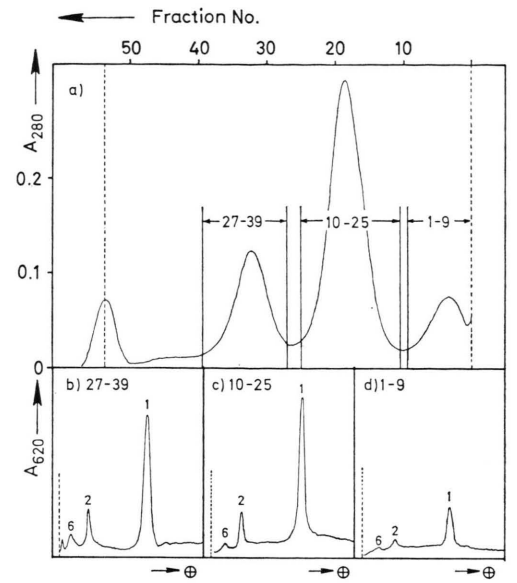

Fig. 3. Behaviour of isolated aminopeptidase forms in SDS gel electrophoresis. Native enzyme (0.75 mg) was run in a gradient buffered with 0.1 M ammonium acetate, pH=6.2. As indicated in Fig. 3 a fractions 1–9 (corresponding to peak I, cf. Fig. 2) fractions 10–25 (peak II) and 27–39, respectively (peak III) were pooled. Samples of these pools were subjected to SDS gel electrophoresis (5% gels containing 0.1% SDS). Densitometric scans of the stained gels are shown in Fig. 4 b–d. The dashed lines mark the top of the gels, the direction of migration was towards the anode. The molecular weights of peaks 1–6 as estimated from standard curves were: 5.5×10^4 (peak 1), 1.1×10^5 (peak 2) and 2.5×10^5 (peak 6).

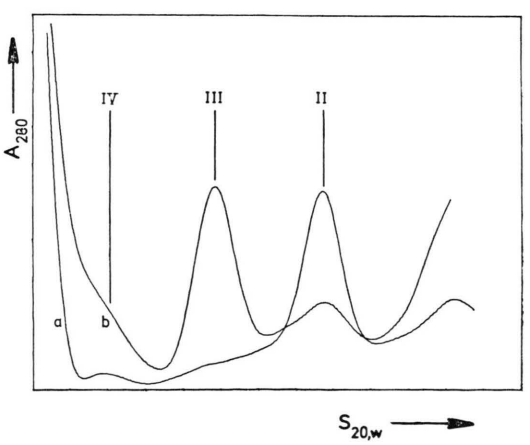

Fig. 4. Spontaneous interconversion of aminopeptidase forms. Samples of pools 10–25 and 27–39 (*cf.* Fig. 3 a) were recentrifuged after concentration and subsequent storage in the cold room for two days. Protein patterns observed with peak II protein (pool 10–25, curve a) and peak III protein (27–39, curve b) are shown.

Molecular weights

The sedimentation coefficients of peak II and peak III proteins were determined in the analytical ultracentrifuge. The values found – $S_{20,w}=21.8$S for peak II and $S_{2,0w}=12.5$S for peak III – are in good agreement with the estimates from the density gradient experiments (see Fig. 1) and also with data of Johnson [8] who reported a sedimentation coefficient of 21.7S and a diffusion constant of 3.07×10^{-7} cm^2 sec^{-1} for the active form of the enzyme. With a partial specific volume of 0.725 cm^3/g [7] the Svedberg equation yields a molecular weight of 640 000 for the active peak II aminopeptidase.

The molecular weight of the 12.5S species was determined by the sedimentation equilibrium technique. Unfortunately, it is impossible to keep peak III material in a strictly homogeneous form for the time necessary to attain equilibrium since, as just mentioned, other forms of the enzyme appear spontaneously. In a sedimentation velocity experiment the peak III preparation used for the equilibrium run was found to contain about 12% of 22S protein and some 6S material. Thus it is not surprising that the M_w-values * found – $335\,000\pm27\,000$ and $337\,000\pm26\,000$ – are characterized by a rather high standard deviation. If one assumes the 22S material to be the only impurity present one can estimate a corrected molecular weight of about 320 000 for the 12.5S aminopeptidase fragment.

Subunit size

In sodium dodecylsulfate (SDS) gel electrophoresis the bulk of the enzyme protein migrates within a single band corresponding to a molecular weight of $53\,000\pm2000$ (mean value from experiments with five different enzyme preparations). In addition, minor bands with higher molecular weights are frequently observed (see Fig. 3 b, d). One of these (band 2) has a molecular weight twice as high as the molecular weight of the main band. Performic acid oxidation of the enzyme does not change its behaviour in SDS electrophoresis.

