Inhibition of Alkaline Phosphatase by L-Phenylalanine

By H. N. FERNLEY and P. G. WALKER

Department of Biochemistry, Institute of Orthopaedics, Stanmore, Middx., U.K.

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In a survey of possible selective inhibitors for mammalian tissue alkaline phosphatases, Fishman, Green & Inglis (1962) reported that L-phenylalanine specifically inhibited the intestinal enzyme but had little or no effect on enzymes from several other rat tissues. It was later shown (Fishman, Green & Inglis, 1963) that phenylalanine had a similar effect on human tissue phosphatases: of the enzymes from bone, intestine, kidney, liver and lung only the intestinal one was affected and only the L-isomer was effective. Further work (Ghosh & Fishman, 1966; Fishman & Ghosh, 1967) established that placental phosphatase was also sensitive to phenylalanine and that the inhibition was of an 'uncompetitive' nature. It was suggested that an inactive ESI complex was formed by the stereospecific combination of L-phenylalanine with the Michaelis complex (ES).

This communication reports some observations with a purified human placental phosphatase indicating that combination with phenylalanine has little effect on the reactivity of ES but prevents the breakdown of a phosphoryl-enzyme intermediate, EX, formed later in the hydrolysis sequence:

where E is free enzyme, S the monester substrate, ES the Michaelis complex, P₁ the non-phosphate product and P₂ inorganic phosphate. The various *k* values are rate constants for the operative steps. This scheme is known to apply under acid conditions (Fernley & Bisaz, 1968), but has not previously been shown to apply under alkaline conditions, where *k*₊₃ is normally greater than *k*₊₂ (Fernley & Walker, 1967).

Alkaline phosphatase was extracted and purified from a single placenta (type S1; Robson & Harris, 1967) by the procedure of Harkness (1968). A 40–60% saturated-ammonium sulphate fraction was obtained, estimated to contain 7–8% of alkaline phosphatase on the basis of one active site per dimer molecule (Gottlieb & Sussman, 1968). D- and L-Phenylalanine were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.,

and used without further purification; 4-methylumbelliferyl phosphate was synthesized as previously described (Fernley & Walker, 1965).

Fig. 1 compares the time-courses of methylumbelliferone liberation by the enzyme in the presence of D-phenylalanine and of L-phenylalanine. The traces were obtained with a stopped-flow apparatus (Gibson & Milnes, 1964) modified for fluorescence measurements as described by Fernley & Bisaz (1968). With D-phenylalanine there is no inhibition and one sees only the steady-state phase of the reaction, the usual observation above pH 8. With L-phenylalanine, however, there is an additional transient phase for which a plot of log Δ*P*_{*t*} (the difference in signal between the observed value and the extrapolated steady-state line at time *t*) against time is used to derive an initial burst value, Δ*P*₍₀₎, and a first-order rate constant, *k*_{*t*} (Fernley & Walker, 1966). Titration of the active sites at pH 4.4

Fig. 1. Reaction of alkaline phosphatase with phenylalanine at pH 8.81 and 20°C. Enzyme (200 μg of protein/ml) in 0.02 *I* tris-acetic acid containing 40 mM-phenylalanine was mixed with 10 μM-4-methylumbelliferyl phosphate and 40 mM-phenylalanine in the same buffer. Trace A, D-phenylalanine; trace B, L-phenylalanine. The signal is fluorescence of released 4-methylumbelliferone and 100 mV corresponds to 0.097 μM-product. The electronic time constant was 1 ms. The dashed section of trace B is an extrapolation of the steady-state line. In the inset log Δ*P*_{*t*} values are plotted against time, indicating that the reaction is first-order for at least 90% of the total burst. Parameters describing the traces are: A, steady-state rate, 1.03 μM/s; B, steady-state rate, 0.098 μM/s; Δ*P*₍₀₎, 0.041 μM; *k*_{*t*}, 16 s⁻¹. Other details are given in the text.

(Fernley & Bisaz, 1968) gave a value of $0.120\ \mu\text{M}$ for this enzyme preparation.

