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CLASSIFICATION OF FRUCTOSE-1,6-BISPHOSPHATE ALDOLASES BASED ON ^{18}O RETENTION IN THE CLEAVAGE REACTION

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Summary

^{18}O was used as a mechanistic probe in the investigation of several different sources of fructose 1,6-bisphosphate aldolases (EC 4.1.2.13) which, due to differences in some physical and chemical properties, could not be clearly put in either Class I or Class II. Aldolases may be identified as belonging to a particular class on the basis of the amount of ^{18}O retained in the dihydroxyacetone phosphate produced in the cleavage of [2-^{18}O]fructose 1,6-bisphosphate. The mechanism of Class I aldolases involves an obligatory exchange of the C-2 oxygen atom of fructose 1,6-bisphosphate, leading to the absence of ^{18}O in the product. For Class II aldolases, the C-2 oxygen atom is retained in the aldol cleavage reaction. Aldolases from spinach and *L. casei* were found to be Class I, suggesting that their mechanisms involve a Schiff-base intermediate. Aldolase from *C. perfringens* was found to be Class II, suggesting a metal-chelate intermediate. Results with *Euglena* aldolase confirmed that this organism contained both types of aldolases with approximately 78% Class II.

The data show that despite a wide variety of physical and chemical properties, there are important mechanistic similarities within each class of enzyme and significant differences between the two classes. The determination of ^{18}O retention in the product of the cleavage reaction using [2-^{18}O]fructose 1,6-biphosphate is an accurate means of classifying these enzymes since it is a measure of a property which is directly related to the mechanisms of the reactions.

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Introduction

Warburg and Christian [1] first observed the presence of two types of fructose-1,6-bisphosphate (Fru-P_2) aldolases (EC 4.1.2.13), a metal-requiring enzyme from yeast and a non-metal requiring enzyme from mammalian muscle. Rutter [2] showed that these two types of Fru-P_2 aldolases have distinct catalytic and molecular properties and designated the enzymes resembling rabbit muscle aldolase as Class I and those resembling yeast aldolase as Class II. These two prototype enzymes have been examined in detail by many workers and have been shown to proceed by way of two different mechanisms. For rabbit muscle Fru-P_2 aldolase, evidence strongly supports a Schiff-base mechanism; (i) inactivation of the enzyme by sodium borohydride in the presence of substrate and identification of β-glyceryllysine isolated from the reduced substrate-enzyme complex [3], and (ii) the obligatory exchange of the 2-keto oxygen atom of Fru-P_2 with the medium in the enzymatic cleavage reaction [4]. Yeast Fru-P_2 aldolase, on the other hand, has been shown to proceed via a different mechanism; (i) complete retention of the 2-keto oxygen atom of Fru-P_2 in the cleavage reaction, (ii) lack of inactivation on treatment with sodium borohydride, and (iii) requirement of a divalent metal cation for activity. These and other results indicate that yeast aldolase, and presumably other Class II aldolases, operate via a metal-chelate intermediate, in a manner analogous to the Schiff-base of Class I aldolases, to cause C_3-C_4 bond cleavage of Fru-P_2.

The experiments cited above in which the fate of the 2-keto oxygen atom of Fru-P_2 was followed through the cleavage reaction showed that the mechanisms of rabbit muscle and yeast aldolases were different. In this work, [2-^{18}O] Fru-P_2 was cleaved with aldolase to produce dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Due to the extremely rapid exchange of the keto oxygen of dihydroxyacetone phosphate with the oxygen of water [5], dihydroxyacetone phosphate was converted to glycerol 1-phosphate by glycerol 1-phosphate dehydrogenase and NADH which was present in the reaction mixture. The resulting glycerol 1-phosphate was derivatized to form the bis(*t*-butyldimethylsilyl) ether, dimethyl ester analog and was subsequently analyzed for ^{18}O enrichment by GC/MS techniques. These results showed complete ^{18}O retention in the reaction catalyzed by yeast aldolase and absence of ^{18}O retention in the reaction catalyzed by muscle aldolase [4].

