Leucine Aminopeptidase. A Calcium Phosphate Gel Complex*

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ABSTRACT: The leucine aminopeptidase of bovine fibroblasts adsorbs strongly and specifically on calcium phosphate gel and retains full activity. Adsorption on hydroxylapatite is not as strong and abolishes enzymatic activity. The stability of the leucine aminopeptidase, expressed as heat resistance, resistance toward organic solvents, and proteolysis, has been decreased in the process of adsorption, indicating a loosening of protein structure. At low temperature (4°) the calcium phosphate gel–leucine aminopeptidase complex is stable for at least 1 year. Supported membranes prepared from the calcium phosphate–aminopeptidase have been used to hydrolyze a large variety of peptides. Amino acid composition, and perhaps sequences of small peptides, may be elucidated readily with such columns or membranes using very high enzyme concentrations.

Insoluble active derivatives of proteolytic enzymes have been prepared by the covalent coupling of trypsin, chymotrypsin, ficin, and papain to various ionic or nonionic polymers (Levin *et al.*, 1964; Hornby *et al.*, 1966). Other investigators have used columns of carboxymethylcellulose-coupled alkaline phosphatase for the continuous hydrolysis of *p*-nitrophenyl phosphate (Weetall and Weliky, 1969).

In contrast to covalent coupling, adsorption of enzymes on calcium phosphate gel or hydroxylapatite has tacitly been assumed to be nonspecific. This paper contains some evidence suggesting that the leucine aminopeptidase of mammalian connective tissue (bovine dental pulp) may orient itself specifically on the calcium phosphate gel particle such as to expose its active site. The adsorption is so strong that the complex has some of the characteristics of covalently linked enzymes, yet is produced by simply mixing the constituents, and can be resolved again. The phenomenon was discovered and investigated subsequent to attempts to purify the connective tissue leucine aminopeptidase by the use of calcium phosphate gel as selective adsorbent.

The strong affinity of this enzyme for calcium phosphate is particularly interesting with respect to the high calcium content of calcifying tissues in general.

Some of the properties of the calcium-bound enzyme are reported in this paper together with an indication of its utility as a tool in peptide chemistry.

Materials and Methods

A leucine aminopeptidase enriched extract was obtained from mammalian connective tissue (bovine dental pulp) as described previously (Schwabe, 1969). Thin-layer chromatography was performed using silica gel G (Merck) on plates prepared in our laboratory. Calcium phosphate gel, purchased from Bio-Rad Laboratories, was suspended in 0.1 M Tris buffer (pH 8.0) and centrifuged at low speed prior to its use as adsorbent. Hydroxylapatite was prepared according to Jenkins (1962). For quantitation and identification of amino acids the Technicon amino acid analyzer (4.5-hr system) was employed. A small acrylic filter holder used to support enzymatically active calcium phosphate–enzyme was built in our departmental workshop. Activity assays were performed as described by Matheson and Tattri (1964). Peptides, trypsin (crystalline), and α-chymotrypsin (crystalline) were purchased from Mann Research Laboratories.

Experimental Section and Results

Adsorption of Leucine Aminopeptidase on Calcium Phosphate Gel. A distilled water extract of a pure, cellular connective tissue (bovine dental pulp) was prepared and separated into acid cathepsins, aminotripeptidase, and leucine aminopeptidase by DEAE chromatography (Schwabe, 1969). The leucine aminopeptidase fraction (referred to as DEAE fraction II in this paper), lyophilized and stored at −20°, served as a convenient starting material. To 500 mg of this leucine aminopeptidase fraction in 10 ml of cold 0.1 M Tris buffer (pH 8.0), 5 g of calcium phosphate slurry was added while the solution was stirred at 0°. After 10 min the suspension was centrifuged at 3000 rpm and the supernatant was decanted. The calcium phosphate was subsequently washed (by resuspension and centrifugation) in succession with the following solutions: (1) 0.1 M Tris (pH 8.0); (2) 0.1 M Tris (pH 8.0), 2 M in NaCl; (3) 0.1 M Tris (pH 8.0), 1 M in Na_2SO_4; and (4) 0.1 M Tris (pH 8.0), 1 M Na_2SO_4 + 2% Triton X-100 detergent. The results are summarized in Table I. The enzyme attaches very strongly to the calcium phosphate gel (as judged by the measures necessary to effect resolubilization) while the activity did not measurably decrease. This can only indicate

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TABLE I: The Adsorption of Connective Tissue Leucine Aminopeptidase on Calcium Phosphate Gel.[a]

