The Purification and Properties of Shikimate Dehydrogenase

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Shikimate dehydrogenase (EC 1.1.1.25) is a key enzyme in the synthesis of many aromatic compounds by plants and micro-organisms. There is, however, no known function for the enzyme in mammalian systems and it was therefore considered to be a desirable site for the rational design of novel herbicides.

The enzyme was isolated from etiolated epicotyls of *Pisum sativum* and purified 400-fold to a specific activity of 2.25 units/mg. Analogues of dehydroshikimate were synthesized that were powerful reversible inhibitors, but surprisingly only in the direction shikimate → dehydroshikimate. This kinetic investigation of the reaction mechanism was made to try to explain these results.

The initial-velocity pattern at 25°C at pH 8.0 in the absence of products was determined for both directions of the reaction and when plotted according to Lineweaver & Burk (1934) gave a family of straight lines intersecting at a point to the left of the ordinate. The K_m values determined by the graphical method of Florini & Vestling (1957) were 0.34 and 0.07 mM for shikimate and $NADP^+$ respectively and 0.36 and 0.28 mM for dehydroshikimate and NADPH respectively. The dissociation constants for $NADP^+$ and NADPH obtained from the initial-velocity data (Frieden, 1957) were 0.11 and 0.017 mM respectively.

The equilibrium constant in the direction dehydroshikimate → shikimate calculated from the above values was in good agreement with that obtained experimentally (6.25 and 6.5 respectively).

In order to obtain more specific information about the kinetic mechanism the product inhibition pattern was determined. For the direction shikimate → dehydroshikimate NADPH gave linear competitive inhibition with respect to $NADP^+$ (K_i 0.033 mM) and dehydroshikimate was a non-linear competitive inhibitor with respect to shikimate (K_i 0.25 mM). In the reverse direction $NADP^+$ gave linear competitive inhibition with respect to NADPH (K_i 0.072 mM) and shikimate was a linear mixed inhibitor with respect to dehydroshikimate (K_i 0.75 mM).

The results are compatible with an ordered Bi Bi reaction mechanism (Cleland, 1963) in which only $NADP^+$ and NADPH can add to free enzyme, but including two dead-end complexes (eqns. 2a and 3a) (E·X·Y represents a reactive ternary complex):

$$E + NADP^+ \rightleftharpoons E \cdot NADP^+ \quad (1)$$

$$E \cdot NADP^+ + \text{shikimate} \rightleftharpoons E \cdot X \cdot Y \quad (2)$$

$$E \cdot NADP^+ + \text{dehydroshikimate} \rightleftharpoons E \cdot NADP^+ \cdot \text{dehydroshikimate} \quad (2a)$$

$$E \cdot X \cdot Y \rightleftharpoons E \cdot NADPH + \text{dehydroshikimate} \quad (3)$$

$$E \cdot NADPH + \text{shikimate} \rightleftharpoons E \cdot NADPH \cdot \text{shikimate} \quad (3a)$$

$$E \cdot NADPH \rightleftharpoons E + NADPH \quad (4)$$

The poor inhibition by dehydroshikimate analogues in the direction of shikimate formation can be explained by such a mechanism.

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A Latent Collagenase Released by Bone and Skin Explants in Culture

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Neutral collagenase activity was assayed on native trypsin-resistant collagen containing [^{14}C]-glycine by the release of soluble radioactivity from reconstituted fibres or by the prevention of gel formation from dispersed ^{14}C-labelled collagen (Nagai, Lapiere & Gross, 1966). In contrast with observations made on other systems (Gross, 1970), no overt activity was found in culture media of mouse bone explants; however, considerable activity could be elicited by exposure of the media to trypsin. Chymotrypsin was 20–40-fold less effective than trypsin as activator. Purified liver lysosomes were active between pH 5 and 7. Skin explants also released latent collagenase.

Activation of bone enzyme followed a sigmoidal course, apparently not explainable by autoactivation. Excess of trypsin inactivated the enzyme. Total latent collagenase was estimated by extrapolation to zero time of the exponential inactivation curve. In absence of substrate latent enzyme was stable at 37°C near neutrality, whereas activated enzyme was rapidly destroyed. Collagenase showed a flat optimum at pH 7.5, required

Ca^{2+} and was inhibited by EDTA, cysteine and serum. EDTA-sensitive activity on insoluble tendon collagen and casein was also elicited by trypsin. Trypsin-activated bone collagenase chromatographed on Sephadex with an apparent molecular weight of 105000 and was slightly retarded with respect to the latent enzyme.

