Phosphoglycollohydroxamic Acid: an Inhibitor of Class I and II Aldolases and Triosephosphate Isomerase. A Potential Antibacterial and Antifungal Agent

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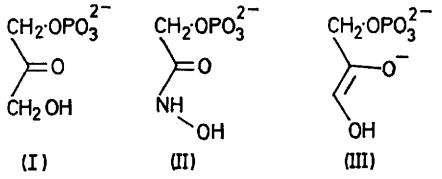
Summary The exceptionally strong binding of phosphoglycollohydroxamic acid to the metallo-aldolase from *Bacillus stearothermophilus* (K_i *ca.* 2×10^{-9}M) compared with rabbit muscle aldolase (K_i *ca.* 2×10^{-6}M) and chicken triosephosphate isomerase (K_i *ca.* 14×10^{-6}M) suggests that it may be a useful antibacterial and antifungal agent.

THE cleavage of D-fructose-1,6-diphosphate (FDP) by FDP-aldolases and the interconversion by triosephosphate isomerase of dihydroxyacetone phosphate (I) and D-glyceraldehyde-3-phosphate so formed, are consecutive steps in glycolysis. FDP-aldolases fall into two distinct classes.[1] Those in class I are found in animals and higher plants, and cleave FDP by way of a Schiff base,[2] whereas those in class II are metalloenzymes and occur in fungi, bacteria, and yeasts, the metal ion binding directly to the carbonyl oxygen of the substrate in the enzyme–substrate complex.[3]

Phosphoglycollohydroxamic acid (II) is a close structural analogue of dihydroxyacetone phosphate (I), but the electron delocalisation in the hydroxamic acid makes formation of a covalent bond with the active site lysine of the class I aldolases unlikely. The hydroxamic acid would be expected to bind exceptionally well to class II aldolases



however because of the known chelating ability of sh groups.[4] Phosphoglycollohydroxamic acid (II) might al be a good inhibitor of triosephosphate isomerase since is an excellent structural analogue of the ene-diolate intermediate (III),[5] which being close in energy to the transiti state[6] should be tightly bound by the enzyme.[7]

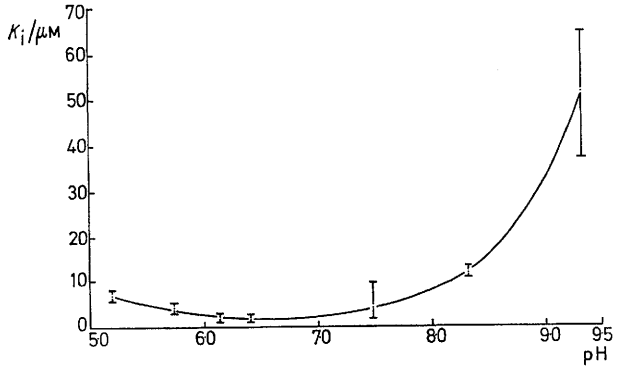


FIGURE 1. The pH-dependence of the inhibition of rabbit muscle aldolase by phosphoglycollohydroxamic acid at 25°, ionic strength = 20 mM.

Phosphoglycollohydroxamic acid (III) was prepared by the following route. Dibenzyl phosphate was esterified with ethyl diazoacetate in the presence of boron trifluoride etherate. The phosphate triester was hydrogenolysed over 10% palladium on charcoal and ethyl phosphoglycollate treated with hydroxylamine. The product was purified by ion exchange chromatography on Dowex 1-×8 (Cl^- form) in order to remove phosphoglycollic acid which is known to be a potent inhibitor of triosephosphate isomerase.[8]

Phosphoglycollohydroxamic acid (II) is a competitive inhibitor of rabbit muscle FDP-aldolase over a wide pH range (Figure 1). The small change in binding constant between pH 5·2 and 6·2 may be associated with the ionisation of the phosphate ester (pK_a 5·85). The increase in K_i at high pH is similar to the increase in K_m for D-fructose-1,6-diphosphate cleavage in this pH range[9] and is possibly associated with the ionisation of a residue on the enzyme involved in binding the phosphate ester. The fluorescence intensity of the enzyme at 325 nm (exciting wavelength 280 nm) is partially quenched by phosphoglycollohydroxamic acid (50·4% when the enzyme is saturated); by this method a dissociation binding constant of 26·5 ± 1 μM was determined at pH 7·6 and 25° (ionic strength = 20 mM). Under similar conditions dihydroxyacetone phosphate has a dissociation binding constant of 25·3 ± 4 μM (with 36·6% fluorescence quenching when the enzyme is saturated). The difference in binding dissociation constant for phosphoglycollohydroxamic acid determined kinetically and by fluorescence quenching could arise for example if the fluorescence quenching were associated with a conformation change of the enzyme after the initial binding.

