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Phosphoglycerate Mutases: Stereochemical Course of the Phosphoryl Group Transfers Catalyzed by the Cofactor-Dependent Enzyme from Rabbit Muscle and the Cofactor-Independent Enzyme from Wheat Germ†

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ABSTRACT: 2-[(*R*)-^{16}O,^{17}O,^{18}O]Phospho-D-glycerate has been synthesized and used to determine the stereochemical course of each of the two mechanistic classes of phosphoglycerate mutases. The enzyme from rabbit muscle requires 2,3-bisphospho-D-glycerate as a cofactor and catalyzes an *inter*molecular phosphoryl group transfer reaction. The enzyme from wheat germ requires no cofactor and catalyzes an *intra*molecular transfer of the phosphoryl group. We have shown that the reaction catalyzed by each of these enzymes proceeds with overall *retention* of the configuration at phosphorus. This stereochemical result is consistent with a double-displacement pathway involving a single phosphorylenzyme, for each of the catalyzed reactions.

Phosphoglycerate mutase catalyzes the interconversion of 2- and 3-phospho-D-glycerate, and two classes of enzymes can be distinguished mechanistically. The mutases from animal sources and from yeast show an absolute requirement for the cofactor 2,3-bisphospho-D-glycerate, and these enzymes catalyze the *inter*molecular transfer of phosphoryl groups amongst the two substrates and the cofactor. This class of mutases has been shown to form a chemically competent phosphorylenzyme, the phosphoryl group of which may be transferred to 2-phospho-D-glycerate or 3-phospho-D-glycerate or (much more slowly) to water. It appears that the cofactor 2,3-bisphospho-D-glycerate is required to maintain the enzyme in its active phosphorylated form.

In contrast to the animal and yeast enzymes, the phosphoglycerate mutases from wheat germ and rice germ show no cofactor dependence, and we have shown earlier that the phosphoryl group is transferred *intra*molecularly by the wheat germ enzyme. While for this enzyme no direct evidence could be obtained to demonstrate the intermediacy of a phosphorylenzyme, the behavior of a number of substrate analogues strongly suggested a pathway involving such a species.

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Pathways involving a cyclic 2,3-phosphate diester, pentacoordinate intermediates (necessarily requiring pseudorotation), and monomeric metaphosphate were all disfavored by the evidence.

In order to delineate more precisely the mechanistic pathways followed by the two classes of phosphoglycerate mutases, we decided to study the stereochemical consequence at phosphorus of these phosphoryl group transfer processes. Our first approach involved the synthesis of the chiral [^{18}O]phosphorothioates of D-glycerate where the phosphate monoesters were rendered chiral by replacement of one peripheral oxygen by ^{18}O and another by sulfur. Despite the fact that all phospho*kinases* tested have been shown to catalyze the intermolecular transfer of *thio*phosphoryl groups (albeit at rather reduced rates compared with their all-oxygen prototypes) and even some phosphatases appear to be capable of thiophosphoryl group transfer to water (i.e., hydrolysis), the phosphoglycerate mutases appeared not to tolerate the substitution of sulfur for oxygen. Indeed, of the ten glycolytic enzymes (from hexokinase down to pyruvate kinase) *only* phosphoglycerate mutase will not accept phosphorothioates as substrates (G. A. Orr and G. J. Chin, unpublished experiments). [No attempt was made to set an upper limit on the rate of catalysis of the phosphorothioates. Suffice it to say that phosphorothioate decomposition (principally by loss of sulfur) interferes with any quantitative assessment of the conceivably nonzero rate of substrate analogue isomerization by phosphoglycerate mutase.]

The recent development in this laboratory of methods for the synthesis and stereochemical analysis of [^{16}O,^{17}O,^{18}O]-phosphate monoesters that are chiral only by virtue of the isotopic labels avoids the ill-behaved phosphorothioates and allows the stereochemical course of the phosphoglycerate mutases to be probed with their natural substrates. We present here the synthesis of 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate and the stereochemical analysis of the chiral 3-[^{16}O,^{17}O,^{18}O]phospho-D-glycerate derived from it in the reactions catalyzed by the phosphoglycerate mutase either from rabbit muscle or from wheat germ.

