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pH DEPENDENCE OF THE KINETIC PARAMETERS OF L-ASPARAGINASE

MARION H. O'LEARY and SUSAN L. MATTES

Department of Chemistry, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

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Summary

The concentration dependence of the rate of hydrolysis of L-asparagine by *Escherichia coli* L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) has been measured over the range pH 4.5 to pH 9.1 by a direct spectrophotometric assay at 220 nm and by a coupled assay utilizing glutamate dehydrogenase to detect the ammonia produced. The velocity of the hydrolysis reaction at saturating levels of substrate is independent of pH over this interval. The plot of V/K_m over the same interval is bell-shaped, being dependent on pK_a values of 6.58 and 8.69. The higher pK_a is attributed to the amino group of asparagine. The lower pK_a is associated with the enzyme active site and is probably due to an imidazole group.

Introduction

In spite of the wide use of L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) in the treatment of acute leukemia [1,2], the mechanism of action of this enzyme is poorly understood [3,4]. Except for studies of the reaction of asparagine with hydroxylamine [5], little attention had been paid to kinetic studies of the enzyme, and a study of the pH dependence of V and K_m has not been reported. The pH dependence of the catalytic activity can provide useful insights into the enzymatic mechanism [6], but progress in this area has been held up by lack of a proper assay.

A variety of assay methods have been used for studies of asparaginase [4], but most of them are not easily adaptable to continuous measurement of substrate or product concentration versus time. We chose to study the hydrolysis of asparagine by means of a coupled assay utilizing glutamate dehydrogenase (L-glutamate:NAD^+ oxidoreductase (deaminating), EC 1.4.1.2) under conditions where the ammonia formed is rapidly converted into glutamate, with concomitant production of NAD^+ from NADH [7]. For experiments at low

pH we used a direct assay utilizing the small change in absorbance at 220 nm which occurs on conversion of asparagine to aspartic acid [8].

Materials and Methods

Purified L-asparaginase from *Escherichia coli* (ATCC 13706) was provided by Eli Lilly and Company. L-Asparagine was recrystallized from water before use. Glutamate dehydrogenase and NADH were obtained from Sigma. Water was purified by means of a Millipore Super-Q water purification system. Buffers used were trimethylamine-*N*-oxide (pH 4.5—5.5), 2(*N*-morpholino)ethanesulfonic acid (pH 5.5—6.6), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 6.6—7.6), and Tris(hydroxymethyl)methylaminopropane sulfonic acid (pH 7.6—9.1).

All assays were carried out at 25°C using 0.1 M buffers in the presence of a small concentration of bovine serum albumin. Absorbances were measured using a Cary 118 spectrophotometer. For coupled assays the solution also contained 1 mM α-ketoglutarate, 0.1 mM NADH, and glutamate dehydrogenase. Absorbance was monitored at 340 nm. For direct assays, absorbance was monitored at 220 nm ($\Delta\epsilon$ = 55). In order to compare results obtained with various samples of enzyme, each enzyme solution was assayed several times under a standard set of conditions and all results were corrected for variations in enzyme activity. Kinetic parameters were calculated by use of the computer programs of Cleland [9].

Results

In the range pH 5.5—9.1 asparaginase was assayed spectrophotometrically by conducting the reaction in the presence of α-ketoglutarate, NADH, and glutamate dehydrogenase. Sufficient glutamate dehydrogenase was used so that the initial lag period (before the system reaches the steady-state) was 1—3 min. Following this initial period, the observed rate was independent of the concentration of glutamate dehydrogenase and varied linearly with the concentration of asparaginase. Care was taken, particularly at the extremes of pH, to show that these criteria were always obeyed. This assay method has significant advantage over other methods because it is continuous and sensitive and readily reproducible. The disadvantage of this assay procedure is, that because of the high K_m for ammonia with glutamate dehydrogenase, rather large quantities of this enzyme are required for assay. Unfortunately, no convenient alternative spectrophotometric procedure for continuous measurement of ammonia exists.

Double-reciprocal plots for the hydrolysis of L-asparagine spanning the concentration range from 0.5 K_m to at least 10 K_m were linear over the entire pH range examined. Typical reciprocal plots are given in Fig. 1.

