Dehydrogenation by gluconate 6-phosphate dehydrogenase of an isosteric phosphonate substrate analogue

Daniel J. W. ROACH* and Roger HARRISON

Department of Biochemistry, University of Bath, Bath BA2 7AY, U.K.

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6,7-Dideoxy-D-glucio-heptonic 7-phosphonic acid, the isosteric phosphonate analogue of gluconate 6-phosphate, was prepared by incubation of the corresponding analogue of glucose 6-phosphate with glucose 6-phosphate dehydrogenase and NADP⁺ in the presence of an enzymic NADPH–NADP⁺ recycling system. The analogue of gluconate 6-phosphate is a substrate for yeast gluconate 6-phosphate dehydrogenase, showing Michaelis–Menten kinetics at pH 7.5 and 8.0. At both pH values the K_m values are approx. 3-fold higher and the V_{max} values approx. 7-fold lower than those of the natural substrate.

Isosteric analogues of carbohydrate phosphates, in which the C–O–P moiety of a naturally occurring phosphate group is replaced by C–CH₂–P, have, over the past few years, been the subject of considerable research activity aimed at investigating the potential of the analogues as metabolic probes (Engel, 1977; Inch, 1978; Roach, 1980). Such potential largely depends on the ability of the analogues to behave as substrates for some enzymes in a metabolic sequence but not for others, so allowing the possibility of selective inhibition of particular biochemical pathways. The similarities in size and shape of the naturally occurring phosphates and their isosteric phosphonate analogues have indeed been found to be reflected in the substrate activities of a number of analogues with relevant enzymes (Adams *et al.*, 1974, 1976; Dixon & Sparkes, 1974; Orr & Knowles, 1974; Stribling, 1974; Webster *et al.*, 1976; Unger *et al.*, 1978; Le Marechal *et al.*, 1980a), whereas in other cases inhibitory but not substrate activity has been demonstrated (Stribling, 1974; Webster *et al.*, 1976; Le Marechal *et al.*, 1980b).

Phosphonate analogues of hexose phosphates are of particular interest in view of the metabolic importance of the phosphates themselves and of the possibility (Dixon & Sparkes, 1974) that the analogues might be used as a source of phosphonate isosteres of ribose 5-phosphate and nucleotides. Webster *et al.* (1976) made use of the availability of the phosphonate analogue of 3-phosphoglycerate by incorporating it, by enzymic

and chemical means, into the corresponding analogue of fructose 6-phosphate. The feasibility of the further conversion of the latter analogue sequentially into isosteres of glucose 6-phosphate, gluconate 6-phosphate and ribulose 5-phosphate was suggested by the generation of NADPH from incubation mixtures containing the 6-phosphonomethylfructose, NADP⁺ and the relevant enzymes. These findings paralleled those obtained by Adams *et al.* (1976), who reported the chemical synthesis of 6,7-dideoxy- α -D-glucio-heptose 7-phosphonic acid (I), the isosteric phosphonate analogue of glucose 6-phosphate (II), and showed that the analogue behaved as a substrate for yeast glucose 6-phosphate dehydrogenase with K_m values some 4–5-fold higher and V_{max} values approx. 50% lower than those of the natural substrate. Evidence that the product of enzymic dehydrogenation is itself a substrate for gluconate 6-phosphate dehydrogenase was obtained in a similar manner to that described by Webster *et al.* (1976).

We now report the synthesis and isolation of 6,7-dideoxy-D-glucio-heptonic 7-phosphonic acid, the isosteric phosphonate analogue of gluconate 6-phosphate, by dehydrogenation of the glucose 6-phosphate analogue in the presence of glucose 6-phosphate dehydrogenase and an NADPH–NADP⁺ recycling system. The analogue is shown to be a substrate for yeast gluconate 6-phosphate dehydrogenase, and its kinetic parameters are compared with those of the natural substrate.

Experimental

Materials

6,7-Dideoxy- α -D-glucio-heptose 7-phosphonic acid

* Present address: Biochemistry/Biophysics Program, Washington State University, Pullman, WA 99164, U.S.A.

