The Function of the Metal Ion in Leucine Aminopeptidase and the Mechanism of Action of the Enzyme

By G. F. BRYCE AND B. R. RABIN

Department of Biochemistry, University College London, Gower Street, London, W.C. 1

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Many workers have speculated on the function of the metal ion in the action of metal ion-activated proteolytic enzymes (Smith, 1951; Malmstrom & Rosenberg, 1959; Klotz, 1954; Vallee, Riordan & Coleman, 1963; Rabin, 1958, 1960). Most authors have assumed, without evidence, that the metal ion is located at the catalytic site of the enzyme and that it is simultaneously bound to the protein and substrate in the transition state of the reaction. However, there are other possible functions for the metal ion, as illustrated by the stabilization of trypsin by Ca^{2+} ions (Nord & Bier, 1953; Green & Neurath, 1953), and we have designed experiments that enable a more rational decision to be made among the possibilities.

MATERIALS AND METHODS

Enzyme, substrates, buffer and sodium chloride. These were as described by Bryce & Rabin (1964).

L-Leucine. This was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

L-Cysteine and p-chloromercuribenzoate. These were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Iodoacetamide. This was a gift from Dr D. C. Watts and had been recrystallized five times from ethanol. It was free from iodine and I^- ions.

Other materials. All other materials were AnalaR or equivalent.

Enzyme assay. This was carried out as described by Bryce & Rabin (1964).

Inactivation experiments. The experiments were conducted at constant pH (8.40) in 0.83 mM-N-ethylmorpholine buffer at 40°, except where stated to the contrary. The ionic strength was constant throughout and approx. 0.08, but its exact value is difficult to compute. Inactivation experiments in the absence of EDTA were carried out in a similar fashion. The incubation mixtures were prepared as follows. A sample (0.5 ml.) of enzyme solution [containing magnesium chloride (5 mM), N-ethylmorpholine buffer, pH 8.40 (5 mM), and sodium chloride (0.1 M)] was added to 2.5 ml. of 10 mM-EDTA, pH 8.4. Samples (usually 0.15 ml.) were removed at various times and pipetted into 2.85 ml. of leucylglycine solution at pH 8.40 (final concn. and ionic strength 1 mM and 0.1 respectively). The assays were conducted at 25°. The initial rates of reaction were used as a measure of the quantity of enzyme remaining. Except where stated to the contrary, magnesium chloride was not included in the assay.

RESULTS

Inactivation experiments. The inactivation of the enzyme at pH 8.4 and 40° in the presence of various concentrations of magnesium chloride is shown in Fig. 1.

To test the reversibility of the inactivation process, assays were conducted in the presence and absence of magnesium chloride. Enough magnesium chloride was included with the substrate to remove all EDTA completely and leave the final assay solution 5 mM with respect to Mg^{2+} ions. The results are shown in Fig. 2. The Mg^{2+} ions added in this way do not bring about a recovery of enzymic activity. However, as shown in Figs. 3 and 4, complete recovery of activity within experimental error is obtained if the enzyme is added to magnesium chloride solution before the substrate. This suggests that the substrate prevents the reassociation of the metal ion with the protein and thus the formation of the active enzyme. Figs. 3 and 4 also

Fig. 1. Inactivation of leucine aminopeptidase at pH 8.4 and 40° as a function of concentration of magnesium chloride. The stock enzyme solution had been stored in 5 mM-magnesium chloride and samples were pipetted into a buffered magnesium chloride mixture to give the appropriate metal ion concentration. The concentrations of magnesium chloride were: ▲, 50 mM; ○, 5 mM; △, 0.25 mM; ●, 0.7 μM (calculated as free concentration in the presence of EDTA).

show that both cysteine and leucine prevent the reconstitution of the enzyme.

Fig. 5 shows that the rates of decay of peptidase and esterase activities are identical.

Fig. 2. Inactivation of leucine aminopeptidase by EDTA at pH 8.0 and 40°. The experimental conditions were as given in the text, with the exception of the pH. O, Enzyme assayed without added magnesium chloride; ●, enzyme assayed in the presence of 5 mM-magnesium chloride.