The demonstration of mechanistic differences for the prototypes of Class I and II Fru-P_2 aldolases using ^{18}O suggests that such experiments could be used as a definitive method of classifying Fru-P_2 aldolases based on a property directly related to their mechanisms. The work presented here describes the results of ^{18}O studies on Fru-P_2 aldolases which do not clearly fit either into Class I or II because of major differences in physical and/or chemical properties with respect to that of the prototypes of the classes.

Spinach Fru-P_2 aldolase is inactivated by sodium borohydride in the presence of substrate and has been proposed to be a Class I enzyme. However, it has a molecular weight of 120 000 [6], intermediate between the 160 000 molecular weight of rabbit muscle aldolase and 70 000 of yeast [2]. Fru-P_2 aldolase of *Clostridium perfringens* is believed to be Class II based on the

requirement of a divalent metal cation and lack of inactivation by sodium borohydride [7]. This enzyme requires Fe^{2+} or Co^{2+} for maximal activity and these ions are freely dissociable, in contrast to the tightly bound Zn^{2+} required for optimal activity in the case of yeast aldolase. Both enzymes show a high degree of specificity toward Fru-P_2, have similar pH profiles, and have similar molecular weights, although the comparative rates of aldol cleavage/proton exchange are quite different, 1.7 for the yeast enzyme and 5.0 for the *C. perfringens* enzyme. Fru-P_2 aldolase of *Lactobacillus casei* was investigated since it appears to be one of a few exceptions to the observation that yeast, bacteria and fungi produce Class II enzymes while higher plants and animals produce Class I enzymes. The prime basis for the suggested classification of this aldolase as Class I was inactivation by sodium borohydride in the presence of substrate [8]. However, Fru-P_2 aldolase from *L. casei* shows only a 32% reduction of activity on treatment with carboxypeptidase A whereas similar treatment with rabbit muscle and yeast aldolases show 82% and 1% reduction of activity, respectively. Also, the molecular weight of *L. casei* aldolase is 100 000 [8], closer to that of yeast than to muscle. Finally, Fru-P_2 aldolase of *Euglena gracilis* was studied since there is evidence which indicates that both Class I and Class II aldolases are produced by these cells, although the relative proportions vary considerably with growth conditions [9].

Materials and Methods

All chemicals were obtained from the Sigma Chemical Company, unless otherwise noted. *t*-Butyldimethylchlorisilane/imidazole reagent was obtained from Applied Science Labs.

$H_2{}^{18}O$ was obtained from Bio-Rad Labs and was analyzed by the combustion technique [10].

Chloroacetol phosphate was prepared according to De LaMare et al. [11]. Diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Co.).

Glycerol-1-phosphate dehydrogenase from rabbit muscle (Sigma) was treated with chloroacetol phosphate prior to use to inhibit residual amounts of triosephosphateisomerase [12]. The preparation was dialyzed for 2 h against 0.05 M glycylglycine buffer (pH = 7.5) containing 0.1 M potassium acetate, and glycerol-1-phosphate dehydrogenase assayed in the same buffer.

Fru-P_2 aldolase was isolated from spinach leaves according to the procedure of Fluri et al. [6] as modified by Davis et al. [13]. *Clostridium perfringens* strain BPGK (kindly supplied by Dr Harold Raidt, Indiana University Medical Center) was grown on enriched media and the Fru-P_2 aldolase isolated according to Groves et al. [7]. *Lactobacillus casei* (ATCC 7469) (kindly supplied by Dr F. Babel, Department of Animal Sciences, Purdue University) were grown on Lactobacilli MRS broth (Difco code 0881). Growth was allowed to proceed for 48 h at 32 °C. After harvest by centrifugation, Fru-P_2 aldolase was isolated and purified according to Kahlij and Nadkarni [14]. *Euglena gracilis* var. *bacillus* were grown from a culture obtained from the Culture Collection of Algae, Indiana University. The cells were grown on an enriched glucose media at pH = 4.5 with 5% CO_2/95% air filtered through Millipore filters. The cells