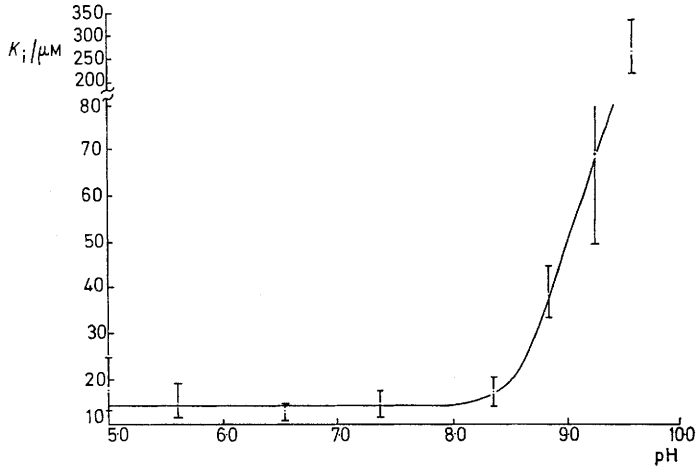


FIGURE 2. The pH-dependence of the inhibition of chicken triosephosphate isomerase by phosphoglycollohydroxamic acid at 30°, ionic strength = 100 mM.

The FDP-aldolase from *Bacillus stearothermophilus* which is a Zn^{II} metalloenzyme, was investigated as the more stable and more active Co^{II} enzyme.[10] At an enzyme and FDP concentration of 7·15 nM and 10 μM respectively and inhibitor concentration of 25 nM, the approach to equilibrium (16·8% residual activity) was readily observable ($t_{\frac{1}{2}}$ *ca.* 2 min). With the same enzyme and substrate concentration and 50 nM of inhibitor, the approach to equilibrium (9·6% residual activity was faster ($t_{\frac{1}{2}}$ *ca.* 1 min). From these experiments of mutual depletion of inhibitor and enzyme,[11] a dissociation binding constant for phosphoglycollohydroxamic acid of 2—4 × 10^{-9}M at pH 7·5 and 25° was calculated. Yeast FDP-aldolase, another class II enzyme, has a dissociation binding constant with phosphoglycollohydroxamic acid of 5 × 10^{-8}M at pH 7·5 and 25°.[12]

The possibility that the hydroxamic acid was extracting Co^{II} from the enzyme was excluded by showing that excess of Co^{II} did not reactivate the enzyme. By contrast, inactivation of the enzyme with EDTA was reversed by the addition of Co^{II}. In the presence of the hydroxamic acid however, the Co^{II} is not extractable by EDTA, indicating as expected that chelation to the metal ion was occurring in the enzyme–inhibitor complex.

Enzymic activity was not recovered even after dialysis of the inhibited enzyme for three days. However, binding

of the hydroxamic acid was readily reversible when the enzyme had been preincubated with EDTA. At pH 2·5 the inhibitor was released from the enzyme, not by enzyme denaturation, but presumably by protonation of the phosphate ester. That the phosphate ester does contribute very significantly to the binding energy was shown by the fact that acetohydroxamic acid is by comparison a rather poor inhibitor ($K_i > 10^{-4}$M).

Phosphoglycollic acid is a potent inhibitor of triosephosphate isomerase, and it has been suggested that it is a 'transition-state analogue' for this enzyme,[8] because of the structural similarity with the *cis*-ene-diolate intermediate in the reaction pathway.[5]

Phosphoglycollohydroxamic acid (II) is a very close structural analogue of the proposed *cis*-ene-diolate intermediate. It was found to be a competitive inhibitor of chicken triosephosphate isomerase over the pH range investigated (Figure 2). This pH-dependence is very similar to the pH-dependence of K_m for dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate with this enzyme.[13] The K_i of $14 \pm 3\,\mu$M at 30° (ionic strength = 0·1M) in the pH-independent region, is in good agreement with the value of K_i (11 μM) determined by u.v. difference spectroscopy at 24° (ionic strength = 0·02M).

Although phosphoglycollohydroxamic acid (II) is an effective competitive inhibitor of rabbit muscle FDP-aldolase (K_i *ca.* 2 μM) at 25° and chicken muscle triosephosphate isomerase (K_i *ca.* 14 μM at 30°), its binding constant to the Co^{II}–aldolase from *B. stearothermophilus* (K_i 2—4 × 10^{-3} μM at 25°) is *ca.* 10^3 times tighter. The possibility that phosphoglycollohydroxamic acid (II) might be an antibacterial and antifungal agent is being investigated. Preliminary tests have shown that two strains of *E. coli* (B and K12) on minimal growth media are inhibited by the hydroxamic acid.

The inhibition binding constants of the ethyl ester, and the amide of phosphoglycollic acid with class I and II FDP-aldolase and with triosephosphate isomerase are being determined.

The enzymes used in all the experiments were dialysed to remove sulphate ions.

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