Treatment	Sample Assayed	Protein (mg/ml at 280 mμ)	Act. (C_0[e] × 10^2)
None	Control	23.2	3.2
Calcium phosphate adsorption	Supernatant	6.9	0
	Suspension	16.3[d]	4.2
Calcium phosphate washed with:			
Tris[b]	Supernatant	2.2	0
Tris + 2 M NaCl	Supernatant	3.2	0
Tris + 1 M Na_2SO_4	Supernatant	4.1	1.5
	Suspension	~9.0[d]	7.5
Tris + 1 M Na_2SO_4 + 2% Triton[c]	Supernatant	~9.0[d]	6.0

[a] The procedure for the adsorption is described in the text. The washes included a 60-min stirring at room temperature in the solvents listed under Treatment. Some activity is lost in the final treatment with Triton. The suspension prior to Triton–Na_2SO_4 treatment contains all of the activity. The increase of the proteolytic coefficient of the adsorbed enzyme is due to the removal of inert proteins during the washing of the calcium phosphate. [b] Tris = 0.1 M pH 8.0. [c] Triton X-100, Rohm & Haas. [d] By difference. [e] $C_0 = K_0E$, where K_0 = zero-order rate constant, E = protein (milligrams per milliliter), determined at 280 mμ. E for calcium phosphate–enzyme was estimated by the difference between solutions before and after adsorption.

that in the process of adsorption the active site of the leucine aminopeptidase remains totally accessible to the substrate. It appears that the calcium phosphate gel interacts with a particular site or area of the enzyme molecule.

Adsorption on Hydroxylapatite. The experimental conditions for the adsorption of connective tissue leucine aminopeptidase on hydroxylapatite were identical with those in the previous experiment. The hydroxylapatite was washed with Tris buffer until the suspension was at pH 8.0 prior to adsorption of the enzyme. The result (Table II) shows clearly that the mode of leucine aminopeptidase on hydroxylapatite differs from adsorption on calcium phosphate. The hydroxylapatite–leucine aminopeptidase complex is inactive and more readily resolved. Since the desorbed enzyme is fully active it can be concluded that in the process of adsorption the active sites of the enzyme are occluded (Table II). This is in sharp contrast to the type of interaction of leucine aminopeptidase with calcium phosphate gel and strengthens the argument for a relatively specific interaction of enzyme and adsorbent.

The Interaction of Alkylleucine Aminopeptidase and p-Hydroxymercuribenzoate-Treated Leucine Aminopeptidase with Calcium Phosphate. In order to explain the specificity of the interaction between connective tissue leucine aminopeptidase and calcium phosphate gel the presence of directive forces has to be assumed. While calcium phosphate should be essentially neutral, relative differences in electron density around the oxygen and calcium atoms might offer some cooperative directional force for the functional groups on proteins. Covering these functional groups on the protein should eliminate or impair the directive forces and lead to a higher percentage of inactive complexes or to nonadsorption of the enzyme. The partially purified aminopeptidase was treated for 2 hr at pH 8.0 with freshly prepared solutions of *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, iodoacetamide, and 1-dimethylaminonaphthalene-5-sulfonyl chloride. The pH was routinely controlled with a pH-Stat during the reaction. Following the reaction, the mixtures were made 1 M in sodium chloride, dialyzed in a very narrow dialysis tubing for several hours at 4° against 0.1 M Tris (pH 8.0), and subsequently treated with calcium phosphate as described in the previous section. The experiments summarized in Table III indicate that adsorption has been severely disturbed subsequent to exposure to the various agents while the activity in solution is not impaired (except with *N*-ethylamaleimide and slightly with *p*-hydroxymercuribenzoate). At a calcium phosphate concentration much higher than that necessary to remove an equal amount of untreated connective tissue leucine aminopeptidase from a solution, a 1-dimethylaminonaphthalene-5-sulfonyl-leucine aminopeptidase could also be adsorbed, but as an inactive complex only.

Activity of the Particle-Bound Enzyme. A calcium phosphate suspension of connective tissue aminopeptidase was used to hydrolyze in succession Leu-Leu, Leu-Gly-Gly, leucinamide, and again Leu-Leu. Of the calcium phosphate–leucine aminopeptidase slurry (0.2 ml of calcium phosphate in 1 ml of Tris buffer, pH 8.0) 25 μl was suspended in 100 μl of Tris buffer (pH 8.0, 10^{-3} M in Mn^{2+}) and 100 μl of one of the above substrates. The particle-bound enzyme was separated from each reaction mixture by low-speed centrifugation, the supernatant was removed for analysis, and the enzyme was stirred with the next substrate. The use of the initial substrate again at the end of the experiment demonstrated clearly that no activity had been lost, *i.e.*, that the enzyme is firmly attached to the particles during their catalytic activity (Table IV).

