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Studies on Aspartase

IV. On the Effect of pH on Aspartase

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The catalytic power of aspartase seems to be highly sensitive to changes of pH. A sharp pH optimum has been found to be situated in the neutral pH-region [1]. From this optimum the acid branch of the pH curve falls steeply [1]. Nothing is known of the character of the alkaline branch of the activity curve.

The present investigation has been undertaken in order to elucidate the influence of pH on the activity and stability of aspartase and thus to find out something of the factors which determine the characteristic features of the pH-activity curve. The aspartase has been obtained from propionic acid bacteria.

EXPERIMENTAL

Enzyme preparation: Propionibacterium peterssonii (strain of this laboratory) was the starting material. The bacteria were cultivated as earlier and prepared as a dry powder [2]. From the dry powder a crude enzyme solution was obtained by *n*-butanol treatment, using a method described earlier [2].

Activity measurements: The initial velocity of the reaction served as measure of the enzymatic activity. It was obtained graphically from a complete experimental curve and expressed in μg NH_3—N liberated or bound within 1 hour at 37° C and in the buffer designated. The activity is expressed as a percentage of the maximum of the initial velocity obtained.

The test solution was exactly the same as used earlier [2] with L-aspartic acid except that enzyme solutions were used instead of dry bacteria. When the enzyme solution was buffered no phosphate buffer was used.

When fumaric acid and ammonia were used as substrates in the synthesis of L-aspartic acid the test solution was the same as in the deamination experiments but instead of 2 ml 0.1 *M* L-aspartic acid, 2 ml 0.1 *M* fumaric acid (pH 7.3) and ammonium chloride were employed.

Ammonia was determined in exactly the same way as described earlier [2].

The hydrogen ion concentration was determined with a glass electrode (Beckman).

The buffer solutions in the experiments were prepared by mixing of the following solutions: sodium acetate (0.1 *M*)-acetic acid (0.1 *M*), sodium dihydrogen (0.33 *M*)-sodium monohydrogen phosphate (0.33 *M*), tris(hydroxymethyl)aminomethane [3] (0.2 *M*)-hydrochloric acid [3] (0.1 *M*) and sodium acetate (1/7 *M*) + the sodium salt of veronal (1/7 *M*)-hydrochloric acid [4] (0.1 *M*).

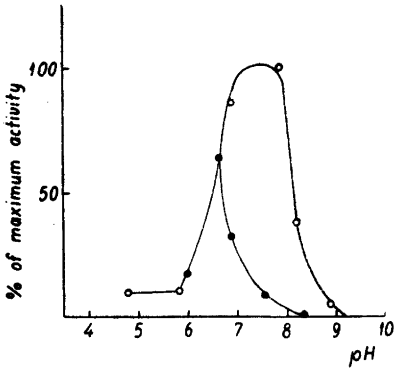

Fig. 1. Influence of pH on aspartase activity. Initial reaction velocities (expressed as percentages of the maximum activity) measured at 37° C in acetate and tris (hydroxymethyl)-aminomethane buffers (○) and in phosphate buffer (●) with L-*aspartic acid as substrate.*

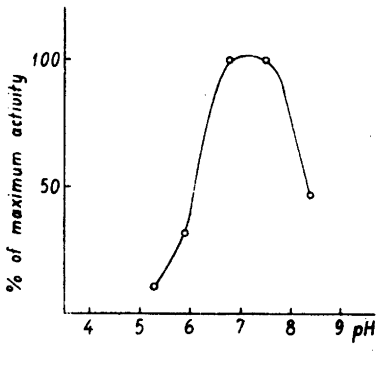

Fig. 2. Influence of pH on aspartase activity. Initial reaction velocities measured at 37° C in veronal-acetate buffer with fumaric acid and ammonia as substrates.

The pH-activity curve. In the deaminating experiments acetate buffer was used in the test solution from pH 4.5 to 5.7 in a total concentration of 0.03 *M* in relation to acetate. Phosphate buffer was used from pH 6 to 8.5 in a total concentration of 0.066 *M* in relation to the PO_4-group. Sodium chloride was added to this buffer to obtain a constant ionic strength equal to 0.2. The tris(hydroxymethyl)aminomethane buffer was used in a final concentration of 0.03 *M* in relation to tris(hydroxymethyl)aminomethane.

