L-Homoarginine

AN ORGAN-SPECIFIC, UNCOMPETITIVE INHIBITOR OF HUMAN LIVER AND BONE ALKALINE PHOSPHOHYDROLASES*

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SUMMARY

It is known that L-homoarginine fails to inhibit human intestinal and placental alkaline phosphohydrolases under conditions which produce inhibition of bone and liver isoenzymes. Now, the mechanism of inhibition by L-homoarginine of human liver and bone alkaline phosphatases has been investigated. Results of inhibition by L-homoarginine were very similar for these two isoenzymes and resembled other organ-specific inhibitors of alkaline phosphatase isoenzymes; L-phenylalanine and L-tryptophan which, however, inhibit intestinal and placental alkaline phosphohydrolases but not liver and bone isoenzymes. Thus, the inhibition by L-homoarginine was independent of pH, but dependent on substrate concentration up to 5 mM. Further increase of substrate concentration, however, had little effect on the inhibition. The double reciprocal plots of 1/v versus 1/s at various concentrations of L-homoarginine showed a series of parallel lines indicating that the mechanism of inhibition was "uncompetitive." Partial denaturation of the enzyme with heat, urea, or papain digestion had no effect on the inhibition but a shift of the optimum temperature for maximum velocity to a higher temperature, from 37-45°C for bone isoenzyme and 39-45°C for liver isoenzyme, was observed when the inhibitor was present in the enzyme digest. Hyperbolic curves were obtained when the percentage of inhibition was plotted against substrate concentration at different temperatures. The n value which is the apparent number of inhibitor molecules combining with one active site of the enzyme molecule was calculated to be 1.02 to 1.04 for bone and 0.90 to 0.94 for liver alkaline phosphatases.

Previous studies on organ specific inhibitors for alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) isoenzymes have yielded two amino acids, L-phenylalanine and L-tryptophan which specifically inhibit placental and intestinal alkaline phosphatases (1-3). The nature of action of these two inhibitors has been found to be very similar; both exhibit stereospecific (i.e. only the L-isomers are active inhibitors), uncompetitive, and nonallosteric inhibitions (4-7).

Now another amino acid, L-homoarginine, has been found to have just the opposite specificity; i.e. to be a strong inhibitor for human bone and liver alkaline phosphatases and to exert virtually no effect on intestinal and placental isoenzymes (3, 8).

The significance of these organ-specific inhibitors of alkaline phosphatase has been intensively explored in the area of serum enzymology applied to the differential diagnosis of disease (9-11). The understanding of the inhibition mechanism, therefore, is important for the design and discovery of stronger or even more specific organ-specific inhibitors of alkaline phosphatase isoenzymes.

It is the primary interest of this study to determine the nature of this inhibition. It was particularly interesting to ascertain that the same type of inhibition which has been observed for the inhibitions by L-phenylalanine and L-tryptophan also occurs in the action of L-homoarginine against bone and liver alkaline phosphatases. Accordingly, in this study, a series of experiments similar to those performed in studies on inhibitions by L-phenylalanine and L-tryptophan were conducted on the L-homoarginine inhibition of both bone and liver alkaline phosphatases.

EXPERIMENTAL PROCEDURE

MATERIALS—Enzyme preparations used in this study were purified from human tissues according to the method of Portmann et al. (9) which included n-butanol extraction of the tissue homogenates followed by ammonium sulfate fractionation (50 to 70% saturation). The preparations have specific activities of 10.5 units per mg of protein for bone, 0.17 units per mg of protein for liver alkaline phosphatase. The unit of enzyme activity is defined as micromoles of substrate (18 mmoles disodium phenylphosphate) hydrolyzed per min at 37°C in 50 mM carbonate-bicarbonate buffer, pH 9.8.

L-Homoarginine and crystalline bovine serum albumin were both obtained from Nutritional Biochemicals Corporation. Disodium phenyl phosphate was obtained from Fisher Scientific Co.

