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Synthesis of 1-Chloro-2-oxohexanol 6-Phosphate, a Covalent Active-Site Reagent for Phosphoglucose Isomerase†

Klaus D. Schnackerz,‡ John M. Chirgwin,§ and Ernst A. Noltmann*

ABSTRACT: A new covalent active site reagent, 1-chloro-2-oxo-6-hexanol 6-phosphate, has been synthesized from glutaric acid monomethyl ester and characterized by NMR spectroscopy. Inactivation of phosphoglucose isomerase, when incubated with various modifier concentrations, was found to be pseudo first order with respect to enzyme concentration (half-life of inactivation 6 h at pH 7.5 (30 °C) and 2.0 μM active site concentration) but showed saturation kinetics for the dependence on inactivator concentration. This saturation phenomenon demonstrates the occurrence of a reversible enzyme–inhibitor complex (K_{diss} = 14.3 mM) preceding the irreversible inactivation via the chloromethyl oxo groups. Substrate or competitive inhibitors such as 6-phosphogluconate or 5-phosphoarabinonate protect against inactivation of the isomerase by the modifying reagent.

Kinetic studies on the effect of pH and temperature on substrate and inhibitor binding to phosphoglucose isomerase [D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9] yielded a proposal for the mechanism of this enzyme that involves both a histidine and a lysine residue as catalytically functional groups of the enzyme protein (Dyson & Noltmann, 1968; Noltmann, 1972). Efforts have been expended over the past several years to find suitable modifying reagents for these two amino acid residues in order to determine the validity of this proposal. Techniques such as dye-sensitized photooxidation (Chatterjee & Noltmann, 1967), carboxamidomethylation (Schnackerz & Noltmann, 1970), and Schiff base formation with pyridoxal 5′-phosphate followed by borohydride reduction (Schnackerz & Noltmann, 1971) have provided supporting evidence for this mechanism. Whereas our proposal calls for the involvement of the imidazolyl nitrogen of a histidine in the proton transfer between carbons 2 and 1 of the hexose-phosphates, Rose's laboratory has advanced the concept that a glutamic carboxyl performs this function (Rose, 1975). For further study of this question and specifically determination of an active-site label that could be used for structural studies, the present investigation was directed toward the synthesis of a halomethyl ketone derivative of the substrate. It was hoped that such a reagent could be covalently bound to the active-site of phosphoglucose isomerase and, after appropriate cleavage of the enzyme molecule into its peptides, serve to identify an amino acid residue involved in the isomerization process, i.e., histidine or glutamic acid or both.

Precedents for the use of halomethyl ketone reagents to label enzymes with similar functions, notably triosephosphate isomerase and aldolase, have been provided by Hartman (1970 a,b; Hartman et al., 1973), Coulson et al. (1970), and Burton & Waley (1966). We are particularly interested in resolving the question of whether a histidine or a glutamic acid residue is involved in the phosphoglucose isomerase reaction. Evidence provided for triosephosphate isomerase at the level of total sequence analysis appears to indicate unequivocally that for this enzyme a glutamic acid is the critical residue. The use of a covalently bound reagent will also be helpful in providing chemical evidence for the hypothesis advanced by Shaw & Muirhead (1976) and Bruch et al. (1976) that the active sites of phosphoglucose isomerase are located at the interface be-

† From the Department of Biochemistry, University of California, Riverside, California 92521, and the Physiologisch-Chemisches Institut der Universität Würzburg, Germany. *Received September 25, 1980.* This is paper 27 in a series dealing with studies on phosphohexose isomerases. The work was supported in part by U.S. Public Health Service Research Grant AM 07203 and by Research Grant Schn 139/6 from the Deutsche Forschungsgemeinschaft. An abbreviated account of this work has been presented at the Joint Meeting of the German and French Societies of Biological Chemistry, Freiburg, October 1977 (Schnackerz et al., 1977).