were harvested one week after innoculation. Light was provided by fluorescent lights. The cells were disrupted by sonication for 14 min in alternate 2 min periods. After removing the cells debris by centrifugation, the pH of the crude extract was lowered to 6.2 with cold 1% acetic acid and the resulting suspension was centrifuged. The supernatant was mixed with Hyflo Supercel and filtered. For the experiments using *Euglena* crude extracts, the solution was then concentrated by lyophilization and dialyzed against 0.05 M glycylglycine buffer at pH = 7.5 containing 0.1 M potassium acetate. For the experiments using purified *Euglena* aldolase, the filtrate was dialyzed overnight against 0.05 M Tris at pH 7.5 and was placed on a 2 cm × 20 cm DEAE-cellulose column equilibrated with this same buffer. The column was washed with 200 ml of buffer and eluted with a linear gradient up to 250 mM NaCl in this buffer. A single peak which showed aldolase activity eluted at a total volume of approximately 900 ml. Fractions containing aldolase activity were pooled and concentrated by lyophilization.

The various Fru-P_2 aldolases were treated with chloroacetol phosphate to inhibit residual triose phosphate isomerase activity as previously described [4].

[2-^{18}O] Fru-P_2 was prepared by dissolving 3 mg of Fru-P_2 tetrasodium salt in 10 µl of 44.68 atom % excess $H_2{}^{18}O$ followed by incubation for 12 h at room temperature in a sealed vial. Since the half-life of exchange of the keto-oxygen atom has been measured to be 29.5 min under similar conditions [5], nearly complete isotope exchange could be expected. Thus, the resulting (2-^{18})Fru-P_2 should contain 44.2 atom % excess ^{18}O after correcting for dilution of the isotope in the medium due to the exchange. In order to confirm this ^{18}O enrichment, in a separate experiment, Fru-P_2 was exchanged in 47.2 atom % $H_2{}^{18}O$ as described above. The [2-^{18}O] Fru-P_2 was hydrolyzed to [2-^{18}O] fructose by treatment with wheat germ acid phosphatase. The fructose was converted to the perfluoroacetyl derivative by reaction with trifluoracetyl 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 Fru-P_2 to contain 46.6 ± 0.1 atom % excess ^{18}O, in close agreement with the expected 46.5 atom % excess ^{18}O after correcting for dilution of the medium.

Aldolase-catalyzed cleavage of [2-^{18}O] Fru-P_2

Five units of aldolase, a variable number of units of glycerol-1-phosphate 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. When *C. perfringens* aldolase was used, the buffer also contained $6.5 \cdot 10^{-3}$ M $CoCl_2$. The solution was warmed to 37°C in a water bath and rapidly added to 6 µmol of [2-^{18}O] Fru-P_2 dissolved in 10 µl of $H_2{}^{18}O$ (prepared as described above) at 37°C. After 90 s the solution was immediately frozen in a dry ice/ethanol slush bath. The frozen solution could then be stored for several days. On thawing at 0°C, it was immediately passed through a 1.5 cm × 20 cm Dowex-50-(H^+) column and fractions containing glycerol 1-phosphate assayed colorometrically [15], and then pooled and lyophilyzed.

Isotope analysis of glycerol 1-phosphate

To the lyophilized glycerol 1-phosphate 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) glycerol 1-phosphate 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 glycerol 1-phosphate 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°C and the column oven programmed from 170 to 210°C at 6°C/min. The mass spectrometer was run at an ionizing electron voltage of 70 eV with ion source and analyzer temperatures at 170°C.

Specific ions from bis(*tert*-butyldimethylsilyl) glycerol 1-phosphate 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 [16]. Each analysis reported in this paper represents an average of at least three independent isotope measurements for a given sample.

