Active-site-directed Inhibition of Triosephosphate Isomerase

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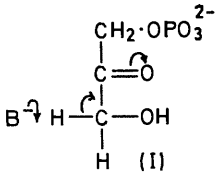
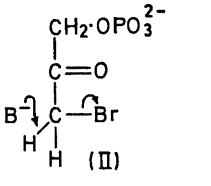
Summary Bromohydroxyacetone phosphate, a close analogue of the substrate dihydroxyacetone phosphate, rapidly and specifically inactivates triosephosphate isomerase leading to the incorporation of one mole of inhibitor per enzyme subunit of molecular weight 25,000.

TRIOSEPHOSPHATE isomerase (TIM) is a widely-distributed glycolytic enzyme which catalyses the isomerisation of D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The studies of Rose,[1] and of Topper[2] and their co-workers have suggested the intermediacy of an enediolate anion or related species formed by the abstraction of a proton from the 3-carbon atom (I) by a basic group on the enzyme. Most bases are also nucleophiles, and we therefore hoped that bromohydroxyacetone phosphate (BHAP)



(I)



(II)

would act as an efficient irreversible inhibitor for TIM (see II).

BHAP was synthesised by the following novel route:

$$Br.CH_2.CO.Br \xrightarrow{CH_2N_2} Br.CH_2.CO.CHN_2 \xrightarrow[BF_3]{H_3PO_4} Br.CH_2.CO.CH_2.O.PO_3H_2$$

1-Bromo-3-diazoacetone[3] was treated with a five-fold molar excess of crystalline orthophosphoric acid in dry ether containing a trace of BF_3. The reactivity and solubility characteristics of BHAP preclude its isolation, though its precursor showed appropriate n.m.r. and mass spectra, and elemental analysis. Moreover, treatment of the ethereal solution of BHAP with diazomethane allowed mass spectrometric analysis of the resulting dimethyl ester of BHAP. Dry ethereal solutions of BHAP appear to be stable indefinitely at 0°.

BHAP is a very potent inhibitor of TIM. Rabbit muscle TIM (C. F. Boeringer und Soehne, Mannheim, Germany), 130 μN (based on a subunit molecular weight of 25,000) in 0·5M-$\beta\beta'$-dimethyl glutarate buffer, pH 6·45, in the presence of an approximately 1·2-fold molar excess of BHAP, is totally inactivated within 5 min. at room temperature. The enzyme activity was assayed by the method of Beisenherz.[4] The reaction of BHAP with the enzyme is too fast for convenient study of the inactivation kinetics. The specificity of the reaction with enzyme (indicated by its very rapid rate) was further demonstrated by observing the course of the reaction in the presence and absence of the highly effective competitive inhibitor, phosphoglycollate (which has a K_i of 6 μM: Wolfenden[5]). Protection of the enzyme by phosphoglycollate against inactivation by BHAP was clearly demonstrated.

In order to determine the stoicheiometry of the reaction [^{32}P]-BHAP was prepared, and used to inactivate TIM as described before. After five min. incubation the protein was separated from the excess of reagent by chromatography on Sephadex G-25 (fine), eluting with 0·05-triethanolamine–HCl buffer, pH 7·8.

To calculate the molar incorporation of radioactive inhibitor into TIM from the ratio of radioactivity to absorbance at 280 nm, we need to know both the molecular weight and the E_{280} for the enzyme. The best values for the molecular weight are 48,000 (from equilibrium ultracentrifugation[6]), 52,000 (from crystallography[7]) and $n \times$ 26,500 (from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate[8,9]). We use a value of 50,000 in what follows. The optical factor E_{280} (0·1%) for commercial rabbit TIM has been determined independently by two groups, who agree on a value of 1·21.[10,11] On this basis, the number of moles of [^{32}P]-BHAP reacting with one mole of TIM was found to be 1·3. This non-integral value could arise if the protein is impure, if the reaction itself is non-stoicheiometric, or if the labelling of the protein is not irreversible. It is unlikely that the protein is impure, since the Lamm plot shows that the enzyme is mono-disperse, and polyacrylamide gel electrophoresis reveals only one sharp band.[8] However, when inactivated enzyme was dialysed against 0·5% ammonium bicarbonate overnight, essentially all the [^{32}P] label was lost from the protein. No enzymatic activity was recovered.

The fact that enzymic activity was not recovered on the loss of the [^{32}P] label suggested that the phosphate group might be lost from the inhibited enzyme more rapidly than the carbon skeleton of the inhibitor. Accordingly, we synthesised [^{14}C]-BHAP of known specific activity from 2-[^{14}C]-bromodiazoacetone.[3] Parallel investigations of samples of TIM inactivated either with [^{14}C]-BHAP or with [^{32}P]-BHAP showed that the [^{14}C] label is indeed much more firmly attached to the protein than is the [^{32}P] group.

These experiments indicated that the [^{14}C]-labelled compound would give a more reliable figure for the stoicheiometry of the reaction of TIM with BHAP. Using [^{14}C]-BHAP, totally inactivated material with a radioactivity equivalent to 2·1 moles BHAP/mole TIM was obtained, and this result has been confirmed several times (to $\pm$0·1).

These results may be compared with those of Hartman[12], who has recently described the inactivation of TIM by [^{32}P]-1-iodo-3-hydroxyacetone phosphate. On the basis of a molecular weight of 60,000 and an optical factor of 1·10, he obtained an uptake value of 1·7. Using the values cited above for the molecular weight and E_{280}, however, produces a value of 1·5.

The evidence presented accords well with the picture of commercial rabbit muscle TIM as a pure, mono-disperse protein with a molecular weight of about 50,000, containing two active sites per molecule, and composed of two identical sub-units.

In attempts to isolate an active-site peptide, [^{14}C]-labelled TIM was digested, both before and after reduction and carboxymethylation, with a number of proteolytic enzymes, and the resulting peptides subjected to paper electrophoresis. A multiplicity of radioactive peptides was found, and more disturbingly, the background of radioactivity along the chromatogram was rather high.

In recent months, crystallographic attention has shifted away from rabbit muscle TIM[7] to the enzyme from chicken muscle. Accordingly, we have inhibited the crystalline chicken muscle enzyme with [^{14}C]-BHAP, and find that the digestion of this material proceeds much more satisfactorily. Thus, after digestion with trypsin, a single radioactive peptide fragment is obtained.

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