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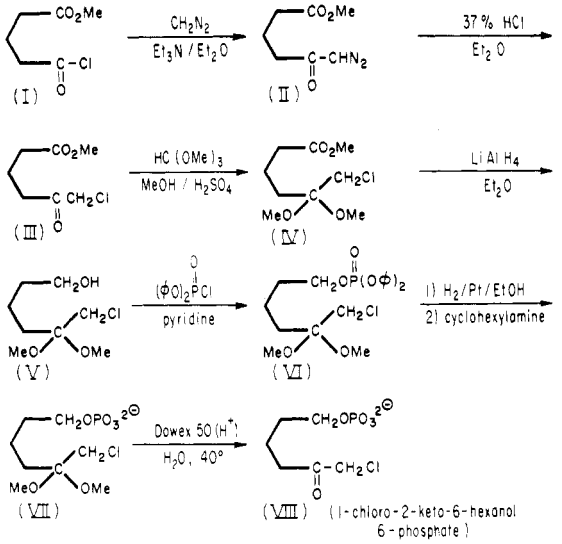
Scheme I



tween its subunits. Finally, as a radioactive marker, such a substratelike irreversible modifier can assist in determining the amino acid sequence of an active-site peptide.

Experimental Procedures

Materials

Chemicals for Organic Synthesis. Monomethylglutaryl chloride was obtained from Research Organic/Inorganic Chemicals Co., Sun Valley, CA; *N,N'*-dimethyl-*N,N'*-dinitrosoterephthalamide and trimethyl orthoformate acquired from Aldrich. Lithium aluminum hydride, diphenyl phosphorochloridate, and platinum dioxide were products of Merck, Darmstadt. Triethylamine was purchased from Fluka, Buchs, Switzerland. General inorganic chemicals were obtained from Mallinckrodt or from Merck, Darmstadt.

For synthesis of tritium-labeled derivatives lithium aluminum [^{3}H]hydride was obtained from New England Nuclear, Boston, MA (e.g., lot 974-298; specific radioactivity, 169.19 μCi/mmol).

Enzymes. Rabbit muscle phosphoglucose isomerase was isolated by the chromatographic procedure of Blackburn et al. (1972) or by substrate elution from cellulose phosphate (Phillips & Gracy, 1976). Enzyme samples used in these experiments had specific activities ranging from 800 to 1000 μmol of fructose 6-phosphate converted to glucose 6-phosphate per min per mg of enzyme at 30 °C.

Enzyme Assay Components. Substrates and enzymes other than phosphoglucose isomerase were obtained either from Sigma Chemical Co. or from Boehringer Mannheim Corp. 6-Phosphogluconate was purchased from Boehringer. 5-Phosphoarabinonate was prepared as described by Chirgwin & Noltmann (1975).

Methods

Synthesis of 1-Chloro-2-oxo-6-hexanol 6-Phosphate.[1] The synthetic procedure follows the outline shown in Scheme I. The initial steps of the synthesis are performed according to the procedure of Lartillot & Baron (1964) for the preparation of the corresponding ethyl ester from glutaric anhydride. In the conversion of intermediate I to intermediate II, etheral diazomethane is prepared from *N,N'*-dimethyl-*N,N'*-dinitrosoterephthalamide as described by Moore & Reed (1961); the diazomethane solution is dried over potassium hydroxide pellets prior to further use.

Conversion to Intermediate III. To a solution of 0.5 mol each of diazomethane and triethylamine in 3 L of ether, which are stirred at −5 °C in a round-bottom flask protected from atmospheric moisture by a drying tube, is added 0.5 mol of monomethylglutaryl chloride in ether dropwise over the course of 1 h. The reaction mixture is brought to room temperature and filtered after 1 h. It is once more cooled with stirring to 4 °C, and 0.5 mol of 37% hydrochloric acid is added dropwise, again over the period of 1 h. The solution is then washed successively with sodium bicarbonate (10% w/v) and with saturated sodium chloride, dehydrated over sodium sulfate, and finally concentrated to dryness. Fractional vacuum distillation [bp 75–80 °C (0.035 mm)] yields intermediate III (recovery approximately 40%); NMR (CCl_4) δ 1.7–3.0 (6 H, m, methylene protons of C-3, C-4, C-5), 3.53 (3 H s, methyl ester protons), 4.04 (2 H, s, methylene protons of C-1).

Conversion to Intermediate IV. Intermediate III is converted to the dimethyl ketal (intermediate IV) by the procedure of Hartman (1970a,b) with almost quantitative recovery.