Enzyme Assay—The method of enzyme assay was the same

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as described in our earlier communications (4, 7), which used 18 mm disodium phenyl phosphate as substrate and 50 mm sodium carbonate-bicarbonate buffer at pH 9.8 and which gave linear initial velocities. The enzyme reaction was terminated by adding an equal volume of 1.5 M formaldehyde to the digest. The liberated phenol was measured as described by Stolbach et al. (13) using aminoantipyrine-ferricyanide reagents (14) instead of a diazotized dye for developing the colored product. The absorbance of the pink color produced was measured at 505 nm with a Coleman spectrophotometer.

To obtain a suitable enzyme activity for assay, bone enzyme preparation was diluted 1:30 and liver 1:15 with 0.1% bovine serum albumin. The albumin at this concentration stabilized the alkaline phosphatase and did not contribute any phosphatase activity (12, 13).

The concentration of the inhibitor L-homoarginine used in most of the assays was 8 mM. The percentage inhibition by L-homoarginine was based on non-homoarginine controls carried out under the same conditions. We were unable to obtain the D isomer of homoarginine at the time when this investigation was conducted which we would have added to the control digests.

RESULTS

Effects of L-Homoarginine Concentration—The organ-specificity of the inhibition by L-homoarginine (Fig. 1) shows the effects of inhibitor concentration (from 2 to 10 mM) on the percentage of inhibition of L-homoarginine on alkaline phosphatase isoenzymes of bone, liver, intestine, and placenta. At 8 mM L-homoarginine, which is the concentration used for the present study, 80% of the activities of both bone and liver isoenzymes were inhibited as compared with less than 10% for placental and intestinal isoenzymes.

Studies on Influence of pH—The effect of pH on inhibition by L-homoarginine (Fig. 2) indicated that the inhibition of liver, bone, and intestinal isoenzymes was relatively pH independent, while that of placental isoenzyme was pH dependent. The inhibition of placental enzyme increased from 5 to 30% when the pH was raised from 9.8 to 10.4. At pH 9.8 (all of the enzyme assays of this study were carried out at this pH), the inhibitions of placental and intestinal isoenzymes were at the minimum, and a maximum differential inhibition between liver-bone type and placental-intestinal type of alkaline phosphatase was observed.

Effect of Substrate Concentration—Fig. 3 shows the identical effect of substrate concentration, ranging from 0.5 to 40 mM, on the inhibition for both bone and liver alkaline phosphatases. The percentage of inhibition was substrate dependent at high substrate concentration also eliminates the
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FIG. 4. Double reciprocal plots of velocity against substrate concentration at different concentrations of L-homoarginine for bone and liver alkaline phosphatases. Enzyme assays were performed at 37° with phenylphosphate concentration varying from 0.33 to 4.0 mM.

1/ν versus 1/s at various concentrations of the inhibitor, a series of parallel straight lines was obtained for both bone and liver alkaline phosphatases (Fig. 4) indicating "uncompetitive" inhibition.

The inhibitor constant (K_i) for the inhibition can also be obtained from this study by plotting 1/\[V_{\text{max}}\]_i versus [L-homoarginine], where 1/\[V_{\text{max}}\]_i is the extrapolated \[V_{\text{max}}\] value at various inhibitor concentrations. The intercept at the axis where 1/\[V_{\text{max}}\]_i equals 0 is 1/K_i. The values of 1/\[V_{\text{max}}\]_i were obtained from Fig. 4 by extrapolating the Lineweaver-Burk plots; the intercepts at the vertical axis were the 1/\[V_{\text{max}}\]_i values for various concentrations of the inhibitor. The plots of 1/\[V_{\text{max}}\]_i versus [L-homoarginine] for both bone and liver alkaline phosphatase are shown in Fig. 5. The K_i values obtained from the plot were 0.36 mM for bone and 0.22 mM for liver alkaline phosphatase, respectively.