(I) was prepared as described in the Appendix (Roach *et al.*, 1981). Before its enzymic dehydrogenation, the analogue was subjected to ion-exchange chromatography on DEAE-Sephadex followed by removal of cations, exactly as described below for the isolation of the dehydrogenated product except that fractions corresponding to the first peak eluted from DEAE-Sephadex were processed. Glucose 6-phosphate (II), gluconate 6-phosphate (IV), 2-oxoglutarate, glucose 6-phosphate dehydrogenase (yeast) (EC 1.1.1.49) gluconate 6-phosphate dehydrogenase (yeast) (EC 1.1.1.44) and glutamate dehydrogenase (ox liver) (EC 1.4.1.3) were all purchased from Boehringer Corp. (London W.5, U.K.). Bovine serum albumin was from Sigma Chemical Co. (Poole, Dorset, U.K.) and DEAE-Sephadex A-25 anion-exchange beads (particle size 40–120 μm) were from Pharmacia (Hounslow, Middlesex, U.K.). All other reagents and chemicals were supplied by BDH Chemicals (Poole, Dorset, U.K.).

Enzymic synthesis of gluconate 6-phosphate and 6,7-dideoxy-D-gluco-heptonic 7-phosphonic acid

Gluconate 6-phosphate (IV) and its isosteric phosphonate analogue (III) were prepared by enzymic dehydrogenation of the corresponding aldose derivatives (II and I respectively) by glucose 6-phosphate dehydrogenase in the presence of NADP^+ . NADPH formed in the reaction was recycled to NADP^+ by using glutamate dehydrogenase in the presence of 2-oxoglutarate and NH_4^+ ions.

A mixture containing 5 mM-2-oxoglutarate, 3 mM-glucose 6-phosphate (II) [or its phosphonate analogue (I)], 25 mM-ammonium acetate, 0.2 mM-ADP, 2 mM- NADP^+ , bovine serum albumin (2 mg/ml), glutamate dehydrogenase (80 $\mu\text{g}/\text{ml}$) and glucose 6-phosphate dehydrogenase (6 $\mu\text{g}/\text{ml}$) in 50 mM-Tris/HCl buffer, pH 8.0 (total volume 50 ml), was incubated at 22°C for 16 h. The reaction was terminated by heating the mixture at 100°C for 5 min and the products were allowed to cool to 22°C when a precipitate of denatured protein had appeared. Activated charcoal (100 mg) was added and the mixture was kept at 4°C for 16 h. The charcoal, together with adsorbed nucleotides and denatured proteins, was removed by filtration, and washed with double-distilled water (65 ml), and the washings were combined with the filtrate.

The combined filtrate and washings were adjusted to pH 8.4 and applied to a column (30 cm $\times$ 17 cm) of DEAE-Sephadex A-25, previously equilibrated with 50 mM- KHCO_3 at 22°C. The column was eluted with a linear gradient (50–400 mM; 400 ml) of KHCO_3 at 22°C with a flow rate of 1.5 ml/min. Fractions (7.5 ml) were collected and samples of each fraction were assayed for phosphate or

phosphonate. The elution pattern obtained starting either from glucose 6-phosphate (II) or from its analogue (I) showed in each case two clearly defined peaks eluted at 210 mM- KHCO_3 and 290 mM- KHCO_3 .

Fractions corresponding to the second peak eluted from DEAE-Sephadex were combined and stirred with an excess of Dowex 50W X1 cation-exchange resin (H^+ form; 50–100 mesh) under vacuum to facilitate escape of CO_2 . When bubbling ceased, the resin was removed by filtration and washed with double-distilled water. Filtrate and washings were combined and freeze-dried to give either gluconate 6-phosphate or its isosteric phosphonate analogue.

