SUBSTRATE SPECIFICITY OF MUSCLE ALDOLASE*

by

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Although many properties of crystalline muscle aldolase have been reported[1], the substrate specificity of this enzyme has been studied mainly with crude preparations[2]. MEYERHOF, LOHMANN AND SCHUSTER[3] demonstrated that dialyzed muscle extracts catalyzed the condensation of dihydroxyacetone phosphate (DAP)§ with any of a variety of aldehydes. It is generally agreed that the enzymically catalyzed aldol condensation directs the newly formed secondary hydroxyl groups to a *trans* configuration with respect to one another as does the base-catalyzed reaction[4].

The specificity of aldolase in the direction of condensation has been studied to clarify the previous finding[5] (p. 349) that only the D component of the FISCHER-BAER ester (DL-glyceraldehyde-3-phosphate) condensed in the presence of isomerase and aldolase from rat muscle. The specificity in the direction of cleavage was studied with several hexose phosphates. In particular the cleavage of fructose-1-phosphate was investigated because MEYERHOF *et al.*[3] and LEUTHARDT *et al.*[6] stated that fructose-1-phosphate was not cleaved by muscle aldolase. HERS AND JACQUES[7], however, reported that rabbit muscle extract cleaved fructose-1-phosphate. Our results show that fructose-1-phosphate is cleaved by recrystallized muscle aldolase and the aldolase of myogen A.

EXPERIMENTAL

Enzyme preparations

Crystalline rabbit muscle aldolase[1] was recrystallized three to five times until free from triosephosphate isomerase. Crystalline D-glyceraldehyde-3-phosphate dehydrogenase was prepared according to CORI, SLEIN AND CORI[8] and freed of triosephosphate isomerase by recrystallization (one to four times). One preparation of myogen A[9] which was recrystallized once contained triosephosphate isomerase. A preparation recrystallized three times was free from detectable isomerase (*cf.* [10]). Triosephosphate isomerase was prepared from rabbit muscle according to MEYERHOF AND BECK[10]. Dialyzed muscle extract was prepared by extracting ground rabbit or rat muscle with 1.5 volumes of water, dialyzing for 2 days at 3° and then diluting the extract with four volumes of water.

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§ The following abbreviations are employed: ATP: adenosine triphosphate; FDP, fructose-1,6-diphosphate; DAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; DPN and DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide. HDP refers to hexose diphosphate in which the exact nature of the hexose is not specified.

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Substrates

Dihydroxyacetone phosphate[11], DL-glyceraldehyde-3-phosphate[12], D-glyceraldehyde[13], L-glyceraldehyde[14], L-sorbose-1- and -6-phosphates[15] and D-tagatose-6-phosphate[16] were synthesized by the methods cited. Glycolaldehyde and glycolaldehyde-2-phosphate were prepared by Dr. B. C. PRESSMAN by adaptations[17] of the procedures of FISCHER AND TAUBE[18] and FLEURY *et al.*[19] respectively. L-Sorbose-1,6-diphosphate was purified from a mixture of its barium salt and barium orthophosphate[15]. D-Mannose-6-phosphate was synthesized in the laboratory of Prof. H. O. L. FISCHER by the procedure which had been developed for glucose-6-phosphate[20]. The tetra-acetyl mannose-6-diphenyl phosphate (m.p. 114°) which is the key intermediate has recently been described also by POSTERNAK AND ROSSELET[21].

D-Fructose-6-phosphate was purified from the commercial barium salt by dissolving in water and removing the barium salts insoluble in 10% ethanol[22] before precipitating with four volumes of acetone. The commercial salt, and the fraction precipitated by 10% ethanol contain appreciable amounts of a substance (presumably HDP) which is cleaved by aldolase. The purified fructose-6-phosphate in the presence of aldolase does not yield any detectable DAP and it is therefore suggested that the apparent cleavage of fructose-6-phosphate reported by DOUNCE *et al.*[23] was due to an impurity in the sample of fructose-6-phosphate tested.

