^{18}O Studies of the Mechanisms of Yeast and Muscle Aldolases†

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ABSTRACT: The mechanisms of action of rabbit muscle and yeast fructose-1,6-diphosphate aldolase have been investigated utilizing ^{18}O to determine the fate of the 2-keto oxygen atom of fructose 1,6-diphosphate in the aldol cleavage reaction. A Schiff-base mechanism would demand a loss of the label from [2-^{18}O]fructose 1,6-diphosphate to the medium producing unlabeled dihydroxyacetone phosphate, while a metal-chelate mechanism should lead to isotope retention. Dihydroxyacetone phosphate produced in the aldolase reaction was converted to α-phosphoglycerol *via* the action of α-phosphoglycerol dehydrogenase to prevent exchange of the keto oxygen atom. ^{18}O abundances were measured by combined gas chromatography-mass spectrometry of bis-(*tert*-butyldimethylsilyl) α-phosphoglycerol dimethyl ester. For rabbit muscle aldolase, no ^{18}O was found in the final product. However, for yeast aldolase, approximately 68% of the ^{18}O was retained in the product. These data are consistent with the proposed mechanisms for these enzymes, *i.e.*, the formation of a Schiff-base intermediate for the rabbit muscle enzyme and a metal-chelate intermediate for the yeast enzyme. Experiments designed to determine the reactions which lead to loss of ^{18}O in the case of yeast aldolase show this loss to be primarily the result of exchange of [2-^{18}O]dihydroxyacetone phosphate with water prior to reduction by α-phosphoglycerol dehydrogenase.

Aldolase-type enzymes catalyze carbon–carbon cleavage-condensation reactions of a variety of substrates and are found in nearly all living cells. FDP[1] aldolase catalyzes the reversible cleavage of FDP to DHAP and glyceraldehyde 3-phosphate. Comparative studies of FDP aldolases isolated from various sources indicate the presence of two types, class I and class II, based on physical and chemical characteristics such as molecular weight, cofactor requirements, pH profile, and substrate specificity (Rutter, 1964). This classification has now been applied to many of the aldolase-type enzymes. FDP aldolase isolated from rabbit muscle is the prototype of class I and has been shown to have a molecular weight of approximately 158,000 with four subunits, to be inactivated by carboxypeptidase treatment, unaffected by metal-chelate agents, unaffected by potassium ions, and have a broad pH optimum between pH 6.9 and 8.8 (Rutter, 1964). FDP aldolase from yeast is the prototype of class II and has been shown to have a molecular weight of approximately 80,000 with two subunits (Harris *et al.*, 1969), to be unaffected by treatment with carboxypeptidase, activated by potassium ions, have an absolute requirement for a divalent metal cation, and have a sharp pH optimum for activity between pH 6.9 and 7.1 (Rutter, 1964).

More direct evidence regarding the mechanism of action of these enzymes has been obtained from studies of the susceptibility of the enzyme–DHAP complex to sodium borohydride reduction. In the case of rabbit muscle aldolase, a covalent intermediate was trapped after this reduction (Grazi *et al.*, 1962a) and was later identified as N^6-β-glyceryllysine (Grazi *et al.*, 1962b). This evidence indicates that the mechanism of action of class I aldolases could involve the formation of a Schiff-base intermediate between the C-2 position of FDP and the ε-amino group of a lysine residue in the enzyme. Further evidence which supports this mechanism showed that rabbit muscle FDP aldolase catalyzes exchange of the keto oxygen atom of FDP with that of water (Model *et al.*, 1968). When the borohydride reduction was attempted with yeast aldolase, a reduced Schiff-base product could not be found. Based on this result and the requirement for a divalent metal cation, it has been proposed that class II aldolases operate *via* a metal-chelate intermediate in which the metal ion serves as the electron donor and acceptor in a role similar to that of the nitrogen atom in the Schiff base (Rutter, 1964). Alternatively, it has been suggested that class II aldolases still may operate *via* a Schiff-base intermediate, but that the metal ion prevents reduction of the intermediate by borohydride (Morse and Horecker, 1968).

