INTERCONVERSION OF THE DIFFERENT HYBRIDS OF AMINOPEPTIDASE I

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1. Introduction

The thermophilic aminopeptidase I from *Bacillus stearothermophilus* has an extraordinary structure. It is composed of two different subunit types [1], and both subunits seem to have aminopeptidase activity [2]. The two subunits (α,β) can combine in varying ratios, resulting in the hybrids $\alpha_6\beta_6$, $\alpha_8\beta_4$ and $\alpha_{10}\beta_2$ [1]. From this point of view the hybrids are isoenzymes. However, the substrate specificity of the two subunit types is different [2] and we have therefore to assume that the two active sites are also different. That means that the three hybrids of aminopeptidase I can equally well be called multienzyme complexes.

Up to now we have not been able to interconvert the different hybrids. It is possible to separate the different subunits, after denaturation of the apoenzyme, by SE-Sephadex chromatography in urea, and to reactivate the α subunits resulting in an α_{12} enzyme. In contrast to this, the β subunit could not be reactivated even in the presence of the α subunit [1].

In this report we show that it is possible to dissociate one hybrid form of the extremely stable aminopeptidase I under special conditions, avoiding denaturation in urea, and to reassociate the enzyme giving a new set of hybrid enzymes.

2. Methods

Aminopeptidase I was purified as described [3, 4].

The hydrolysis of leucine *p*-nitroanilide was followed at 405 nm to test the activity of the α subunit [2]. A 1 mM solution of leucine *p*-nitroanilide in 0.05 M imidazole buffer, pH 7.5, containing 1 mM cobalt(II)chloride was incubated with the enzyme at 65°C. All pH values were adjusted at room temperature. Glutamic acid 1-(4-nitroanilide) hydrolysis was followed to estimate the activity of the β chain [2]. A 0.25 mM solution of this substrate in 0.05 M Tris buffer, pH 7.5 and 1 mM cobalt(II)chloride was incubated with the enzyme at 65°C.

Sucrose density gradient centrifugation was done according to Martin and Ames [5] in a Spinco SW 39 rotor. A gradient of 5–25% sucrose was applied. The following buffers were used in the different gradient tubes at a concentration of 0.05 M: Sodium formiate, pH 4.0; imidazole-HCl, pH 7.2; Tris–HCl, pH 9.0, 0.2 ml of enzyme solution containing about 0.3 mg of protein were added on top of the gradients. Centrifugation was performed for 10 hr at 38 000 rpm. Fractions of 0.25 ml were collected into tubes already containing 1 ml of imidazole buffer, pH 7.0, and 0.5 mM cobalt(II)chloride for the reactivation of the apoenzyme. The enzyme was allowed to reassociate overnight. 5 ml of 0.05 M Tris buffer, pH 7.2, with 1 mM cobalt(II)chloride were added afterwards to dilute out the sucrose.

The different hybrids were separated by polyacrylamide gel electrophoresis according to Davis [6].

Metal free aminopeptidase I (apoenzyme) was prepared by dialysis of the enzyme against 2.5 mM EDTA, buffered with 10 mM acetate, pH 5.2.

3. Results and discussion

Aminopeptidase I is an extremely stable protein which remains fully active at 80°C [4]. The enzyme

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requires a metal ion (Zn^{2+} or Co^{2+}) for catalytic activity [7]. It has been shown that the removal of the metal atom from a metalloenzyme may affect the quaternary structure (e.g. [8, 9]). It seemed possible that a similar situation exists with aminopeptidase I since the apoform of this enzyme is rather unstable [10]. In addition the quaternary structure of an enzyme may be affected by changes in pH (e.g. [11–13]). We therefore investigated the sedimentation behaviour of the apoenzyme of aminopeptidase I at different pH values on sucrose density gradients. For comparison native aminopeptidase I was always added to one gradient tube. Fig. 1 shows a typical experiment. It is evident that the apoenzyme at pH 7.2 shows the same sedimentation behaviour as the native aminopeptidase. The same is true for the apoenzyme at pH 4.0 (not shown). However, the apoenzyme at pH 9.0 is obviously dissociated. The

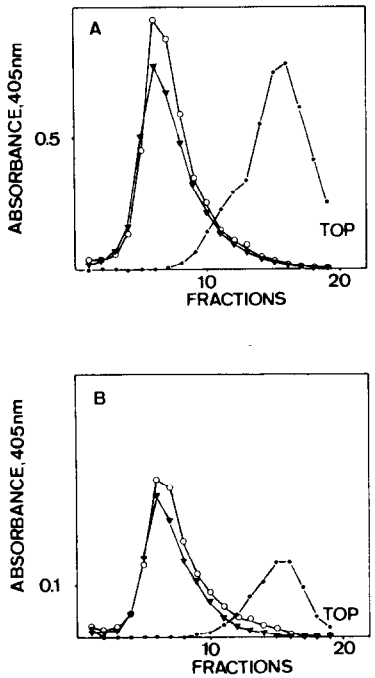

Fig. 1. Identical amounts of apoenzyme were layered on top of sucrose density gradients, containing 0.5 mM EDTA and different buffers (see methods). A reference run contained native aminopeptidase I and 0.2 mM cobalt(II)-chloride. (A) Shows the activity of the reactivated enzyme due to the α subunit; (B) shows the activity of the β subunit. (▲) Native enzyme, pH 7.2; (○) apoenzyme, pH 7.2; (●) apoenzyme, pH 9.0.