Effects of Heat, Urea, and Papain Digestion on Inhibition—In examining the effect of heat denaturation on the inhibition, enzyme preparations were diluted with water to a suitable concentration and heated at 55° in a water bath; aliquots were removed at different time intervals and immediately cooled in ice to stop the heat denaturation process. Activities of the heated enzyme samples were then assayed along with the unheated control samples, with or without 8 mM L-homoarginine. The results showed that both bone and liver alkaline phosphatases progressively lost their activities correlating with the duration of heating while the percentage inhibition by L-homoarginine remained fairly constant (Fig. 6).

Effects of urea on enzyme activities and inhibition by L-homoarginine were examined by incubating the enzyme preparations in the presence of urea at 37°. At various time intervals of the incubation, portions of the enzyme-urea mixture were withdrawn, diluted with cold water, and immersed in the ice bath to stop the denaturation. The effects of urea (3 mM for bone, 4 mM for liver isoenzymes) on enzyme activity and inhibition by L-homoarginine (Fig. 7) were similar to those obtained from the heat denaturation study in that both isoenzymes were progressively inactivated by incubating with urea, while the percentage inhibition by L-homoarginine remained constant.
Effects of urea on enzyme activity (o) and inhibition by l-homoarginine (◇) of bone and liver alkaline phosphatases. Urea concentrations were 3 M for bone and 4 M for liver isoenzymes.

FIG. 7. Effects of urea on enzyme activity (o) and inhibition by l-homoarginine (◇) of bone and liver alkaline phosphatases. Urea concentrations were 3 M for bone and 4 M for liver isoenzymes.

The effects of digestion with papain on liver alkaline phosphatase and inhibition by l-homoarginine are shown in Fig. 8. In this study, 0.4 ml of liver enzyme preparation was diluted with 0.6 ml of water and incubated with 1.0 ml of papain-glutathione solution (50 mg of crystalline papain in 5 ml of 0.4 M glutathione) at 37°. An aliquot (0.2 ml) of this enzyme-papain mixture was withdrawn at various time intervals, diluted with 1.4 ml of cold water to stop the digestion, and assayed for phosphatase activity with or without 8 mM l-homoarginine. It was found that 50% of the enzyme was inactivated after 45 min of incubation with papain. The percentage of inhibition by l-homoarginine was not affected by papain digestion, registering 78% throughout the 45 min of incubation.

Attempts to digest bone alkaline phosphatase with papain failed. A precipitate formed upon the addition of the papain solution to the enzyme preparation and the enzyme activity remained unchanged at the end of incubation, indicating that a contaminant interfered with papain action.

Effects of l-Homoarginine Inhibition on Optimum Temperature for Maximum Velocity—The effect of inhibition by l-homoarginine on the optimum temperature for $V_{\text{max}}$ (the temperature where the highest $V_{\text{max}}$ value is observed) of both bone and liver alkaline phosphatases was studied. The $V_{\text{max}}$ values were measured as described in Fig. 4 as a function of temperature ranging from 5 to 50°, in the presence or absence of 8 mM l-homoarginine. Fig. 9 showed that l-homoarginine caused a shift in the optimum temperature of $V_{\text{max}}$ from 37-45° for bone isoenzyme and 37-45° for liver isoenzyme. This shift toward a higher optimal temperature for $V_{\text{max}}$ in the presence of the inhibitor indicates that the binding of the inhibitor to the enzyme-substrate complex may cause a change in the enzyme molecule to a more thermostable conformation or the inhibitor may stabilize the enzyme in its active form.

Inhibition by l-Homoarginine as Function of Inhibitor Concentration at Different Temperatures—The inhibition by l-homoarginine was further studied by examining the effect of l-homoarginine concentration (ranging from 0.5 to 80 mM) on the inhibition at four different temperatures: 25°, 37°, 42°, and 50°. Plots of the resulting percentage of inhibition against l-homoarginine concent...
centration for each temperature gave an apparent hyperbolic curve in each case (Fig. 10). It is also observed from this finding that the extent of inhibition by L-homoarginine decreased with increasing temperature.