Extensive succinylation of aminopeptidase converts the enzyme almost quantitatively to a form which has a molecular weight well below 100 000 and is eluted as a single peak from Sephadex G 75 columns. Succinyl aminopeptidase sediments with

* $M_w=\sum c_i M_i/\sum c_i$; mean value based on weight.

about 2.5S and migrates as a sharp band with $M_r = 53\,000$ in SDS gels. A similar dissociation may be achieved by lowering the pH (see below) and by addition of 1 – 2 M guanidine·HCl or of 5 – 7 M urea. Finally, extensive desintegration of the enzyme accompanies lyophilization of dilute aminopeptidase solutions.

In all of these cases particles with sedimentation constants of 2 – 3 are formed. If the 3S fraction isolated from gradients run in the presence of guanidine is freed from the denaturing agent by dialysis and concentrated by ultrafiltration, a considerable fraction of the initial aminopeptidase activity is recovered. Thus, spontaneous reaggregation of the enzyme seems to occur under favourable conditions.

The bifunctional reagent dimethyl suberimidate is known to crosslink protein subunits via ε-amino groups of lysine residues [15]. If native aminopeptidase is modified in this way and then analyzed by SDS gel electrophoresis at least 6 peaks are found. As is seen from Fig. 5 all species encountered have molecular weights close to whole integers of 53 000. Besides the protomer (peak 1, see Fig. 5 a) peak 6 with a molecular weight of about 300 000 is most prominent.

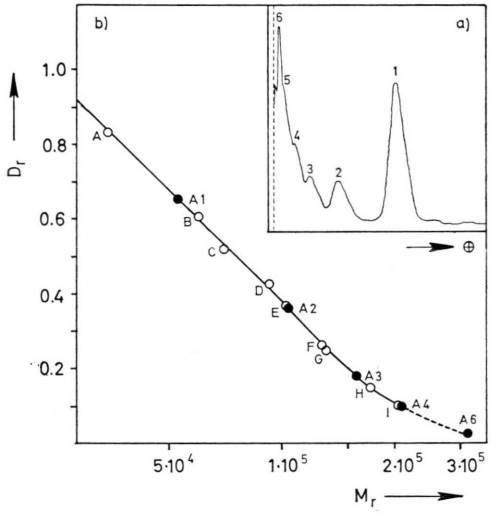

Fig. 5. SDS gel electrophoresis of products formed by crosslinking of native aminopeptidase with dimethyl suberimidate. The enzyme was reacted with dimethyl suberimidate as described in 'methods'. a) Densitometric scan of a gel showing the distribution of protein. The dashed line indicates the top of the gel; migration was from the left to the right. b) The relative mobilities D_r (distances migrated relative to the mobility of the marker dye bromophenol blue) of aminopeptidase species 1 – 6 (●, *cf.* Fig. 5 a) are compared with a standard curve constructed from the mobilities of a series of reference proteins run in the same experiment. Besides subunits of untreated proteins oligomers of the L-asparaginase subunit, obtained by dimethylsuberimidate treatment of native asparaginase were employed as molecular weight standards. The reference proteins (○) and the corresponding molecular weights are: A) L-asparaginase subunit $(Asn)_1$ — 35000, B) bovine catalase — 58000, C) $(Asn)_2$ — 70000, D) phosphorylase A — 94000, E) $(Asn)_3$ — 105000, F) β-galactosidase — 130000, G) $(Asn)_4$ — 140000, H) $(Asn)_5$ — 175000, I) $(Asn)_6$ — 210000.

Taken together these findings suggest that all forms of yeast aminopeptidase are made up from a single type of subunits with a molecular weight of 53 000. On the other hand, as reported previously [7] multiple active peaks with identical specific activities are observed after DEAE-cellulose chromatography and also in isoelectric focusing. As a reason for such a polymorphism the existence of at least two different classes of subunits with different net charges has to be taken into account. If this was the case protein species divergent with respect to their ion exchange properties should also differ in their amino acid composition. No significant differences were found, however, in amino acid analyses performed with samples which were taken at equal intervals from the active fractions of a DEAE-cellulose eluate. In particular, the difference between the sum of acidic amino acid (Asn and Gln counted as Asp and Glu) and the sum of the basic residues was fairly constant amounting to 42 ± 1.5 on the average.

