Immobilized Derivatives of Leucine Aminopeptidase and Aminopeptidase M

APPLICATIONS IN PROTEIN CHEMISTRY*

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GARFIELD P. ROYER† AND JOHN P. ANDREWS

From the Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210

SUMMARY

Leucine aminopeptidase and aminopeptidase M have been covalently bound to an arylamine derivative of porous glass. The bound forms of both enzymes retain 100% of their activities at saturating levels of substrate (leucine p-nitroanilide). For the hydrolysis at pH 7.3 and 25°C, kcat values for bound and free leucine aminopeptidase are 46 ± 5 s⁻¹ and 46 ± 2 s⁻¹, respectively; for aminopeptidase M (pH 7.5 and 25°C), kcat values are 23 ± 2 s⁻¹ and 21 ± 0.4 s⁻¹, respectively. Although the Michaelis constants for both enzymes increase on binding, the pH and temperature dependencies of the bound enzymes remain unchanged. These data suggest that the environments and conformations of the enzymes are not significantly changed after coupling to the solid support. The apparent decrease in the binding of substrate could be explained by a decrease in the effective diffusion coefficient of the substrate.

Both insoluble enzymes are active against polypeptide substrates. After treatment for removal of contaminating endopeptidases, the immobilized derivatives of leucine aminopeptidase and aminopeptidase M were used successfully in NH₂-terminal sequence determination. The bound aminopeptidase M appears to be the better of the two for this purpose.

Both bound enzymes will catalyze the hydrolysis of the aminoethylated A and B chains of insulin nearly to completion (≥87% recovery of free amino acids in all cases). These digests are carried out at pH values near neutrality in a volatile buffer with no activating metal. Immobilized pronase (ROYER, G. P., AND GREEN, G. M. (1971) Biochem. Biophys. Res. Commun. 44, 426) was used in concert with bound leucine aminopeptidase and bound aminopeptidase M for the hydrolysis of β-lactoglobulin. In both cases the recovery of free amino acids was 93%. These bound enzymes should be quite useful in amino acid composition determinations when acid-labile residues such as tryptophan, glutamine, asparaginc, or certain “affinity labeled” side chains are present.

Possible applications for immobilized enzymes exist in a number of areas including therapeutics, synthesis, analysis, and food processing (3-6). It is also apparent that immobilized enzymes may serve as research tools. One area for such use would be protein structure studies. Specifically, we hope to prepare and employ a series of immobilized proteolytic enzymes for total hydrolysis, sequence determination, and proof of optical purity. Enzyme immobilization would permit the use of mixed proteases in high concentration for total hydrolysis. In all of the applications, the convenient separation of enzyme from digest is an advantage. Also, the insoluble enzymes are stable, reusable, and may be used in high concentration without the risk of contaminating the digest.

Aminopeptidases are extensively used for amino acid sequence determination (7-9) and have also been used in attempts to hydrolyze proteins and peptides to free amino acids (10-12). Although the importance of NH₂-terminus sequencing by aminopeptidases has been diminished by the development of automatic sequencing systems, immobilized aminopeptidase derivatives could be quite useful in placement of amides, tryptophan determination, proof of optical purity, identification of acid-labile catalytic intermediates, and identification of affinity labeled side chains.

EXPERIMENTAL PROCEDURE

Materials

Leucine aminopeptidase was type IV from Sigma Chemical Company. Aminopeptidase M was the product of the Henley Company. The arylamine derivative of porous glass (13) was obtained from the Corning Glass Works. Tris (ultrapure), Leu-p-NO₂-anilide¹ and DFP were obtained from Schwarz-Mann. Insulin (Lilly Lot No. T2842) was the generous gift of Dr. Bruce Frank. Aminocarboxylatation was carried out as described by Cole (14); the chains were separated by chromatography on IRC-50 (Bio-Rad) according to Humble et al. (15). Pronase bonded to

¹ The abbreviations used are: Leu-p-NO₂-anilide, leucine p-nitroanilide; DFP, diisopropylphosphorofluoridate.
glass was prepared as previously described (16). All other chemicals were reagent grade.

