The bisphosphonomethyl analogue of 2,3-bisphosphoglycerate inhibits yeast but not wheat-germ phosphoglycerate mutase

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The bisphosphonomethyl analogue of 2,3-bisphosphoglycerate [4-phosphono-2-(phosphonomethyl)butanoate] was a potent competitive inhibitor of cofactor-dependent phosphoglycerate mutase from yeast, with a K_i of 0.8 mM. In contrast, the analogue did not affect the activity of cofactor-independent phosphoglycerate mutase from wheat germ. It is considered that this compound should be particularly useful for n.m.r. spectroscopic studies on the mechanism of action of cofactor-dependent phosphoglycerate mutases.

INTRODUCTION

Phosphoglycerate mutases (EC 5.4.2.1) comprise a family of enzymes that catalyse reactions involving the transfer of phospho groups among the three carbon atoms of phosphoglycerates. There are at least five types of phosphoglycerate mutase that are kinetically and structurally distinct, but nevertheless have many features in common (e.g. see Oh & Freese, 1976; Rose, 1980; Price *et al.*, 1985a). In glycolysis, phosphoglycerate mutase catalyses the interconversion of 3-phosphoglycerate and 2-phosphoglycerate. Two main types of glycolytic phosphoglycerate mutase are known. One type is dependent on the cofactor 2,3-bisphosphoglycerate, and is isolated from yeast (*Saccharomyces cerevisiae*), from vertebrates, from certain invertebrates such as molluscs and annelids, and from *Escherichia coli* (Sutherland, *et al.*, 1949; Rodwell *et al.*, 1956; Torralba & Grisolia, 1966; D'Alessio & Josse, 1971; Carreras *et al.*, 1982). The activity of this type of mutase requires the transfer of a phospho group from the cofactor to an active-site histidine in an initial priming reaction (reviewed by Rose, 1980). The other commonly occurring type of phosphoglycerate mutase does not require 2,3-bisphosphoglycerate, and is found in organisms such as plants, certain algae and fungi, and certain invertebrates such as sponges, arachnids and echinoderms (Grisolia & Joyce, 1959; Carreras *et al.*, 1980, 1982; Price *et al.*, 1983).

The detailed structure of yeast phosphoglycerate mutase is available from amino acid sequence and crystallographic studies (Winn *et al.*, 1981; Fothergill & Harkins, 1982). The C-terminal ten residues are known to be essential for activity (Sasaki *et al.*, 1966; Winn *et al.*, 1981; Price *et al.*, 1985b), but their function is not understood. These residues do not contribute to the electron-density map, presumably because they form a flexible tail in the non-ligated form of the enzyme that was crystallized. The present study was undertaken by a desire to find cofactor or substrate analogues that would be suitable for investigating the function of the C-terminal tail. The analogue used in this study is isosteric with the natural ligand, and involves only the

replacement of $-O-PO_3H_2$ by $-CH_2-PO_3H_2$. This bisphosphonomethyl analogue of 2,3-bisphosphoglycerate may thus be expected to bind to the same site on the enzyme as the cofactor, but will be unable to phosphorylate the enzyme. We have previously shown that the phosphonomethyl analogue of 3-phosphoglycerate is a potent competitive inhibitor of both cofactor-dependent and cofactor-independent phosphoglycerate mutases (McAleese *et al.*, 1985).

EXPERIMENTAL

4-Phosphono-2-(phosphonomethyl)butanoate

The bisphosphonomethyl analogue of 2,3-bisphosphoglycerate, i.e. DL-4-phosphono-2-(phosphonomethyl)butanoic acid

was synthesized in the form of its tris-cyclohexylammonium salt as follows. Tetraethyl 2,2-biscarbethoxy-1,4-bisphosphonate

was made by the method of Pfeiffer *et al.* (1974) by alkylation of diethyl malonate with diethyl bromomethylphosphonate and with diethyl 2-bromoethylphosphonate. A sample (4.7 g, 10 mmol) was de-esterified by treatment with bromotrimethylsilane (9.5 g, 60 mmol) in dry chloroform (15 ml) for 2 h at room temperature, followed by removal of the volatile materials *in vacuo*. The residual oil was boiled with HCl (2 M, 20 ml) for 4 h to effect decarboxylation, and the solution was evaporated to dryness and treated with cyclohexylamine (3.3 g, 0.33 mol) in methanol (20 ml) to generate the tris-amine salt of the product. On evaporation, a colourless solid was obtained, which was crystallized from ethanol to give the product (3.63 g, 65% yield), m.p. 150 °C (Found: C, 49.36; H, 8.92; N, 7.37;

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$C_{23}H_{51}N_3O_8P_2$ requires C, 49.36; H, 9.19; N, 7.51%). 1H n.m.r. δ (p.p.m.) (2H_2O) 1.56–1.64 (m, H-3, H-3'), 1.7–1.78 (m, H-1', H-4, H-4'), 2.00 (ddd, J_{HH} 16 Hz and 9 Hz, J_{PH} 17.5 Hz, H-1'') and 2.61 (m, H-2); ^{31}P n.m.r. δ (p.p.m.) (2H_2O) 26.95 and 29.75 (singlets).

