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Identification of a Carboxyl group at the Active Site of Barley Malt Phosphatase

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Identification of a Carboxyl group at the Active Site of Barley Malt Phosphatase[1]

Biochemistry 512 Class of 1965[2]

(D. J. Graves[3], Instructor)

Abstract. The dependence of the maximal velocity, V_M, with pH for barley malt phosphatase has been studied from pH 4.0 to 5.4. The data show that an ionizable group in the enzyme-substrate complex with a pK_a of 4-4.6 is important for enzymic activity. These results suggest strongly the involvement of a carboxyl group of a glutamic or aspartic acid residue at the active site of this enzyme.

Introduction

To understand the mechanism of enzymic catalysis, it is essential to determine what amino acid residues of the enzyme are involved in the interaction with substrate and to delineate how this interaction results in the formation of free enzyme and product. Although we know rather little about the latter, much information is presently being accumulated about the nature of amino acid residues at the active sites of various enzymes. One approach to this problem is through the study of enzyme kinetics and the pH dependence of the kinetic parameters, K_M and V_M. The particular advantages of this probe are that pure enzyme is not essential for this study and the experi-

[1] This research was supported by Science Education (701-04-30) from the College of Science and Humanities, Iowa State University.

[2] This work was completed during the tenure of the Biochemistry 512 laboratory class, March 9 - May 28, 1965. Coauthors on this paper are: F. X. Aherne, G. A. Bennett, D. L. Berner, M. E. Burke, N. E. Busch, W. Carrothers, V. K-P. Chan, A. R. Conradie, J. D. Daniels, F. L. Dunn, T. L. Fisher, L. T. Frobish, M. W. Ganfield, M. E. Hallahan, D. C. Hillls, L. A. Hodgin, J. R. Howard, D. J. Hume, J. L. Huntington, R. R. Husted, J. W. Isenhart, R. J. Johnson, W-H. Kao, P. J. Killos, R. D. F. Lachica, J. C Linden, D. K. Nelson, S. C. Patnode, M. C. Qureshi, R. S. Rambo, L. F. Richardson, R. J. Rose, R. A. Sair, S. Scharffenberg, M. L. Shonka, R. L. Smith, F. B. Stifel, G. E. Treadwell, Jr., Y. Tsuda, J. L. Wise, Jr., M. Feider.

[3] Recepient of a Research Career Development Award of the U.S. Public Health Service (1-K3-GM-6753-01).

mental procedures for such research are relatively simple. The major disadvantage is that this procedure results only in pK values and not functional groups. Although the assignment of a functional group from enzyme kinetic data is complicated by the fact that pK values for amino acid residues in proteins may vary with environment (1), these assignments have been in most instances corroborated by other studies (2).

The present work is with barley malt phosphatase, an enzyme which has a pH optimum of approximately 5.3 (3), and indicates that loss of enzymic activity on the acid limb of the pH activity curve is due to the protonation of a carboxyl group in the enzyme-substrate complex.

Materials and Methods

Disodium phenylphosphate and Malt diastase (phosphatase) were purchased from Sigma Chemical Co., St. Louis, Missouri. All other chemicals were reagent grade and obtained at the chemistry stockroom at Iowa State University.

Enzyme (50 gms) was suspended in 500 mls of distilled water and then centrifuged to remove insoluble matter. The supernatant fluid was dialyzed against three changes of cold distilled water (100 ml each) and stored frozen. The adjustment of pH of buffers and substrates were carried out on pH meters with a precision of ±0.01 pH unit. For the determination of maximum velocity, V_M, at any pH, the following reaction mixtures were prepared: 8 mls of 0.01M ethylenediamine citrate buffer, 1 ml of disodium phenylphosphate (10^{-1}M — 5 x 10^{-3}M), and 1 ml of enzyme. At various intervals 1 ml aliquots were removed and added to 8 ml of 10% Na_2CO_3 to quench the reaction, after which 1 ml of Folin's reagent (4) was added for the detection of liberated phenol. After 10 minutes the developed colors were read in a Klett colorimeter utilizing a #66 filter. For each pH, initial velocities were determined for five different substrate concentrations by construction of a tangent at t_0 for each of the progress curves. The maximum velocity was then deduced by extrapolation of a double reciprocal plot of Lineweaver and Burke (5) to the velocity at infinite substrate concentration.

All kinetic experiments were performed in constant temperature water baths with a temperature control of ±0.1°C.