Fig. 3. Effect of magnesium chloride and cysteine on the reconstitution of leucine aminopeptidase at pH 8.4 and 40°. O, Inactivation curve in the presence of EDTA; ●, at the time denoted by the full arrow a portion of the enzyme was removed and made 5 mM with respect to magnesium chloride; ▲, at the times denoted by the broken arrows a portion of the enzyme was added to cysteine solution (final concn. 1 mM) and magnesium chloride (final concn. 5 mM) was added after a further 10 min.

The enzyme is slowly inactivated by *p*-chloro-mercuribenzoate, as shown in Fig. 6. *p*-Chloro-mercuribenzoate does not enhance the rate of loss of enzyme in the presence of EDTA, but the activity loss becomes irreversible and no activity can be recovered on incubation with magnesium chloride. The enzyme was not inhibited by 1 mM-iodoacetamide, and the presence of this concentration of reagent did not prevent the restoration of activity lost in the presence of EDTA.

Fig. 4. Effect of magnesium chloride and L-leucine on the reconstitution of leucine aminopeptidase at pH 8.4 and 40°. O, Inactivation curve in the presence of EDTA; ●, at the time denoted by the full arrow a portion of the enzyme was removed and made 5 mM with respect to magnesium chloride; ▲, at the times denoted by the broken arrows a portion of the enzyme was added to leucine solution (final concn. 1 mM) and magnesium chloride (final concn. 5 mM) was added after a further 30 min.

Fig. 5. Progress curve for the loss of peptidase and esterase activities of leucine aminopeptidase at pH 8.4 and 40° in the presence of EDTA. Samples were removed at the times indicated and assayed in the usual way against: O, L-leucylglycine (1 mM); ●, L-leucine benzyl ester (1 mM).

Fig. 7 shows that the rate of inactivation is significantly increased by the presence of L-leucine. The results also show that the inactivation process follows a first-order law. The inactivation rate increases with temperature (Fig. 8). The ratio of rates of inactivation at 40° and 25° was found to be 8.9, corresponding to an Arrhenius activation energy of 27 kcal./mole.

Activation by manganese ions. The enzyme in 5 mM-magnesium chloride and buffer was pipetted

into manganese chloride solution at the same pH to give final concentrations of manganese chloride and magnesium chloride of 2 mM and 0.8 mM respectively. The activity increase was followed by removing portions and assaying in the usual way (Fig. 9). The approximate value of the ratio of the initial rates of increase of activity at 40° and 25° is 8.6, corresponding to an Arrhenius activation energy of 26 kcal./mole.

Fig. 6. Effect of *p*-chloromercuribenzoate on the inactivation of leucine aminopeptidase by EDTA at pH 8.4 and 40°. The inactivation mixtures contained, in addition to enzyme: ▲, *p*-chloromercuribenzoate (0.1 mM) and magnesium chloride (5 mM), no EDTA; ●, EDTA alone, no *p*-chloromercuribenzoate; ○, *p*-chloromercuribenzoate (0.1 mM) and EDTA; △, at the time indicated by the arrow a portion of the enzyme solution containing EDTA and *p*-chloromercuribenzoate was made 5 mM with respect to magnesium chloride.

Fig. 7. Effect of L-leucine on the inactivation of leucine aminopeptidase by EDTA at 40° and pH 8.4. ●, Enzyme and EDTA; ○, enzyme, EDTA and L-leucine (1 mM).

Fig. 8. Effect of temperature on the inactivation of leucine aminopeptidase by EDTA at pH 8.4. ●, 25°; ○, 40°.

Fig. 9. Effect of temperature on the activation of leucine aminopeptidase by manganese chloride at pH 8.4. ○, 25°; ●, 40°.

DISCUSSION

It is clear from the results in Fig. 5 that the esterase and peptidase activities are due to the same catalytic entity.

The loss of catalytic activity in the presence of EDTA is a slow process that could be due to slow dissociation of the metal ion or slow conformational changes in the protein subsequent to removal of the metal ion. Reassociation is much faster than dissociation, in keeping with the value reported (Smith & Spackman, 1955) for the association constant. The activation energies for activation by Mn^{2+} ions and activity loss are very nearly the same. This suggests that the slow step in inactivation is dissociation of Mg^{2+} ions, and this dissociation is also the rate-limiting step for Mn^{2+} ion-activation.

