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The Interaction of the Phosphonate Analogue of 3-Phospho-D-glycerate with Phosphoglycerate Kinase

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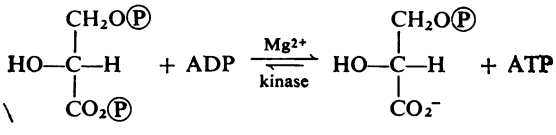
The methylene analogue of 3-phospho-D-glycerate, 2-hydroxy-4-phosphono-DL-butyric acid, is a substrate for phosphoglycerate kinase. The pK_a values for the final dissociation of the natural substrate and its methylene isostere are 6.20 and 7.45 respectively. The kinetic parameters K_m and $k_{cat.}$ for the enzyme-catalysed reaction were determined at pH 6.9 and 8.5 by using low substrate concentrations. Although the $k_{cat.}$ values for the two substrates at each pH are similar, there is a 60-fold increase in the K_m value for the methylene isostere on going to the lower pH. The results are most readily interpreted in terms of a dianionic group on C-3 being required for efficient substrate binding to the enzyme.

Phosphoglycerate kinase (ATP–3-phospho-D-glycerate 1-phosphotransferase; EC 2.7.2.3) catalyses the reversible transfer of a phosphoryl group between 1,3-diphospho-D-glycerate and ADP:



Krietsch & Bücher (1970) have concluded that the enzyme exhibits absolute specificity for the phosphate acceptor, 3-phosphoglycerate, in the back reaction. This specificity does not extend to the phosphate donor, ATP, in the site for which a number of nucleotides such as ITP, GTP and to a lesser extent dATP and dGTP are tolerated (Krietsch & Bücher, 1970). It has been found, however, that 2-hydroxy-4-phosphono-DL-butyric acid, the methylene analogue of 3-phosphoglycerate, is a substrate for phosphoglycerate kinase (Dixon & Sparkes, 1974).

Alkyl phosphonates, in general, have higher pK_a values for the final deprotonation than their phosphate monoester isosteres. In the present case, the second pK_a values for 3-phosphoglycerate and the phosphonate analogue are 6.20 and 7.45 respectively. As phosphoglycerate kinase is still fully active around the pK_a of the phosphonomethyl analogue (Krietsch & Bücher, 1970), we have the opportunity of finding out how the state of ionization of the phosphoryl group affects the kinetic parameters of the enzyme-catalysed reaction.

Experimental

Materials

Enzymes. Phosphoglycerate kinase was isolated from yeast as described by Scopes (1971). Rabbit D-glyceraldehyde 3-phosphate dehydrogenase was purchased from Boehringer Corp. (London W5 2TZ, U.K.). Both enzymes were dialysed at 4°C against 200mM-triethanolamine–HCl buffer, pH 7.8, to remove $(NH_4)_2SO_4$; the dialysis buffer for the dehydrogenase also contained 20mM-2-mercaptoethanol. The concentrations of the kinase solutions were calculated from E_{280} by using $E^{0.1\%}_{280} = 0.49$ for a 10mm light-path (Krietsch & Bücher, 1970). A molecular weight of 47000 was assumed.

NADH and ATP. These were obtained from Boehringer Corp. as their disodium salts.

3-Phospho-D-glyceric acid. This was purchased as the disodium salt from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. The concentration of 3-phosphoglycerate was determined enzymically. A typical assay mixture (3.0ml) contained: 200mM-triethanolamine–HCl buffer (pH 7.8); EDTA (32mM); $MgCl_2$ (40mM); neutral hydrazine (2mM); ATP (6mM); NADH (0.14mM); glyceraldehyde 3-phosphate dehydrogenase (200μg/ml); 3-phosphoglycerate kinase (8 or 40μg/ml); and 3-phosphoglycerate (1.0–3.0mM). The reaction was followed by the decrease in absorbance of NADH at 340nm, assuming an extinction of $6.22 \times 10^3 M^{-1} \cdot cm^{-1}$ (Horecker & Kornberg, 1948).