In the range pH 4.5—5.5 the hydrolysis of asparagine was studied by a direct spectrophotometric assay employing the small ($\Delta\epsilon$ = 55) absorbance change at 220 nm which occurs on hydrolysis of asparagine. Results obtained by this method were in good agreement with those obtained by the coupled assay. At higher pH the method is not sufficiently sensitive to allow studies below the K_m of asparagine; thus, the coupled assay must be used in that case for the de-

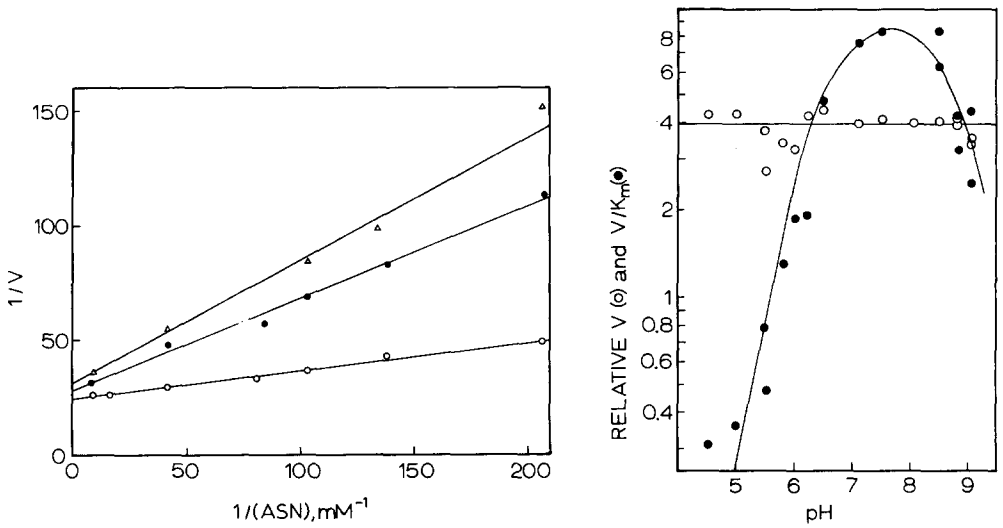

Fig. 1. Double reciprocal plots for the rate of hydrolysis (in arbitrary units) of L-asparagine by L-asparaginase from *E. coli* at 25°C. Each point represents the average of three determinations by the coupled assay method using glutamate dehydrogenase. △, data at pH 6.01 in 0.1 M 2(*N*-morpholino)ethanesulfonic acid buffer; ○, pH 7.52 in 0.1 M *N*-2-hydroxyethanepiperazine-*N'*-2-ethanesulfonic acid buffer; ●, pH 9.07 in 0.1 M tris(hydroxymethyl)methylaminopropane sulfonic acid buffer. ASN, L-asparagine.

Fig. 2. pH Dependence of V(○) and V/K_m(●) (in arbitrary units) for the hydrolysis of L-asparagine by L-asparaginase from *E. coli* at 25°C.

termination of kinetic parameters. Because of the many problems associated with the measurement of small absorbance changes at wavelengths near 220 nm, this assay procedure is of limited usefulness. Only releatively pure enzyme preparations can be measured in this way, and small amounts of turbidity can wreak havoc with the assays. We attempted to extend the range of this assay procedure by working in 10-cm cells but were unable to obtain reproducible results.

The kinetic parameters V and V/K_m obtained in this study are summarized in Fig. 2. V is independent of pH over the range pH 4.5—9.1. The V/K_m profile is bell-shaped. The pK_a on the low side is 6.58, and that on the high side is 8.69.

The parameters given in Fig. 2 are the proper ones for consideration of enzyme mechanism [6], but it is also interesting to note the pH dependence of the Michaelis constant. This constant is approx. 0.005 mM in the range pH 7.0—8.5 and increases rapidly above and below this range. The K_m values are approximately 0.1 mM at pH 5.0 and 0.01 mM at pH 9.0. Earlier studies of the pH dependence of the hydrolysis of L-asparagine by this enzyme showed a maximum in the pH-activity profile at approximate pH 7.5 [16]. However, those studies were conducted at only a single substrate concentration, and it is likely that the enzyme was not fully saturated with substrate at the extremes of pH.