In the work reported in this paper, the proposed mechanisms have been tested by determining the fate of the keto oxygen atom of [2-^{18}O]FDP in the aldolase-catalyzed cleavage reaction. A Schiff-base mechanism would cause an obligatory exchange of the ^{18}O label with the ^{16}O of the water medium. The DHAP product would therefore not contain ^{18}O in excess of natural abundance. On the other hand, if a metal-chelate mechanism operates, there would not be an obligatory exchange of the keto oxygen of the substrate and [2-^{18}O]DHAP would be formed. We have studied rabbit muscle and yeast FDP aldolase in this manner and have measured the retention of ^{18}O in the DHAP produced in the cleavage reaction. DHAP was not directly analyzed but was converted to αPG because of the extremely rapid nonenzymatic exchange of free DHAP ($t_{1/2} \simeq 10$ sec) (Reynolds *et al.*, 1971). αPG dehydrogenase was included in the aldolase reaction solution to rapidly convert

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[1] Abbreviations used are: FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; αPG, α-phosphoglycerol; BDMS, *tert*-butyldimethylsilyl; and TFA, trifluoroacetyl.

DHAP to αPG. The αPG was analyzed for ^{18}O by first converting it to a volatile derivative followed by direct isotope analysis by combined gas chromatography–mass spectrometry. A preliminary report of this work has been published (Caprioli and Heron, 1973).

Materials and Methods

Bakers yeast was obtained from Nutritional Biochemical Corp. *tert*-Butyldimethylchlorosilane–imidazole reagent was obtained from Applied Science Labs. All other reagents were obtained from the Sigma Chemical Co. unless otherwise noted.

$H_2{}^{18}O$ was obtained from Miles-Yeda and was analyzed by the combustion technique (Rittenberg and Ponticorvo, 1956).

Chloroacetol phosphate was prepared according to a published procedure (De La Mare *et al.*, 1972). Diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Co.).

αPG dehydrogenase from rabbit muscle (Sigma) was treated with chloroacetol phosphate prior to use to inhibit residual amounts of triosephosphate isomerase (Hartman, 1970). The preparation was dialyzed for 2 hr against 0.05 M glycylglycine buffer (pH 7.5) containing 0.1 M potassium acetate. αPG dehydrogenase was then assayed in the same buffer containing 0.4 mM DHAP and 0.1 mM NADH.

Rabbit muscle FDP aldolase was obtained from Sigma and contained no measurable amount of triosephosphate isomerase. Yeast FDP aldolase was isolated from bakers yeast (Mildvan *et al.*, 1971). Prior to use, the enzyme was dialyzed against 0.05 M glycylglycine buffer (pH 7.5) containing 0.1 M potassium acetate. Sufficient chloroacetol phosphate was added to inhibit residual triosephosphate isomerase. The solution was allowed to stand for 1 hr and was dialyzed as described above. Both aldolases were assayed prior to use.

[2-^{18}O]FDP was prepared by dissolving 3 mg of Na_4FDP salt in 10 μl of 47.02 atom % $H_2{}^{18}O$ and allowing the solution to stand for 12 hr at room temperature in a sealed vial. The half-life of exchange of the keto oxygen atom has been measured to be 29.5 min under similar conditions (Model *et al.*, 1968). Thus, the [2-^{18}O]FDP should contain 46.5 atom % excess ^{18}O after accounting for dilution of the $H_2{}^{18}O$ caused by the exchange. In order to confirm the ^{18}O enrichment, FDP was hydrolyzed to fructose by treatment with wheat germ acid phosphatase. The fructose was converted to the perfluoroacetyl derivative by reaction with perfluoroacetyl anhydride and analyzed for ^{18}O. To determine ^{18}O exchanged out of the keto group during the phosphatase hydrolysis and subsequent isolation, control reactions were run in $H_2{}^{18}O$. The result of this assay showed the original FDP to contain 46.6 atom % excess ^{18}O, in close agreement to that expected for complete isotopic exchange.