Conversion to Intermediate V. In the following reductive step 206.6 mg of lithium aluminum hydride (10% calculated excess over intermediate IV) is dissolved in 40 mL of anhydrous ether in a 100-mL, three-arm flask fitted with mechanical stirrer, addition funnel, reflux condenser with $CaCl_2$ drying tube, and nitrogen flushing line. Intermediate IV (1 g = 4.45 mmol dissolved in 6 mL of ether) is added dropwise with vigorous stirring to the reaction flask which is cooled at 4 °C over a period of 30 min. Stirring is then continued at room temperature for an additional 30 min. The resulting suspension is treated as described by Mikovic & Mihailovic (1953) which allows removal of aluminum salts in a granular state. The filtrate is dried over anhydrous sodium carbonate. After evaporation of the ether, the resulting intermediate V is dried over P_2O_5 for several hours at room temperature. Thin-layer chromatographic analysis in benzene/ethanol (see below) indicates complete conversion of intermediate IV (R_f 0.58) to intermediate V (R_f 0.31). Recovery at this step is usually 0.8 g of intermediate V; NMR ($CDCl_3$) δ 1.2–1.9 (6 H, m, methylene protons of C-3, C-4, C-5), 3.23 (6 H, s, methyl protons of ketal), 3.49 (2 H, s, protons of C-1), 3.66 (2 H, t, protons of C-6).

Conversion to Intermediate VI. For phosphorylation of intermediate V, the product of the previous step (0.8 g) is dissolved in 20 mL of dry pyridine. Diphenyl phosphorochloridate (0.92 mL) is added slowly with a Hamilton microsyringe, the pyridine solution being kept stirred at 0 °C. After an additional 1.5 h of stirring at 0 °C, the temperature is raised to 25 °C with continued stirring. The reaction is terminated after 15 min through addition of 110 μL of water. After 1 h the reaction mixture is concentrated to dryness of 35 °C with the use of a rotary evaporator. The residue is dissolved in 45 mL of ether and washed twice with 10 mL of sodium bicarbonate (5%) and subsequently 3 times with 10 mL of distilled water. The ether phase is dried with anhydrous sodium carbonate.

After evaporation of the ether, the oily reaction product is kept for several hours under vacuum of at least 0.05 mmHg. Final traces of water are removed by storing in a desiccator over P_2O_5. Thin-layer chromatographic analysis in a benzene/ethanol system in which intermediate IV has an R_f of 0.50–0.58 and intermediate V an R_f of 0.25–0.31 shows in-

[1] Abbreviations used: CKHP, 1-chloro-2-oxohexanol 6-phosphate; G6P, glucose 6-phosphate; 6-PG, 6-phosphogluconate; 5-PA, 5-phosphoarabinonate.

termediate VI, the reaction product of this step, at an R_f of 0.42–0.45. In the event that some intermediate V has not been converted, it can be removed by preparative column chromatography on silica gel (see below); NMR (CCl_4) δ 1.0–1.9 (6 H, br, methylene protons of C-3, C-4, C-5), 3.02 (6 H s, methyl protons of ketal), 3.25 (2 H, s, protons of C-1), 4.0–4.4 (2 H, br, protons of C-6), 7.2 (10 H, s, protons of phenyl ester).

Conversion to Intermediate VII. The protecting phenyl groups are removed from the phosphotriester (intermediate VI) by hydrogenolysis in the presence of platinum dioxide. Prior to introducing the sample, 150 mg of platinum dioxide is suspended in 20 mL of absolute ethanol and the mixture is placed in a hydrogenation bulb attached to a mechanical shaking device. After reduction of the catalyst, which usually consumes about 25 mL of hydrogen, the product from the previous step (1.5 g), dissolved in 6 mL of absolute ethanol, is added to the hydrogenation suspension. Vigorous shaking is continued until no further hydrogen consumption occurs (8 equiv of hydrogen is required to accomplish both removal of the two phenyl groups and to account for the reduction of the resulting benzene ring to cyclohexane), which takes approximately 2.5 h at room temperature. The catalyst is removed by filtration and the filtrate is concentrated to dryness. It is then taken up in 5 mL of water. The suspension has a pH of about 1.3, and sufficient triethylamine is added to bring it to 6.8. The resulting solution, which is colorless and slightly opalescent, constitutes intermediate VII. Thin-layer chromatography in 1-butanol/acetic acid/water (see below) yields an R_f value of 0.70 for the hydrogenation product.