Discussion

Over the range pH 4.5—9.1, L-asparaginase from *E. coli* shows simple saturation kinetics. Although the enzyme is a tetramer [4], we see no evidence for

subunit interactions in the kinetics. Prior kinetic studies in the presence of hydroxylamine at a single pH were consistent with the intervention of an acyl-enzyme intermediate [5], but our results provide no evidence for or against the existence of this intermediate.

The maximum velocity of hydrolysis of L-asparagine is independent of pH over the range 4.5—9.1. This lack of a pH dependence indicates that functional groups in the enzyme · substrate complexes do not undergo ionization of importance to catalysis in that range.

The reaction rate extrapolated to zero substrate concentration, V/K_m, shows a bell-shaped pH-rate profile, apparently dependent on the basic form of a group with pK_a 6.6 and on the acidic form of a group with pK_a 8.7. The latter group can be readily identified. The pH dependence of V/K_m reflects ionization of groups in the free enzyme, the free substrate, and in enzyme · substrate complexes prior to the first irreversible step. The pK_a of the amino group of asparagine is 8.8 [10], which is within experimental error of the value of 8.7 determined in this study. Thus, the decrease in V/K_m at high pH is almost certainly due to the fact that asparaginase requires the amino group of asparagine to be protonated before binding occurs. This is consistent with the fact that this enzyme, unlike the asparaginase from some sources [8], is unable to catalyze the hydrolysis of succinamide [11].

The group showing a pK_a of 6.6 in the V/K_m profile must be in the unprotonated state for catalysis to occur. Based on its pK_a, it is most likely that this group is either an imidazole group of a histidine residue or a carboxyl group of a glutamic acid or aspartic acid residue. Two independent lines of evidence indicate that a histidine is present at the active site of the enzyme [12—14]; thus it is logical, though not imperative, that we associate that histidine with the ionization observed in our kinetic studies. A serine hydroxyl group has recently been shown to be present at the active site of the enzyme [15], but the value of the pK_a observed in the present study makes it unlikely that this serine is undergoing ionization in the pH range under study.

The presence of serine and (presumably) histidine at the active site of asparaginase immediately calls to mind the presence of these same two residues at the active sites of the serine proteases. It is possible that asparaginase may function by a mechanism similar to that postulated for chymotrypsin and other serine proteases.

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References

1 Kidd, J.G. (1953) J. Exptl. Med. 98, 565—571
2 Broome, J.D. (1961) Nature 191, 1114—1115
3 Wriston, Jr., J.C. (1971) The Enzymes, 3rd edn., 4, 101—121
4 Wriston, Jr., J.C. and Yellin, T.O. (1973) Adv. Enzymol. 39, 185—248
5 Ehrman, M., Cedar, H. and Schwartz, J.H. (1971) J. Biol. Chem. 246, 88—94

6 Cleland, W.W. (1977) Adv. Enzymol. 45, 273—387
7 Boyd, J.W. and Phillips, A.W. (1971) J. Bacteriol. 106, 578—587
8 Howard, J.B. and Carpenter, F.H. (1972) J. Biol. Chem. 247, 1020—1030
9 Cleland, W.W. (1967) Adv. Enzymol. 29, 1—32
10 Jencks, W.P. (1968) in Handbook of Biochemistry, (Sober, H.A., ed.), p. J-161
11 Herrmann, V., Röhm, K-H. and Schneider, F. (1974) FEBS Lett. 39, 214—217
12 Makino, H. and Inada, Y. (1973) Biochim. Biophys. Acta 295, 543—548
13 Menge, U. and Jaenicke, L. (1974) Z. Physiol. Chem. 355, 603—611
14 Homer, R.B. and Allsopp, S.R. (1976) Biochim. Biophys. Acta 434, 100—109
15 Peterson, R., Richards, F.F. and Handschuhmacher, R.E. (1977) J. Biol. Chem. 252, 2072—2076
16 Ho, P.P.K., Milikin, E.B., Bobbitt, J.L., Grinnan, E.L., Burck, P.J., Frank, B.H., Boeck, L.D. and Squires, R.W. (1970) J. Biol. Chem. 245, 3708—3715