The mass spectrum of bis(*tert*-butyldimethylsilyl)glycerol 1-phosphate dimethyl ester, shown in Fig. 1, 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 glycerol 1-phosphate 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) glycerol 1-phosphate dimethyl ester, the electron impact

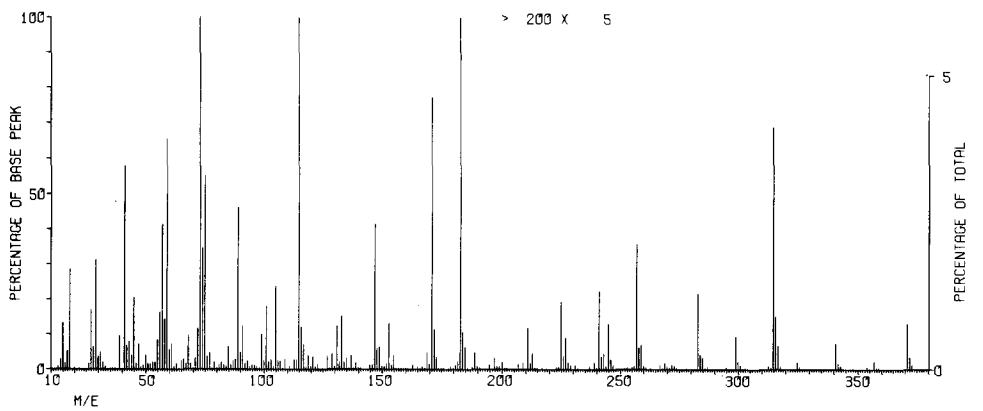

Fig. 1. Mass spectrum of bis(*t*-butyldimethylsilyl) glycerol 1-phosphate dimethyl ester.

fragmentation of which was the subject of a detailed report with particular attention to the identification of individual oxygen atoms in the fragment ions [17].

^{18}O Analysis of pentakis(trifluoracetyl)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 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

The ^{18}O enrichments of glycerol 1-phosphate obtained from the cleavage of [2-^{18}O] Fru-P_2 by various aldolases are given in Table I. In these experiments, the number of units of glycerol-1-phosphate dehydrogenase were varied between 5 and 200 units while using 5 units of aldolase to insure that the [2-^{18}O] dihydroxyacetone phosphate produced in the aldolase catalyzed reaction was converted as rapidly as possible to glycerol 1-phosphate. The C-2 oxygen atom of glycerol 1-phosphate does not exchange under the reaction conditions, while that of dihydroxyacetone phosphate was a half-life of exchange of approximately 10 s [18]. For those aldolases giving ^{18}O retention in the glycerol 1-phosphate, the results show that the maximum amount of isotope was retained using approximately 25—50 units of glycerol-1-phosphate dehydrogenase, with no further retention at higher concentrations. When less than 20 units of glycerol-1-phosphate dehydrogenase were used, a decrease in the isotope enrichment was observed due to the exchange the C-2 oxygen atom of [2-^{18}O] dihydroxyacetone phosphate with the oxygen of water prior to reduction to glycerol 1-phosphate.

The data given in Table I have not been corrected for the slight enrichment of the reaction medium caused by mixing 10 μl of 44.7 atom % excess $H_2{}^{18}O$ with 2.2 ml of H_2O containing ^{18}O at natural abundance. This effect would lead to the reaction medium containing approximately 0.2 atom % excess ^{18}O, and, therefore, even complete loss of the C-2 oxygen atom in the reaction would result in incorporation of this amount of ^{18}O.

The maximum ^{18}O retained in glycerol 1-phosphate from the spinach aldolase reaction was approximately 0.3 atom % excess, or about 0.1 atom % excess after correcting for enrichment of reaction medium. Since this is well within the overall experimental error, it is concluded that no excess ^{18}O was retained in this experiment.

For aldolase from *C. perfringens*, the effect of varying the concentration of Co^{2+} was studied to confirm this divalent cation as an activator and to determine the optimal concentration. Maximum activity was observed using $6.5 \cdot 10^{-3}$ M $CoCl_2$, similar to that observed by Groves et al., [7] and this concentration of Co^{2+} was used in the aldolase experiments. A maximum of 31.2 atom % excess ^{18}O was observed in the glycerol 1-phosphate, or a retention of approximately 70% of the C-2 oxygen atom of Fru-P_2.