Conversion to Compound VIII. The aqueous solution (pH 6.8) of intermediate VII is placed on a small Dowex 50-X8W column (1.0 × 18.5 cm) and elution is initiated with water. The effluent is collected in 3–4-mL fractions and the tubes containing the intermediate are identified by spotting on cellulose sheets followed by spraying with Hanes–Isherwood reagent (Hanes & Isherwood, 1949). Normally, fractions 4–10 are phosphate positive; their pH is between 1 and 2. These fractions are combined and incubated for 12 h at 40 °C. Alternatively, after removal of ethanol in the hydrogenation step, intermediate VII can directly be converted to intermediate VIII by dissolving it in water and keeping it for 12 h at 40 °C. Thereafter, the solution is titrated with 0.5 M LiOH until 2 equiv of lithium hydroxide has been consumed. The product is then concentrated in vacuo; yield, 0.7 g. When subjected to thin-layer cellulose chromatography in 1-butanol/acetic acid/water (see above), a single spot with an R_f value of 0.40 is obtained for the final product, as compared with 0.70 for intermediate VII; NMR (D_2O) δ 1.69 (4 H, m, methylene protons of C-4 and C-5), 2.76 (2 H, t, protons of C-3), 3.82–3.89 (2 H, q, protons of C-6), 4.55 (2 H s, protons of C-1). Anal. Calcd for $C_6H_{10}O_5ClPLi_2 \cdot 0.5H_2O$ (M_r 252.43): C, 28.55; H, 4.39; Cl, 14.04. Found: C, 28.67; H, 4.27; Cl, 14.47.

Characterization of Synthetic Intermediates. Thin-Layer Chromatography. Intermediates IV through VII were identified by thin-layer chromatography on silica gel sheets in benzene/96% ethanol (9:1 by volume); intermediates VII and VIII were chromatographed on cellulose sheets in 1-butanol/acetic acid/water (2:0.25:0.5). Reaction products were located by spraying with either Hanes–Isherwood spray (visualized under long wavelength ultraviolet light) or with a special keto spray containing 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid (Mehlitz et al., 1963).

Radioactivity Measurements. Radioactivity of tritium-labeled intermediates up to step VI was measured by mixing suitable aliquots with 15 mL of standard toluene-based scintillation fluid. Radioactive intermediates VII and VIII were counted in Bray solution (Bray, 1960). Reagents for scintillation counting were purchased from either Zinsser (Frankfurt) or Beckman Instruments.

Chemical Quantitation of the Synthetic Product. Chlorooxohexanol 6-phosphate in solution was determined as organic phosphate by the microprocedure of Ames & Dubin (1960). Elemental analyses, including Cl, were performed by Robert Glier, Mikro-Elementaranalysen, SchweinfurtRöthlein.

Nuclear Magnetic Resonance Spectroscopy. Intermediates were characterized throughout by nuclear magnetic resonance with either a Varian T-60 or a Bruker 90-MHz spectrometer. Nuclear magnetic resonance solvents were carbon tetrachloride, chloroform-*d* (Merck), or D_2O (>99.5%). The results are expressed as δ shifts downfield from internal or external standard tetramethylsilane or 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate (Na^+).

Inactivation of Phosphoglucose Isomerase. Enzyme and Protein Measurements. Enzyme assays were performed in the direction from fructose 6-phosphate to glucose 6-phosphate by a coupled spectrophotometric assay utilizing yeast glucose-6-phosphate dehydrogenase and NADP (Noltmann, 1966). Protein concentrations were measured at 280 nm with the use of an absorbance factor of 1.32 for a 10-mm light path, for a solution containing 1 mg of enzyme/mL. Enzyme molarities are based on active site equivalents corresponding to a subunit molecular weight of 66 000 (Pon et al., 1970; Blackburn & Noltmann, 1972).