The synthesis experiments were performed in the buffer of Michaelis [4] at a final concentration of 0.01 *M* in relation to sodium acetate and the sodium salt of veronal. The pH was measured at the beginning and the end of the experiment and the average was used for the activity curve.

The pH-stability curve: 4 ml enzyme solution was used in all the experiments. The pH was adjusted with 0.1 *M* acetic acid, 0.1 *M* sodium hydroxide and tris(hydroxymethyl)aminomethane buffer with a final concentration of 0.033 *M*. The enzyme solution was then incubated at 37° C for 24 hours. During this time pH was controlled and, if it changed, adjusted to its original value. The pH was measured at the beginning and the end of the incubation and the average was used for the stability curve. After incubation pH was adjusted to 7.2 and the activity of the preparation was measured at this pH as described above.

RESULTS

The correlation between pH and activity of aspartase is illustrated in Fig. 1 with L-aspartic acid and in Fig. 2 with fumaric acid and ammonia as substrates. No greater difference can be observed between these two curves although a lesser shift of the acid branch of the synthesis curve towards the acid side may be noticed. Fig. 1 shows the strong inhibitory effect of phosphate, which causes an apparent shift of the pH-optimum towards the acid side. Since the total concentration of phosphate (PO_4) and ionic strength were kept constant in all pH values the only change with rising pH was a shift of dihydrogen phosphate to monohydrogen phosphate. The increasing inhibitory effect with the rise in

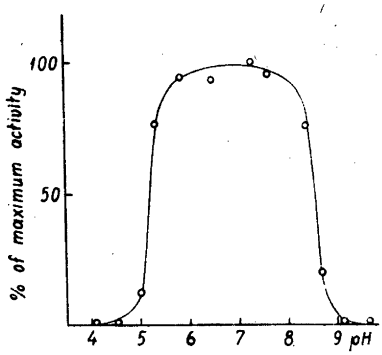

Fig. 3. Influence of pH on the stability of aspartase at 37° C.

the concentration of the monohydrogen ion of phosphate is strongly indicative of the existence of two valencies available for combination with the inhibitor.

The same concentration of phosphate as used here affects the activity of fumarase too [5]. But contrary to aspartase, the pH-optimum of fumarase is shifted towards the alkaline side by phosphate and a considerable activation is observed over most of the pH-range studied [5].

In an earlier paper no inhibitory effect of phosphate was observed [6]. Then, however, the concentration of monohydrogen phosphate was low, since the experiments were performed at pH 7.

The effect of pH on enzymatic activity is considered to be a summation of at least three factors: the stability of the enzyme, the ionization of the groups involved in the formation of the enzyme-substrate compound, and the ionization of the substrate.

In order to discover the extent to which the effect limiting the activity of aspartase at acid and alkaline pH values is due to enzyme destruction, the pH stability of aspartase was studied (Fig. 3). Aspartase seems to be stable between pH 6 and 8 but on the acid side of 5.5 and the alkaline side of 8.5 aspartase is irreversibly changed to an inactive protein. Comparing the pH-activity curves with the pH-stability curve reveals that the alkaline branch of the pH activity curve depends on instability of the enzyme at these pH values. The acid branches exhibit no such similarities as those of the alkaline branches.

DISCUSSION

Since the enzymes are proteins it seems rather obvious that their activity must depend on the electrical charge or on the state of dissociation of the enzyme. The variation of the activity with pH has therefore been interpreted in terms of acceptance and donation of protons by the active enzyme. This concept was further expanded to include ionization of the substrate.

Aspartase was found to exercise its catalytic power within the pH-range 6 to 9. Accordingly, only the zwitter-ion of L-aspartic acid forms the active substrate. (The pK values of aspartic acid are as follows: pK_1 = 1.88, pK_2 = 3.75 and pK_3 = 9.60) [7]. Hence it is quite evident that the ionization of the

substrate did not influence the shape of the pH-activity curve to any greater extent although a decrease of active substrate took place at the highest alkaline values (*e.g.* 10 % at pH 8.6). Since initial velocity is proportional to the product of the active substrate concentration and the active enzyme concentration, the pH-activity curve in Fig. 1 represents the concentration of active enzyme only.

An evaluation of the dissociation constants of the active groups of the enzyme may be possible from data in the pH-activity curve, which may give indications as to the possible structure of the functional groups in the active surface.