Synthetic D-fructose-1-phosphate was prepared by catalytic hydrogenation (2.8 g PtO) of 13.1 g 2,3; 4,5-di*iso*propylidene-D-fructopyranose-1-diphenylphosphate[24] in 75 ml methanol containing 5 ml glacial acetic acid. When the theoretical amount of hydrogen had been consumed the catalyst was filtered off and the solvent evaporated *in vacuo*. Removal of *iso*propylidene groups and isolation of the barium salt were accomplished by the procedure used for the analogous sorbose-1-phosphate[15]. The fructose-1-phosphate, obtained in a yield of 68%, was free of hexosediphosphate as measured by enzymic assay and by ion exchange chromatography on Dowex 1.

D-Tagatose-1,6-diphosphate was synthesized from D-tagatose-6-phosphate and ATP using partially purified phosphohexokinase[25] which had been prepared from acetone dried rabbit muscle to avoid contamination by aldolase[26]. When the enzymic synthesis was complete, adenine nucleotides were removed with mercuric nitrate and after removal of excess mercury with sulfide and, after aeration, tagatose diphosphate was precipitated together with a small amount of inorganic phosphate as the barium salt.

DPNH was prepared chemically[27] or enzymically[28] from commercial DPN.

Analytical methods

Total and inorganic phosphate were determined according to KING[29]. Alkali-labile phosphate was measured by determining inorganic phosphate before and after treatment of samples with 1 *N* NaOH for 20 minutes at room temperature. Spectrophotometric methods for the determination of dihydroxyacetone phosphate[9] and D-glyceraldehyde-3-phosphate[1,30] were rendered specific by using enzymes free from triosephosphate isomerase.

RESULTS

Condensation of DL-*GAP* by dialyzed extracts of rat or rabbit muscle proceeded under the conditions described in Table I, to an equilibrium* at which about 50% of the alkali-labile phosphate had disappeared. It had been assumed that the isomerase present would convert D-GAP to DAP which the aldolase present should have condensed with L-GAP to yield L-sorbose-1,6-diphosphate. Since only one-half of the triosephosphate disappeared it appeared likely that the L-isomer of GAP did not react with aldolase. The data in Table II demonstrate that the remaining triosephosphate is not DAP since it is oxidized by iodine to an alkali-stable compound, and is not D-GAP since it is not oxidized by the dehydrogenase specific for this substrate. It must therefore be assumed to be L-GAP.

That isomerase-free aldolase is capable of condensing L-GAP with DAP is shown in experiments D and E of Table I. Under the conditions of the experiment (a slight molar excess of dihydroxyacetone phosphate was present) the disappearance of alkali-

* Analyses after various periods of incubation at 4° yielded constant values for alkali-labile phosphate. The equilibrium of the reaction is strongly influenced by temperature, low temperatures favoring HDP formation[2].

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labile phosphate at equilibrium corresponded to essentially complete condensation of the added DL-GAP.

TABLE I

CONDENSATION OF TRIOSE PHOSPHATES

0.5 ml of the enzyme solution (10 γ of protein in the case of aldolase) were incubated with solutions of the substrate neutralized to pH 7.0 in a final volume of 4.5 ml at room temperature for 10 minutes and at 4° for 30 minutes. The mixture was then frozen, perchloric acid (0.2 ml of 60%) was added and the denatured protein separated by centrifugation before analysis of the solution.

Expt.	*Enzyme*	*Substrate added*		*Alkali-labile P at equilibrium*	*Per cent condensation of* DL-*glyceraldehyde-3-phosphate*
		DL-*Glyceraldehyde-3-phosphate*	*Dihydroxyacetone phosphate*		
		μg P	μg P	μg P	
A	Dialyzed rat muscle extract*	2100	0	1120	47
B	Dialyzed rabbit muscle extract	105	0	50	52
C	Dialyzed rabbit muscle extract	94	0	49	52 (50)**
D	Crystalline aldolase	74	82	8	100
E	Crystalline aldolase	59	90	22	107 (99)***
F	Crystalline aldolase	72	DL-glycer-aldehyde§	9	
G	Crystalline aldolase	78	L-glycer-aldehyde§	15	
H	Crystalline aldolase	90	D-glycer-aldehyde§	11	

* The extract was prepared from the cold acetone precipitate of dialyzed rat muscle extract.
** Average of 5 experiments with rabbit muscle extract.
*** Average of 6 experiments with varying amounts of the two triose phosphates.
§ Approximately 2 μmoles of aldehyde were added in each experiment and the condensation was approximately complete.