Experimental Conditions for Enzyme Inactivation by Chlorooxohexanol 6-Phosphate. For a test of the effectiveness of the modifying reagent, phosphoglucose isomerase (concentrations ranging from 0.5 to 10 μM) was incubated in 0.05 or 0.1 M triethanolamine–HCl buffer (pH 7.5) with reagent concentrations ranging from 1 to 30 mM. The incubation mixture was pipetted into capped 1-mL reaction vials which were placed for the duration of the experiment in a water bath at 30 ± 1 °C. Small aliquots were withdrawn at various time intervals, diluted into ice-cold 50 mM sodium EDTA, pH 8.0, and assayed for enzymatic activity. Experiments in which substrates or substrate analogues were tested for their protective effect against inactivation by CKHP were performed analogously except that the protectant was present at concentrations indicated in the figure legends. The results of the inactivation experiments were plotted semilogarithmically, i.e., log residual activity vs. time. The slopes of the straight lines (fitted by unweighted linear least-squares analysis) in such a plot equal–k'/2.303, where k' is the apparent pseudo-first-order rate constant of inactivation.

Results and Discussion

Preparation and Characterization of 1-Chloro-2-oxo-6-hexanol 6-Phosphate. An outline of the synthesis described in detail under Methods is shown in Scheme I [see also Chirgwin (1974)]. This synthesis involves the introduction of two different functional groups at the distal ends of a straight, six-carbon chain. These groups are a phosphorylated hydroxyl and an α-chloromethyl ketone. The principle of this synthesis involves the use of commercially available monomethylglutaryl chloride as starting compound into which an α-chloromethyl ketone is introduced at the acid chloride end. For stabilization of the chlorine and prevention of reduction of the ketone, the latter group is protected by conversion to the dimethyl ketal. The methyl ester function at the other end of the carbon chain is reduced to the primary alcohol and is subsequently phosphorylated. A key step in the synthesis is

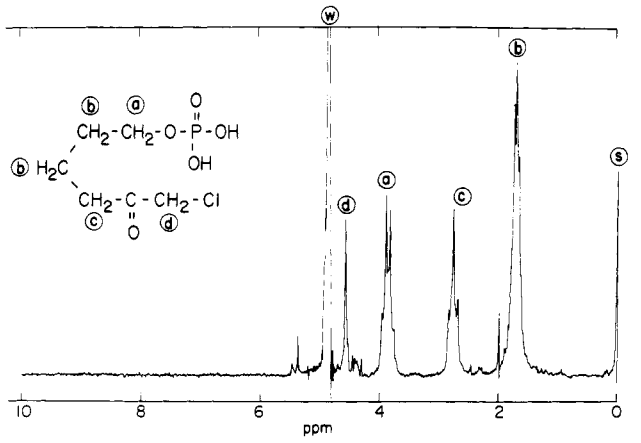


FIGURE 1: Proton magnetic resonance spectrum of 1-chloro-2-oxo-6-hexanol 6-phosphate synthesized as described in the text. Sample concentration was approximately 0.2 M in D_2O (pD 7). The data are expressed in parts per million downfield from 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate (Na^+), labeled *s*. Assignments of proton resonances to specific carbons of CKHP are shown by the letters a–d on the structure drawn in the figure. The peak at 4.8 ppm (w) stems from the solvent D_2O.

the use of diphenyl phosphorochloridate as phosphorylating agent so as to arrive at the diphenyl phosphate ester as intermediate VI (Fischer, 1960).

The synthesis has been carried through without detailed purification at each intermediate step. Thin-layer chromatography and proton magnetic resonance spectroscopy were employed to verify the nature of each intermediate and the purity at each step. Reference NMR data are included at the end of the description of each synthetic step (see above).

Characterization of Compound VIII as 1-Chloro-2-oxo-6-hexanol 6-Phosphate. The final reaction product was determined to be pure by the criteria of thin-layer chromatography in 1-butanol/acetic acid/water. In the system defined under Methods a single spot with an R_f of 0.40 was obtained without any indication of the presence of contaminants. The same spot gave a positive reaction with both the Hanes–Isherwood phosphate spray and the special keto reagent. Elemental analyses of compound VIII for Cl (see above) were in excellent agreement with the theoretical value. In addition, proton magnetic resonance spectra were indicative of a single compound of the structure $CH_2ClCO(CH_2)_4PO_4$ (see Figure 1). The following assignments apply to the peaks shown in Figure 1: The singlet at approximately 4.55 ppm corresponds to the C-1 methylene as the lowest field of resonance. The triplet centered around 2.76 ppm reflects the methylene adjacent to the carbonyl. Its triplet character results from peak splitting because of its location between a carbonyl and a methylene group. The peak with a center at approximately 1.69 ppm corresponds to the methylenes of C-4 and C-5. By necessity they are at the highest field because they are maximally shielded in the area of highest electron density with no electronegative neighbors. They are essentially within one peak because their chemical shifts are expected to be identical. The quadruplet at 3.82–3.89 ppm is produced by the C-6 methylene; its downfield location is expected from the oxygen's electron-withdrawal capacity. The complete absence of any signal between 7 and 8 ppm suggests the absence of any phenyl groups attached to the phosphoryl oxygen, indicating that the hydrogenolysis went to completion.

