INHIBITION STUDIES OF THE ENANTIOMERS OF β CHLOROALANINE ON PURIFIED ALANINE RACEMASE FROM B. SUBTILIS

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SUMMARY

Detailed kinetic studies of the inhibition of Alanine Racemase (5.1.1.1.) by the enantiomers of β chloroalanine reveal that the two enantiomers inhibit the enzyme at strikingly different levels and by different mechanisms. D chloroalanine inhibits at very low concentrations (K_i = .005 mM) in a competitive fashion. L chloroalanine inhibits only at much higher concentrations (K_i = 1.71 mM) and in a noncompetitive fashion. From these results it is postulated that the active site of the Alanine Racemase reacts asymmetrically with the enantiomers of the substrate and has a conformation which greatly favors the D enantiomer.

INTRODUCTION

Previous work in this laboratory has been carried out on the purification and mechanism of action of Alanine Racemase from B. subtilis (1-3). Dunathan first suggested on a theoretical basis that pyridoxal phosphate catalyzed racemase may react preferentially with one of the enantiomers (4). Kinetic studies of the racemization reaction and the α hydrogen exchange reaction with D_2O with each of the two enantiomers of alanine have lead us to postulate that the D alanine is the more natural enantiomer in its interaction with the enzyme since the D enantiomer has a smaller K_m, a smaller V_m, and a more rapid α hydrogen exchange than the L enantiomer (3,5).

Although a distinct difference in the ability of the active site of the enzyme to interact with the D alanine enantiomer is thus established, the rapid interconversion of the enantiomers at the active site limits the extent to which one can determine the asymmetry of the enzyme for the two enantiomers by using the natural substrate. Manning has shown that both the

D and L isomers of β chloroalanine inhibit the growth of several bacteria by inactivation of the Alanine Racemase required for their cell wall synthesis (6). We have found that β chloroalanine inhibits the enzyme catalyzed α hyrodgen exchange reaction. Furthermore, β chloroalanine does not exchange its α hydrogens in the presence of the racemase. Since, β chloroalanine inhibits Alanine Racemase and is not racemized itself, we initiated a more detailed study of the reactions of the two enantiomers of β chloroalanine with Alanine Racemase to ascertain the binding affinities of the D and L forms separately and thus deduce more about the asymmetric nature of the active site of the enzyme.

The kinetic studies reported here reveal an inhibition constant for L chloroalanine 342 times greater than for the D as well as different mechanisms of inhibition for the two enantiomers (competitive for the D and noncompetitive for the L).

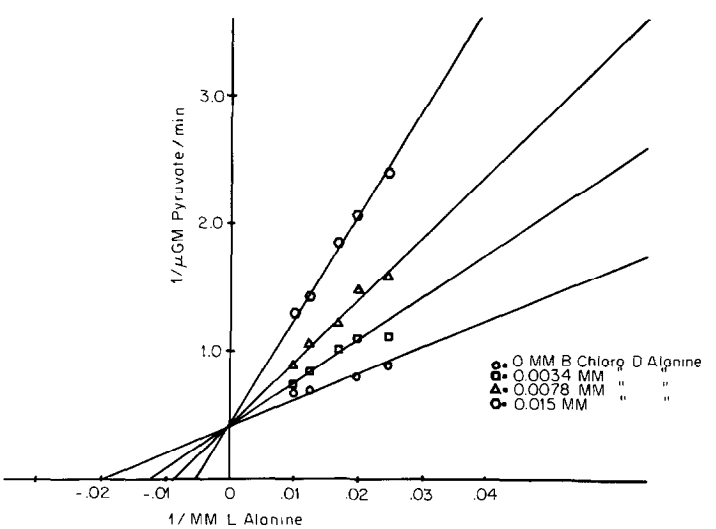

FIGURE 1. Inhibition of the racemization of L alanine by Alanine Racemase from B. Subtilis by varying concentrations of β chloro D alanine. The reaction mixtures of total volume 1.25 ml contained 0.25 ml of a solution of 0.02% pyridoxal phosphate and 0.051 M glutathione, 0.50 ml of 0.1 M phosphate buffer pH 8.1, and 0.25 ml of a solution of β chloro D alanine and L alanine of appropriate concentrations. After initiating the reaction by addition of the racemase, the mixtures were incubated at 37°C. At varying intervals of time, samples were inactivated by heating in a boiling water bath for 10 minutes. The extent of racemization was determined as indicated in methods section.