When log \( \left( \frac{V_0 - v}{v} \right) \) was plotted against the log [L-homoarginine] where \( v \) is the enzyme activity in the presence of 8 mM L-homoarginine with 18 mM of phenylphosphate as substrate, and \( V_0 \) the activity without the inhibitor at the same condition, a series of parallel straight lines was obtained (Fig. 11). According to the data of Taketa and Pogell (15), the slopes of these lines are equal to the \( n \) value, which is the apparent number of inhibitor molecules combining with 1 molecule of enzyme assuming only 1 active site per molecule. The slopes obtained from Fig. 11 ranged from 1.02 to 1.04 for bone alkaline phosphatase and 0.90 to 0.94 for the liver isoenzyme at different temperatures studied. From these data one therefore concludes that approximately 1 molecule of L-homoarginine combines with 1 active site of the bone or liver enzyme during the inhibition reaction.

**DISCUSSION**

The experimental data demonstrate that the organ-specific inhibition by L-homoarginine of bone and liver alkaline phosphatase is uncompetitive as indicated by the observations that high substrate concentration had no effect on the inhibition and that the double reciprocal plot of \( 1/e \) versus \( 1/s \) showed a series of parallel lines.

The L-homoarginine inhibition marks the third example of amino acid organ-specific inhibition observed with alkaline phosphatase isoenzymes. The other two are the inhibitions by L-phenylalanine and L-tryptophan of placental and intestinal alkaline phosphatases (4-7). All of the three inhibitions exhibit organ-specific inhibitors and the possibility that a hydrophobic site exists in the isoenzymes of alkaline phosphatase is uncompetitive in nature. This suggests that the same mechanism will possibly occur in the inhibition by L-homoarginine.

The observation that the optimum temperature for maximum velocity was increased in the presence of the inhibitor is consistent with the homosteric mechanism defined by McElroy et al. (17), who suggested that a conformational change occurred in the enzyme protein as a result of the binding of a specific modifier to the active site and this led to a modification of the reaction of the bound intermediate. The effect of L-homoarginine on the conformation of alkaline phosphatase has not yet been examined.

In certain cases of competitive inhibition, the enzyme active site and the allosteric site respond independently to denaturation. Thus, papain treatment can remove the inhibition of AMP on liver fructose 1,6-diphosphatase while it retains its enzyme activity (15). In our experiments with papain, for example, the enzyme loses its activity but not its ability to be inhibited by L-homoarginine. However, since partial inactivation can result from an incomplete denaturation of each molecule, or complete denaturation of a portion of the molecules, a conclusion that the enzyme is not allosteric cannot be drawn solely from our observations with papain.

The significance of the hydrophobic ring or side chain of the organ-specific inhibitors and the possibility that a hydrophobic site exists in the isoenzymes of alkaline phosphatase have been connected by Fishman and Sie (3). The structure of the hydrophobic site of the enzyme then becomes an important factor in determining the organ specificity of the inhibition by different inhibitors. The hydrophobic region of the inhibitor must be position timed close to the active center to hinder its catalytic action but may not permit the substrate to compete with it.

From the results of the kinetic studies of the three organ-specific inhibitors on four different alkaline phosphatase isoenzymes discussed above, it is interesting to observe that all the inhibitors are uncompetitive in nature. This suggests a possible correlation among hydrophobic bindings, uncompetitive inhibition mechanism, and organ-specific inhibitions.

**REFERENCES**

I-Homoarginine: AN ORGAN-SPECIFIC, UNCOMPETITIVE INHIBITOR OF HUMAN LIVER AND BONE ALKALINE PHOSPHOHYDROLASES
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