TABLE II: The Adsorption of Connective Tissue Leucine Aminopeptidase on Hydroxylapatite.[a]

Treatment	Sample Assayed	Protein (mg/ml at 280 mμ)	Act. ($C_0 \times 10^2$)
None	Control	40.0	3.2
Hydroxylapatite adsorption	Supernatant	20.0	0
	Suspension	~20.0[c]	0.6
Hydroxylapatite washed with:			
Tris[b]	Supernatant	2.0	0
Tris + 1 M NaCl	Supernatant	8.5	0
Tris + 0.2 M Na_2SO_4	Superantant	10.2	5.0

[a] See text for conditions. The enzyme–apatite complex is inactive. The activity is largely recovered at 0.2 M Na_2SO_4 wash, a less drastic condition than necessary to resolve the calcium phosphate gel–enzyme complex (Table I). [b] Tris = 0.1 M Tris (pH 8.0). [c] By difference.

TABLE III: Distribution of the Activity following Treatment of Modified Enzyme and Control with Calcium Phosphate.[a]

Modifying Agent	ΔOD 570/min	
	Act. Remaining in Soln	Act. Adsorbed in Calcium Phosphate
None	0	1.6
N-Ethylmaleimide	0.1	0.1
p-Hydroxymercuribenzoate	1.0	0
Iodoacetamide	1.5	0.4
1-Dimethylaminonaphthalene-5-sulfonyl chloride	1.6	0

[a] The connective tissue leucine aminopeptidase (DEAE fraction II) was treated with the agents listed in the table. The column "Activity Remaining in Solution" is an indication of the inhibition of adsorption of leucine aminopeptidase onto calcium phosphate under conditions leading to the adsorption of untreated enzyme. *N*-Ethylmaleimide and *p*-hydroxymercuribenzoate appear to inhibit the enzyme activity in solution as well as the adsorption on calcium phosphate gel.

The "particulate" enzyme is identified as a leucine aminopeptidase by its action on leucinamide. While adsorbed on the gel the activity is absolutely dependent upon manganous ions, insensitive to diisopropylfluorophosphate, and strongly inhibited by copper ions.

The pH Optimum of Calcium Phosphate–Leucine Aminopeptidase. Calcium phosphate gel–leucine aminopeptidase suspension was prepared as described in the previous section. Aliquots (2 ml) were centrifuged at 3000 rpm and the pellet was resuspended in Tris buffer at pH values from 6.0 to 11.0 in 0.5 pH unit intervals. Tris buffer above pH 9.0 and below pH 7.0 has no buffering capacity but the pH range occurring in 3 min in the reaction mixture was so small that it could be ignored. Other buffers were avoided to eliminate the possibility of specific buffer effects. The graph (Figure 1) shows that the pH–activity curve has not been changed drastically due to the adsorption of the leucine aminopeptidase on the calcium phosphate gel. A slight change in the ascending slopes of the curves between pH 6.0 and 8.0 is noted. If a functional group with a pK at 7.5 is indeed important for binding of the substrate (Schwabe, 1969) the result (Figure 1) suggests that the ionization of that group might be altered by the calcium phosphate gel.

The Stability of the Calcium Phosphate–Leucine Aminopeptidase. The relative stability of the active calcium phosphate–leucine aminopeptidase complex is an interesting property as far as protein chemistry and enzymology are concerned. Secondly, the applicability of this quasi particulate enzyme to sequence analysis

TABLE IV: The Successive Hydrolysis of Several Substrates by a Single Sample of Particle-Bound Leucine Aminopeptidase in Suspension.[a]

Sequence of Assay	Substrate	μmoles Hydrolyzed/min ml of Suspension
1	Leucylleucine	3.3
2	Leucylglycylglycine	0.6
3	Leucinamide	0.3
4	Leucylleucine	3.3

[a] The hydrolysis of $LeuNH_2$ identifies the enzyme as leucine aminopeptidase. The rate of hydrolysis of Leu-Leu at the beginning and end of the experiment indicates that no activity had been desorbed. See text for description.

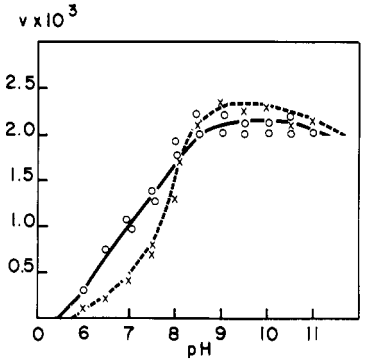
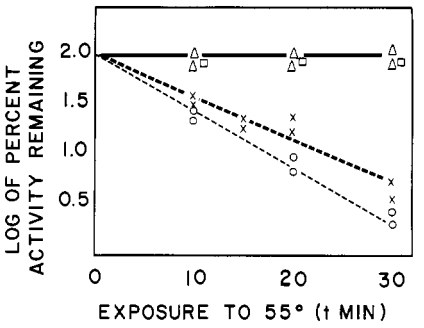


FIGURE 1: The pH optimum of calcium phosphate–leucine aminopeptidase and soluble enzyme. Enzyme and substrate were adjusted to the corresponding pH values and assayed at 40° with 5 mM Leu-Leu as substrate. (○—○) Calcium phosphate–leucine aminopeptidase and (x---x) soluble enzyme control.