Inactivation of Phosphoglucose Isomerase by 1-Chloro-2-oxo-6-hexanol 6-Phosphate. The objective of this work has been to prepare a covalently bound substrate analogue that could serve as an active-site label in future structural studies.

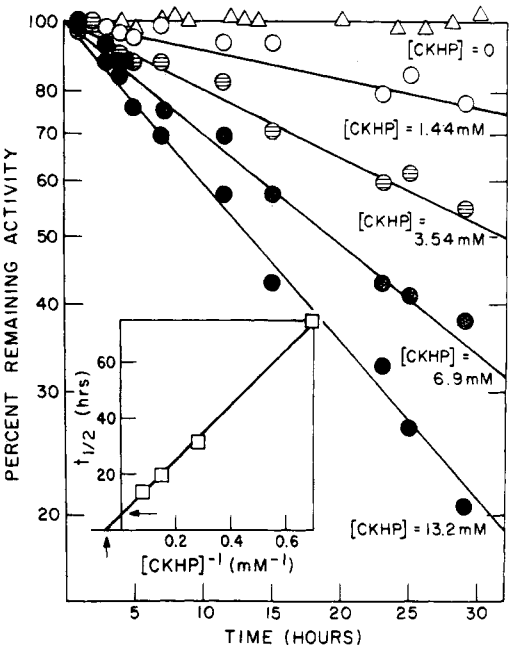


FIGURE 2: Inactivation of rabbit muscle phosphoglucose isomerase by CKHP. Reaction conditions: Isomerase, 2 μM active sites; inactivator concentrations as indicated; triethanolamine–HCl, 100 mM, pH 7.5; temperature, 30 °C. Aliquots were removed at the designated time intervals, diluted in 50 mM EDTA, pH 8.0, and assayed for enzyme activity in the coupled spectrophotometric assay. The half-lives obtained from the solid lines corresponding to the activity loss at various inactivator concentrations, which are proportional to the reciprocal pseudo-first-order rate constants for inactivation, are plotted in the inset as a function of the reciprocal inactivator concentration. The limiting apparent rate constant for inactivation and the apparent dissociation constant for the reversible enzyme–inhibitor complex (see arrows) are 0.11 h^{-1} and 14.3 mM, respectively.

For a compound to fulfill the requirements prerequisite to irreversible stoichiometric binding at the enzyme active site, kinetics of inactivation need to prevail that follow either one of two basic patterns. One type of inactivation proceeds as an uncomplicated, direct bimolecular reaction in which the rate of activity loss is first order with respect to both enzyme and modifying reagent concentrations. Following simple mass action law, doubling the modifying reagent concentration will double the rate of inactivation.

The other possible type of inactivation process is preceded by the formation of a reversible enzyme–inhibitor complex which manifests itself kinetically in a rate saturation effect. As a result the inactivation rate depends on the inhibitor concentration in the same manner the velocity of an enzymatically catalyzed process depends on the substrate concentration (Kitz & Wilson, 1962; Baker, 1967; Shaw, 1970; Visser et al., 1971).