Another method which may reveal the existence of subunits with different primary structures is the determination of end groups. We failed, however, in detecting an aminoterminal residue by dansylation. The only derivative appearing in the expected amounts was ε-dansyl lysine. Attempts to identify the COOH-terminus with carboxypeptidase A were also unsuccessful.

Cyanogen bromide cleavage of yeast aminopeptidase yielded 6 – 7 peptides (the unreacted protomer not included). One fragment with a molecular weight of about 27 000 and 2 – 3 peptides with molecular weights between 5000 and 12 000 were identified by SDS gel electrophoresis. Three smaller peptides with molecular weights below 2000 were separated by chromatography on Sephadex G 25. Since the enzyme contains 6 mol of methionine per 53 000 g of protein [7] this result is consistent with the view that all subunits are identical or closely similar to each other with respect to their primary structure.

pH-dependence of the subunit composition

The sedimentation pattern shown in Fig. 2 a is subject to little change at any pH between 5.5 and 8.5, regardless whether Zn^{2+} and Cl^--ions – both potent activators of the enzyme – are present or not. Below and above this range of pH, however, pronounced alterations of the enzyme's quaternary structure are taking place. This is clearly reflected by the results of the experiment depicted in Fig. 6. Residual activities measured after storage of dilute enzyme solutions at various pH for different periods of time are shown. The marked decrease of activity, observed at both low and high pH cannot be due to an irreversible denaturation of the enzyme protein, since it is terminated after a few days and no further loss of activity occurs for a long time. From Fig. 6 b it is clear that Zn^{2+} and Cl^- have no effect at low pH, whereas these ions completely prevent the inactivation upon storage at high pH under the conditions of this experiment.

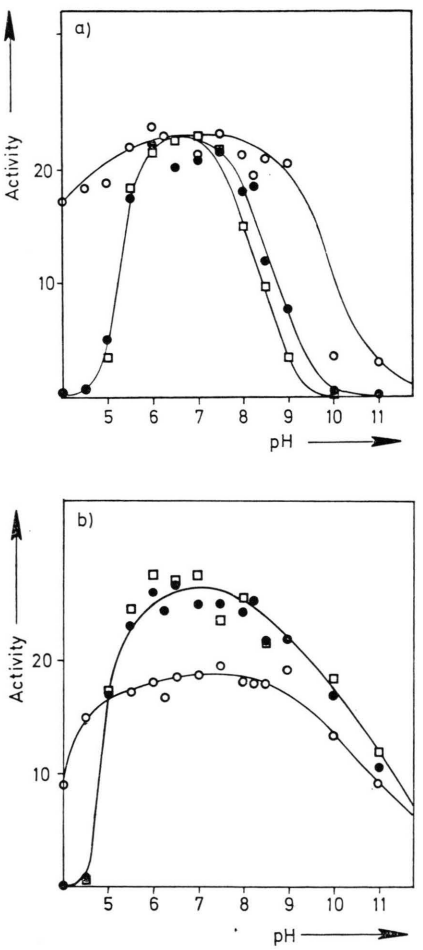

Fig. 6. Residual activities after prolonged storage of aminopeptidase at different pH. Samples of an aminopeptidase solution (0.9 mg/ml in 10 mM ammonium acetate buffer, pH=6.2) were diluted with buffers of different pH to a final concentration of 0.11 mg/ml. Activities against L-Leu-p-nitroanilide were determined under standard conditions 1.) after 30 min (○), 2.) after 1 week of standing at 4 °C (●) and 3.) after 5 weeks of standing in the cold room (□). Fig. 6 a shows residual activities determined in this way as a function of the pH of preincubation. The buffers used (all 0.1 M) were: sodium acetate, pH 4.0–4.5; succinate/borate, pH 5.0–5.5; phosphate/borate, pH 6.0–9.0; borate/NaOH, pH 10.0–11.0. The data of Fig. 6 b was obtained in the same way but with enzyme solutions containing 75 mM Br^- and 12.5 μM Zn^{2+}.