Fru-P_2 aldolase from *L. casei* showed a 40% activation by 10^{-1} M potas-

TABLE I

OXYGEN-18 ABUNDANCE OF GLYCEROL 1-PHOSPHATE OBTAINED FROM THE CLEAVAGE OF [2-^{18}O] FRUCTOSE 1,6-BISPHOSPHATE BY ALDOLASE

^{18}O enrichment of [2-^{18}O] Fru-P_2 cleaved in the reaction was 44.2 atom % excess in the C-2 oxygen atom, except in the cases of yeast and rabbit muscle which were 46.6 atom % excess.

Aldolase source	Units of glycerol-1-phosphate dehydrogenase	Atom % excess ^{18}O
Spinach	10	0.3
	50	0.4
	100	0.3
	200	0.4
C. perfringens	10	25.6
	25	31.1
	50	30.6
	90	32.4
	200	32.2
L. casei	10	0.3
	25	0.1
	50	0.4
	100	0.3
	200	0.3
E. gracillis (purified)	10	24.6
	25	29.3
	50	27.8
	100	30.8
	200	29.5
E. gracillis (crude extract)	10	14.2
	35	23.8
	50	22.2
	100	25.6
Yeast*	5	20.3
	10	21.7
	27	29.7
	50	30.7
	85	29.7
	120	31.5
	200	24.9
Rabbit muscle*	25	0.1
	50	0.0
	85	0.1
	100	0.4
	200	0.2

* These data, in part, have appeared in an earlier publication (ref. 4).

sium acetate and also a 20% activation by $3 \cdot 10^{-3}$ M EDTA. On cleavage of [2-^{18}O] Fru-P_2, approximately 0.3 atom % excess ^{18}O was found in glycerol 1-phosphate.

For *Euglena*, both Fru-P_2 aldolase purified by DEAE-cellulose chromatography and a crude enzyme extract were studied. The purified preparation was found to be approximately 20% activated by 10^{-1} M potassium acetate and 80% inhibited by $5 \cdot 10^{-3}$ M EDTA. Cleavage of [2-^{18}O] Fru-P_2 resulted in the retention of approximately 29.4 atom % excess ^{18}O, or 67% of the C-2 oxygen atom of Fru-P_2. Since the above results indicated that only a Class II aldolase emerged from the DEAE-cellulose column, a crude enzyme extract was examined in order to determine if the organism also produced a Class I enzyme. The

Euglena used for this experiment were grown on continuous light to increase the amount of Class I aldolase [9]. The data shows a maximum of approximately 23.9 atom % excess ^{18}O, or a retention of 54% of the C-2 oxygen atom of Fru-P_2.

Comparative data for yeast (*S. cerevisiae*) and rabbit muscle have been previously published in part by Heron and Caprioli [4] and are included in Table I. For yeast aldolase, 30.9 atom % excess ^{18}O was measured in the glycerol 1-phosphate, or 67% retention of the C-2 oxygen of Fru-P_2 and for rabbit muscle aldolase, 0.2 atom % excess ^{18}O, or complete exchange of this oxygen atom.

Discussion

The results given in Table I show that ^{18}O may be employed to detect and quantitate exchange of the C-2 oxygen atom of Fru-P_2 and that this method provides a clear distinction between the two classes of aldolases. The data show that there is a mechanistic difference between Fru-P_2 aldolases obtained from different sources and that this difference as determined by ^{18}O studies may be used as a direct method of classifying these enzymes. These results are consistent with the suggested mechanisms of action of the prototypes of the two classes, a Schiff-base intermediate for the rabbit muscle enzyme and a metal-chelate intermediate for the yeast enzyme.

Spinach Fru-P_2 aldolase gave complete exchange of the C-2 oxygen atom of Fru-P_2, similar to muscle aldolase, and can be clearly classified as Class I. The low molecular weight of the enzyme shows that this property alone is a poor indicator of the class to which it belongs.