Methods

Enzyme Coupling—Three hundred milligrams of the arylamino glass were suspended in 50 ml of 1 N HCl at 0°C with mechanical stirring. Drops of sodium nitrite (0.5 x) were added until an excess was indicated with starch-iodide paper. After 15 min, the glass was collected on a filter and washed with 200 ml of 3% sulfamic acid and 400 ml of distilled water. The coupling was carried out by adding the glass to a test tube containing a solution of aminopeptidase (3 mg of enzyme in 2 ml of 0.1 M Tris (pH 7.3) which was 1 mm in MnCl₂. The tube, connected to a constant torque stirrer, was rotated in an ice-water bath. The stirrer was stopped periodically, and aliquots of supernatant were removed for assay. When reaction was complete the glass-enzyme derivative was filtered and washed with 200 ml of buffer. The preparation was stored at 4°C in a moist cake.

Determination of Amount of Enzyme Bound—The depletion of enzyme activity in the supernatant is used to calculate the amount of enzyme bound to the glass. Consideration should be made of any activity in the washings which represents loosely bound enzyme. For this particular system all of the activity was accounted for either in the form of tightly bound enzyme or free enzyme.

The amount of protein bound was also determined by amino acid analysis (17). A control and enzyme glass derivative was accounted for either in the form of tightly bound enzyme or bound enzyme. For this particular system all of the activity was accounted for either in the form of tightly bound enzyme or free enzyme.

The amount of protein bound was also determined by amino acid analysis (17). A control and enzyme glass derivative were hydrolyzed with 6 N HCl in an evacuated, sealed tube at 110°C for 30 hours.

Enzyme Assays—The hydrolysis of Leu-p-NO₂-anilide was followed by monitoring the appearance of p-nitroaniline at 405 nm with a Cary model 15 spectrophotometer fitted with a thermostated cell holder. The extinction coefficient employed was 9.9 X 10⁴ M⁻¹ cm⁻¹. The temperature was controlled to ±0.2°C.

The insoluble enzyme was assayed by filtering the reaction mixture at given time intervals and measuring the optical density of the filtrate. The samples were stirred at uniform rates in a water bath with a “Tri-R” Ms. 7 immersible stirrer. Drops of sodium nitrite (0.5 N) were added until an apparent loss of activity.

Experimental errors in the rate determinations were within ±5%. The kinetic constants, k_cat and k_m(app), were calculated by the method of Wilkinson (18). These constants occur in the Michaelis-Menten equation

\[
dp = \frac{k_{cat} E_0 S_0}{K_m + S_0}
\]

which applies to the scheme

\[
E + S \rightleftharpoons k_i \rightarrow ES \rightarrow k_{cat} \rightarrow E + P
\]

E₀ and S₀ are the initial concentrations of enzyme and substrate, respectively.

The molecular weight for the calculation of E₀ was 280,000 for both aminopeptidase M (19, 20) and leucine aminopeptidase (21-23). In the latter case, 280,000 represents an average of reported values.

Peptide Hydrolysis—Digestions were performed with 1 mm solutions of peptide in 0.2 N N-ethylmorpholine acetate (pH 7.3 or 7.5). A screw cap tube (15 ml) with baffled sides was used as a reaction vessel. The tube containing 5 ml of protein solution and 150 mg of insoluble enzyme was rotated in a constant temperature bath (35°C) by a constant torque motor. Samples (0.2 ml) withdrawn at various time intervals were lyophilized, dissolved in citrate buffer (pH 2.2), and subjected directly to amino acid analysis (16). No interference from peptides was evident. The enzymes employed in sequencing experiments were treated with DFP and iodoacetate by standard procedures (8) and stored under Tris buffer containing 1 mm MnCl₂.

For the hydrolysis of β-lactoglobulin, 5 ml of a solution which was 1 mm in protein and 0.2 N in N-ethylmorpholine-acetate buffer (pH 7.5) were placed in a screw cap tube with baffled sides. Immobilized Pronase (75 mg) was added, and the tube was rotated by a constant torque motor for 6 hours at room temperature. The bound Pronase was removed by filtration, and bound aminopeptidase (150 mg) was added. The tube was rotated for 24 hours, and the contents were filtered, lyophilized, and subjected to amino acid analysis.