Experimental Section

Materials

Enolase (from yeast, as a freeze-dried powder) and phosphoglycerate mutase, lactate dehydrogenase, pyruvate kinase, and glyceraldehyde-3-phosphate dehydrogenase (each from rabbit muscle, as crystalline suspensions) were obtained from Sigma Chemical Co. Wheat germ phosphoglycerate mutase was prepared by P. E. Johnson according to the method described by Leadlay et al. (1977). NADH (sodium salt) and ATP (disodium salt) were obtained from Sigma. (*S*)-Propane-1,2-diol was prepared as described earlier (Abbott et al., 1979) and had 86% enantiomer excess.

*Benzyl 3-O-Benzyl-*D*-glycerate* (**2**). 3-*O*-Benzyl-D-glycerate was prepared according to the method of Ballou & Fischer (1954). This material was then esterified by using benzyl bromide in dimethylformamide according to Wagenknecht et al. (1972). Benzyl 3-*O*-benzyl-D-glycerate (**2**), after purification by chromatography on silica gel (eluting with hexane–ethyl acetate, 4:1 v/v), was obtained in 62% yield from methyl 6-*O*-benzyl-α-D-galactopyranoside: ^{1}H NMR ($CDCl_3$) δ 3.15 (d, 1 H, J = 7 Hz), 3.72 (d, 2 H, J = 3.5 Hz), 4.31 (t, d, 1 H, J = 3.5 and 7 Hz), 4.49 (br s, 2 H), 5.19 (s, 2 H), 7.23 (s, 5 H), and 7.29 (s, 5 H); $[\alpha]^{25}_D$ +21.5° (*c* 4.0, $CHCl_3$).

*2-[(R)-^{16}O,^{17}O,^{18}O]Phospho-*D*-glycerate* (**7**). Benzyl 3-*O*-benzyl-D-glycerate was phosphorylated by the general synthetic method for chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters described earlier (Abbott et al., 1978, 1979). A solution of benzyl 3-*O*-benzyl-D-glycerate (**2**) (1.28 g, 4.48 mmol) in dry tetrahydrofuran (150 mL) at −78 °C was treated with lithium diisopropylamide [freshly prepared in 5 mL of tetrahydrofuran from diisopropylamine (4.6 mmol) and *n*-butyllithium (4.48 mmol) in hexane]. The solution was stirred at −78 °C for 30 min and then transferred over 10 min through a cannula to a solution of (2*R*,4*S*,5*S*)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-[2-^{17}O]oxazaphospholidin-2-one (**4**) [this is the major product from the reaction of (−)-ephedrine (**3**) with [^{17}O]-$POCl_3$] [the $H_2{}^{17}O$ used to prepare the [^{17}O]$POCl_3$ had ^{16}O (31.89%), ^{17}O (41.01%), and ^{18}O (27.01%): see Abbott et al. (1978, 1979)] (4.48 mmol) in tetrahydrofuran (30 mL) at −78 °C. The mixture was brought to 0 °C and stirred for 30 min. The solution was then poured into cold water (100 mL) and the product was extracted into ether. The combined ether extracts were washed with cold water, dried over $MgSO_4$, and concentrated under reduced pressure. Purification of the product by preparative thin-layer chromatography on silica (eluting with ethyl acetate) gave the cyclic phosphoramidate diester **5** as a pale yellow oil in 66% yield from the chloro compound **4**: ^{31}P NMR ($CDCl_3$) δ −19.04 ppm (s); ^{1}H NMR ($CDCl_3$) δ 0.77 (d, 3 H, J = 7 Hz), 2.62 (d, 3 H, J = 10 Hz), 3.90–3.32 (m, 1 H), 3.90 (d, 2 H, J = 5 Hz), 4.48 (d, 1 H, J = 13 Hz), 4.64 (d, 1 H, J = 13 Hz), 5.24 (t, d, 1 H, J = 5 and 9 Hz), 5.28 (s, 2 H), 5.61 (d, d, 1 H, J = 6 and 2 Hz) and 7.27 and 7.26 (s and s, 15 H together).