FIGURE 2: The heat stability of the calcium phosphate–leucine aminopeptidase complex. Samples were incubated at 55° for various time intervals and assayed immediately at 40° with 5 mM Leu-Leu as substrate. The calcium phosphate–leucine aminopeptidase is labile compared to the soluble enzyme, particularly in the presence of manganous ions. (△—△) Purified leucine aminopeptidase, (□—□) DEAE fraction II, (x---x) calcium phosphate–leucine aminopeptidase without Mn^{2+}, and (○---○) calcium phosphate–leucine aminopeptidase with Mn^{2+} (10^{-3} M).



of peptides requires that the stability of the complex under a range of conditions be known.

The Effect of Heat. Aliquots of the calcium phosphate–leucine aminopeptidase with and without Mn^{2+}, the DEAE fraction II (from which the calcium phosphate–leucine aminopeptidase had been prepared), and purified leucine aminopeptidase (Schwabe, 1969), all from connective tissue, were incubated at 55°. At 0 time, 10, 15, 20, and 30 min 10 μl was withdrawn and assayed immediately at 40° with 5 mM Leu-Leu as substrate. The result of this experiment is depicted in Figure 2. The DEAE fraction II and the pure leucine aminopeptidase are stable over the test period. In contrast, the calcium phosphate–leucine aminopeptidase loses more than 90% of its activity under identical conditions. The presence of manganous ion (10^{-3} M) appears to slightly enhance denaturation. The linear relationship between the log of velocity of denaturation and time indicates first-order kinetics and is typical for

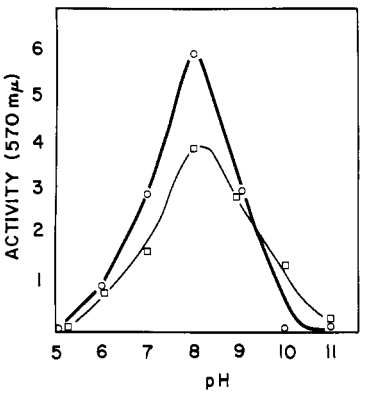


FIGURE 3: The stability of the calcium phosphate–leucine aminopeptidase at various hydrogen ion concentrations. The samples were exposed to the various pH values at 40° for 30 min and readjusted to pH 8.0 and an aliquot from each sample was assayed at 40° with 5 mM Leu-Leu as substrate.

the denaturation of soluble proteins. The calcium phosphate must interact with the leucine aminopeptidase in a way which labilizes its structure substantially, yet permits typical first-order kinetics of denaturation to occur.

The Effect of pH. Aliquots of calcium phosphate–leucine aminopeptidase were adjusted to pH values from 5.0 to 11.0 in pH unit intervals with small amounts of HCl or NaOH and kept at 40° for 30 min. Thereafter the samples were readjusted to pH 8.0 and 25-μl aliquots were assayed with Leu-Leu as substrate. A sample of DEAE fraction II was subjected to the same treatment and assayed for comparison. The sensitivity of the calcium phosphate–leucine aminopeptidase to higher hydrogen ion concentration is unchanged while the enzyme appears slightly more labile in high hydroxyl ion concentrations (Figure 3). From the pH–activity curve (Figure 1) it is apparent that substrate does protect the enzyme effectively at high pH values.

The Effect of Organic Solvents. Aliquots of calcium phosphate gel–leucine aminopeptidase were incubated at 50° with 30% solutions of ethanol, methanol, acetone, and dioxane in 0.1 M Tris buffer (pH 8.0). After various time intervals (0, 10, 15, and 30 min) samples were removed and assayed for activity with Leu-Leu as substrate. For comparison data obtained with purified leucine aminopeptidase (soluble) under identical conditions are included in Figure 4. The destabilization of the connective tissue leucine aminopeptidase occurring in the process of adsorption on calcium phosphate gel is apparent. Dioxane is most noxious for the adsorbed and the free enzyme but the 90% denaturation level is reached much sooner for the calcium phosphate–leucine aminopeptidase. Acetone and ethanol have a slightly beneficial effect. The soluble purified enzyme is somewhat deactivated in the presence of ethanol but stable in 30% acetone for the period tested.