As shown in Figure 2, the inactivation of phosphoglucose isomerase by CKHP (for experimental details refer to the legend of the figure) conforms definitely to the second case, i.e., involves complex formation prior to attainment of irreversible inactivation. From the data presented in Figure 2, an apparent dissociation constant of 14.3 mM may be calculated for the reversible enzyme–CKHP complex. Also, from the intercept of the second-order plot with the ordinate (inset to Figure 2), an apparent maximal inactivation rate for infinite CKHP concentration may be calculated to be 0.11 h^{-1}, corresponding to a half-life for inactivation of 6.3 h. The fact that the inactivation of phosphoglucose isomerase by CKHP obeys rate saturation kinetics may be taken as strongly supporting evidence for its indeed being a genuine active-site reagent that initially behaves like a substrate analogue at-

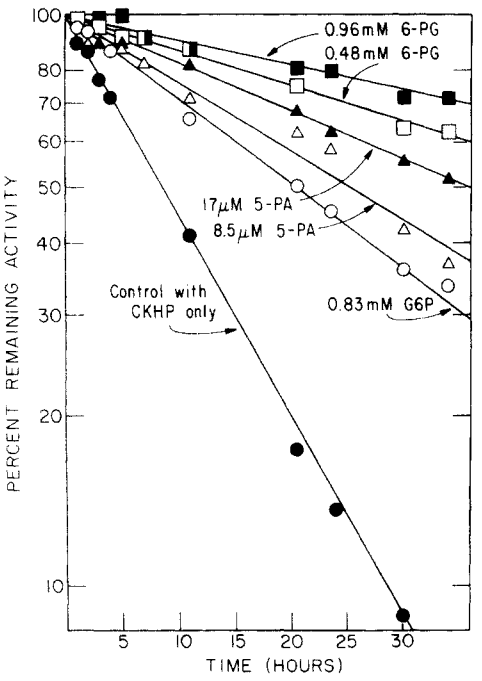


FIGURE 3: Protection of rabbit muscle phosphoglucose isomerase by substrate or substrate analogues against inactivation by CKHP. Reaction conditions: Isomerase, 12 μM active sites; CKHP concentration, 25.2 mM; protectant concentrations as indicated; all other conditions were as stated in the legend to Figure 2.

tempting to engage in reversible binding at the active site before its chloromethyl oxo function causes its irreversible attachment to the enzyme protein.

Similar supporting evidence is provided through the protection afforded by substrate or competitive inhibitors against inactivation by CKHP. Figure 3 represents a comparison of the inactivation rates in the presence of 6-phosphogluconate or 5-phosphoarabinonate or of a substrate equilibrium mixture (in the figure represented as glucose 6-phosphate) with the rate obtained in the absence of any protectant. The fact that the inactivation goes to completion is consistent with active-site modification. Also, the very low concentrations of the protectants, especially of the transition state analogue 5-phosphorarabinonate (Chirgwin & Noltmann, 1975), that result in protection are further indication of the specificity of both the inactivation and the protection effects.

For the purpose of showing that the protection is due to true competition between protectant and modifying reagent, a series of experiments was initiated in which inactivation rates were studied as a function of both varying protectant and varying inactivator concentrations. This elegant approach was suggested by Meloche (1967) who applied it to the inactivation of 2-oxo-3-deoxy-6-phosphogluconic aldolase by bromopyruvate and its protection by pyruvate. If binding of the two antagonists occurs at the same site, a plot of the half-life of inactivation as a reciprocal of the inactivator concentration will yield a family of straight lines for varying protectant concentrations that intercept the ordinate at a common point, which is equivalent to the extrapolated half-life for infinite inactivator concentration. Great difficulty was experienced in attempts to utilize this kinetic method for the protection of phosphoglucose isomerase by 5-phosphoarabinonate or 6-phosphogluconate because their high affinity for the enzyme produced a degree of protection that is so large that concentrations small enough to afford only partial protection in the competition experiment had to be at levels of the same order of magnitude of or lower than the enzyme concentration. As