The cyclic phosphoramidate diester **5** (2.95 mmol) in dry dioxane (6 mL) was added to freshly distilled trifluoroacetic anhydride (200 μL) in $H_2{}^{18}O$ (2 mL, of the following isotopic composition: ^{16}O, 2.29%; ^{17}O, 0.43%; ^{18}O, 97.28%). After 2 min, solid NH_4HCO_3 (500 mg) was added and the solution was diluted with H_2O–EtOH (50 mL, 1:1 v/v). [In a previous experiment the solution after ring opening was evaporated to dryness and the ^{31}P NMR ($CDCl_3$) showed one singlet at +1.63 ppm.] This solution of acrylic phosphate diester **6** was then subjected to hydrogenolysis over Pd/C at 3.4 atm for 2 h. The catalyst was removed by filtration through Celite. The filtrate was brought to pH 7.5 with solid NH_4HCO_3 and then concentrated under reduced pressure to about 2 mL. Water (23 mL) was added and the solution was washed 4 times with ether. Ethanol (25 mL) was added and the solution was subjected to further hydrogenolysis over Pd/C at 3.4 atm for 17 h. Evaporation to dryness gave the ammonium salt of 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate (**7**) [2.46 mmol by enzymic assay (Bergmeyer, 1974), 83% yield from the cyclic phosphoramidate diester **5**]. No 3-phospho-D-glycerate could be detected by enzymic assay (Bergmeyer, 1974). A portion of this material was converted to the tricyclohexylammonium salt which was recrystallized from water–acetone and had mp 155–156 °C. ^{31}P NMR (D_2O) showed a singlet that was slightly broadened (presumably deriving from the ^{18}O-labeled species), centered at −3.03 ppm; $[\alpha]^{25}_D$ (tricyclohexylammonium salt) +4.2° (*c* 1.14, H_2O); $[\alpha]^{25}_D$ (free acid) +22.5° (*c* 0.52, 1 N HCl) [Ballou & Fischer (1954) give +13.0°, Meyerhof & Kiessling (1935) give +24.3°, and Neuberg (1943) gives 23.2°]; $[\alpha]^{25}_D$ (in saturated aqueous ammonium molybdate) +15.4° (*c* 0.64, H_2O) [Ballou & Fischer (1954) give +5.0°]. 3-Phospho-D-glycerate under these conditions has $[\alpha]^{25}_D$ −720° (*c* 0.56, H_2O) [Meyerhof & Schulz (1938) give −745°].

Methods

^{1}H NMR spectra were recorded on a Varian XL-100 or a Varian CFT-20 instrument. Proton-decoupled ^{31}P NMR spectra were taken on a Varian XL-100 machine, chemical

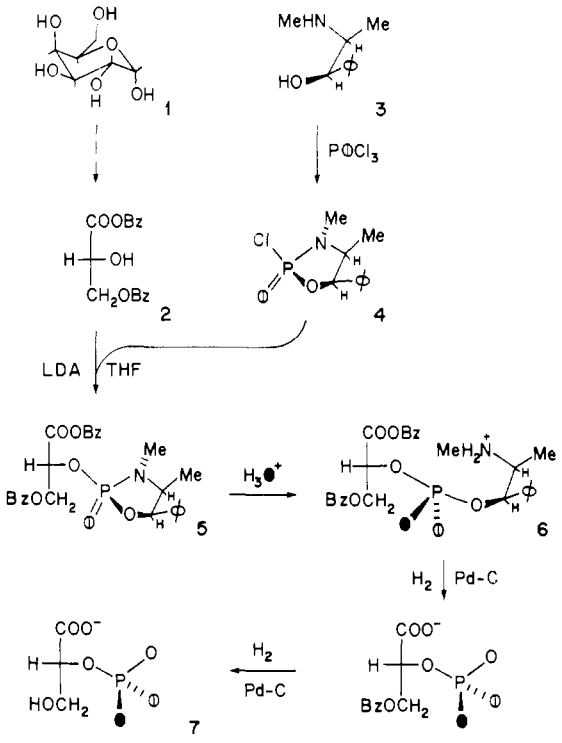
shifts being measured from external 85% H_3PO_4 (downfield was negative). Ion-exchange chromatography was done by using DEAE-cellulose (DE-52, from Whatman), Dowex 1 (200–400 mesh, 8% cross-linked) and Dowex 50 (100–200 mesh, 8% cross-linked). High-pressure liquid chromatography and mass spectrometry in both normal and linked-scan modes were done as described earlier (Abbott et al., 1979).