[2-^{18}O]αPG was prepared by converting 100 mg of DHAP, dimethylketal, dimonocyclohexylamine to the acid form, lyophyllizing the solution, and dissolving the residue in 50 μl of 47.02 atom % $H_2{}^{18}O$. The ketal was then hydrolyzed by incubation at 40° for 4 hr. To this solution was added 1045 units[2] of αPG dehydrogenase and 200 mg of NADH dissolved in 8.0 ml of 0.5 M glycylglycine buffer (pH 7.5). The solution was allowed to stand for 7 min and was then passed through a 1.5 × 20 cm Dowex-50 (Na^+) column. Fractions found to contain αPG by enzymatic assay (Hohorst, 1963) were pooled, diluted by a factor of 10, and passed through a 1.5 × 48 cm Dowex-1 (formate) column (Bartlett, 1959). Fractions containing αPG were neutralized with ammonium hydroxide and lyophilized. The isolated αPG was analyzed for isotope enrichment as described later and was found to contain 15.88 atom % excess ^{18}O in the C-2 oxygen atom.

Aldolase-Catalyzed Cleavage of [2-^{18}O]FDP. Five units of either yeast or rabbit muscle aldolase, the proper number of units of αPG dehydrogenase, 10 μmol of NADH, and sufficient buffer (0.05 M glycylglycine–0.1 M potassium acetate (pH 7.5)) were added to make a total volume of 2.2 ml. This mixture was warmed to 37° in a water bath and rapidly added to 6 μmol of [2-^{18}O]FDP dissolved in 10 μl of $H_2{}^{18}O$ (prepared as described above). After 90 sec of incubation at 37°, the solution was immediately frozen in a Dry Ice–ethanol bath. The frozen solution could then be stored for several days. On thawing, it was immediately passed through a 1.5 cm × 20 cm Dowex 50 (H^+) column and fractions containing αPG were pooled and lyophilized.

Non-Aldolase Catalyzed Exchange of [2-^{18}O]DHAP. NADH (10 μmol) and the proper amount of αPG dehydrogenase were dissolved in 2.2 ml of 0.05 M glycylglycine–0.1 M potassium acetate buffer (pH 7.5) and were warmed to 37°. The solution was mixed vigorously using a vortex mixer and 2.0 μl of 47 atom % excess $H_2{}^{18}O$ containing 6 μmol of [2-^{18}O]DHAP was added at a constant rate over a period of 60 sec using a 2-μl syringe. After a total of 90 sec, the solution was quickly frozen and the αPG was isolated.

αPG Dehydrogenase Catalyzed Exchange of [2-^{18}O]αPG. αPG (30 μmol), 25 μmol of NAD^+, and 25 μmol of NADH were dissolved in 0.05 M glycylglycine buffer (pH 7.5) containing 0.1 M potassium acetate. The proper amount of αPG dehydrogenase was added in sufficient buffer to make a final volume of 11 ml. The solution was incubated at 37° and 2.2-ml samples were removed at various times. The samples were immediately frozen in a Dry Ice–ethanol bath to stop the reaction. On thawing, the solution was passed through a 1.5 × 20 cm Dowex 50 (H^+) column. The fractions containing αPG were pooled and lyophilized.

Isotope Analysis of αPG. To the lyophilized αPG samples dissolved in 1 ml of methanol was added dropwise a solution of diazomethane in diethyl ether until the yellow color persisted. The solution was taken to dryness under vacuum and the residue dissolved in 20 μl of *tert*-butyldimethylchlorosilane–imidazole reagent. After standing 15 min at room temperature, 1-μl portions of the solution containing bis(*tert*-butyldimethylsilyl) α-phosphoglycerol dimethyl ester were analyzed by gas chromatography/mass spectrometry.