An alternative interpretation is that inactivation is associated with a rapid removal of the metal ion followed by a slow conformational change in the protein. The experiments with *p*-chloromercuribenzoate would seem to argue against this, since an enhanced rate of inactivation in the presence of *p*-chloromercuribenzoate might be expected and is not, in fact, observed.

The observation that *p*-chloromercuribenzoate renders inactivation irreversible strongly suggests the involvement of a thiol group in binding the metal ion, as has been demonstrated for carboxypeptidase (Vallee, Coombs & Hoch, 1960; Coleman & Vallee, 1960). The stability constants for the Mn^{2+} ion complexes of a number of peptidases are given in Table 1. Some of these constants need to be corrected for metal ion-substrate interactions (Rabin & Crook, 1956). Allowing for this, and differences in temperature and pH, the values are all remarkably similar, suggesting that the same metal ion-binding sites are involved. On the basis of studies on carboxypeptidase (Coleman & Vallee, 1961), these sites are amino and thiol groups.

The substrate and related compounds prevent the reassociation of the protein and metal ion. This has been observed by a different technique for carboxypeptidase (Coleman & Vallee, 1962*a, b*), and shows a further similarity between the two enzymes. The substrate must interact with the

metal ion-free enzyme in such a manner as to prevent access of the metal ion to its sites of coordination on the protein.

For carboxypeptidase, substrate analogues prevent dissociation of the metal ion, in contrast with the finding for leucine aminopeptidase. The addition of ligands to a metal ion-protein complex involves two separate and opposite effects on the metal ion-protein interaction. The electronegativity of the metal ion will be decreased and hence the metal ion-protein bonds weakened. On the other hand, if direct ligand-protein interactions also occur then there will be a stabilizing effect on the metal ion-protein combination. For carboxypeptidase stabilization results because only weak interactions between the metal ion and substrate can occur and substrate-binding to other sites is the dominating effect. For leucine aminopeptidase fairly strong interactions between the substrate and metal ion are possible with only weak interactions at other sites on the protein. The electronegativity effect is dominant, and hence the metal ion-leucine complex is more easily removed from the protein than the free metal ion.

Reaction mechanism. The evidence presented above is consistent with the metal ion mediating the binding of the substrate to the enzyme and the existence of secondary interactions between the substrate and protein. Studies on metal ion-peptide complexes (Rabin, 1956, 1958, 1960; Datta, Leberman & Rabin, 1959) indicate that the likely sites of interaction are the uncharged amino group and the peptide amide or ester oxygen atom. The formation of a ternary substrate-metal ion-enzyme complex would not be expected to enhance the reaction rate very much (Rabin, 1958), and additional interactions of a catalytic nature must occur. For ribonuclease (Findlay, Herries, Mathias, Rabin & Ross, 1962) and chymotrypsin (Bernhard, Coles & Nowell, 1960; Bender & Glasson, 1960) there is excellent evidence for the existence of a site binding water. An interaction of this sort is also possible for leucine aminopeptidase, and the alcohol-inhibition studies are consistent with this idea. The pH-activity curves suggest the existence of two groups required in the base form for catalysis (Smith & Spackman, 1955); one of these could be

Table 1. *Stability constants of manganese ion complexes of proteolytic enzymes*

Enzyme	Temperature	pH	log K_s	Reference
Leucine aminopeptidase	40°	8.6	4.5*	Smith & Spackman (1955)
Glycylglycine dipeptidase	35	8.3	5.5	Wilcox & Fried (1963)
Glycyl-leucine dipeptidase	40	7.8	3.8*	Smith (1948)
Prolidase	40	7.7	4.3*	Adams & Smith (1952)
Carnosinase	40	8.0	4.4*	Rosenberg (1960)
Carboxypeptidase	4	8.0	5.6	Coleman & Vallee (1961)

* Constants uncorrected for metal ion-substrate interactions; the corrected values will be 0.5–1.0 unit higher.

the amino group of the substrate and the other, which has been suggested to be an imidazole, could be the water-binding site. We suggest that the mechanism for the addition of water to the susceptible bond is as shown in Scheme 1.