2-Hydroxy-4-phosphono-DL-butyric acid. The bis-(monocyclohexylammonium) salt was a kind gift from Dr. H. B. F. Dixon (Department of Biochemistry, University of Cambridge) and was prepared as described in the preceding paper (Dixon & Sparkes, 1974). The concentration of the D-enantiomer was determined by the same procedure as described for 3-phospho-D-glycerate.

Buffers. These were prepared from analytical-grade reagents and twice-distilled deionized water.

Methods

pH measurements were made with a Radiometer TTTlc pH-meter fitted with a pHA 630 scale-expander, standardized against standard buffer solutions from BDH Ltd., Poole, Dorset, U.K. For the determination of the second pK_a values of 3-phosphoglyceric acid and its methylene isostere, a titrator SBR 2c was attached to the above instrument. The pK_a values were determined at 30°C in the presence of 100mM-NaCl. Substrate concentrations were approx. 5mM. The free acids were liberated from their respective salts by using a Dowex 50 (H^+ form) sulphonic acid resin. Under these conditions the pK_a values for 3-phospho-D-glycerate and 2-hydroxy-4-phosphono-DL-butyrate were 6.20 and 7.45 respectively. Larsson-Raznikiewicz (1972) has determined the pK_a value for the final dissociation of the phosphate substrate in 0.25M-NaCl at 25°C to be 6.25. The dissociation constants for the complexes between Mg^{2+} and the fully dissociated substrates were determined in 100mM-NaCl at 30°C; the method of Larsson-Raznikiewicz (1972) was used.

All u.v.-absorption measurements were taken on a Unicam SP. 1800 instrument with an AR 25 linear chart recorder.

Kinetic and computational techniques. All kinetic runs were carried out spectrophotometrically at 30±0.5°C. The kinetic parameters for the kinase-catalysed reaction of both substrates were determined at pH6.90 and 8.50. In all experiments the concentration of free $MgCl_2$ was maintained at 1.0mM. The following dissociation constants for complexes with Mg^{2+} were used: ATP, 1.45×10^{-5}M (Morrison & Hyde, 1972), 3-phosphoglycerate, 1.41×10^{-2}M, and 2-hydroxy-4-phosphono-DL-butyrate, 9.88×10^{-3}M (this paper).

The cuvette contained, in 3.0ml: 200mM-triethanolamine–HCl, pH6.90 or 8.50; ATP (2.0mM); NADH (0.3mM); glyceraldehyde 3-phosphate dehydrogenase (approx. 200μg/ml); and phosphoglycerate kinase (35.4ng/ml). The concentration of 2-hydroxy-4-phosphono-DL-butyrate at pH6.90 ranged from 0.5 to 20mM. Otherwise the concentration of the substrates ranged from 0.05 to 3mM. Under these conditions it was shown that the observed rate of reaction was due solely to catalysis by the target enzyme (phosphoglycerate kinase).

Initial rates were measured at each substrate concentration, from the decrease in extinction at 340nm. The kinetic parameters K_m and $k_{cat.}$ were obtained from an unweighted least-squares analysis of the plots of v_0 against $v_0/[S_0]$ (the gradient of which equals $-K_m$) and $[S_0]$ against $[S_0]/v_0$ (the gradient of which equals $k_{cat.}$) where v_0 is the initial velocity and $[S_0]$ is the initial substrate concentration.

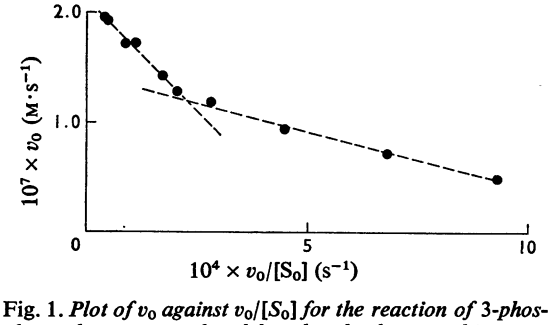


Fig. 1. *Plot of v_0 against $v_0/[S_0]$ for the reaction of 3-phospho-D-glycerate catalysed by phosphoglycerate kinase at pH6.9, 30°C*

For details see the text.