Some culture fluids contained a non-competitive thermostable collagenase inhibitor of high molecular weight (at least 120 000). The latent enzyme is, however, unlikely to be a simple combination of active collagenase with this inhibitor for the following reasons: (1) mixtures of enzyme and inhibitor are not reactivated by trypsin; (2) the decrease in molecular weight accompanying activation is much too small; (3) up to 100-fold dilution caused no activation whatsoever of latent enzyme; (4) on exposure to trypsin the inhibitor is apparently destroyed before significant activation of latent enzyme has occurred.

However, considerably more information is needed on this inhibitor and on the activation phenomenon before it can be stated that, as our results suggest, collagenase is released as an inactive proenzyme that is activated by limited proteolysis. In any case it appears that regulation of extracellular collagenase activity is achieved by complex interactions involving the production of latent enzyme, of activating enzymes (possibly via lysosomes) and of protein inhibitors.

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An Altered Erythrocyte Acetylcholinesterase Associated with Hereditary Muscular Dystrophy in Mice

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When the cholinesterases from the blood of normal and dystrophic Bar Harbor mice are compared the plasma pseudocholinesterases (EC 3.1.1.8) appear to be similar whereas the erythrocyte acetylcholinesterases (EC 3.1.1.7) show a number of differences: in the dystrophic mice both plasma and erythrocyte cholinesterase activities are about half the normal values.

Blood from normal and dystrophic mice was centrifuged to separate the plasma, which was used for investigation of pseudocholinesterase activity. The erythrocytes were used to prepare erythrocyte 'ghosts' (Dodge, Mitchell & Hanahan, 1963), from which acetylcholinesterase was solubilized with 0.5% Triton X-100 in 67mM-sodium phosphate buffer, pH7.4, the debris being centrifuged off.

In the assay method described by Das & Liddell (1970) acetylcholinesterase was found to hydrolyse acetylthiocholine but not butyrylthiocholine. Comparison of the Michaelis curves for the normal and 'dystrophic' enzymes showed that $V_{max.}$ was reached when the acetylthiocholine concentrations were 0.18 and 0.45mM respectively, yielding corresponding $V_{max.}/2$ values of 0.04 and 0.1mM. Lineweaver–Burk plots gave K_m values of 0.06 and 0.19mM for normal and 'dystrophic' respectively, the difference from $V_{max.}/2$ values reflecting the effect of inhibition by excess of substrate.

The two enzymes were also affected to different extents by several inhibitors. Eserine when added to the assay mixture to give a final concentration of 0.5μM has less effect on normal acetylcholinesterase activity but inhibited the 'dystrophic' enzyme by about 50%. Inhibition values obtained with other inhibitors were for the normal and 'dystrophic' preparations respectively: with 5μM-compound C51 [1,5-bis-(4-allyldimethylammonium phenyl)-pentan-3-one dibromide] 90 and 78%; with 6% butanol, 23 and 52%. Other inhibitors tested, 0.3mM-sodium fluoride, 0.01μM-compound RO2–0683 [dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)trimethylammonium bromide], 0.1mM-dibucaine hydrochloride and 10mM-nialamide hydrochloride, inhibited both enzymes to the same extent within the limits of experimental error.

Electrophoresis of normal mouse acetylcholinesterase on starch gels at pH 8.6 with a tris–citrate/borate buffer system (Watts & Moreland, 1970) revealed three or four enzymically active components. 'Dystrophic' preparations contained only one or two with slightly different electrophoretic mobility.

Criteria similar to those reported here, taken in conjunction with family or population studies (reviewed by Harris, 1970), have been used as evidence for genetic variants of the pseudocholinesterases. Because of the complexity of expression of hereditary muscular dystrophy a similar inference for acetylcholinesterase must be treated with caution until better characterization of the purified enzyme is possible.

The pseudocholinesterase of plasma from both normal and dystrophic mice was equally active towards acetylthiocholine and butyrylthiocholine. No significant differences could be detected with the inhibitors mentioned above, but electrophoresis revealed an additional band of low electrophoretic mobility in dystrophic-mouse plasma. This minor component probably originates in muscle.