TABLE II

CHARACTERIZATION OF TRIOSE PHOSPHATE REMAINING AFTER CONDENSATION OF FISCHER-BAER ESTER BY RABBIT MUSCLE EXTRACT

Expt.	*Alkali-labile P*		D-*glyceraldehyde-3-phosphate*
	before I_2 *oxidation*	*after* I_2 *oxidation**	
	μg	μg	$\Delta \log (I_0/I)$ at 340 mμ
B	50	2	0.000**
C	49	4	0.000

* The alkaline iodine oxidation was carried out by modification[31] of the procedure of MEYERHOF[32].
** The assay system for D-GAP (neutralized solution) was based on the reduction of DPN[1,30] in the presence of 100 γ crystalline D-GAP dehydrogenase[8]. With $4 \cdot 10^{-6}$ M GAP the value for $\Delta \log (I_0/I)$ at 340 mμ was 0.660 per minute.

The finding that aldolase does bring about the condensation of L-GAP caused us to reinvestigate the condensation of DL-GAP by muscle extracts. It was found that greatly prolonging the incubation period during which the temperature is dropped from

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24° to 4° permitted condensation of an appreciable part of the L-isomer. Thus it appears that in the earlier experiments a pseudo equilibrium was obtained because D-GAP reacts with DAP at a much greater rate than does the L-isomer. It is also possible that the equilibrium constant for the reaction yielding D-fructose diphosphate is different from that yielding L-sorbose diphosphate.

Glycolaldehyde and Glycolaldehyde-2-phosphate condense with DAP in the presence of isomerase-free aldolase to yield phosphorylated pentoses (Table III), the characterization of which is described in the following paper.

TABLE III

CONDENSATION OF DIOSES WITH DIHYDROXYACETONE PHOSPHATE BY ISOMERASE-FREE ALDOLASE

Dihydroxyacetone-P incubated with:	*Alkali-labile phosphate*		*"Pentose" formed*
	before incubation	*after incubation*	
	μmoles	μmoles	μmoles
Glycolaldehyde	2.4	0.3	2.0
Glycolaldehyde-2-phosphate	2.4	2.3	1.6

Experimental conditions as in Table I. Pentose was determined by the method of MEJBAUM[33] using xylose as a standard.

Cleavage of ketohexose-1,6-diphosphates. Although aldolase is assumed to form only ketoses with a *trans* configuration of hydroxyls on carbon atoms 3 and 4, it is not so highly specific in the direction of cleavage. D-Tagatose-1,6-diphosphate, in which the hydroxyl groups on carbons 3 and 4 are *cis* to one another, is cleaved by muscle aldolase (Table IV). This confirms earlier results obtained with an extract of beef brain[16]. Sorbose-1,6-diphosphate is also cleaved. It must be pointed out that the data of Table IV provide only qualitative evidence for the cleavage and do not permit deductions concerning the relative rates with different hexose-diphosphates.

TABLE IV

CLEAVAGE OF KETO-HEXOSE DIPHOSPHATES

Substrate	*$\Delta \log I_0/I$ first minute*
D-Fructose-1,6-diphosphate	0.408
L-Sorbose-1,6-diphosphate	0.328
D-Tagatose-1,6-diphosphate	0.045

The reaction mixture contained 60 μmoles substrate, 70 μmoles phosphate buffer pH 7.0, 10 μg aldolase, and after standing 5 minutes, 0.4 μmole DPNH and 120 μg myogen A were added. Final volume 3 ml. Oxidation of DPNH by DAP measured in the Beckman spectrophotometer at 340 mμ.