Results and Discussion

The kinetics of the reaction of phosphoglycerate kinase with 3-phospho-D-glycerate and its methylene isostere have been studied at two pH values, 6.9 and 8.5. The steady-state kinetic parameters have been obtained from plots of v_0 versus $v_0/[S_0]$, and $[S_0]$ versus $[S_0]/v_0$ (Dowd & Riggs, 1965). These plots have been used instead of the more common Lineweaver–Burk method, since the latter tends to suppress any deviations from linearity, especially at low $[S_0]$ values. The plots of v_0 versus $v_0/[S_0]$ are clearly non-linear (see, e.g., Fig. 1). Non-linear double-reciprocal plots have also been observed for phosphoglycerate kinase-catalysed reactions by Larsson-Raznikiewicz (1967), Krietsch & Bücher (1970) and Dr. R. Scopes (personal communication). It has been suggested that the activation by excess of substrate evident from Fig. 1 may be a consequence of phosphoglycerate kinase containing multiple binding sites for each substrate (Larsson-Raznikiewicz, 1967). Whatever the reason, however, we take the view that the kinetic parameters determined at low substrate concentrations are less likely to be perturbed by non-productive or non-competitive factors, and the values quoted in Table 1 relate to the linear portions of the reciprocal forms of the Michaelis equation at low $[S_0]$ values.

The results in Table 1 show that the replacement of the oxygen atom of the C–O–P linkage of 3-phosphoglycerate by a methylene group has little effect on the absolute values or the ratios of the $k_{cat.}$ values at pH8.5 and 6.9. The ratio of the K_m values, however, shows a dramatic increase at the lower pH. Although K_m is, of course, a complex function, for present purposes it is considered as some measure of substrate binding to the enzyme. As the K_m value for 3-phosphoglycerate and its methylene isostere are of the same order of magnitude at pH8.5, where both substrates are essentially fully deprotonated, it appears that the

Table 1. *Steady-state kinetic parameters for the phosphoglycerate kinase-catalysed reaction of 3-phosphoglycerate and 2-hydroxy-4-phosphonobutyrate*

For details see the text. The kinetic parameters quoted have probable errors of less than 10%.

	3-Phospho-D-glycerate		2-Hydroxy-4-phosphono-DL-butyrate	
pH	6.9	8.5	6.9	8.5
$10^{-2} \times k_{cat.}$ (s^{-1})	2.81	2.15	1.53	1.45
K_m (mM)	0.10	0.19	6.3	0.49

linking oxygen atom of the natural substrate is not required for binding. Comparison of the K_m values at pH 6.9 suggests that one of the main contributions to the specificity of substrate binding for phosphoglycerate kinase is the electrostatic interaction between the dianionic phosphoryl group of the substrate and one or more positively charged groups on the enzyme. This view is substantiated by the finding that the sulphate analogue of 3-phospho-D-glycerate (prepared as described by Fitzgerald *et al.*, 1971), although of similar size but carrying only one negative charge, is not a substrate for phosphoglycerate kinase (G. Orr, unpublished work).

It should be noted that the use of racemic 2-hydroxy-4-phosphono-DL-butyrate may affect both K_m and $k_{cat.}$ if the non-productive (L) enantiomer also binds to the enzyme (see, e.g., Huang & Niemann, 1951). However, in the present case we are comparing the relative magnitudes of the steady-state parameters at two pH values, and the absolute magnitudes of these parameters are not at issue.

The accompanying papers deal with methylene isosteres of phosphate ester intermediates of the glycolytic pathway (Dixon & Sparkes, 1974; Stribling, 1974; Adams *et al.*, 1974). Although the phosphonate analogue of dihydroxyacetone phosphate is not a substrate for triose phosphate isomerase (EC 5.3.1.1) (Dixon & Sparkes, 1974), it is a substrate for aldolase (EC 4.1.2.13) and for glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) (Stribling, 1974). From Stribling's (1974) results it may be suggested that, in contrast with phosphoglycerate kinase, neither aldolase nor the dehydrogenase requires a dianionic group in the C-3 position of the substrate for effective binding (though the monoanionic methyl phosphonate analogue of glycerol phosphate is not a substrate for the dehydrogenase; Adams *et al.*, 1974). More generally, the present results point to the possibility of exploiting the significant difference in the pK_a values of phosphates and phosphonates in investigations of enzyme–substrate interaction.

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