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Short Communications

Specificity of Glycerol Kinase

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The activity of a number of alcohols was examined as substrates or inhibitors of glycerol kinase (ATP–glycerol phosphotransferase; EC 2.7.1.30) from *Candida mycoderma*. On the basis of these and other results, a modified model is proposed to account for the substrate specificity of the enzyme.

The stereospecific phosphorylation of glycerol catalysed by glycerol kinase yields *sn*-glycerol 3-phosphate exclusively. This reaction is often cited as an example of the high steric selectivity of enzymes (Mahler & Cordes, 1971; Alworth, 1972). A model requiring three hydroxy groups in particular mutual orientation has been proposed (Gancedo *et al.*, 1968) to account for the substrate specificity and stereoselectivity of the enzyme. However, the reported substrate activity of certain propanediols (Eisenthal *et al.*, 1972) suggests that this model requires modification. A series of glycerol analogues have been investigated to redefine the structural requirements for substrates and competitive inhibitors of glycerol kinase. In the present paper, to clarify the interrelationships involved, the propanediols are named as glycerol derivatives. Stereospecific numbering, as recommended by the IUPAC–IUB Commission (1967), is used for these compounds.

Experimental

L(−)-Threitol was prepared from dimethyl L(+)-tartrate by reduction with borohydride as described by Kent & Barnett (1964); it had m.p. 88–89°C, $[\alpha]_D^{22}$ −4.0±0.4° (*c* 5.0 in water).

D(+)-Threitol was obtained from 2,3-*O*-isopropylidene-D-threitol prepared from D(−)-tartaric acid by using the method described by Carmack & Kelley (1968) for its enantiomer. It had m.p. 88–89°C, $[\alpha]_D^{22}$ +4.0±0.4° (*c* 5.0 in water).

2-Methylglycerol (2-methylpropane-1,2,3-triol) was prepared from 2-amino-2-methylpropane-1,3-diol. Sodium nitrite (22.5g) in water (100ml) was added dropwise over a period of 1h to a stirred solution of 2-amino-2-methylpropane-1,3-diol (31.5g) in 200ml of 1M-H_2SO_4. The temperature was maintained below 10°C throughout the addition, after which the mixture was allowed to warm to room temperature over a period of 1h, stirred with $BaCO_3$ (35g), filtered and concentrated to 100ml under reduced pressure. The resulting solution was diluted with ethanol (500ml) and the precipitated inorganic salts were removed by filtration. The filtrate was concentrated to 50ml under reduced pressure, deionized on a column of Zerolit DM-F mixed-bed resin (500ml) and reconcentrated to a syrup (14.5g). Fractional distillation gave 2-methylglycerol, b.p. 79–81°C/2.7Pa (0.02mmHg) (Found: C, 45.28; H, 9.44; $C_4H_{10}O_3$ requires C, 45.28; H, 9.44%). N.m.r. data (^2H_2O): δ 1.45 (singlet, 3 protons, methyl); 3.80 (singlet, 4 protons, hydroxymethyl). Gas chromatography (at 100°C) of the trimethylsilyl derivative showed less than 0.5% impurity. Hearne & de Jong (1941) obtained 2-methylglycerol by hydrolysis of 2,3-epoxy-2-methylpropan-1-ol and quote b.p. 115–120°C/213Pa (1.6mmHg).

L(+)-Butane-2,3-diol was a gift from Dr. A. H. Haines, University of East Anglia, U.K. 2-Amino-2-methylpropane-1,3-diol and D(−)-tartaric acid were purchased from Ralph N. Emmanuel Ltd., Wembley, U.K., dimethyl-L(+)-tartrate was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and erythritol was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and enzymes were from Boehringer Corp., Darmstadt, W. Germany. All compounds used in kinetic studies were either recrystallized or distilled before use.

Initial rates of enzyme reactions were determined by coupling the rate of ADP formation to the oxidation of NADH, by using phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase as described previously (Eisenthal *et al.*, 1972).

Results and discussion

2-Methylglycerol (III) behaves as a substrate of glycerol kinase showing Michaelis–Menten kinetics

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with K'_m 5.7mM and V (relative to glycerol) 0.10. None of the compounds listed in Table 1 showed substrate activity at concentrations up to 400mM; however, they all competitively inhibited the phosphorylation of glycerol with the K_i values shown.

Orientation of the C-1 and C-2 hydroxy groups of glycerol relative to the phosphorylated hydroxy group at C-3 is shown for *sn*-glycerol (I). 1-Deoxy-*sn*-glycerol (IV) [K'_m 45mM, V (relative to glycerol) 0.018] and 2-deoxyglycerol (V) [K'_m 88mM, V (relative to glycerol) 0.012] are weak substrates (cf. K_m for glycerol 0.05mM) whereas 3-deoxy-*sn*-glycerol (VI) is a competitive inhibitor but not a substrate of glycerol kinase (Eisenthal *et al.*, 1972). Deoxyglycerols (IV), (V) and (VI) are therefore behaving as analogues of *sn*-glycerol (I) in which the hydroxy groups at C-1, C-2 and C-3 respectively have been replaced by hydrogen. Thus the lack of a hydroxy group at C-1 and C-2 does not necessarily abolish substrate activity. The presence of two hydroxy groups is, however, a minimum requirement since, although propan-1-ol, propan-2-ol and ethanol appear to bind weakly (Table 1) to the active site of the enzyme, they are not substrates.