Production of 3-Phospho-D-glycerate by Rabbit Muscle Phosphoglycerate Mutase. 2-[(*R*)-^{16}O,^{17}O,^{18}O]Phospho-D-glycerate ammonium salt (1 mmol) was dissolved in 50 mM *N*-ethylmorpholine hydrochloride buffer, pH 7.0 (100 mL), and the mutase reaction was initiated by the addition of 2,3-bisphospho-D-glycerate (5 μmol) and rabbit muscle phosphoglycerate mutase (500 units). After 4 h at room temperature, the reaction mixture was poured into boiling methanol (300 mL), and after 5 min the methanol was removed by evaporation under reduced pressure. The remaining solution was passed through a column (20 mL) of Dowex 50 (H^+ form). After neutralization of the eluate with NEt_3, the solution was applied to a column (200 mL) of DEAE-cellulose (DE-52, equilibrated with 50 mM triethylammonium bicarbonate, pH 7.2) which was eluted with a linear gradient (800 mL plus 800 mL) of 50–200 mM triethylammonium bicarbonate. Fractions containing the mixture of 3-phospho-D-glycerate (772 μmol) and 2-phospho-D-glycerate (67.2 μmol) were pooled. The recovery of phosphoglycerate was 84%. The remaining 2-phospho-D-glycerate was removed from this mixture by incubation with NADH (100 μmol), ADP (100 μmol), pyruvate kinase (250 units), lactate dehydrogenase (260 units), and enolase (240 units), in 50 mM *N*-ethylmorpholine hydrochloride buffer, pH 7.0 (20 mL), containing $MgCl_2$ (100 μmol) and KCl (1 mmol). After 1 h at room temperature the reaction mixture was passed through a column (20 mL) of Dowex 50 (H^+ form). After neutralization of the eluate with NEt_3, the phosphoglycerates were isolated by chromatography on DEAE-cellulose as described above. The pooled fractions contained 3-phospho-D-glycerate (599 μmol) and 2-phospho-D-glycerate (7.4 μmol) (i.e., the 3-phospho compound was contaminated with 1.2% of 2-phosphoglycerate).

Production of 3-Phospho-D-glycerate by Wheat Germ Phosphoglycerate Mutase. The isomerization of 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate (1 mmol) by the wheat germ mutase was done analogously. No bisphosphoglycerate cofactor is required by this enzyme, and the reaction was run in 50 mM Tris-HCl buffer, pH 8.6 (100 mL), with 50 units of mutase. The incubation was for 23 h at room temperature and yielded a mixture of 3-phospho-D-glycerate (776 μmol) and 2-phospho-D-glycerate (67.4 μmol) (recovery was 84%). The removal of remaining 2-phospho-D-glycerate was done as described above (except using somewhat higher levels of NADH and ADP), to yield the 3-phospho compound (776 μmol) contaminated by 5.7 μmol (0.73%) of 2-phosphoglycerate.

Transfer of the Chiral Phosphoryl Groups to (S)-Propane-1,2-diol. The whole of the sample of 3-[^{16}O,^{17}O,^{18}O]phospho-D-glycerate from the rabbit muscle mutase reaction was dissolved in 0.3 M $KHCO_3$–K_2CO_3 (1:1, 2.4 mL) containing $Mg(OAc)_2$ (7.5 μmol) and $Zn(OAc)_2$ (75 nmol). (*S*)-Propane-1,2-diol (2.4 mL) was added, and the transfer reaction was initiated with alkaline phosphatase (42 units, in 100 μL of buffer). After 13 h at room temperature the reaction was stopped, and the mixture of 1- and 2-phospho-(*S*)-propane-1,2-diols was isolated as described earlier (Blättler & Knowles, 1979b). [The yield of phosphopropanediols after one recrystallization of the dicyclohexylammonium salt from acetone–water was 7% based on the phosphoryl group donor 3-phospho-D-glycerate.] The transfer reaction using the 3-phospho-D-glycerate sample from the wheat germ mutase reaction was done analogously, using proportionately larger volumes and 54 units of alkaline phosphatase. [The yield of phosphopropanediols was 7.5% based on the donor 3-phospho-D-glycerate.]

Scheme I: Synthetic Scheme for 2-[(*R*)-^{16}O,^{17}O,^{18}O]Phospho-D-glycerate



Analysis of the absolute configuration of the phosphoryl groups in the two samples of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (each of which contained 20% of the 2-phospho isomer as determined by ^{1}H NMR) was done by the method described earlier (Abbott et al., 1979).