Results and Discussion

The dependence of the V_M of barley malt phosphatase with pH is illustrated in Figure 1. According to Dixon and Webb (6),

V_M may be expressed by: $$V_M = \frac{ke}{1 + \frac{H}{K_{ES_1}} + \frac{K_{ES_2}}{H}}$$

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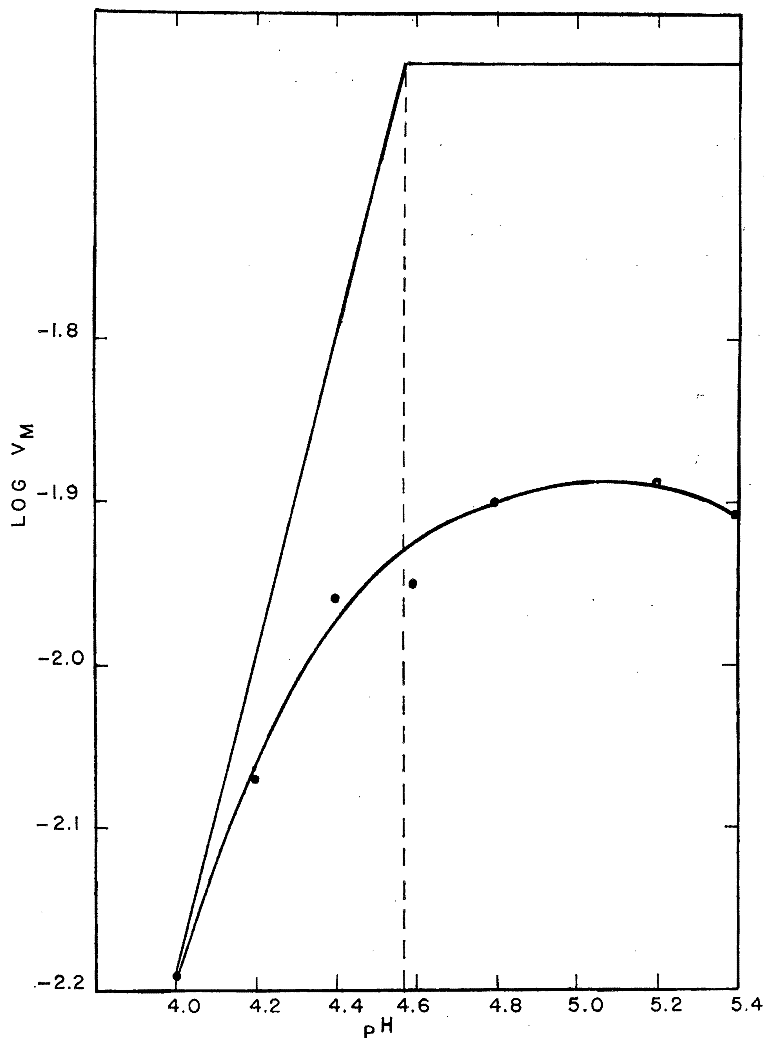

Figure 1. Dependence of the maximal velocity with pH at 37° for barley malt phosphatase.

and by analysis of log V_M curves against pH, the dissociation constants K_{ES_1} and K_{ES_2} for functional groups in the enzyme-substrate complex of equation 1 may be determined. The constant, k, is a rate constant for the decomposition of the ES complex to enzyme and product and e is the total enzyme concentration. The acid limb of the pH activity curve may be expressed by:

$$\log V_M = \log ke + pH - pK_{ES_1}$$

The data show that the slope of the experimental curve ap-

proaches unit slope at pH's below 4.5 as predicted by the above equation. If the loss of activity on the acid limb was due to an unfolding of the enzyme molecule rather than due to a protonation of a specific amino acid residue, it might be expected that the experimental slope would be greater than unity. Davis and Metzler (7) observed a slope of approximately three for dependence of K_M with pH for threonine dehydrase and postulated a reversible denaturation for loss of enzymic activity.

For the analysis of pK_{ES_1}, Dixon and Webb (6) showed that from equation 1 the theoretical curve of unit slope should intersect a curve of zero slope and this intersection point should occur 0.3 units above the experimental curve. These data show that a functional group with a pK of 4.5-4.6 is implicated at the active site of barley malt phosphatase (Figure 1).

In two other experiments of 37° pK values of 4.4 and 4.6 were obtained. Since the β-carboxyl and γ-carboxyl group of aspartic and glutamic acid residues, respectively, have pK values from 3-4.7 (6) and no other functional groups in proteins have pK values in this range, it would appear that the observed variation of V_M with pH for malt phosphatase is due to the ionization of a carboxyl group. Similar conclusions were reached by Alvarez (8) with potato phosphatase. Although the mode of participation of this residue in phosphate ester hydrolyzis is unknown, study of models for enzymic reactions shown that carboxylate groups stimulate ester hydrolysis (9). In one case a cyclic anhydride has been isolated as an intermediate for intramolecular catalysis by a carboxylate group (10).

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