Cleavage of hexosemonophosphates. Since aldolase catalyzes the condensation of DAP with D- and L-glyceraldehyde ([3] and Table I) it must perforce catalyze the reverse reaction, the cleavage of D-fructose-1-phosphate and L-sorbose-1-phosphate. According to MEYERHOF *et al.*[3] the reaction in the direction of cleavage is not detectable by analysis for triosephosphate. Removal of one of the cleavage products should permit detection of the reaction although LEUTHARDT *et al.*[6] were unsuccessful in doing so. Using a similar

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test system, we have found that both fructose-1-phosphate and sorbose-1-phosphate are cleaved by the aldolase in myogen A (Table V) and by crystalline aldolase (Fig. 1), although at a very slow rate.

TABLE V

CLEAVAGE OF KETOHEXOSE MONOPHOSPHATES

Substrate	$\Delta \log I_0/I$
D-Fructose-1-phosphate	0.065
L-Sorbose-1-phosphate	0.051
D-Fructose-6-phosphate	0
D-Tagatose-6-phosphate	0
D-Mannose-6-phosphate	0

Conditions as in Table IV for the -1-phosphate esters. Cleavage of the -6-phosphate esters ($3 \cdot 10^{-5}$ *M*) was tested for, in the presence of the enzymes aldolase and triose phosphate dehydrogenase, together with other adjuvants as described in the legend of Fig. 1.

The myogen A test system (Table V) measures oxidation of DPNH by DAP, catalyzed by the α-glycerophosphate dehydrogenase in myogen A[9]. By reducing DAP as rapidly as it is formed from ketose-1-phosphate the reversibility of the reaction is clearly demonstrated.

The cleavage of fructose-1-phosphate by the crystalline aldolase of TAYLOR *et al.*[1] can be demonstrated by the use of DPN, D-glyceraldehyde-3-phosphate dehydrogenase, and triosephosphate isomerase which converts DAP to GAP (Fig. 1). The fact that no reduction of DPN occurs before addition of isomerase attests to the absence of FDP in the synthetic preparation of fructose-1-phosphate.

HERS AND JACQUES[7] have explained the negative results of LEUTHARDT *et al.*[6] as resulting from a much lower affinity of muscle aldolase for fructose-1-phosphate ($K_M = 1.1 \cdot 10^{-1}$ *M*) than for fructose-diphosphate ($K_M = 10^{-3}$ *M*). Under the conditions of our assay*, the MICHAELIS constants, estimated

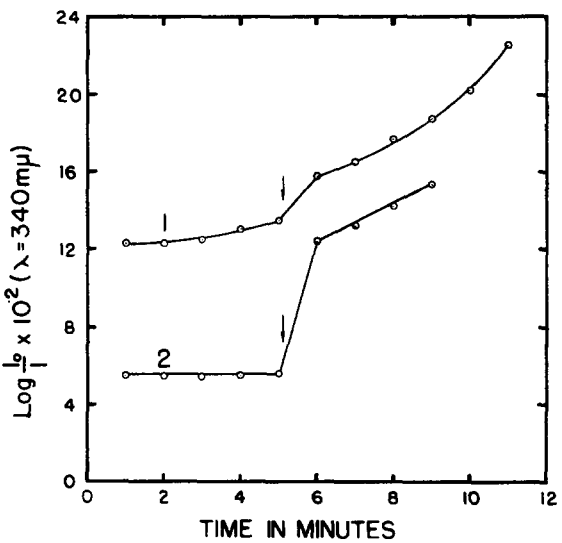

Fig. 1. Cleavage of fructose-1-phosphate by crystalline aldolase. The reaction mixture contained, in moles per 3 ml tris HCl buffer $1 \cdot 10^{-4}$ (pH 8.0), DPN $1 \cdot 10^{-6}$, Na-Arsenate $1 \cdot 10^{-5}$, fructose-1-phosphate $3.6 \cdot 10^{-5}$, and enzymes as indicated below. Temperature, 26°. Curve 1: With isomerase (1.88 mg) and triosephosphate dehydrogenase (1.48 mg). Aldolase (0.128 mg) was added at the time indicated by arrow. Curve 2: With triosephosphate dehydrogenase (1.48 mg) and aldolase (0.128 mg). Isomerase (1.88 mg) was added at the time indicated by arrow. The density increase during the minute immediately following the addition of isomerase is due to the turbidity of this preparation.