RESULTS

Preparation and Characterization of Immobilized Aminopeptidases—A representative time course of coupling appears in Fig. 1. The amount of enzyme coupled was determined both by depletion of activity in the supernatant and by amino acid analysis. Good agreement was found for the two methods. The average values were 1.2% (w/w) and 0.8% (w/w) for leucine aminopeptidase and aminopeptidase M, respectively. We have used these enzyme preparations over many months without apparent loss of activity.

Both soluble and insoluble forms of the enzymes follow Michaelis-Menten kinetics (Fig. 2 and 3). Values of k_m(app) and k_cat appear in Table I. These data point out the importance of establishing the substrate dependence of the enzyme-catalyzed reaction for purposes of comparing the activities of soluble and insoluble enzyme forms. For instance, in the case of leucine aminopeptidase the Michaelis-Menten equation describes both reactions, but v_max/v_sol varies as a function of substrate concentration until saturation is reached. This behavior reflects the differences in K_m for the two enzyme forms; i.e. when V_max is unchanged, Equation 1 holds. A plot of v_max/v_sol against S₀ is in fact hyperbolic and approaches unity as expected.

A change in pH dependence often accompanies insolubilization of enzymes (24). It is therefore important also to compare the activities of soluble and insoluble forms of an enzyme as a func-

![FIG. 1. Representative time course of enzyme coupling. Diazinated arylamino glass was reacted at 0°C with 2 ml of leucine aminopeptidase solution (1.5 mg per ml in 0.1 M Tris-HCl buffer (pH 7.3), 1 mm in MnCl₂). Activity was measured spectrophotometrically with leucine p-nitroanilide as substrate.](http://www.jbc.org/Downloaded from)
Fig. 2. Lineweaver-Burk plots for the hydrolysis of leucine p-nitroanilide at pH 7.3 and 25°C by soluble and insoluble forms of leucine aminopeptidase.

Fig. 3. Lineweaver-Burk plots for the hydrolysis of leucine p-nitroanilide at pH 7.5 and 25°C by the bound and free forms of aminopeptidase M.

Fig. 4. pH profiles for hydrolysis of leucine p-nitroanilide catalyzed by soluble and insoluble leucine aminopeptidase. Tris buffer, 0.1 M and 1 mM in MnCl₂, was used for the entire pH range.

Table I

| Parameter                  | Leucine aminopeptidase | Aminopeptidase M |
|----------------------------|------------------------|------------------|
|                            | Soluble                | Bound            | Soluble                | Bound            |
| $k_{cat}$ (s⁻¹)            | 46                     | 46               | 21                     | 23               |
| $K_{m(app)}$ (mM)          | ± 2                    | ± 5a             | ± 0.4b                 | ± 2              |
| $E_{0}$ (kcal/mole)        | 86.8                   | 35.4             | 75.0                   | 21.5             |
| pH optimum                 | 7.3                    | 7.3              | 7.5                    | 7.5              |

a pH 7.3, 25°C.

b pH 7.5, 25°C.

c pH 7.3.

d pH 7.5.

Peptidase Sequencing—The results of sequencing experiments appear in Figs. 6 and 7. The enzyme preparations used in these experiments were treated once with DFP and iodoacetate and used repeatedly. When enzymes are stored in moist cake in the presence of 1 mM MnCl₂, no activating metals need be added to the digests. Note that the amino acids occur in the predicted order and that the separation of isoleucine and valine is less well defined in the digest with immobilized leucine aminopeptidase.

Total Hydrolysis of Aminoethylated Chains of Insulin and β-Lactoglobulin—The hydrolysis of the aminoethylated A chain goes to the extent of 93 and 96% for bound leucine aminopeptidase and bound aminopeptidase M, respectively (Table II).
FIG. 7. Release of amino acids from the aminomethylated A chain of insulin catalyzed by glass-bound aminopeptidase M. The expected sequence is Gly-Ileu-Val-Glu.