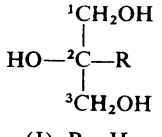
Ethanediol is not phosphorylated, indicating that a minimum chain length of three carbons is required for substrate activity. It is, however, an effective inhibitor (Table 1). This would be consistent with alignment of the hydroxy groups at the active site in positions corresponding to those on C-1 and C-2 of *sn*-glycerol. A preference for this orientation over one corresponding to C-2 and C-3 of *sn*-glycerol suggests that the C-1 hydroxy group of the *sn*-glycerol makes a greater contribution to binding than that at C-3. This would also be consistent with alignment of the non-substrate propan-1-ol with its hydroxy group

Table 1. *Competitive inhibition of glycerol kinase by substrate analogues*

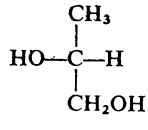
Details are as described in the Experimental section. K_i values were calculated from plots of [S]/v versus [S] at fixed inhibitor concentrations.

Inhibitor	[I] (mM)	K_i (mM)
3-Deoxy-*sn*-glycerol (VI)*	—	4.6
L-Threitol (VII)	180	188
D-Threitol (VIII)	100	21.0
Erythritol (IX)	100	34.3
L(+)-Butane-2,3-diol (X)	100	1000
D-Butane-1,3-diol (XI)	100	>210†
L-Butane-1,3-diol (XII)	100	>210†
Ethanediol	34	13.4
Propan-1-ol	250	250
Propan-2-ol	250	1500
Ethanol	500	900

* From Eisenthal *et al.* (1972).
† Calculated from racemic mixture.



(I) R = H
(II) R = OH
(III) R = CH_3



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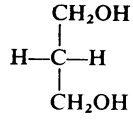

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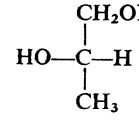

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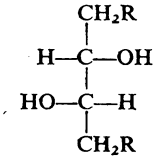
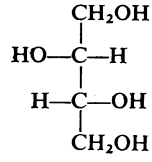


(VII) R = OH
(X) R = H



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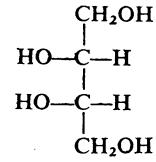

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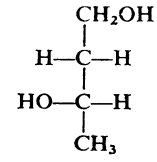

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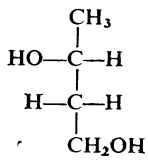

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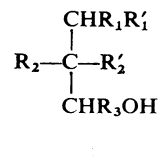

(XIII)

in a position corresponding to that of C-1 of *sn*-glycerol. It is noteworthy that ethanediol stabilizes the enzyme.

The tetritols (structures VII–IX) can be regarded as analogues of *sn*-glycerol with a hydrogen atom at C-1 or C-3 replaced by a hydroxymethyl group. L-Threitol (VII) and D-threitol (VIII) are respectively C-1 and C-3-substituted analogues of *sn*-glycerol whereas erythritol (IX) can be regarded as either a C-1 or a C-3 analogue. Similarly L(+)-butane-2,3-diol (X) can be formally derived from 3-deoxy-*sn*-glycerol (VI) and the butane-1,3-diols (XI and XII) from 2-deoxyglycerol (V) by replacement of a C-1 or C-3 hydrogen atom by a methyl group. It is evident that lengthening the carbon chain at either end of the glycerol or 2-deoxyglycerol molecule results in loss of substrate activity. However, all *n*-butane derivatives tested behave as competitive inhibitors of glycerol phosphorylation. The very much higher K_i value of L-threitol (VII) compared with that of either D-threitol (VIII) or erythritol (IX) (Table 1) suggests that the presence of a bulky group at C-1 of the *sn*-glycerol molecule is more deleterious to binding than the presence of the corresponding group at C-3. A dramatic decrease in binding on introduction of a carbon atom at C-1 is also reflected in the K_i values of 3-deoxy-*sn*-glycerol (VI) and its C-1-substituted analogue L(+)-butane-2,3-diol (X) (Table 1). The greater loss of affinity caused by substitution at C-1 of *sn*-glycerol over corresponding replacement at C-3 further supports the suggestion that the hydroxy group at C-1 contributes more to overall binding than that at C-3.

In the model for substrate specificity proposed by Gancedo *et al.* (1968) three hydroxy groups in 'glycerol-like' orientation are required. Since dihydroxyacetone and D- and L-glyceraldehyde are substrates (Gancedo *et al.*, 1968) this necessitates postulating that the substrate activity of these compounds is due to their hydrated forms. Whereas substitution of hydrogen by methyl (or hydroxymethyl) at either C-1 or C-3 of *sn*-glycerol or its substrate analogues abolishes substrate activity in all cases studied, corresponding replacement at C-2 gives the substrate 2-methylglycerol (III). The ability of this C-2-substituted glycerol analogue to act as a substrate is consistent with the proposal that dihydroxyacetone is phosphorylated as its hydrate, i.e. 2-hydroxyglycerol (II).

The revised structural requirements for glycerol kinase substrates on the basis of our results are: a chain length of three carbons, at least two of which must carry hydroxy groups; if a hydroxy group at C-2 is present then it must be correctly oriented relative to the primary hydroxy group (as in IV). On the basis of structure (XIII), substrate activity will be obtained if one of the groups R_1, R_1' or R_2 is an OH group. Compounds with R_2' as large as methyl ac tas substrates, but R_1, R_1' or R_3 cannot be larger than OH. It may be noted that interaction of the ligand with the enzyme at two points is sufficient to define the stereospecificity of glycerol kinase.

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