important point is that the dissociation under these conditions is reversible. The curves in fig. 1A represent activity due to the α chain after reactivation, and it is easy to see that the activity recovery from the pH 9.0 experiment is quite good compared to the pH 7.2 run. Table 1 shows the activity of the reactivated enzyme we obtained by integration of the different curves. The dissociation of the enzyme at pH 9.0 clearly does not affect the activity recovered in the case of the α chain. The situation is different in the case of the β chain. Fig. 1B shows the activity due to the β subunit. The recovery even under these conditions avoiding denaturation in urea is not very good. But nevertheless it is obvious that the reassociation of the β chains took place. The experiment shown in fig. 1 was done with the $\alpha_8\beta_4$ hybrid, which is by far the most abundant hybrid in the *B. stearothermophilus* cell [1]. Since this enzyme in the metal free form is dissociated at pH 9.0, we have to assume that after reactivation in buffer containing cobalt(II)-chloride a set of new hybrids would appear. In addition, since the recovery of the activity due to the β subunit was only 50% we have to expect that primarily β-poor species would be formed. That is exactly what we find. Fig. 2 shows the electrophoretic separation of the reassociated enzymes (disc I). Three main bands can be seen, representing the α_{12} enzyme [12], the $\alpha_{10}\beta_2$ [10, 2] and the $\alpha_8\beta_4$ [8, 4] hybrid. The $\alpha_6\beta_6$ hybrid is hardly visible. On the other hand the metal free $\alpha_8\beta_4$ hybrid did not dissociate at pH 7.2. Therefore after the reactivation only the original hybrid can be detected (disc II).

These results show that it is possible to dissociate aminopeptidase I under special conditions into low molecular weight components. We don't yet know whether these components are monomeric subunits or dimers. Although the β chain is much more diffi-

Table 1
Reactivation of the centrifuged apoenzyme: % activity recovered

	pH 7.2	pH 9.0
α-subunit Leucine *p*-nitroanilide test	96%	100%
β-subunit glutamic acid 1-(4-nitroanilide)test	100%	51%

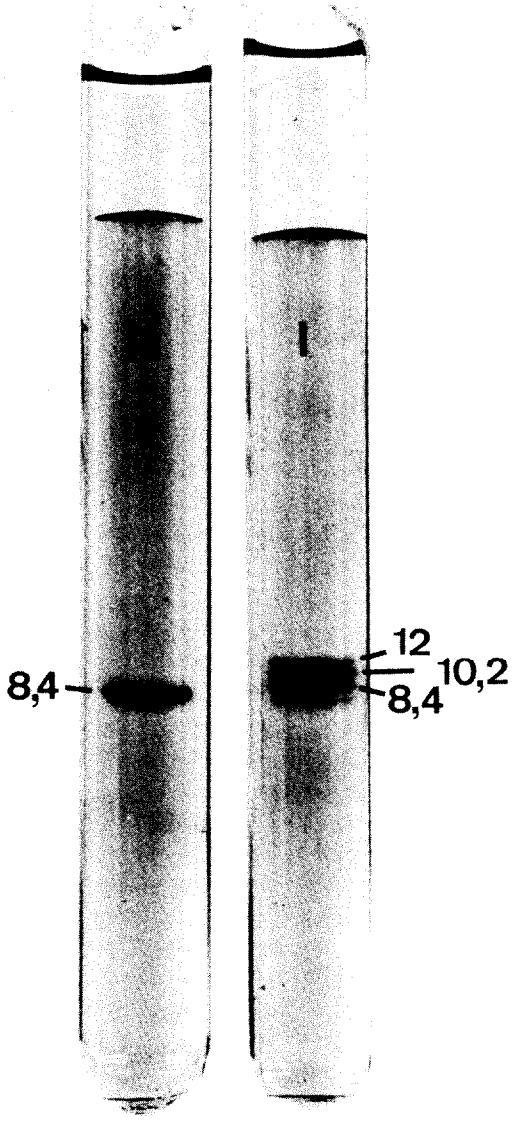

Fig. 2. Electrophoretic separation of the reactivated aminopeptidase I after centrifugation. Authentic hybrids were used to identify the different bands. (I) Sample from fraction 16, pH 9.0 gradient; (II) Sample from fraction 6, pH 7.2 gradient.

cult to reactivate than the α subunit, it is possible to get recovery of the β chain activity. We still hope to find conditions under which the β subunit can also be reactivated in good yields. This would enable us to answer the question whether β rich species ($\alpha_4\beta_8$, $\alpha_2\beta_{10}$, β_{12}), which we do not find in the *B. stearothermophilus* extracts can be prepared, or if for energetic reasons they cannot exist.

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