The order of increasing polarity of the solvents tested is acetone, ethanol, methanol, and water. The favorable influence on the stability of the calcium

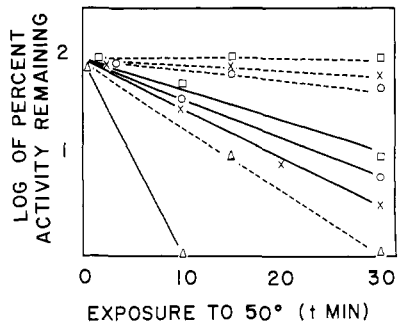


FIGURE 4: The effect of organic solvents on the calcium phosphate–leucine aminopeptidase. Samples of the absorbed and the purified connective tissue leucine aminopeptidase were incubated at 50° with 30% solutions of acetone (□), ethanol (○), dioxane (△) in pH 8.0 Tris buffer (0.1 M). The controls (x) received Tris buffer alone. The broken lines denote purified soluble enzymes, solid lines the calcium phosphate–leucine aminopeptidase complex. Immediately after heating in the solvents, 25 μl of samples was withdrawn and assayed at 40° (in a total volume of 200 μl) in pH 8.0 Tris buffer with 5 mM Leu-Leu as substrate.

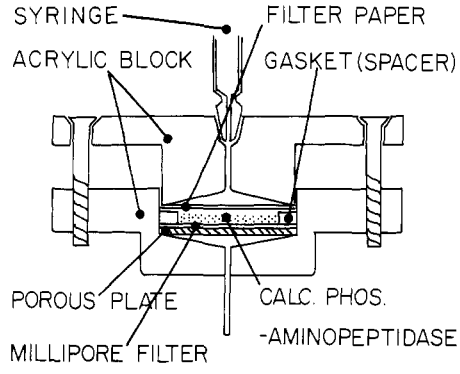


FIGURE 5: Cross section of the acrylic filter holder used to support a calcium phosphate–leucine aminopeptidase membrane. The total volume is 200 μl if one spacer is used (0.5-mm calcium phosphate–leucine aminopeptidase layer). A small polyethylene tubing was connected to the outlet during experiments.

phosphate–leucine aminopeptidase complex decreases with increasing polarity. Methanol is not included in the figure but is identical with the water control. Dioxane denatures soluble and adsorbed leucine aminopeptidase rapidly.

The Effect of Proteolytic Enzymes on the Activity of the Calcium Phosphate–Enzyme. Calcium phosphate–leucine aminopeptidase (200 μl) and DEAE fraction II (200 μl) were incubated at 35° with 100 μl of trypsin and chymotrypsin (1 mg/ml) for 4 hr. Controls containing no protease were carried along. After 4 hr samples were withdrawn and tested for activity in the usual system using Leu-Leu as substrate. No leucine aminopeptidase activity was lost from the soluble enzyme incubated with proteases by comparison with the control. The calcium phosphate–enzyme had lost 40% of its activity by comparison with its control. It appears that adsorption on the calcium phosphate gel changes the structure of the leucine aminopeptidase sufficiently to render it susceptible to proteolytic enzymes. Chymotrypsin appears somewhat more effective in destroying the leucine aminopeptidase activity than trypsin.

Calcium Phosphate–Leucine Aminopeptidase Membranes. Since the leucine aminopeptidase of connective tissues remains fully active when adsorbed on calcium phosphate and does not desorb when exposed to normally used buffers, it became possible to experiment with "solid leucine aminopeptidase reactors" or supported membranes. An acrylic filter holder was constructed which had exactly 1-cm^2 filter area and could be adjusted to various heights (thickness of the calcium phosphate membrane) in 0.5-mm intervals (Figure 5). The filter holder was immersed partially in a 40° water bath during experiments and stored in a moist chamber in the refrigerator between experiments.

A 0.5-mm thick calcium phosphate–leucine aminopeptidase membrane was prepared and a 1 mM solution of Met-Leu, 10^{-3} M in Mn^{2+}, was forced through the membrane (with the aid of a syringe) at a rate of 1 ml/min. The contact with the enzyme was thus limited to about 6 sec for each substrate molecule. In such short intervals an estimated 2–5% hydrolysis had occurred (Figure 6). The same figure also shows the result of experiments with Leu-Leu and Leu-Gly, all in 1 mM solution. Figure 7 shows the complete hydrolysis of Met-Leu in three passes through the same membrane, each at a rate of 0.2 ml/min. Note the complete absence of any protein stain at the spot site, indicating that no enzyme had been lost during these experiments.