Enzymes and substrates

Cofactor-dependent phosphoglycerate mutase was prepared from dried baker's yeast (*Saccharomyces cerevisiae*) by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography and gel filtration as described previously (McAleese *et al.*, 1985). A specific activity of about 10000 units/mg was usually obtained. A modification of the method of Britton *et al.* (1971) was used to purify cofactor-independent phosphoglycerate mutase from wheat germ (see McAleese *et al.*, 1985). The cofactor-independent mutase used in the present study has a specific activity of 4000 units/mg.

D-3-Phosphoglycerate disodium salt (grade I) and D-2-phosphoglycerate sodium salt were from Sigma Chemical Co. (Poole, Dorset, U.K.). D-2,3-Bisphosphoglycerate was from Boehringer (Mannheim, Germany).

Measurement of enzyme activity

The cofactor-dependent mutase was assayed by the enolase-coupled assay method described by Grisolia (1962), with a total assay volume of 1 ml. Routinely, a solution (940 μ l) containing the following reagents was equilibrated at 30 °C: 50 μ l of 200 mM-3-phosphoglycerate containing 6 mM-2,3-bisphosphoglycerate, 50 μ l of 1 M-Tris/HCl buffer, pH 7.0, and 50 μ l of 100 mM-MgSO₄. The reaction was started by the addition of 10 μ l of enolase (10 units) (type III from yeast; Sigma Chemical Co.) and 50 μ l of suitably diluted mutase. Enzyme solutions were diluted for assay with 0.2% bovine serum albumin (fraction V; Sigma Chemical Co.) in water. The formation of phosphoenolpyruvate was followed by an increase in absorbance at 240 nm. The rate was calculated over 2 min. An increase in absorbance of 0.1/min correspond to 1 unit. We prefer to define enzyme activity in these terms rather than in katal, because it is known that there is not a stoichiometric conversion of 2-phosphoglycerate into phosphoenolpyruvate under the conditions of assay (Grisolia & Cleland, 1968). As an approximation, 1 unit corresponds to about 1.4 nmol of 3-phosphoglycerate converted/s. When appropriate, the cofactor analogue was included in the solution equilibrated at 30 °C before the addition of enzymes.

The cofactor-independent mutase was assayed in the same manner except that the 2,3-bisphosphoglycerate was omitted, and the buffer was 50 mM-Tris/HCl, pH 8.7.

In each experiment the rates are expressed relative to that obtained in the presence of 5 mM substrate, which is taken as unity. All the experimental points are averages of duplicate measurements.

RESULTS

The effects of the bisphosphonomethyl analogue of the cofactor 2,3-bisphosphoglycerate on the activities of phosphoglycerate mutases have been studied. Both the cofactor-dependent and cofactor-independent phosphoglycerate mutases were assayed in the presence of this compound. The analogue was a racemic mixture; the

Fig. 1. Inhibition of yeast phosphoglycerate mutase by the bisphosphonomethyl analogue of 2,3-bisphosphoglycerate

The enzyme was assayed in the presence of different concentrations of 4-phosphono-2-(phosphonomethyl)butanoate as described in the text. The inhibitor concentration values refer to the concentration of the D-isomer. ●, No inhibitor; ■, 0.2 mM inhibitor; ▲, 0.5 mM inhibitor; ○, 1.0 mM inhibitor. Lines were fitted by use of the microcomputer program GENFIT (Powell, 1970). In each experiment, the amount of enzyme used gave a velocity of about 0.02 unit in 5 mM substrate. All rates are expressed relative to this value, which was taken as unity.

concentrations cited are those of the D-isomer, and we assume that the presence of the L-isomer does not affect the results, as such isomers bind very weakly to the enzyme (Pizer & Ballou, 1959).

The analogue inhibited the yeast cofactor-dependent mutase, but had no effect on the activity of the wheat-germ cofactor-independent enzyme.

The effects of different concentrations of the bisphosphonomethyl analogue on yeast phosphoglycerate mutase are shown in Fig. 1. The data are presented as a series of double-reciprocal plots of the reaction velocity as a function of the concentration of the substrate, 3-phosphoglycerate. The analogue causes an increase in the value of K_m from 0.86 ± 0.07 mM (no analogue) to 1.7 ± 0.2 mM (1 mM analogue). These data were replotted (Fig. 2) as described by Cornish-Bowden (1979) to distinguish between the competitive and uncompetitive components of the inhibition, and to obtain values of K_i (the competitive component) and (if appropriate) K_i' (the uncompetitive component). The value for K_i is about 0.8 mM, and the inhibition appears to be largely competitive, as the value for K_i' calculated from the point of intersection of the lines in Fig. 2(b) is about 3 mM.