Results

Isotopically labeled 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate was synthesized by the general route we have described earlier (Abbott et al., 1978, 1979) in which the chiral phosphoryl group is elaborated on a free hydroxyl function (in this case, that of benzyl 3-*O*-benzyl-D-glycerate), as shown in Scheme I. Although the neighboring carboxyl group gave minor cause for concern, it was shown in control syntheses that there is no washout of isotopic label from the 2-phosphoglycerate **7** into the solvent during preparation and isolation. On the basis of the proven stereochemical validity of the synthetic scheme [this has been shown by independent determinations of the absolute stereochemistry of the chiral phosphoryl group in both 1-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (Abbott et al., 1978, 1979) and γ-[(*S*)-^{16}O,^{17}O,^{18}O]adenosine triphosphate (Blättler & Knowles, 1979a,b)], it is evident that **7** is 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate.

The sample of 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate was used as a substrate in the reactions catalyzed by the phosphoglycerate mutases from rabbit muscle and from wheat germ. Incubation with the mutases produced the equilibrium mixture of 2- and 3-phospho-D-glycerate in a ratio of 1:11.5 [see also Clarke et al. (1974)]. Most of the residual 2-phospho compound was removed enzymically, and the configuration

Table I: Results from Linked-Scan Metastable Ion Mass Spectrometry of the Isotopically Labeled Trimethyl Phosphate Ions Derived from the Samples of [^{16}O,^{17}O,^{18}O]Phospho-(*S*)-propane-1,2-diol[a]

	ratio of granddaughter ion intensities at 111 and 113[b]		% of (*R*)-phospho compound derived from the ratio of granddaughter ion intensities		
	anti[c]	syn[c]	anti[c]	syn[c]	average[d]
ideal case for					
(*R*)-phosphopropanediol	0	0.5	100	100	100
racemic mixture	0.25	0.25	50	50	50
(*S*)-phosphopropanediol	0.5	0	0	0	0
observed for phosphopropanediol from					
rabbit muscle mutase product	0.165	0.350	67.1	70.0	68.5[e]
wheat germ mutase product	0.170	0.384	66.1	76.7	71.4[f]
predicted[g] for (*R*)-phosphopropanediol from					
rabbit muscle mutase product	0.135	0.353	73.1	70.5	71.8
wheat germ mutase product	0.135	0.353	73.1	70.5	71.8

[a] Samples of [^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol were analyzed as described by Abbott et al. (1978, 1979). After cyclization and methylation, the resulting syn and anti cyclic triesters were separated by high-pressure liquid chromatography and subjected to methanolysis. Linked-scan metastable ion mass spectrometry of the resulting acyclic triesters allowed the fragmentation from the labeled trimethyl phosphate ion of *m/z* 143 (which contains *both* ^{17}O and ^{18}O) to be investigated (Abbott et al., 1978, 1979). [b] The metastable fragmentation of the labeled trimethyl phosphate ion at *m/z* 143, which can lose [^{16}O]formaldehyde (to *m/z* 113), [^{17}O]formaldehyde (to *m/z* 112), or [^{18}O]-formaldehyde (to *m/z* 111), was measured. [c] That is, results, deriving from investigation of the anti or syn cyclic triesters after their separation by high-pressure liquid chromatography and subsequent methanolysis. [d] Average of the results from the separate measurements on the syn and the anti cyclic triesters. [e] This value should be compared with the predicted value of 71.8% and shows that the phosphopropanediol from the rabbit muscle mutase product is 95.4% *R* at phosphorus. [f] This value should be compared with the predicted value of 71.8% and shows that the phosphopropanediol from the wheat germ mutase product is 99.4% *R* at phosphorus. [g] Predicted results on the basis of the known isotopic composition of the labeled water samples used in the synthesis of 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate, the measured enantiomeric purity of the (*S*)-propane-1,2-diol used, the measured cross contamination of the syn and anti cyclic triesters after separation, and the measured ratio of 1- to 2-phosphopropanediol. The use in the analysis only of the ratio of granddaughter ion intensities at 111 and 113 obviates any need for corrections for "downward cross talk" in the mass spectrometer or for interference from natural-abundance ^{13}C and ^{3}H. For a full explanation of these effects, see Abbott et al. (1979).