MATERIALS AND METHODS

Alanine Racemase was obtained from B. subtilis after ammonium sulfate precipitation, followed by CM-Sephadex ion exchange chromatography and Sephadex G-100 gel chromatography. The details of purification will be reported elsewhere. The β chloroalanine was obtained from Cyclo Chemical and the other materials were the same as previously described (1,2). The L-chloroalanine, the D chloroalanine and the DL chloroalanine show identical IR and NMR spectra.

The activity of Alanine Racemase was measured by the method of R. B. Johnston (1). The amount of D alanine formed from L alanine as a result of racemization was determined by the D Amino Acid Oxidase (1.4.3.3.) method (1,2).

RESULTS

The results of studies with purified Alanine Racemase from B. subtilis demonstrate that D chloroalanine is a much more potent inhibitor of the enzyme than L chloroalanine. Kinetic studies of the two enantiomers of β chloroalanine were carried out on the reaction proceeding in the L alanine to D alanine direction, at varying concentrations of inhibitor. The initial

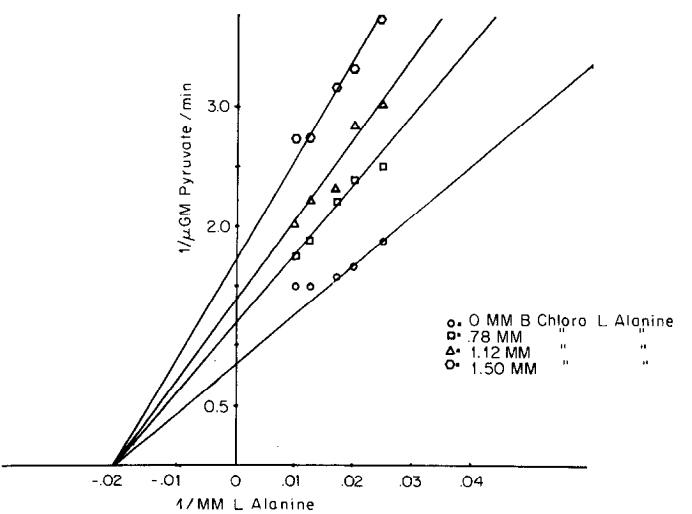

FIGURE 2. Inhibition of the racemization of L alanine by Alanine Racemase from B. Subtilis by varying concentrations of β chloro L alanine. Conditions and methods same as figure 1.

velocities were calculated then plotted as their reciprocals against the reciprocals of L alanine concentrations. These plots revealed a competitive type pattern of inhibition by the D chloroalanine (Fig. 1). A noncompetitive type pattern of inhibition by the L chloroalanine is shown in Fig. 2. K_i values were obtained by replotting intercept against inhibitor concentration for the L chloroalanine and slope against inhibitor concentration for the D chloroalanine (Fig. 3). The extrapolated values as indicated correspond to a K_i of .005 mM for the D chloroalanine and 1.71 mM for L chloroalanine. Thus the enzyme exhibits a very strong affinity for the D form of the inhibitor and this can be displaced by the substrate. The inhibition constant for the L enantiomer is 342 times greater than for the D.

DISCUSSION

The different patterns of inhibition for the D and L forms of β chloroalanine, competitive for the D and noncompetitive for the L, was an unexpected observation. At the higher concentration at which the L-chloroalanine inter-