Analyses were performed on a Finnigan Model 3100D gas chromatograph–mass spectrometer equipped with a Finnigan Model 6000 data system. Samples were introduced into the instrument *via* the gas chromatograph inlet in order to achieve separation of the derivatized αPG from other volatile components. A packed column of 3% dexsil 300 on 80–100 mesh Chromosorb W was used in a 0.2 mm × 2 m column with He as a carrier gas. The injector temperature was maintained at 130° and the column oven programmed from 170 to 210° at 6°/min. The mass spectrometer was run at an ionizing electron voltage of 70 eV with

[2] Units of enzyme activity used in this work are international units expressed in micromoles of substrate converted to product per minute.

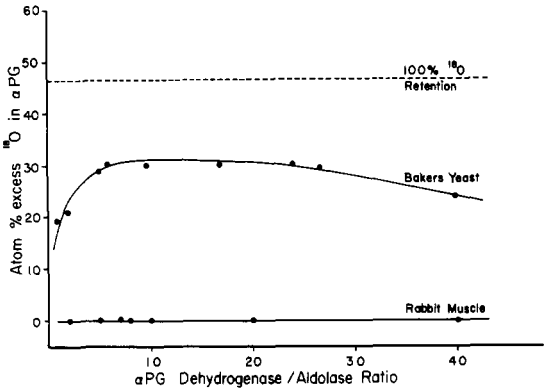


FIGURE 1: ^{18}O enrichment of α-phosphoglycerol isolated from reaction mixtures containing either bakers yeast or rabbit muscle aldolase, varying amounts of α-phosphoglycerol dehydrogenase, NADH, and [2-^{18}O]fructose 1,6-diphosphate containing 46.6 atom % excess ^{18}O.

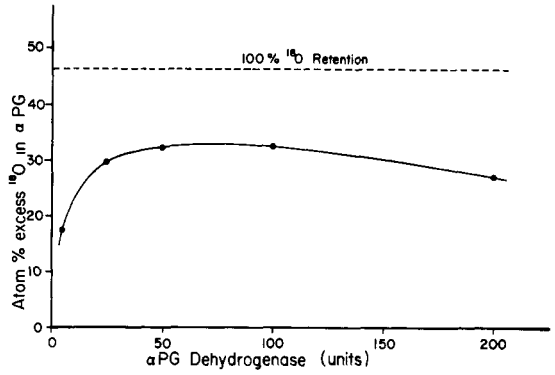
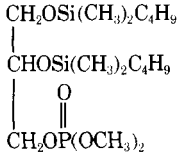


FIGURE 2: ^{18}O enrichment of α-phosphoglycerol isolated from reaction mixtures containing α-phosphoglycerol dehydrogenase, NADH, and [2-^{18}O]dihydroxyacetone phosphate containing 46.5 atom % excess ^{18}O.

ion source and analyzer temperatures at 170°.

Specific ions from bis(*tert*-butyldimethylsilyl) α-phosphoglycerol dimethyl ester were continuously monitored throughout the analysis. In this multiple ion mode, the mass spectrometer continuously cycles, collecting ions from up to four separate masses. Details of this method of analysis, which provides accuracies of 0.2–0.4%, have been published elsewhere (Caprioli *et al.*, 1974). Each analysis reported in this paper represents an average of at least three independent isotope measurements for a given sample.

The mass spectrum of [2-^{18}O]bis(*tert*-butyldimethylsilyl) α-phosphoglycerol dimethyl ester has no molecular ion



but shows a relatively intense ion at *m/e* 371 corresponding to the loss of a butyl group. This ion and its isotope at *m/e* 373 were used to measure ^{18}O incorporation in the isolated αPG samples. Other ions were used to verify that the isotope was in the C-2 oxygen atom. The mass spectrum is analogous to that of bis(trimethylsilyl) α-phosphoglycerol dimethyl ester, the electron impact fragmentation of which was the subject of a detailed report with particular attention to the identification of individual oxygen atoms in the fragment ions (Caprioli and Heron, 1972).