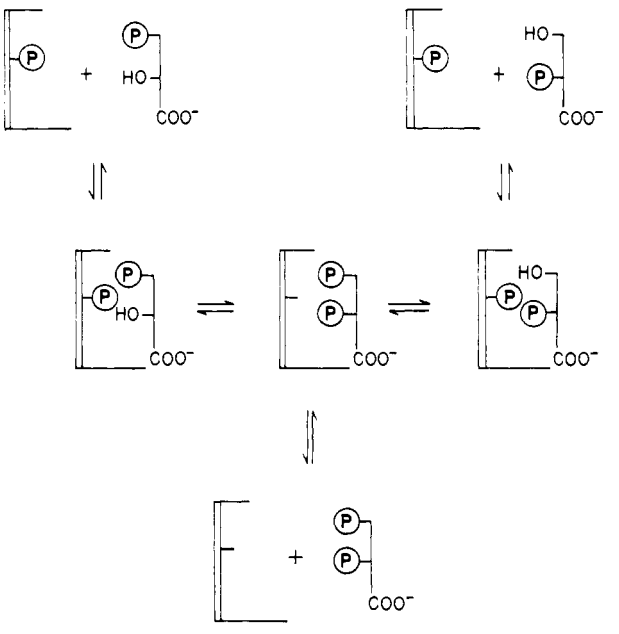
at phosphorus in the product 3-phospho-D-glycerate was determined. This analysis was achieved by transfer of the phosphoryl group with retention of configuration (Jones et al., 1978) to (*S*)-propane-1,2-diol catalyzed by alkaline phosphatase, followed by the determination of the absolute configuration of the phosphoryl group in the resulting samples of phosphopropanediol (Abbott et al., 1978, 1979).

In each sample (that is, the products from the reaction catalyzed by each of the phosphoglycerate mutases), the configuration at phosphorus is *R* (95.4 ± 8% *R* for the reaction of the rabbit muscle enzyme and 99.4 ± 8% *R* for the reaction of the wheat germ enzyme). *Each of the mutases therefore transfers the phosphoryl group with overall retention of configuration.* The results are presented in detail in Table I.

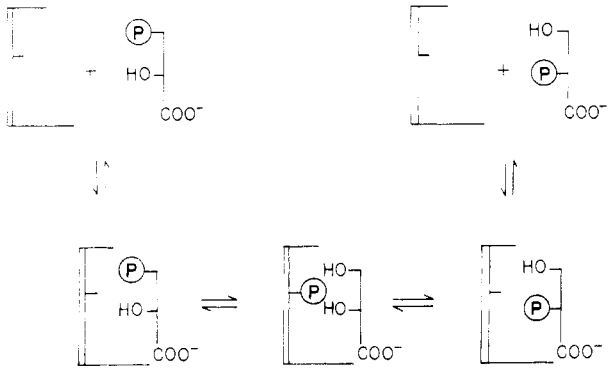
Discussion

The phosphoglycerate mutases from rabbit muscle and from wheat germ provide a nicely complementary pair of enzymes for mechanistic comparison. The rabbit enzyme requires the presence of 2,3-bisphosphoglycerate as a cofactor and catalyzes the *inter*molecular transfer of phosphoryl groups amongst the two substrates (2- and 3-phospho-D-glycerate) and the cofactor. The most probable pathway followed by this enzyme is shown in Scheme II, which illustrates the role of the cofactor in maintaining the enzyme in its phosphorylated form and the intermolecular shuttling of phosphoryl groups amongst different carbon skeletons. A chemically competent phosphorylenzyme has been isolated from the rabbit and yeast enzymes in which a histidine residue is phosphorylated (Rose, 1970, 1971), and the peptide containing the phosphorylated residue in the chicken enzyme has been characterized (Rose et al., 1975). In the case of the chicken enzyme, the rates of enzyme phosphorylation and dephosphorylation are fast enough to validate the kinetic competence of this phosphorylenzyme (Rose & Dube, 1976). A mechanistic pathway via a phosphorylenzyme is also indicated by steady-state kinetic studies (Grisolia & Cleland, 1968; Rose & Dube, 1978) and by the flux kinetics approach pioneered by Britton (Britton et al., 1972; Britton & Clarke, 1972). The absence of induced transport in the latter experiments further required that any isomerization of the phosphorylenzyme have a rate constant in excess of 10^6 s^{-1}. These experiments are all consistent with the pathway [somewhat simplified: see Grisolia & Cleland (1968)] shown in Scheme II. The stereochemical results presented here are also consistent with this scheme since, in two turnovers of the enzyme (which is what is needed to transfer a given phosphoryl group from 2-phosphoglycerate to 3-phosphoglycerate), the transferred phosphoryl group suffers two transfers. Whether these each proceed by retention or by inversion [though inversion seems to be the general rule for single enzyme-catalyzed phosphoryl transfers: see Blättler & Knowles (1979a,b)], the overall consequence will be re-

Scheme II: Pathway for the Reaction Catalyzed by Rabbit Muscle Phosphoglycerate Mutase



Scheme III: Pathway for the Reaction Catalyzed by Wheat Germ Phosphoglycerate Mutase



tention, which is what is observed experimentally.