The effects of different concentrations of the bisphosphonomethyl analogue on the activity of wheat-germ phosphoglycerate mutase are given in Table 1. This analogue has no inhibitory effect on the cofactor-independent enzyme. The slight activation at low concentrations is reminiscent of the activation caused by

Fig. 2. Determination of K_i for the bisphosphonomethyl analogue of 2,3-bisphosphoglycerate

The data shown in Fig. 1 were replotted. The inhibitor concentrations refer to the D-isomer. (a) Plot of $1/\text{velocity}$ against inhibitor concentration at various 3-phosphoglycerate concentrations. (b) Plot of 3-phosphoglycerate concentration/velocity against inhibitor concentration at various 3-phosphoglycerate concentrations. ●, 5 mM Substrate; ■, 2 mM substrate; ▲, 1 mM substrate; ○, 0.5 mM substrate. Lines were fitted by linear-regression analysis.

Table 1. Activity of cofactor-independent phosphoglycerate mutase in the presence of the bisphosphonomethyl analogue of 2,3-bisphosphoglycerate

The analogue concentration values refer to the concentration of the D-isomer. The relative activity values are the averages of duplicate measurements.

[3-Phosphoglycerate] (mM)	[Analogue] (mM)	Relative activity (%)
1.0	0	100
1.0	0.1	106
1.0	0.5	117
1.0	1.0	102
1.0	5.0	101

similar low concentrations of 2,3-bisphosphoglycerate (McAleese *et al.*, 1985).

Preliminary kinetic measurements in the presence of an arsonomethyl/phosphonomethyl analogue of 2,3-bisphosphoglycerate[4-arsono-2-(phosphonomethyl)butanoate showed that this compound was also an inhibitor

of cofactor-dependent phosphoglycerate mutase, but had no effect on the activity of the cofactor-independent enzyme.

DISCUSSION

An intriguing and as yet ill-understood feature of the mechanism of action of phosphoglycerate mutases concerns the role played by the C-terminal ten residues. Proteolysis of yeast phosphoglycerate mutase leads to complete loss of mutase activity, with the concomitant loss of the sequence Ala-Ala-Gly-Ala-Ala-Val-Ala-Asn-Gln-Lys-Lys-Gly from the C-terminus (Sasaki *et al.*, 1966; Winn *et al.*, 1981; Price *et al.*, 1985b). Examination of the structure of phosphoglycerate mutase together with modelling studies indicates that the C-terminal tail could modulate access to the active site (Winn *et al.*, 1981), and could thus exclude water during phospho transfer. The cluster of residues with small side chains would permit the flexibility suggested by crystallographic results. Moreover, the two consecutive lysine residues may help to bind the phospho groups at the active site, as otherwise the substrate molecules are not maximally ligated. The provision of a ligand for the otherwise exposed third oxygen atom of the transferred phospho

group would be of particular importance for stabilizing the transition-state complex.

Preliminary ^1H -n.m.r. spectroscopic measurements have indicated that this should be a rewarding experimental approach for studying the C-terminal tail (G. C. K. Roberts, personal communication). A requirement for more extensive studies is the availability of a ligand that will bind tightly to the enzyme, but will not be rapidly metabolized. Moreover, the ligand must be able to entice the C-terminal tail to adopt its position at the active site where it participates in catalysis. In this context it is relevant to note that 2,3-bisphosphoglycerate helps to protect phosphoglycerate mutase from proteolysis, whereas 3-phosphoglycerate provides no protection (Price *et al.*, 1985b). Apparently the presence of two phospho groups is required to draw the tail into the active site.

The cofactor analogue used in this study is isosteric with the natural cofactor, and our results show that it acts as a competitive inhibitor of the cofactor-dependent mutase. We presume that the analogue binds to this enzyme in the same manner as the cofactor, but is unable to undergo phospho transfer. Thus it is likely that this analogue and also the arsonomethyl/phosphonomethyl analogue will be suitable ligands for studying the role of the C-terminal tail in cofactor-dependent phosphoglycerate mutase by n.m.r. methods.

Neither analogue inhibits the cofactor-independent mutase from wheat germ. Indeed, at low concentrations (1 mM or less) the bisphosphonomethyl analogue causes a slight increase in enzymic activity, as does 2,3-bisphosphoglycerate itself (McAleese *et al.*, 1985). A suitable ligand for further studies on this enzyme would be the phosphonomethyl analogue of 3-phosphoglycerate, which we have previously shown to be a good competitive inhibitor of both cofactor-dependent and cofactor-independent phosphoglycerate mutases (McAleese *et al.*, 1985).

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