^{18}O Analysis of (TFA)$_5$-fructose: The sample was introduced *via* the reservoir into a CEC 21-110 double focusing mass spectrometer. The ions at *m/e* 533 and 535 were slowly scanned 8–10 times to measure the isotope ratio. The ion at *m/e* 533 is produced by the loss of $CF_3COOCH_2\cdot$ from the molecular ion and was verified by high-resolution mass spectral measurements. Standard [2-^{18}O]fructose of known isotope enrichment was derivatized and analyzed to ensure the validity of the isotope measurements.

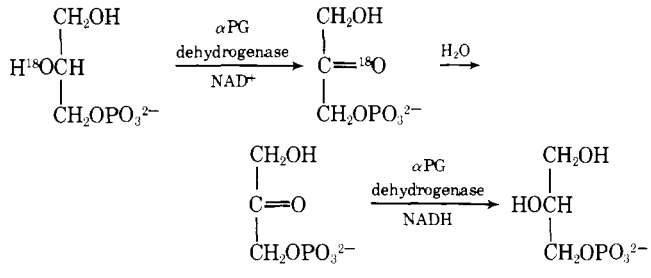
Results

When [2-^{18}O]FDP was cleaved by yeast aldolase, the major portion of the label was retained in the αPG isolated in the experiment. Figure 1 shows the ^{18}O enrichment found in αPG as a function of the αPG dehydrogenase/aldolase ratio. ^{18}O retention was measured as a function of the enzyme ratio to determine the point at which the rate of conversion of DHAP to αPG was maximized. Since DHAP exchanges extremely rapidly with the medium and αPG does not, this maximum rate of conversion should give maximum ^{18}O retention. The low enrichment values for the initial portion of the curve, *i.e.*, where the enzyme ratio is less than 5, are the result of exchange of DHAP prior to reduction. A maximum ^{18}O retention of approximately 31 atom % excess occurred at an enzyme ratio of approximately 15:1. This represents retention of 68% of the 2-keto oxygen atom of the substrate.

When [2-^{18}O]FDP was cleaved by rabbit muscle aldolase, the αPG contained no significant excess ^{18}O throughout the range of αPG dehydrogenase/aldolase ratios tested. These results are also shown in Figure 1. The small amount of label found in the αPG, approximately 0.2 atom % excess ^{18}O, was due to the enrichment of the reaction medium at the start of the reaction when 10 μl of 47 atom % excess $H_2{}^{18}O$ was added to 2.2 ml of buffer containing normal water.

Several experiments were performed to elucidate the processes which give rise to ^{18}O loss in the yeast aldolase reaction. In order to measure total non-aldolase catalyzed ^{18}O exchange, [2-^{18}O]DHAP was added to the normal aldolase reaction mixture, with varying dehydrogenase concentration but lacking aldolase. The labeled DHAP was added slowly simulating the rate it would be produced by aldolase. The results are shown in Figure 2. The ^{18}O retention in the αPG isolated reaches a maximum between 40 and 110 units of αPG dehydrogenase. The maximum of 32.9 atom % excess ^{18}O measured represents a 70% retention of the 2-keto oxygen atom of DHAP.

A study was performed to determine the rate of exchange of [2-^{18}O]αPG in the presence of αPG dehydrogenase. Since this enzyme is reversible, a certain amount of DHAP would be present at equilibrium, eventually leading to isotopic equilibration between H_2O of the medium and the 2-keto oxygen of DHAP. To measure the extent of this effect,



[2-^{18}O]αPG was incubated with αPG dehydrogenase in the

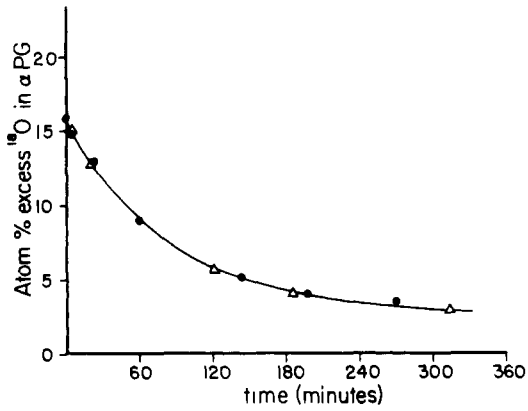
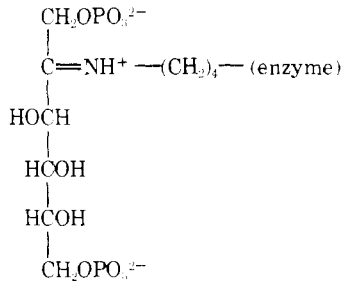