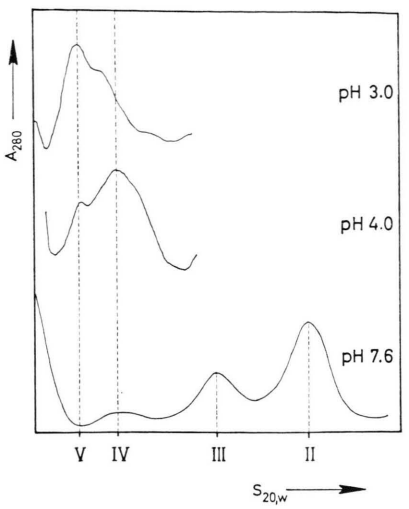

Fig. 7. Sedimentation behaviour of aminopeptidase at acidic pH. Density gradient centrifugations were performed and evaluated as described in 'methods'. The bottom trace shows the protein pattern of a gradient containing 0.1 M phosphate/borate buffer, pH=7.6 and run at 36 000 rpm for 16 h. The middle curve represents the protein distribution in a gradient buffered with 0.1 M sodium acetate buffer, pH=4.0. To achieve a better resolution this gradient was run for 38 h at 36 000 rpm; the data was then recalculated to the abscissa scale of the lower curve. The upper trace was obtained under the same conditions but with sodium acetate buffer, pH=3.0. Peaks II–IV are the same as shown in Figs 1 and 2. Peak V which does not appear above pH 4.5 has a sedimentation coefficient of about 3S.

In view of this observation, it is not surprising that the sedimentation patterns obtained at low and high pH, are quite different. At pH values below pH 5, the 22S and 12.5S peaks rapidly disappear (see Fig. 7). At pH 4.0 most of the protein sediments within a band at 6S, small amounts of which are already present at neutral pH (peak IV in Fig. 2 a). A shoulder in the 6S peak at about 3S becomes predominant if the pH is lowered still

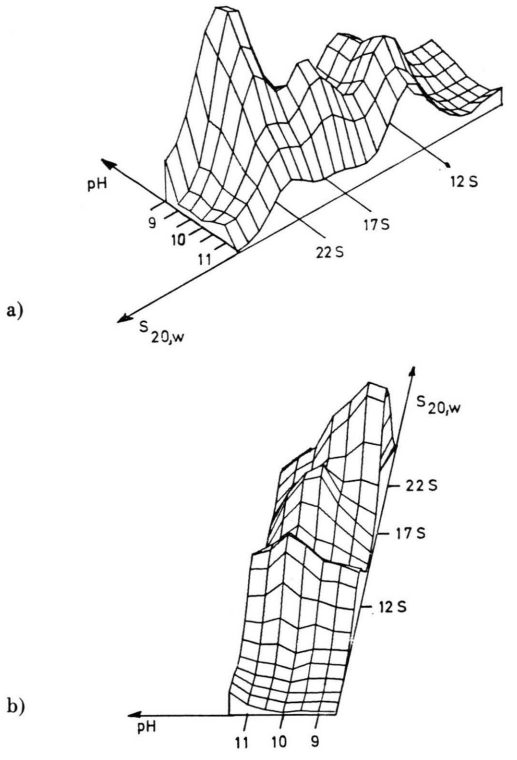

Fig. 8. Sedimentation behaviour of aminopeptidase I at alkaline pH. The figure summarizes the protein patterns measured with seven different density gradients run at various values of pH between 8.5 and 11.5. Equal amounts of enzyme were applied to each gradient after a 16 h-pre-incubation at the respective pH which was maintained by 0.1 M glycine/NaOH buffers. Three-dimensional representations, both derived from the same set of data but viewed from different directions were drawn by computer. For the sake of clarity, the range of sedimentation constants below 7S has been omitted. The notation of peaks is the same as in Fig. 2.

further. At pH 3.0 the bulk of the enzyme protein is found in the 3S fraction (peak V).

Above pH 8.5 a peak with a sedimentation coefficient of about 17S appears which is absent at lower pH. As is seen from Fig. 2 b its specific activity is the same as measured with the native 22S enzyme. Fig. 8 summarizes the changes in the quaternary structure of the enzyme taking place between pH 8.5 and 11.5. With increasing pH the native 22S species is gradually replaced by the 17S form, the relative amount of which increases up to pH 10. At still higher pH the 17S protein disappears too. The 12.5S aminopeptidase subfragments seem to be relatively alkali stable. It dissociates only after prolonged storage at high pH.

Discussion

The main features of the various molecular forms which yeast aminopeptidase I can take are compiled in Table I. From the experiments described in the present study it seems clear that all forms of the enzyme are assembled from a single type of subunits with a molecular weight of 53 000. Never smaller fragments are observed, not even upon performic acid oxidation. This rules out the existence of disulfide bonds within the protomer connecting different protein chains. In contrast to other multisubunit aminopeptidases [16, 17] complete dissociation of the yeast enzyme to give protomers is easily achieved by any of the usual denaturation methods (*i. e.* extreme pH, guanidine·HCl, urea, succinylation). The slightly different sedimentation coefficients (between 2 and 3S) of the resulting products may be explained by the varying degree to which the subunit tertiary structure is destroyed by the denaturant.