The adduct should be bound more firmly to the metal ion than is the substrate. There must be considerable changes in geometry on adduct forma-

Scheme 1. M^{2+} , Mg^{2+} or Mn^{2+} ion.

Scheme 2. M^{2+} , Mg^{2+} or Mn^{2+} ion.

tion, since the adduct would have a tetrahedral structure whereas the peptide group of the substrate is planar. The second stage of the reaction is decomposition of the adduct. This could be assisted by interaction of the peptide nitrogen (or ester oxygen) with an acid group. An interaction of this sort would not be expected for the substrate because of resonance in the peptide bond, which makes the lone pair of electrons on the nitrogen unavailable. No such resonance exists in the adduct and interaction with an acid group should occur much more readily. The mechanism we suggest for decomposition of the adduct is shown in Scheme 2.

It is possible that B and B' are in fact the same group, or different nitrogen atoms of a single imidazole group. If so, conformational changes during the reaction could be of great importance for catalysis.

Two factors favour the dissociation of the products: the higher pK of the amino acid favours protonation of the amino group and decomposition of the metal ion complex, and secondary interactions at R and R' are in different molecules in the products and no longer mutually stabilizing.

SUMMARY

1. Leucine aminopeptidase is reversibly inactivated by incubation with EDTA. In the presence of *p*-chloromercuribenzoate this inactivation is irreversible.
2. Substrate, leucine and cysteine prevent the reconstitution of the metal ion-free enzyme with Mg^{2+} ions.
3. Evidence has been obtained that the esterase and peptidase activities occur at the same catalytic site.
4. The activation of the enzyme by Mn^{2+} ions is a slow process, the rate-limiting step of which is the dissociation of the Mg^{2+} ion-enzyme complex.
5. Evidence is presented that the metal ion-binding sites are the same for leucine aminopeptidase, glycylglycine dipeptidase, glycyl-leucine dipeptidase, prolidase, carnosinase and carboxypeptidase.
6. A mechanism is presented for the action of leucine aminopeptidase.

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Fatty Acids of Brown and Yellow Fat in Rats

By A. M. CHALVARDJIAN*

Department of Pathology, Washington University School of Medicine, St Louis 10, Mo., U.S.A.

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It is common knowledge that the mammalian body stores fat in its adipose tissue, mainly as triglycerides. This fat can be drawn upon whenever need arises to be utilized as an efficient source of energy. Adipose tissue in a well-nourished animal has a glistening pale-yellow appearance and microscopically the fat is present as one large vacuole within the cell, distending it and pushing the nucleus to one side against the cellular limiting membrane. In some mammals a type of tissue can be identified which also contains large amounts of lipids, again mainly as triglycerides, and which can be found in certain well-defined anatomic locations. This tissue is lobulated, and because of its tanned appearance it has come to be known as brown fat. In contradistinction to the 'usual' yellow adipose tissue, the fat in this case is found as small regular droplets within the cell with the nucleus at or near the centre of the cell. Other morphological differences have been demonstrated by histochemical techniques (Fawcett, 1952) and by electron microscopy (Lever, 1957; Napolitano & Fawcett, 1958). Brown fat is particularly rich in mitochondria and its colour has been shown to be due to its high cytochrome content (Joel & Ball, 1962). Although

it is found most frequently in hibernating mammals, its exact role in the process of hibernation is still obscure. The subject has been reviewed extensively by Johansson (1959).

Although lipids constitute the major component of yellow and brown fat, detailed characterization is lacking except for brown fat of the mouse (Spencer & Dempster, 1962).

The purpose of the present paper is to present analyses of the epididymal fat pad and the interscapular brown-fat pad of albino rats that were maintained on a laboratory chow diet. Results are also included that were obtained on animals deprived acutely of food for comparatively short periods of time.

EXPERIMENTAL

Materials

Animals. Male albino rats weighing 190–290 g. were maintained on pellets of Rockland rat diet for at least 2 weeks before any analysis was performed. They were killed by opening the chest cavity after light ether anaesthesia.

The study was divided into two parts: in the first portion the epididymal fat pad (yellow fat) and the interscapular fat pad (brown fat) were analysed in eight rats that were housed in a group cage and had free access to the food

* Present address: The Research Institute, The Hospital for Sick Children, Toronto, Canada.