The acid branch of the activity curve shows a pK of 6.2—6.5 (the pH value corresponding to half-maximum activity). As the pH rises increasing quantities of the enzyme are changed into an active form till its concentration again begins to decrease. The alkaline branch of the pH activity curve indicates a pK value of 8.2—8.5. The alkaline branches of the pH activity and pH stability curves seem to be nearly identical. It may at least be assumed that the same groups are responsible for the decrease in activity in both cases. No similar consonance is observable in the acid branches.

The acid group (s) in aspartase with pK = 6.2—6.5 permits the exclusion of hydroxyl groups (the pK of the phenolic group in tyrosine is equal to 10.07) [7], sulphydryl groups (the pK of this group is 8.18[8] or 10.28[7], *cf.* below), the guanidine group (the pK of the guanidine group in arginine is equal to 12.48) [7]. The dissociation constants of most amino groups differ considerably from the value of the acid group in aspartase, although in special peptidic combinations such as tyrosylarginine (with pK equal to 7.39) [7] and phenylarginine (with pK equal to 7.57) [7] the constants are not so far from that of the acid group. Nor is it likely that carboxyl groups of aspartic acid and glutamic acid are responsible for the dissociation constant above. The free second carboxyl group of aspartic and glutamic acid have the pK value 3.65 and 4.25, respectively [7]. In special peptides these values are slightly higher (*e.g.* in aspartylglycine the pK is equal to 4.53 and in lycylglutamic acid to 4.47) [7].

Ring nitrogen, however, as in imidazole derivatives seems to have dissociation constants which concur well with the value of the acid group in aspartase. The pK value of imidazole nitrogen in histidine is equal to 6.00 [7] (in histidylhistidine the pK values of the imidazole groups are 5.60 and 6.80) [7].

Free histidine in acid solution exists in the form of imidazolium ion, in which there is resonance of the double bond [9]:

The imidazolium ion is capable of dissociating a proton (with a p$K \approx 6$), giving rise to the following resonating structure in alkaline solutions [9]:

A B

Since B is less stable than A it is almost completely suppressed in the imidazole.

The importance of the acid group in the enzyme is explained by the suggestion that aspartase is a metal protein [6], a suggestion strongly confirmed in this study by the observations concerning the inhibitory effect of phosphate. If the metal is bound by ionic bonds to any group in the enzyme it replaces a proton. From the discussion above it can be concluded that the metal atom must be bound to imidazole groups, *i.e.* to the negatively-charged N-atom in the imidazole nucleus (formula B above). After coupling with the positive metal the unstable form (B) is stabilized by induction from the metal.

Histidine has been found to bind many divalent metals very readily, surpassed only by cysteine in this respect, with the surprising exception of Mn and Mg[10]. These data, however, are not valid for the combination of metal with histidine residues in a protein since the carboxyl and amino groups present in the histidine are not available when the histidine forms part of a peptide chain.

The dissociating basic group of aspartase with a pK value equal to 8.2—8.5, responsible for the alkaline branch of the activity curve, rules out carboxylic guanidium and phenolic ions. The pK value of this group seems to be in the sulphydryl or amino group range. There seems, however, to be some doubt about the pK values of the sulphydryl group in cysteine. The treatise of Schmidt [8] favours pK 8.18, whereas Cohn and Edsall [7] give pK 10.28. The most recent investigation shows up the difficulties of attributing the pK values to either of the thiol groups or the amino groups respectively [11].

In the case of aspartase it is known from inactivation by specific reagents that free sulphydryl groups are essential to the enzymatic action. The alkaline group with a pK value 8.2—8.5 is therefore assumed to indicate the dissociation of a sulphydryl group. The undissociated form of the group seems to be essential to the activity of the enzyme.

SUMMARY

1. Aspartase activity exhibits a pH optimum at 7.5 with a steep decrease towards the acid and alkaline sides.

2. Phosphate buffer exercises an inhibitory effect on the catalytic action of aspartase.

3. Aspartase is stable between pH 6 and 8. On the acid side of pH 5.5 and the alkaline side of 8.5 the enzyme is irreversibly changed to an inactive protein.

4. The results are discussed and the dependence of aspartase activity on pH is interpreted in terms of acid and basic groups in the active centre. On

the basis of dissociation constants it is suggested that histidine residues and undissociated sulphydryl groups are functional groups in the active aspartase. The histidine residues are assumed to link the active metal to the enzyme.

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