Enzymic determination of glucose 6-phosphate (II) and of 6,7-dideoxy- α -D-gluco-heptose 7-phosphonic acid (I)

Glucose 6-phosphate (II) or its isosteric phosphonate analogue (I) were assayed by determining the amount of excess of NADP^+ that was reduced by the test sample in the presence of glucose 6-phosphate dehydrogenase. Reaction mixtures contained 0.8 mM- NADP^+ , glucose 6-phosphate dehydrogenase (4 μg) and approx. 0.05 mM-glucose 6-phosphate (or its phosphonate analogue) in 100 mM-Tris/HCl buffer, pH 7.5 (total volume 1.22 ml). Reactions were started by the addition of enzyme, and NADPH was measured by total increase in absorbance at 340 nm in a Unicam SP.1800 spectrophotometer at 25°C.

Enzymic determination of gluconate 6-phosphate (IV) and of 6,7-dideoxy-D-gluco-heptonic 7-phosphonic acid (III)

Gluconate 6-phosphate (IV) and its isosteric phosphonate analogue (III) were assayed exactly as described above for the corresponding aldoses except that gluconate 6-phosphate dehydrogenase replaced glucose 6-phosphate dehydrogenase.

Determination of phosphate or phosphonate

Organic compounds containing phosphate or phosphonate groups were ashed as described by Ames & Dubin (1960), after which inorganic phosphate was determined by the procedure of Chen *et al.* (1956).

Kinetic determinations

Initial rates of reactions catalysed by gluconate 6-phosphate dehydrogenase were followed by determining the formation of NADPH from NADP^+ by measuring increase in A_{340} as described above. Reaction mixtures contained various concentrations of substrates in 100 mM-Tris buffer adjusted to the required pH with 2 M-HCl, and readings were made in 4 cm-path-length cells (total volume 4.005 ml). Reactions were initiated by the addition of enzyme

[1 μ g (for gluconate 6-phosphate) or 5 μ g (for 6,7-dideoxy-D-gluco-heptonic 7-phosphonic acid)] in buffer (5 μ l) containing 1% (w/v) bovine serum albumin. A standard assay for gluconate 6-phosphonate dehydrogenase activity with 0.45 mM-NADP⁺ and 1.2 mM-gluconate 6-phosphate was performed at regular intervals during each experiment to check the stability of the enzyme. Initial rates were determined for five concentrations of one substrate at each of five different fixed concentrations of the alternative substrate. Substrate concentrations used covered the ranges 0.006–0.4 mM for NADP⁺, 0.07–0.5 mM for gluconate 6-phosphate and 0.14–1.0 mM for 6,7-dideoxy-D-gluco-heptonic 7-phosphonic acid. Individual rate measurements were reproducible to better than

conversion. This had the advantage, not only of sparing valuable phosphonate analogue during the development of the method, but also of providing a control with which to check the purity of the aldonic acid product. As NADPH is known to act as a product inhibitor of glucose 6-phosphate dehydrogenase (Afolayan, 1972), a scheme was sought whereby NADPH could be recycled to NADP⁺. That chosen is based on the method described by Lowry *et al.* (1961) for the measurement of low concentrations of NADPH, which involves the cycling of NADPH and NADP⁺ by using glutamate dehydrogenase coupled to glucose 6-phosphate dehydrogenase. The use of the cycling process in the presently described preparation of gluconate 6-phosphate can be represented as:

$\pm 5\%$. Values of K_m and V_{max} were obtained by extrapolation to infinite concentration of alternative substrate as described by Florini & Vestling (1957) by using slopes from $[S]/V_0$ -versus- $[S]_0$ plots, which were calculated by weighted regression by using a computer program based on the procedure described by Cornish-Bowden (1976).

Results and discussion

In view of previous observations that the isosteric phosphonate analogue (I) of glucose 6-phosphate (II) is a substrate for yeast glucose 6-phosphate dehydrogenase (Adams *et al.*, 1976; Webster *et al.*, 1976), we investigated the use of this enzyme to catalyse the conversion, on a preparative scale, of the phosphonate (I) into the corresponding analogue (III) of gluconate 6-phosphate (IV). The method was established by using, in the first instance, glucose 6-phosphate itself as substrate for the enzymic

The procedure as described in the Experimental section was found to be suitable for conversion of concentrations of glucose 6-phosphate (II) (or of its phosphonate analogue, I) of up to 5 mM.