Sequence Analysis. The utility of calcium phosphate–leucine aminopeptidase membranes for sequence analysis was tested using the C-terminal tetrapeptide of gastrin (Trp-Met-Asp-PheNH$_2$). A 2 mM solution of the peptide in Tris buffer at pH 8.0 was forced through the 0.5-mm thick calcium phosphate–leucine aminopeptidase membrane at a rate of 0.2 ml/min. After every pass through the membrane samples (5 μl) were spotted on a thin-layer plate; after the third pass 100 μl was removed and applied together with 0.1 μmole of Hyp and Nor-Leu to a Technicon amino acid analyzer column (4.5-hr system). Tryptophan was readily demonstrated as the first amino acid to appear. Thin-layer chromatography gave initially a clearer picture since tryptophan on the single-column system (Technicon) appeared immediately behind the peptide (substrate) peak in the histidine region. A very small peak of methionine was seen in the first chromatogram. After six cycles methionine was clearly recognized on thin-layer plates as well as on the AutoAnalyzer record, while tryptophan appeared as a larger peak next to the diminishing substrate peptide (Figure 8). After the tenth cycle through the active leucine aminopeptidase membrane tryptophan,[1] methionine, and aspartic acid were present in nearly equal quantities while phenylalanine appeared together with traces of a peptide (Asp-

[1] Tryptophan values were low.

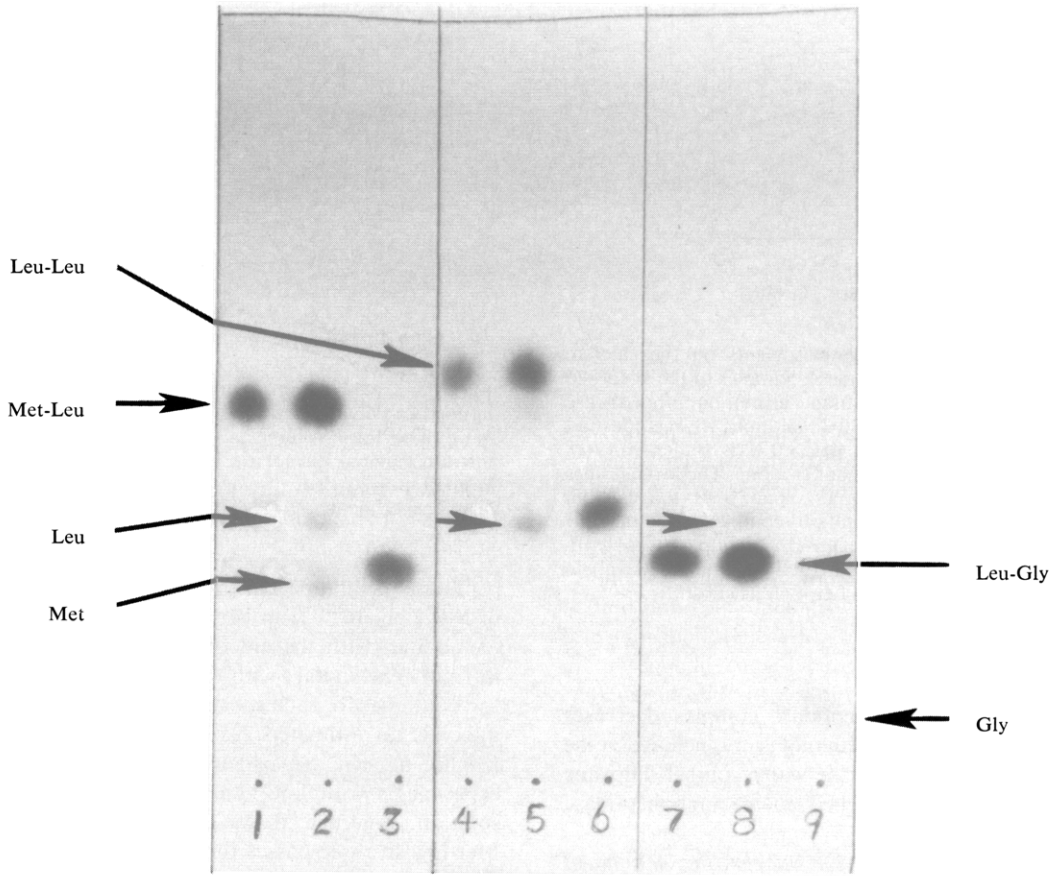


FIGURE 6: Thin-layer plate of the effluent of the calcium phosphate–leucine aminopeptidase membrane. Methionylleucine, leucylleucine, and leucylglycine solutions (1×10^{-2} M) were forced through the membrane at a rate of 1 ml/cm^2 per min. Numbers 2, 5, and 8 are the solutions 1, 4, and 7 after hydrolysis,

$PheNH_2$) and some $PheNH_2$. The latter was slowly converted into phenylalanine and ammonia. Quantitative analysis and thin-layer chromatography gave an unequivocal picture of the sequence of the peptide.

The samples taken after treatment with calcium phosphate–leucine aminopeptidase were applied without further treatment to the amino acid analyzer. The speed (very high enzyme concentration in the membrane) and lack of contamination by enzyme protein are significant advantages of this system.

Discussion

Adsorption of enzymes on hydroxylapatite and calcium phosphate gel is a well-known method for enzyme purification. The interaction of proteins with the adsorbent has tacitly been assumed to occur randomly, since no distinct interacting functional groups could be identified. Yet, evidence presented in this paper can only be interpreted to be the consequence of a specific interaction.