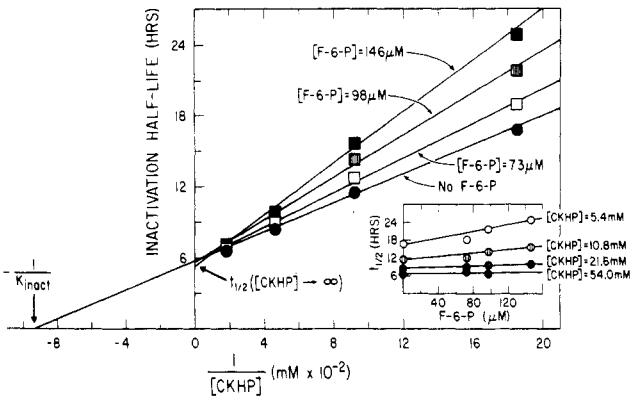


FIGURE 4: Protection of rabbit muscle phosphoglucose isomerase by substrate against inactivation by CKHP as a function of both substrate and CKHP concentrations. Reaction conditions as stated in the legend to Figure 2 except that the isomerase concentration was 12 μM (as for the experiments represented in Figure 3). Concentrations of CKHP and fructose 6-phosphate (F-6-P) as shown in the figure. Note that F-6-P stands for an equilibrium mixture of fructose 6-phosphate and glucose 6-phosphate attained immediately on addition of fructose 6-phosphate to the reaction mixture.

a result, the requirement for the derivation to be valid, i.e., that the enzyme concentration is small compared with that of the ligand, would no longer be met, and the Meloche plots became nonlinear. This difficulty was finally overcome when substrate was used as protectant. Because of its lower dissociation constant (about 10^{-4} M), inactivation rates at varying substrate concentrations could be obtained at concentration ratios of substrate:enzyme high enough for the kinetic derivations to be applicable. These data are shown in Figure 4. It was gratifying to find that they confirmed the conclusions drawn from the simple protection experiments with 5-phosphoarabinonate or 6-phosphogluconate shown in Figure 3.

While this paper was in preparation a communication was published by Gibson et al. (1977) in which the inactivation of human phosphoglucose isomerase by *N*-(bromoacetyl)-ethanolamine phosphate [an active-site reagent designed for aldolase (Hartman et al., 1973)] was reported. These authors found 3-(carboxymethyl)histidine after hydrolysis of the inactivated enzyme and interpreted this finding to be in support of our earlier proposal (Dyson & Noltmann, 1968) of an active-site histidine. It should be kept in mind, however, that O'Connell and Rose (1973) identified a glutamic acid residue covalently linked to 1,2-epoxymannitol 6-phosphate after reaction of yeast phosphoglucose isomerase with this reagent.

Acknowledgments

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Binding of Divalent Cations to Dipalmitoylphosphatidylcholine Bilayers and Its Effect on Bilayer Interaction†

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ABSTRACT: We have confirmed that $CaCl_2$ swells the multilayer lattice formed by dipalmitoylphosphatidylcholine (DPPC) in an aqueous solution. Specifically, at room temperature 1 mM $CaCl_2$ causes these lipid bilayers to increase their separation, d_w, from 19 Å in pure water to >90 Å. $CaCl_2$ concentrations >40 mM cause less swelling. We have measured the net repulsive force between the bilayers in 30 mM $CaCl_2$ at T = 25 °C (below the acyl chain freezing temperature). For interbilayer separations between 30 and 90 Å, the dominant repulsion between bilayers is probably electrostatic; Ca^{2+} binds to DPPC lecithin bilayers, imparting a charge to them. The addition of NaCl to $CaCl_2$ solutions decreases this repulsion. For d_w < 20 Å, the bilayer repulsion appears to be dominated by the "hydration forces" observed previously between both neutral and charged phospholipids. From the electrostatic repulsive force, we estimate the extent of Ca^{2+} binding to the bilayer surface. The desorption of bound Ca^{2+}, apparent when bilayers are pushed together, is more rapid than one would expect if an association constant governed Ca^{2+} binding. The association affinity does not appear to be a fixed quantity but rather a sensitive function of ionic strength and bilayer separation.

There is a great deal of curiosity about the extent and kind of interaction between mobile ions and the components of biological membranes. Among the more accessible of these interactions is the affinity of Ca^{2+} and related alkaline earth ions for the zwitterionic phospholipid lecithin.[1] Electrophoretic studies by Bangham & Dawson (1962) and McLaughlin et al. (1978) have shown that the presence of Ca^{2+} in the bathing medium induces a positive charge on lecithin vesicles. Several NMR studies indicate Ca^{2+} binding to egg lecithin (McLaughlin et al., 1978; Hauser et al., 1975, 1977; Hutton et al., 1977; Grasdalen et al., 1977). Structural consequences of alkaline earth ion binding to lecithins were detected by Inoko

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; Ca^{2+}, calcium ion; T_m, gel to liquid crystal transition temperature.

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