The separation of gluconate 6-phosphate from residual glucose 6-phosphate was initially attempted by differential elution with ammonium tetraborate from an anion-exchange resin (Lefevre *et al.*, 1964). Although a good separation of the sugar phosphates was achieved by these means, however, complete removal of borate salts from the separated carbohydrates proved to be very difficult, and the procedure was abandoned in favour of the alternative use of bicarbonate elution from DEAE-Sephadex (Loesche *et al.*, 1974).

Elution from DEAE-Sephadex of the product mixture from incubations containing either glucose 6-phosphate (II) or 6,7-dideoxy- α -D-gluco-heptose 7-phosphonic acid (I), its phosphonate analogue, gave two clearly separated peaks when the fractions

Table 1. Comparison of kinetic parameters for glucose 6-phosphate and its phosphonate analogue
Conditions are as described in the Experimental section. Values are $\pm$ s.e. (Bliss, 1967).

Substrate	K_m (μ M) for compound (III) or (IV)		K_m (μ M) NADP^+		$V_{\max}$. (relative to that of gluconate 6-phosphate)	
	pH 7.5	pH 8.0	pH 7.5	pH 8.0	pH 7.5	pH 8.0
6,7-Dideoxy-D-glucio-heptonic acid 7-phosphonate (III)	435 $\pm$ 64	736 $\pm$ 74	5.4 $\pm$ 0.5	6.5 $\pm$ 2.5	0.14	0.12
Gluconate 6-phosphate (IV)	119 $\pm$ 6	276 $\pm$ 18	4.3 $\pm$ 0.5	3.6 $\pm$ 0.7	1.0	1.0

were ashed and assayed for phosphate. Fractions corresponding to the first peak showed substrate activity with glucose 6-phosphate dehydrogenase but not with gluconate 6-phosphate dehydrogenase, whereas the reverse was true for fractions corresponding to the second peak. Removal of cations from pooled fractions of the second peak allowed isolation of gluconate 6-phosphate or of its isosteric phosphonate analogue in 56% overall yield from the corresponding aldose precursor. The same value was obtained either by enzymic assay or by assay for phosphate (or phosphonate) content.

6,7-Dideoxy-D-glucio-heptonic 7-phosphonic acid (III) prepared by these means was dehydrogenated by NADP^+ in the presence of yeast gluconate 6-phosphate dehydrogenase, showing Michaelis-Menten kinetics, and the kinetic parameters for this reaction are compared with those of the natural substrate, gluconate 6-phosphate (IV), in Table 1. The latter parameters, obtained by using commercially supplied gluconate 6-phosphate, were unchanged when gluconate 6-phosphonate prepared by the enzymic dehydrogenation procedure was used in its stead. This shows that the enzymically prepared gluconate 6-phosphate (IV) and, by implication, also its phosphonate analogue (III) are free from minor impurities, arising from the preparation or purification procedures, that affect the measured kinetic parameters. The similarity of the $K_m^{\text{NADP}^+}$ values for gluconate 6-phosphate (IV) and for its analogue (III) suggests that the mechanism of the enzymic reaction is the same in the two cases, as was found for glucose 6-phosphate dehydrogenase and its corresponding substrates (II) and (I) (Adams *et al.*, 1976). The K_m^{III} values for the phosphonate (III) are similar to, but significantly higher than, the corresponding values for the natural substrate (IV) at both pH values tested, whereas the $V_{\max}$. values of analogue (III) are both an order of magnitude less than those for gluconate 6-phosphonate (IV). These differences in kinetic parameters between phosphonate analogue and natural substrate are similar to those observed in the case of glucose 6-phosphate dehydrogenase, although the corresponding $V_{\max}$. values differed less in the latter example (Adams