The picture for the yeast mutase has become more teasing, however, with the refinement of the crystal structure of this enzyme to high resolution (Campbell et al., 1974) and the fitting of the recently determined amino acid sequence to the electron density map [S. I. Winn, H. C. Watson, R. N. Harkins, and L. A. Fothergill, private communication; these results are also briefly referred to in Fersht (1977)]. The active site of the yeast mutase evidently contains two almost parallel histidine residues, one pointing toward each of the phosphate binding loci (which have been located from the position of bound 3-phosphoglycerate in the crystal). It has been suggested by the above authors that either of these two histidines may be phosphorylated and that in the phosphorylenzyme there is a rapid ($>10^6$ s^{-1}) shuttling of the phosphoryl group between them. For this seductive postulate to be consistent with our stereochemical evidence, we should require that if each of the phosphoryl group transfers between enzyme and substrate occurs with inversion, the interhistidine phosphate shuttling must occur with retention [which in turn requires a pseudorotation: see Westheimer (1968)]. The alternative is that there is only one phosphorylenzyme [i.e., only the one that has been characterized by Rose (1970, 1971)] and that the second histidine has a general acid–base role in the phosphoryl group transfer. This postulate avoids the two problems of the unreasonably rapid interhistidine phosphate transfer and the fact that one phosphorylenzyme has to have a significantly lower free energy than the other. The fact that only one phosphorylenzyme (in which histidine-8 is labeled) has been detected, which after denaturation hydrolyzes with a single rate constant, means that if two phosphorylenzymes exist, they must differ in thermodynamic stability by more than 50-fold (Han & Rose, 1979). Our finding of overall retention makes the pathway involving a single phosphorylenzyme the preferred one.

In contrast to the rabbit muscle and yeast enzymes, the phosphoglycerate mutase from wheat germ catalyzes a cofactor-independent *intra*molecular phosphoryl group transfer (Britton et al., 1971; Gatehouse & Knowles, 1977). No phosphorylenzyme has been detected, though from flux kinetics (Britton et al., 1971) and from studies on the "unnatural" phosphoryl group transfer from substrate analogues to D-glycerate (Breathnach & Knowles, 1977) it was concluded that a phosphorylenzyme was an intermediate on the reaction pathway (see Scheme III). To account for the absence of isotopic exchange with [^{14}C]-D-glycerate and the failure of attempts to isolate the phosphorylenzyme directly, we presumed that (a) the phosphorylenzyme was of relatively high free energy and (b) D-glycerate binds extremely tightly to this phosphorylenzyme. The stereochemical results reported here for the wheat germ enzyme are in accord with this notion. If, as we are coming to accept, inversion is the course of single enzyme-catalyzed phosphoryl transfers, then the overall retention of configuration of the transferred phosphoryl group is consistent with a double-displacement mechanism.

Although the two phosphoglycerate mutases studied here are superficially so different, it is evident that there are fundamental similarities in the pathways they follow. Each appears to involve a phosphorylenzyme intermediate, and as far as the group transfer is concerned the only difference is that in the yeast and animal systems (Scheme II) the transfer occurs from the enzyme to a *phospho*glycerate, whereas in the plant system (Scheme III) the transfer occurs from the enzyme to glycerate itself. The only other difference is that the intermediate between 2- and 3-phosphoglycerate can be lost in one case (2,3-bisphosphoglycerate: Scheme II) and not in the other (glycerate: Scheme III). Access of the 2,3-bisphospho compound for the priming of the free yeast and animal enzymes is essential, of course, since only the phosphorylenzyme can mediate the interconversion of the monophospho substrates by these enzymes.