Table II

Hydrolysis of the aminomethylated A chain of insulin catalyzed by immobilized derivatives of leucine aminopeptidase (Column A) and aminopeptidase M (Column B)

| Amino acid        | Known residues/mole A chain | Recovery |
|-------------------|-----------------------------|----------|
|                   |                             | A        | B        |
| Aminoethylcysteine| 4                           | 90       | 93       |
| Serine            |                             | 100      | 100      |
| Glutamine         | 2                           | 90       | 90       |
| Glycine           | 1                           | 100      | 100      |
| Alanine           | 1                           | 90       | 95       |
| Valine            | 2                           | 90       | 95       |
| Isoleucine        | 1                           | 90       | 95       |
| Leucine           | 2                           | 90       | 95       |
| Tyrosine          | 2                           | 100      | 100      |
| Aspartic acid     | 0.4                         | 90       | 103      |
| Mean              |                            | 93       | 96       |

Table III

Hydrolysis of the aminomethylated B chain of insulin catalyzed by immobilized derivatives of leucine aminopeptidase (Column A) and aminopeptidase M (Column B)

| Amino acid         | Known residues/mole B chain | Recovery |
|--------------------|-----------------------------|----------|
|                    |                             | A        | B        |
| Lysine             | 1                           | 80       | 60       |
| Histidine          | 2                           | 105      | 100      |
| Arginine           | 1                           | 100      | 100      |
| Aminoethylcysteine| 2                           | 90       | 90       |
| Threonine          | 1                           | 80       | 60       |
| Serine             | 2                           | 95       | 100      |
| Glutamine          | 3                           | 100      | 100      |
| Glycine            | 2                           | 96       | 95       |
| Alanine            | 3                           | 97       | 100      |
| Valine             | 4                           | 103      | 98       |
| Leucine            | 2                           | 100      | 100      |
| Tyrosine           | 3                           | 100      | 100      |
| Phenylalanine      | 4                           | 99       | 99       |
| Proline            |                             | 93       | 97       |
| Mean               |                             | 93       | 97       |
93 and 87% with leucine aminopeptidase and aminopeptidase M, respectively. Notice that the residues on the carboxyl side of proline, lysine, and alanine, are released to a greater extent than proline. In the case of aminopeptidase M hydrolysis, one would expect the complete release of threonine, this is not observed. In the case of the digestion by immobilized leucine aminopeptidase, one would expect the release of threonine to be at the same level as proline recovery. This is not the case. Notice that alanine, the COOH-terminal residue of the insulin B chain, is released completely by bound leucine aminopeptidase but not by bound aminopeptidase M.

For the hydrolysis of β-lactoglobulin, we chose to pretreat with immobilized Pronase (16). Pronase is a mixture of proteolytic enzymes secreted by Streptomyces griseus; the mixture contains enzymes which encompass a very broad range of specificity. After several hours of incubation of β-lactoglobulin with bound Pronase, we see approximately 50 spots on a peptide map. The number of spots then decreases slowly as more peptides are reduced to free amino acids. The degree of hydrolysis is about 65% after 6 hours. Subsequent treatment with bound leucine aminopeptidase or aminopeptidase M increases the extent of hydrolysis to 93% in both cases; recoveries of proline are 25 to 11%, respectively (Table IV).

**Table IV**

Recovery of free amino acids from a digest of β-lactoglobulin

| Amino Acid | Known residues/mole of β-lactoglobulin | Recovery |
|------------|--------------------------------------|----------|
|            |                                      | A | B |
| Tryptophan | 2                                    | 100 | 105 |
| Lysine     | 15                                   | 101 | 97 |
| Histidine  | 2                                    | 80 | 85 |
| Arginine   | 3                                    | 103 | 90 |
| Asparagine | 8                                    | 94 | 95 |
| Threonine  | 8                                    | 110 | 113 |
| Serine     | 14                                   | 92 | 105 |
| Glutathione| 10                                   | 95 | 112 |
| Glycine    | 4                                    | 103 | 110 |
| Alanine    | 4                                    | 94 | 93 |
| Valine     | 22                                   | 96 | 98 |
| Methionine | 4                                    | 103 | 98 |
| Isoleucine | 10                                   | 98 | 98 |
| Leucine    | 8                                    | 25 | 11 |
| Tyrosine   | 8                                    | Mean 93 | Mean 93 |
| Phenylalanine | 4                             | Mean 93 | Mean 93 |
| Proline    | 8                                    | Mean 93 | Mean 93 |