Since the reaction rate of adsorbed enzyme does not change significantly from that of the free enzyme, it seems reasonable to assume that nearly all the bound enzyme molecules expose their active sites.

If one assumes a spherical enzyme molecule and an equal affinity of each surface sector for the adsorbent, the probability of covering the active site during this process should be roughly proportional to a constant times the surface area of the active site. A 100,000 molecular weight molecule (we believe that the smallest functional subunit of connective tissue leucine aminopeptidase has a molecular weight close to 100,000 (Schwabe, 1969)) has a diameter of approximately 100 Å; the substrate used in our experiments is about 5–8 Å long. Allowing for various angles of approach for the substrate we could conceive of a 60-Å wide circle around the active site as the active sphere, occupying 10% of the total surface of the molecule. This implies that 10% of all random approaches to the surface of the calcium phosphate gel should lead to inactive complexes. Such reduction in total activity in a particular volume of enzyme solution before and after adsorption to the calcium phosphate gel has not been observed.

Hydroxylapatite forms an inactive complex with connective tissue leucine aminopeptidase. Reversing the above argument, the formation of an inactive complex at every encounter of an enzyme molecule with the adsorbent cannot occur by chance. If we assume the active sphere to be very small in order to explain the

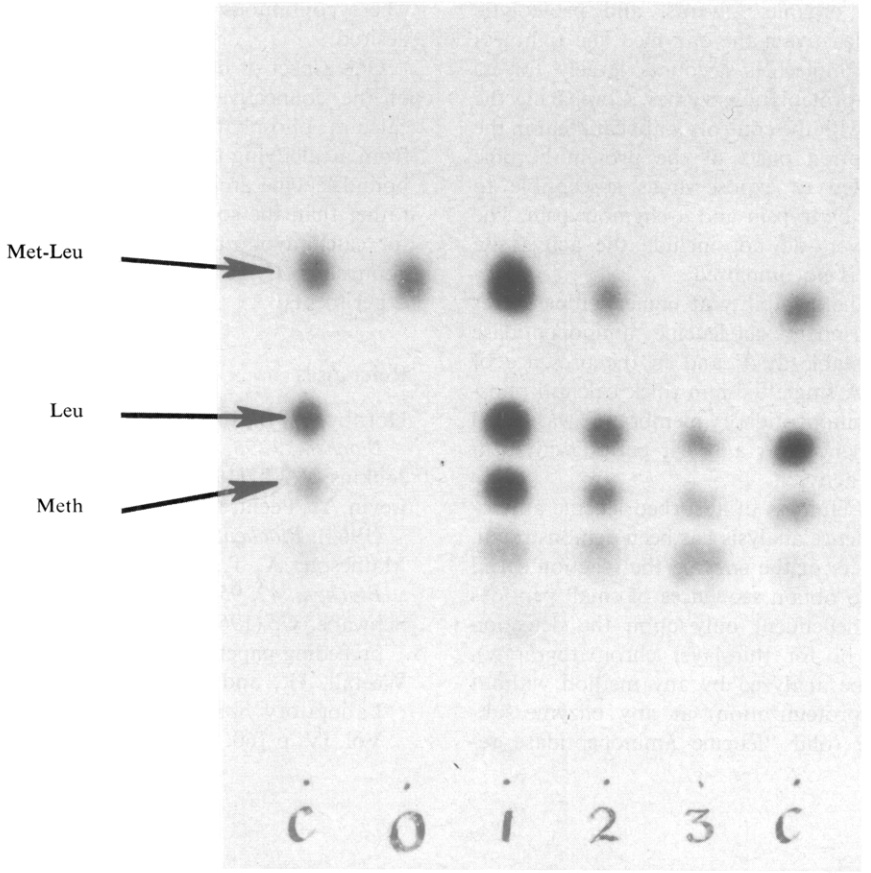


FIGURE 7: Hydrolysis of methionylleucine at a flow rate of 0.3 ml/cm^2 per min. Three passes through the calcium phosphate–aminopeptidase are sufficient to hydrolyze methionylleucine (1–3). C and O designate appropriate controls. Spots seen below methionine are artifacts from the buffer.