The Fru-P_2 aldolase from *C. perfringens* gave a 70% retention of the C-2 oxygen atom of Fru-P_2. A control experiment described in an earlier publication [4] showed that under the conditions used in these experiments, approximately 30% of the label would be exchanged from [2-^{18}O]dihydroxyacetone phosphate after release from aldolase, but before conversion of glycerol 1-phosphate. A maximum of 1.4% of the label is exchanged from [2-^{18}O] Fru-P_2 during the reaction period and an additional 1.5% by exchange after conversion to [2-^{18}O]glycerol 1-phosphate due to the reversibility to the glycerol-1-phosphate dehydrogenase catalyzed reaction, i.e. the reoxidation of [2-^{18}O] glycerol 1-phosphate to [2-^{18}O]dihydroxyacetone phosphate, exchange with $H_2^{16}O$, followed by reduction once again to form glycerol 1-phosphate. Therefore, in total, it would be expected that if the C-2 oxygen atom of Fru-P_2 were 100% retained in the aldolase cleavage step itself, the ^{18}O analysis of glycerol 1-phosphate would show about 69% of the ^{18}O originally present in Fru-P_2. For the experiments with aldolase from *C. perfringens*, this value was determined to be 70% or essentially complete retention in the aldolase step. This result is similar to that obtained for yeast aldolase (67% retention) and therefore verifies that the *C. perfringens* enzyme is a Class II aldolase.

L. casei aldolase gave a 0.2% retention of the C-2 oxygen atom of Fru-P_2, showing it to be similar in mechanism to rabbit muscle aldolase. This enzyme was tentatively placed in Class I based on inhibition of activity when treated with sodium borohydride in the presence of substrate. On the other hand, the

present work found that it was activated by 0.1 M potassium ions, an effect characteristic of yeast aldolase. There is no doubt that *L. casei* aldolase should be considered Class I even though most other bacterial aldolases investigated are Class II. On the basis of ^{18}O studies, the results obtained with this enzyme suggest that some specific catalytic and structural properties of Fru-P_2 aldolases are not satisfactory as phylogenetic probes, i.e. *L. casei* aldolase is mechanistically more similar to rabbit muscle aldolase than to yeast aldolase.

Euglena are thought to produce both Class I and Class II aldolases. An enzyme extract obtained from cells grown on alternating periods of light (16 h) and dark (8 h) gave only a single aldolase containing peak on purification using DEAE-cellulose chromatography. When analyzed using [2-^{18}O] Fru-P_2, it was found that 67% of the C-2 oxygen atom was retained, the same as that for yeast. Thus, this fraction contained a Class II aldolase, but no Class I. However, this does not preclude the possibility that a Class I enzyme was produced by the organism but was inactivated by the purification procedures or that it was not eluted from the column. A second experiment was carried out with *Euglena* grown on continuous light since it has been shown that these conditions appear to increase the amount of Class I aldolase [9]. In this case, the [2-^{18}O] Fru-P_2 cleavage was carried out with the enzyme extract with no attempt at separating the aldolases, avoiding the possibility of inhibiting one or both. The ^{18}O present in the glycerol 1-phosphate shows a 54% retention of the C-2 oxygen atom of Fru-P_2 significantly less than that for the purified Class II enzyme. These results show that *Euglena* do produce both a Class I and Class II aldolase rather than either alone or an intermediate form. Since it has been shown that approximately 67% ^{18}O retention was obtained for the purified Class II enzyme from *Euglena* and that from other work the Class I enzyme would be expected to give complete exchange, it was calculated that the extract studied contained 78% Class II. Such a calculation, of course, assumes that both aldolases are fully extracted and fully active under the conditions of the reaction. Groves et al. [7] also found a predominance of Class II aldolase in *Euglena* grown on enriched media with continuous light; sucrose density-gradient centrifugation studies indicated approximately twice as much Class II aldolase as Class I.

The work presented here demonstrates the use of the ^{18}O isotope in providing a method of determining the class to which a particular source of aldolase belongs. It has the advantage of utilizing a property which is a direct function of the mechanism to distinguish between mechanisms of action without altering the reaction conditions or the enzyme itself. Other methods based on physical and chemical properties are in many cases inadequate and can lead to uncertain classifications.

Acknowledgements

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