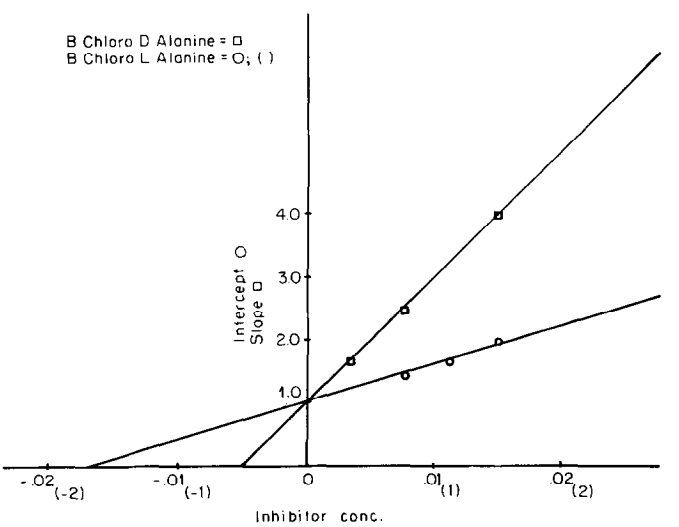

FIGURE 3. Determination of inhibitor constants. Slopes (for β chloro D alanine) and intercepts (for β chloro L alanine) are those obtained from the respective Lineweaver-Burke plots.

acts with the enzyme there is no assurance that the interaction of the L form of the inhibitor with the enzyme is at the active site or is bound to the enzyme in an analogous fashion as the L alanine.

The significance of the strong preference of the enzyme for the D enantiomer is at the present not clearly understood. However, this characteristic feature of the enzyme may have an important role in the regulation of cell wall synthesis or the germination of bacterial spores. The difference could suggest that the mode of action of inhibition at the relatively higher levels of concentration required by the L enantiomer may be different than the mode of action of the D enantiomer at low concentrations. Thus not only do we observe a striking difference in the concentration of the two enantiomers required to inhibit the enzyme but also the mechanism of inhibition may be different for the two enantiomers.

One simplified model which we have used in our interpretation of the mechanism of action could be called a swinging door mechanism (Fig. 4). In this system, the planar pi system of the Schiff base complex with the pyridoxal

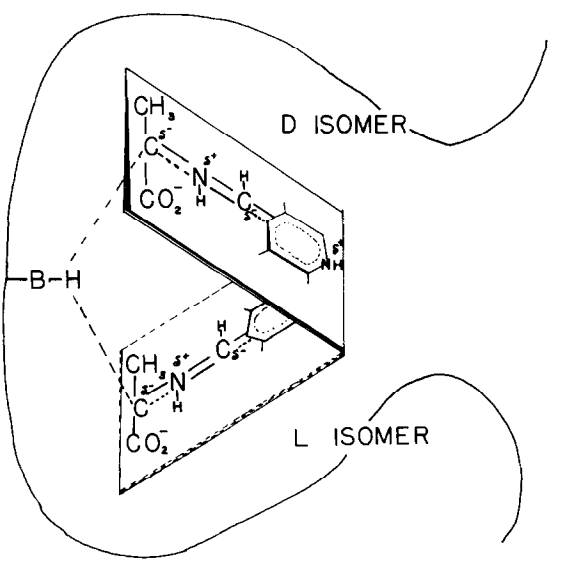

FIGURE 4. Schematic representation of the "Swinging Door" mechanism of alanine racemase.

phosphate which extends to the α hydrogen of alanine would swing on both sides of the point of protonation and deprotonation. The position of the door relative to the point of protonation will determine whether a D or L configuration is interacting with the enzyme. This model could easily fit into our present understanding of the mechanism of alanine racemase. One position of interaction of the planar system with the enzyme could be more stable, hence exhibiting a stronger binding of the substrate, a more rapid α hydrogen exchange with the solvent and a slower rate of racemization - this would correspond to the D alanine enantiomer. The L alanine form, however, could have less stably bound planar system to the enzyme, hence as soon as the α hydrogen was exchanged it would preferentially go to the more stable D form. Thus the D alanine would exchange at a much faster rate than it racemizes and the L alanine would racemize at about the same rate it exchanges. The conformation of the enzyme is such that it shows a preference for the D form and thus not easily interact with the L form of the inhibitor.

Manning observed that D chloroalanine is much more potent in inhibiting the growth of microorganisms than L chloroalanine (6). Our studies revealing the much stronger interaction of D chloroalanine with Alanine Racemase could explain his results.

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