et al., 1976). As was found for glucose 6-phosphate and its phosphonate analogue, it is difficult to explain the present results in terms of a predominance of the fully ionized form of the natural substrate compared with the phosphonate, as the ionization states of the two substrates are likely to be similar at pH 8.0, where the differences in kinetic parameters persist. The relationship between the kinetic parameters of natural substrate and phosphonate analogue observed here is similar to a number of such relationships reported for isosteric phosphonate analogues of a range of molecular types, including phospholipids, nucleotides and simple carbohydrates (Roach, 1980). When analyses have been performed, it is commonly found that either K_m or $V_{\max}$, particularly $V_{\max}$, is less favourable in the analogue, a situation that can best be explained in terms of enzyme-substrate interactions involving the ester oxygen atom and/or critically dependent on precise bond angles in the natural substrate.

Nevertheless 6,7-dideoxy-D-glucio-heptonic 7-phosphonic acid (III) is an effective substrate for glucose 6-phosphate dehydrogenase, confirming the feasibility of an enzymic route from the phosphonate analogues of hexoses to those of the pentoses and potentially of nucleotides. Indeed, preliminary experiments (Roach, 1980) have demonstrated that sequential incubation of 6,7-dideoxy- α -D-glucio-heptose 7-phosphonic acid (I), the phosphonate analogue of glucose 6-phosphate (II), with glucose 6-phosphate dehydrogenase and with gluconate 6-phosphate dehydrogenase in the presence of the cyclic NADP^+ -regenerating system described in the present paper, can lead to phosphonate product with the chromatographic behaviour to be expected from the analogue of ribulose 5-phosphate. Experiments of this type are dependent on reliable supplies of starting material in the form of chemically synthesized phosphonate analogues. Although general methods for such syntheses are now becoming available (Adams *et al.*, 1974, 1976; Dixon & Sparkes, 1974, 1976; Engel, 1977), improvements in yield and convenience, of what can be very time-consuming reaction sequences, are of con-

siderable value, and an improved synthesis of the isosteric analogue (I) of glucose 6-phosphate (II) is described in the Appendix (Roach *et al.*, 1981).

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APPENDIX

An improved synthesis of 6,7-dideoxy- α -D-glucopyranose 7-phosphonic acid

Daniel J. W. ROACH,* Roger HARRISON,* C. Richard HALL† and Thomas D. INCH†

*Department of Biochemistry, University of Bath, Bath BA2 7AY, U.K., and †Chemical Defence Establishment, Porton, Salisbury, Wilts. SP4 0JQ, U.K.

6,7-Dideoxy- α -D-glucopyranose 7-phosphonic acid (I), the isosteric phosphonate analogue of glucose 6-phosphate (II), was previously synthesized (Adams *et al.*, 1976) by treatment of the crude 6-aldehyde sugar benzyl 2,3,4-tri-O-benzyl- α -D-glucopyranoside (III) with the Wittig reagent diphenyl triphenylphosphoronylidene-methylphosphonate (Jones *et al.*, 1968), followed by transesterification of the reaction product to the fully benzylated compound and catalytic hydrogenolysis to the free phosphonic acid (I). The method suffers from the major disadvantage that diphenyl triphenylphosphoronylidene-methylphosphonate is difficult to prepare, involving reaction of the unreactive triphenylphosphine with diphenyl chloromethylphosphonate, itself not readily available. Moreover, the transesterification step, necessary because of the unpredictable behaviour of phenyl esters in hydrogenolysis reactions (Adams *et al.*,

1974, 1976), requires a column-chromatographic separation of product from benzyl alcohol and proceeds in relatively low yield.

An alternative synthetic route to the phosphonate (I) is now described (Scheme 1). This avoids the diphenyl ester intermediate, proceeding instead via the corresponding diethyl derivative (IV), which is more readily prepared and undergoes ready dealkylation to the phosphonic acid in the presence of bromotrimethylsilane.

Method

Benzyl 2,3,4-tri-O-benzyl-6,7-dideoxy- α -D-glucopyranose 7-phosphonic acid diethyl ester (IV)

n-Butyl-lithium (15 ml) was added dropwise to a solution of tetraethyl methylene-diphosphonate (9 g) [prepared by the procedure of Ford-Moore &