FIGURE 3: Loss of ^{18}O from [2-^{18}O]-α-phosphoglycerol in a reaction mixture containing either 50 units (△) or 150 units (●) of α-phosphoglycerol dehydrogenase, NAD^+, and NADH. The isotope enrichment in the initial α-phosphoglycerol was 15.88 atom % excess ^{18}O.

presence of both NAD^+ and NADH. Samples were taken with time and the ^{18}O excess in αPG was analyzed. Two experiments were performed, one with 50 units of αPG dehydrogenase corresponding to the absolute amount present in the aldolase experiment where the enzyme ratio was 10 and the other with 150 units of dehydrogenase, corresponding to the aldolase experiment where the enzyme ratio was 30 (see Figure 1). The results are given in Figure 3. The two curves are coincident, *i.e.*, loss of ^{18}O from αPG through the enzyme-catalyzed reaction is not dependent on enzyme concentration in the range examined. From the initial slope of the curve, the maximum rate of exchange was calculated to be 0.165 atom % excess ^{18}O/min starting with 15.88 atom % ^{18}O excess in [2-^{18}O]αPG, or a loss of 2-keto oxygen atom at the rate of approximately 1.0%/min.

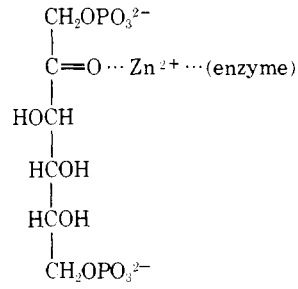
Discussion

Comparison of the percentage of the 2-keto oxygen atom retained for yeast and for rabbit muscle, *i.e.*, 68 and 0%, respectively, shows that their mechanisms of action are different. The complete loss of ^{18}O in the cleavage reaction catalyzed by rabbit muscle aldolase is consistent with existing data which suggest a Schiff-base intermediate. Such a



mechanism would demand loss of the keto oxygen of FDP as an obligatory part of the aldol cleavage reaction. An analogous loss of the keto oxygen of 2-keto-3-deoxy-6-phosphogluconate has been observed in the aldol cleavage catalyzed by 2-keto-3-deoxy-6-phosphogluconate aldolase. The mechanism of action of this enzyme is also thought to involve a Schiff-base intermediate (Rose and O'Connell, 1967).

Retention of ^{18}O in the cleavage reaction involving yeast aldolase shows that whatever the proposed mechanism, the keto oxygen atom of FDP cannot be lost to the medium as an obligatory part of the mechanism. The ^{18}O data support the proposed mechanism of action of this enzyme as involving a metal-chelate intermediate. Such an intermediate would involve the formation of a bond between the keto oxygen atom of FDP and the zinc cation (Rutter, 1964).



Since ^{18}O retention was not complete, *i.e.*, approximately 32% of the total was lost, exchange must take place at one or more points in the reaction sequences. A small amount of label is known to be lost as a result of nonenzymatic exchange of [2-^{18}O]FDP with water of the medium. This exchange rate has a $t_{1/2}$ of 29.5 min under conditions similar to those used here (Model *et al.*, 1968) and therefore accounts for a loss of less than 2% of the total label present.