Finally, it may be noted that the present work relates to the stereochemical consequence at phosphorus of members of the third general class of enzymes that transfer phosphoryl groups: phosphomutases. We have earlier studied the stereochemical course of a phosphatase (Jones et al., 1978) and of two phosphokinases (Blättler & Knowles, 1979a,b) using the ^{16}O,^{17}O,^{18}O methodology. Additionally, using [^{18}O]-phosphorothioates, we (Orr et al., 1978) and Frey and coworkers (Richard & Frey, 1978; K. F. R. Sheu, J. P. Richard, and P. A. Frey, private communication) have studied a number of other phosphokinases, and phosphorothioates have been used widely in work on displacements at phosphate diesters [see Eckstein (1979)]. So far, all of the results from these investigations are consistent with *inversion* being the preferred stereochemical course for enzyme-catalyzed displacements at phosphate monoesters and diesters, even if the mechanistic reasons for this apparent stereochemical imperative are still hazy.

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Methylation of Histidine-48 in Pancreatic Phospholipase A_2. Role of Histidine and Calcium Ion in the Catalytic Mechanism†

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ABSTRACT: It is known that His-48 is part of the active center in pancreatic phospholipase. To further elucidate the role of histidine-48 in the active center of pancreatic phospholipase A_2, we have modified the enzyme with a number of bromo ketones and methyl benzenesulfonates. Rapid methylation occurred with methyl *p*-nitrobenzenesulfonate. Methylated phospholipase shows total loss of enzymatic activity whereas binding of substrate and the cofactor Ca^{2+} remains intact. Amino acid analysis of methylated equine phospholipase showed the loss of the single molecule of histidine and the formation of one molecule of 2-amino-3-(1-methyl-5-imidazolyl)propanoic acid (1-methylhistidine). Equine phospholipase was also modified by [^{13}C]methyl *p*-nitrobenzenesulfonate and the methylated enzyme was studied by ^{13}C NMR. The results indicate that the proton on the nitrogen in position 3 of the imidazole ring is involved in a strong interaction with a buried carboxylate group, thereby hindering rotation of the imidazole ring, and that the nitrogen in position 1 is involved in catalysis. These data are in full agreement with the three-dimensional structure at 1.7-Å resolution of bovine pancreatic phospholipase. A catalytic mechanism is proposed in which a water molecule which is close to the nitrogen at position 1 of the imidazole ring of the Asp-99-His-48 couple acts as the nucleophile. A comparison is made between phospholipase A_2 and the serine esterases.

Phospholipase A_2 (EC 3.1.1.4) has been isolated from several venoms as well as from pancreatic tissue or juice (Rosenberg, 1979). From the latter source the enzyme is isolated as a zymogen which in contrast to the phospholipase does not degrade natural phospholipids in aggregated structures (de Haas et al., 1971; Pieterson et al., 1974). Synthetic short-chain lecithins, however, are hydrolyzed at concentrations below the cmc[1] not only by pancreatic or snake venom phospholipases but also by the zymogens (Pieterson et al., 1974). From these studies it was concluded that pancreatic phospholipases and their zymogens possess an active site with comparable efficiency. The conclusion was supported by inhibition experiments with *p*-bromophenacyl bromide which alkylates His-48 with the same velocity in both enzyme and zymogen (Volwerk et al., 1974). The introduction of the *p*-bromophenacyl group completely abolishes enzymatic activity and binding of monomeric analogues. Moreover, the binding of the essential cofactor Ca^{2+} is severely distorted. The conclusion of Volwerk et al. (1974) that His-48 is an active center residue is supported by the finding of several investigators that also in snake venom phospholipases modification of His-48 is accompanied by loss of enzymatic activity (Fohlman et al., 1979). Although the data support the importance of His-48 which is preserved in the primary structure of all vertebrate phospholipases, they do not specify its catalytic role.

The participation of His-48 in catalysis would be more readily demonstrated by the introduction of a small modifying group neither perturbing Ca^{2+} binding nor substrate binding and by the measurement of the pK of His-48 in the Michaelis

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[1] Abbreviations used: cmc, critical micellar concentration; 1-methylhistidine (π-methylhistidine), 2-amino-3-(1-methyl-5-imidazolyl)propanoic acid; 3-methylhistidine (τ-methylhistidine), 2-amino-3-(1-methyl-4-imidazolyl)propanoic acid; thialaminine, *S*-[2-(*N*,*N*,*N*-trimethylamino)ethyl]cysteine; thialamininated phospholipase, phospholipase A_2 in which all cysteines have been converted into thialamines by reduction and alkylation; Hepes, 2-[4-(2-hydroxymethyl)-1-piperazine]ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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