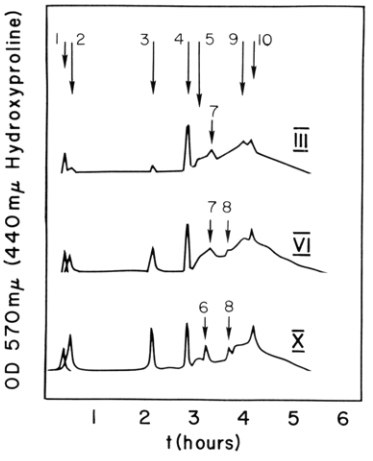


FIGURE 8: The effluent record from the amino acid analyzer of successive samples obtained after III, VI, and X cycles of Trp-Met-Asp-PheNH$_2$ solution (2 mM) through the leucine aminopeptidase membrane (Figure 5). Peptide digests (100 μl) were mixed with 0.1 μmole of Hyp and Nor-Leu standards and applied to the column in 10^{-3} M HCl and 12% sucrose. Cycle III shows the presence of original peptide (9 in the figure) and tryptophan (10). A trace of methionine (3) is visible. Number 7 refers to a peptide perhaps with Met-Asp-PheNH$_2$. Cycle VI shows an increase in tryptophan (10) and methionine (3) as well as some aspartic acid (2). In cycle X peptide 7 has diminished and phenylalanine (6) is observed which appears about 5 min earlier than the peptide. Number 8 could be PheNH$_2$ which is only very slowly deaminated by the connective tissue leucine aminopeptidase. Numbers 1 and 4 are hydroxyproline and norleucine standards. The elevated base line between 3.5 and 5 hr is the ammonia plateau usually observed when single column systems are employed.

maintenance of enzymatic activity in spite of random adsorption on calcium phosphate gel, then we must also assume the adsorption on hydroxylapatite to be so specific that a very small active site would be covered at every protein–adsorbent encounter. The alternate possibility that, upon adsorption on hydroxylapatite, the leucine aminopeptidase might partially denature is unlikely since fully active molecules are eluted from the hydroxylapatite under appropriate conditions.

While our experiments do not suggest mechanisms of interaction between adsorbent and enzyme, a random process seems to be excluded as their result.

A second common assumption (true in many cases perhaps) is that enzymes are stabilized on adsorbent surfaces, particularly on calcium phosphate gel. Results presented here clearly negate this assumption

as far as connective tissue leucine aminopeptidase is concerned. Heat, organic solvents, and proteolytic enzymes readily deactivate the enzyme. The tightness of native protein molecules accounts largely for its resistance toward proteolytic enzymes. Comparing the adsorbed enzyme with the controls it appears that in the process of adsorption parts of the protein become sufficiently distorted to expose areas susceptible to proteolytic attack by trypsin and α-chymotrypsin. The distortions, however, do not include the active site since the activity is not impaired.

In contrast to the instability at higher temperatures the calcium phosphate gel–leucine aminopeptidase complex is very stable at 4° and in the presence of substrate at 40°. A single 0.5-mm thick calcium phosphate–leucine aminopeptidase membrane was used for many experiments over a 1-year period without a detectable loss of activity.

The potential usefulness of adsorbed leucine aminopeptidase for sequence analysis has been demonstrated. Using thicker layers of the enzyme the reaction speed can be adjusted to obtain sequences of small peptides in a time span dependent only upon the detection method used (1 hr for thin-layer chromatography). The filtrate can be analyzed by any method without pretreatment (deproteinization) at any enzyme/substrate ratio. The solid "leucine aminopeptidase reactor" is potentially useful in peptide analysis systems where continuous hydrolysis of part of the eluate is desired.

One aspect of biological interest is the great affinity of the connective tissue leucine aminopeptidase for calcium phosphate. Since the enzyme is extracted from a calcifying tissue the question arises whether the bound leucine aminopeptidase is the physiologic form rather than the soluble enzyme. The very high affinity for calcium phosphate appears to be unique for the connective tissue enzyme according to preliminary experiments.

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Hydrolysis of Nucleoside Triphosphates by Myosin during the Transient State*

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ABSTRACT: The production of hydrogen ions during the hydrolysis of nucleotide triphosphates by myosin was determined in a stopped-flow spectrophotometer by measuring the optical density change of an indicator dye. At pH 8, in 0.5 M KCl with MgATP, MgITP, or CaATP as substrates, hydrogen ions were liberated during the transient phase at rates which greatly exceeded the steady-state rates of hydrolysis. The time course for MgATP at 0° or MgITP at 20° could be fitted by a single rate constant, while two rate constants were required for MgATP at 20° and CaATP at 0°.

In the case of MgATP at 20°, both rate constants were proportional to substrate concentration over a range of low substrate concentrations. As substrate concentration was increased above 10^{-4} M the rates approached limiting values corresponding to rate constants of approximately 130 and 20 sec^{-1}.

Numerous studies have been made of the enzymatic properties of myosin but with few exceptions (Nanninga and Mommaerts, 1960; Kanazawa and Tonomura, 1965; Tokiwa and Tonomura, 1965; Imamura *et al.*, 1965) analyses of the kinetics have been restricted to the steady-state behavior.

The production of hydrogen ions during hydrolysis of nucleotide triphosphates at pH 8 was determined by measuring the change in optical density of an indicator dye in a stopped-flow spectrophotometer.

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