Several reactions may be responsible for the partial loss of ^{18}O. First, exchange of the keto-oxygen atom of FDP and/or DHAP may occur in an enzyme-catalyzed reaction which is not an obligatory part of the mechanism. Second, exchange of the keto oxygen of DHAP may occur after release from aldolase, but prior to reduction by αPG dehydrogenase and NADH. Third, exchange of DHAP may result from oxidation of αPG catalyzed by αPG dehydrogenase. Although equilibrium lies in favor of αPG by several orders of magnitude, isotopic equilibrium could take place between the oxygen atom of water and the C-2 oxygen atom of αPG through exchange of DHAP as the product of the reoxidation reaction. Finally, lower than theoretical yields of ^{18}O in the yeast aldolase reaction could be the result of dilution of [2-^{18}O]αPG with normal αPG produced from glyceraldehyde 3-phosphate by the action of residual amounts of triosephosphate isomerase present in the reaction mixture.

The experiment in which [2-^{18}O]DHAP was added to varying amounts of αPG dehydrogenase in a simulated aldolase reaction (see Figure 3) produced a curve which is remarkably similar to that for the actual yeast aldolase reaction. The retention of ^{18}O in both cases is approximately the same, 70% of the total. Since this experiment measures retention from the point where [2-^{18}O]DHAP is released in the medium, it suggests that the major portion of the ^{18}O lost in the yeast aldolase reaction is due to exchange of [2-^{18}O]DHAP either before conversion to αPG or from reoxidation of αPG. It eliminates a significant aldolase-catalyzed exchange reaction or dilution of [2-^{18}O]αPG *via* the action of triosephosphate isomerase.

The final series of experiments were performed in order to measure loss of label resulting from reoxidation of [2-^{18}O]αPG to [2-^{18}O]DHAP followed by exchange of the 2-keto oxygen atom (see Figure 3). The amounts of αPG dehydrogenase used in these experiments was the same as those in the yeast aldolase experiments where the ratio of dehydrogenase/aldolase was 10 and 30. The results show that a maximum of only 1%/min of the 2-keto oxygen could be lost in this manner, and therefore only a total loss of less than 2% of the label. It can be concluded from these data that the major portion of ^{18}O lost in the yeast aldolase experiment is the result of exchange of [2-^{18}O]DHAP after

release from aldolase but prior to reduction by αPG dehydrogenase.

In conclusion, the results from this work demonstrate a mechanistic difference between yeast and muscle aldolase. Presumably, this difference also applies to the two classes these enzymes represent. The techniques used for this work present an unambiguous test for distinguishing between class I and class II aldolases. Investigations are currently in progress to determine if other aldolases now considered to be class I or class II behave in the same manner as their prototypes. Those aldolases which cannot clearly be placed in either class according to their chemical and physical properties are of particular interest.

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Physical–Chemical Studies of Phospholipids and Poly(amino acids) Interactions†

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ABSTRACT: The interactions of phospholipid vesicles with poly(L-glutamic acid) and poly(L-tyrosine) were investigated as a model for the molecular interactions between proteins and phospholipids in biological membranes. We have used the spin-label and glucose permeability techniques to study the interactions between poly(amino acids) and phosphatidylcholine. The spin-labels that we used are the spin-labeled stearic acids and the spin-labeled phosphatidylcholines. The spin-label results suggest that these two poly(amino acids) interact on the surface of the phosphatidylcholine vesicles and that this interaction might cause a lateral tightening up of the polar region of the phospholipid molecule, but the flexibility gradient in the methylene chain is still preserved in the model membranes. In addition, the slower rate of glucose permeability in the complexes provides another piece of evidence that there is a tightening up of the bilayer structure of the phosphatidylcholine vesicles upon complex formation with negatively charged poly(amino acids) in aqueous solution.

For many years the structure and function of biological membranes have been the object of intensive research. A typical membrane is known to contain both proteins and lipids. From the time Danielli and Davson (1935) first proposed the classical lipoid model of a plasma membrane, a number of membrane models have been proposed. These range from the unit membrane structure which consists of a continuous biomolecular lipid leaflet surrounded by a layer of protein on either side of the membrane as proposed by Robertson (1964) to a structure which consists of associated, repeating proteolipid structural units as proposed by Green and Purdue (1967). Due to the complexity in the molecular structure as well as the diversity of the functional properties of membranes, it is unlikely that any one of the

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