Steady-state studies under conditions similar to those employed in Fig. 1 indicated that inhibition by L-phenylalanine was strictly uncompetitive: percentage activities (V_{max} values) were 52, 35 and 21 for 0.5 mM-, 1.0 mM- and 2.0 mM-L-phenylalanine respectively; K_m in the absence of inhibitor was $5.6\ \mu\text{M}$. It was also established that the time-course was linear over the 30 min assay period. These experiments were carried out at pH 9.04, the lower pH in the stopped-flow experiment being due to the buffering action of the phenylalanine.

With regard to the above data, two types of mechanism can be formulated. (1) The transient state may represent a slow reaction between ES and inhibitor to give an inactive ESI complex, while ES transforms directly into E and P1, P2. This mechanism predicts that the transient phase will be of longer duration the lower the concentration of inhibitor I. (2) The transient state may represent phosphoryl-enzyme formation with ES transforming into EX: inhibitor may combine with E, ES or EX, but only EXI is inactive. Mechanism (2) predicts that the transient phase will be shorter the lower the concentration of inhibitor. The inhibitor acts by decreasing k_{+3} according to the relationship $k_{+3(\text{app.})} = k_{+3}/(1 + [I]/K_i)$, K_i being the lumped dissociation constant for the system EI, ESI, EXI. The exponential term (k_t) is equal to $k_{+2(\text{app.})} + k_{+3(\text{app.})}[k_{+2(\text{app.})} = k_{+2}/(1 + [S]/K_s)$; $K_s = (k_{-1} + k_{+2})/k_{+1}$ (Fernley & Bisaz, 1968)] and must increase as $k_{+3(\text{app.})}$ approaches k_{+3} . In addition mechanism (2) imposes a constraint on the magnitude of the burst, since this is now related to the active-site concentration $[E_0]$ by $\Delta P_{(0)} = [E_0](k_{+2(\text{app.})})^2/(k_{+2(\text{app.})} + k_{+3(\text{app.})})^2$. It is possible therefore to distinguish experimentally between these two mechanisms by varying the inhibitor concentration.

A stopped-flow experiment similar to that described in Fig. 1 but with 5 mM-L-phenylalanine and an enzyme preparation containing $0.138\ \mu\text{M}$ -active sites gave the following result: steady-state rate, $0.370\ \mu\text{M/s}$; $\Delta P_{(0)}$, $0.031\ \mu\text{M}$; k_t , $26\ \text{s}^{-1}$. The significant features are that the burst is both smaller and faster than with 40 mM-phenylalanine (cf. Fig. 1 legend). Further support for mechanism (2) is given by analysis of the data in terms of

phosphoryl-enzyme formation and breakdown. Thus values for $k_{+2(\text{app.})}$, $k_{+3(\text{app.})}$ and $[E_0]$ with 5 mM- and 40 mM-phenylalanine respectively are: $18\ \text{s}^{-1}$, $8\ \text{s}^{-1}$ and $0.064\ \mu\text{M}$ ($0.069\ \mu\text{M}$ by titration); $14\ \text{s}^{-1}$, $2\ \text{s}^{-1}$ and $0.054\ \mu\text{M}$ ($0.060\ \mu\text{M}$ by titration).

Confirmation that L-phenylalanine acts by changing the rate-limiting step might be obtained through the isolation of a phosphoryl-enzyme, by using ^{32}P -labelled substrate in conjunction with a suitable quenching technique. The sensitivity of certain tissue phosphatases to this reagent may be due not only to differences in affinity for phenylalanine but also to differences in the k_{+2}/k_{+3} ratio, and one would predict that for two substrates with different k_{+2} values under the same conditions inhibition would be more marked with the higher-activity substrate. An important use could be in investigating the action of other modifiers; for instance, Mg^{2+} ions activate to exactly the same degree with and without L-phenylalanine, suggesting that the effect is on $[E_0]$ rather than on k_{+2} or k_{+3} . Finally, one might speculate that phenylalanine inhibits by preventing access of water to its acceptor site on the phosphoryl-enzyme.

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