DISCUSSION

There are at least two types of explanations which could account for complete retention of activity when an enzyme is immobilized. The side chains must accessible for coupling to the insoluble carrier could be sufficiently distant from the active center to allow covalent attachment without reaction of the groups at the active center. One might easily envisage available tyrosine and lysine residues on one side of the protein molecule and the active center on the opposite side. Alternatively, reaction of the carrier with the enzyme could occur relatively close to the active center, if the catalytic groups were recessed in a crevice or hole. One related picture would be a toroid or donut-shaped molecule with the active site(s) on the inner wall of a hollow center. Again, reaction could occur at the active site only if a sufficiently small chemical modification reagent were used. Although no mention was made of the active site on the basis of low angle x-ray diffraction, Kretschmer and Kollin (20) suggested such a toroid structure for bovine lens aminopeptidase.

A second possibility would involve the inactivation of 1 molecule or subunit with commensurate activation of others. This situation would require the fortuitous coincidence of $V_{max}$ values of soluble and insoluble forms. Similarities in pH dependences and activation energies would also tend to rule out this explanation. Moreover, Schwabe (27) found that a nonequivalent complex of calcium phosphate gel and leucine aminopeptidase from dental pulp retained full activity. Although no comparisons of kinetic parameters for the soluble and insoluble forms of this enzyme were made, Schwabe suggested that the interaction of enzyme with support results in a specific orientation of the active site away from the support to face the solvent. Although our system involves a different support and an enzyme from another source, we feel a similar orientation prior to formation of a covalent complex could also explain the kinetic results presented here.

The differences in the $K_m$ values of the soluble and insoluble enzyme forms could be explained by diffusion limitations on the substrate. The approach of substrate to the enzyme might be impeded by the support itself or an "unstirred layer" on the support particle (28, 29).

The use of insoluble enzyme supports as chemical modification reagents seems attractive. It is our hope to look also at soluble chemical modification reagents of low molecular weight to further probe the three dimensional structure of leucine aminopeptidase and aminopeptidase M. Results of both approaches taken together could yield valuable information on the topography of these and other proteins.

The data presented here indicate that bound leucine aminopeptidase and bound aminopeptidase M could be quite useful in peptide sequencing and total hydrolysis. Figs. 6 and 7 illustrate the use of bound aminopeptidases in sequencing. Isoleucine and valine are somewhat better resolved when bound aminopeptidase M is employed. These immobilized enzymes would be especially valuable when examination of the residual peptide was desired. In this case, the reaction, termination, and enzyme removal is accomplished simply by filtration. Also, the feature of enzyme reuse is valuable in that leucine aminopeptidase is relatively expensive. We are hopeful that a multiple aminopeptidase sequencing system can be devised in which other bound aminopeptidases, such as aminopeptidase P and proline aminopeptidase, for example, could be used in concert with bound leucine aminopeptidase. When release of amino acids is slow because of X-Pro or Pro-X linkages, the digest could be exposed to aminopeptidase P or proline aminopeptidase. Enzyme insolubility would considerably reduce the operational difficulties inherent in such a system.

It is evident that bound aminopeptidases could be quite useful in the total hydrolysis of peptides and proteins. When proline, α-N-acyl residues, and β isomers are absent, the digestion should be complete. All of the advantages of enzyme immobilization given above would apply in total hydrolysis studies as well. In addition, mixed immobilized enzymes could be used without danger of contamination or enzyme inactivation. In addition, for total hydrolysis impurities of the enzyme preparation to be
immobilized could be an advantage. It is clear that our leucine aminopeptidase has a carboxypeptidase contaminant, since lysine and alanine are released to the extent of 80 and 95%, respectively, from the aminoethylated B chain of insulin. These residues are on the carboxyl side of proline and should, therefore, be recovered to the same extent as proline (60%). Delange, et al. (30) have demonstrated the presence of a carboxypeptidase contaminant in aminopeptidase M. Our results (Table III) indicate closer agreement among recoveries of proline, lysine, and alanine: 50, 60, and 65%, respectively. The soluble preparation could be of higher purity or perhaps a carboxypeptidase contaminant could have been inactivated during coupling or simply coupled to a limited degree.

Our present direction is, of course, to solve the problem presented by the resistance of imide and amide bonds involving proline. Much the same approach applicable in sequencing applies to total hydrolysis, except that bound derivatives of proline-releasing enzymes could be used simultaneously.

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