With other high-molecular weight aminopeptidases it was demonstrated that two different types of subunits of similar size but different primary structure are involved in the assembly of the native enzyme [16, 18]. The electrophoretic polymorphism of yest aminopeptidase I points to a similar phenomenon. From our experiments there is no evidence, however, suggesting that there are protomers with sequences so divergent to be detectable by amino acid analysis. Moreover, the number of CNBr-peptides does not give support to the assumption of grossly different subunits. Apart from the existence of genetically different protomers there is quite a variety of reasons which may give rise to electrophoretically polymorphous protein preparations. In the first place, artifacts originating from the purification procedure have to be considered. As reported previously [7] aminopeptidase I is a glycoprotein. Since in our large-scale isolation procedure the enzyme is liberated from the cells by autolysis, not only proteinases but also glycosidases present in the autolysate could effect minor changes in the structure of the enzyme. Likewise, molecules differing in their amide bond content (Asn, Gln) would be electrophoretically different but could not be distinguished by amino acid analysis. Finally, it cannot be excluded that the active enzyme can assume several conformational states with slightly different net charges, equilibrating so slowly that they

Table I. Molecular forms of yeast aminopeptidase I. Roman numerals correspond to the denotation of peaks observed in sedimentation experiments. Sedimentation coefficients $S_{20\,w}$, molecular weights (M_r) the presumed number of component subunits n and the conditions under which a given species occurs in highest amounts ('conditions') are given in the table.

Form	$S_{20,w}$ [S]	Molecular weight	Activity	n	Conditions
I	>30	$>10^6$	+	m·12 [a]	5 <pH<11
II	21.8	640 000±30 000 [b]	+	12	5 <pH< 8.5
IIa	17	?	+	?	9 <pH<12
III	12.5	320 000±20 000 [c]	−	6	4.5<pH<12
IV	6	~100 000 [d]	−	2	3.5<pH< 4.5
V	2−3	53 000±2000 [e]	−	1	pH< 3.5 denat. agents

[a] Aggregates of 22S particles. [b] Calculated from sedimentation velocity data. [c] Measured by equilibrium centrifugation. [d] Estimated from the sedimentation constant. [e] Determined by SDS gel electrophoresis.

appear as separate entities in electrophoretic or ion exchange experiments.

From the known molecular weights the numbers of 3S protomers from which the various aminopeptidase forms are made up may be derived quite unequivocally. Although we did not determine the molecular weight of the peak IV protein in a straightforward manner, there is little doubt, that this molecule corresponds to the dimeric form of the basic subunit with a molecular weight of 100 000 − 110 000 which is also found in SDS gels (see Fig. 3). This assumption is compatible with the sedimentation coefficient of peak IV since most proteins sedimenting with 6S have molecular weights close to 10^5.

From its molecular weight the 12.5S subfragment is identified as a hexameric structure. This is in accordance with results of the crosslinking experiment depicted in Fig. 5. Clearly, the hexamer accumulates in the reaction mixture; this indicates that this molecule is an outstanding intermediate in the assembly of subunits to the active enzyme form. Thus the active 22S molecule most probably is a dodecamer. This agrees excellently with the molecular weight as determined from its sedimentation velocity. Further support for this assignment will be provided by the electron microscopic evidence presented in the following paper [10].

The aminopeptidase activity associated with peak I and larger particles could be due to aggregates of the native peak II enzyme. As will be shown too in the accompanying report, in fact, such an aggregation may be observed with concentrated aminopeptidase solutions.

The nature of the 17S molecule which is formed from peak II protein at alkaline pH has not been established so far. Either a part of the native dodecamer is lost under these conditions or alternatively, the conformation of the molecule shifts in such a way that its hydrodynamic properties are markedly changed. The observation that the specific activity of the enzyme remains constant upon the 22 − 17S conversion contradicts the first explanation. We therefore tentatively conclude that a conformational change is responsible for the formation of the 17S band, possibly triggered by the loss of Zn^{2+} which was shown to stabilize aminopeptidase activity against heat inactivation [7] and moreover, specifically prevents the loss of activity at alkaline pH (*cf* Fig. 6 b).

Although complex subunit structures are not uncommon with aminopeptidases, yeast aminopeptidase I seems to be rather unique in that its substantial molecular forms are in equilibrium with each other at physiological pH. Moreover, it is quite rare that the smallest active species of an enzyme has a molecular weight of 600 000. So far we do not know how many active sites are operative in yeast aminopeptidase. Determinations of the amount of essential zinc indicate that there are only a few of them [7]. More detailed studies on this point are going on in our laboratory.

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