* Tris buffer (pH 8.0), $1 \cdot 10^{-4}$ moles; DPNH, $3 \cdot 10^{-7}$ moles; myogen A (containing isomerase), 30 μg; volume = 3 ml.

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from LINEWEAVER-BURK plots were $3.4 \cdot 10^{-3}$ *M* for fructose-1-phosphate and $5 \cdot 10^{-5}$ *M* for FDP. Although the affinity for both substrates appears to be higher than reported by HERS AND JACQUES, the differences between the two substrates are similar.

None of several aldo- and keto-hexose-6-phosphates were cleaved by aldolase (Table V). An explanation for a previous report[23] that fructose-6-phosphate is cleaved is offered in the EXPERIMENTAL section under *Substrates*.

SUMMARY

A study of the substrate specificity of rabbit muscle aldolase led to the following observations.

1. Both the D and L isomers of glyceraldehyde-3-phosphate can be condensed with dihydroxyacetone phosphate, but the former appears to react much more rapidly than the latter.

2. Tagatose-1,6-diphosphate, which possesses a *cis* configuration of hydroxyl groups on carbon atoms 3 and 4, is cleaved but at a slower rate than D-fructose-1,6-diphosphate or L-sorbose-1,6-diphosphate.

3. The condensation of D- or L-glyceraldehyde with dihydroxyacetone phosphate to yield fructose- or sorbose-1-phosphates respectively is reversible. Crystalline rabbit muscle aldolase haə a much greater affinity for fructose diphosphate ($K_M = 5 \cdot 10^{-5}$ *M*) than for fructose-1-phosphats ($K_M = 3.4 \cdot 10^{-3}$ *M*).

RÉSUMÉ

Une étude des substrats spécifiques de l'aldolase du muscle de lapin a conduit aux observations suivantes:

1. Les isomères D et L du glycéraldéhyde-3-phosphate peuvent être condensés avec le dihydroxyacétone phosphate, mais le premier réagit beaucoup plus vite que le second.

2. Le tagatose-1,6-diphosphate, dont les groupes hydroxyles portés par les carbones 3 et 4 sont en *cis*, est hydrolysé mais plus lentement que le D-fructose-1,6-diphosphate ou le L-sorbose-1,6-diphosphate.

3. La condensation du D ou du L-glycéraldéhyde avec le dihydroxyacétone phosphate en fructose- ou sorbose-1-phosphate, respectivement, est réversible. L'aldolase cristallisée du muscle de lapin a une affinité beaucoup plus grande pour le fructose diphosphate ($K_M = 5 \cdot 10^{-5}$ *M*) que pour le fructose-1-phosphate ($K_M = 3.4 \cdot 10^{-3}$ *M*).

ZUSAMMENFASSUNG

Das Studium der Substratspezifität von Kaninchenmuskelaldolase führt zu folgenden Beobachtungen:

1. Sowohl die D- wie die L-Form von Glycerinaldehyd-3-phosphat kann mit Dihydroxyacetonphosphat kondensiert werden. Jedoch reagiert die D-Form viel schneller als die L-Form.

2. Tagotose-1,6-diphosphat, das eine *cis* Konfiguration der Hydroxylgruppen am 3. und 4. Kohlenstoffatom besitzt, wird gespalten, jedoch mit einer kleineren Rate als D-Fruktose-1,6-diphosphat oder L-Sorbose-1,6-diphosphat.

3. Die Kondensation von D- oder L-Glycerinaldehyd mit Dihydroxyacetonphosphat, die zu Fruktose- oder Sorbose-1,6-phosphat führt, ist reversibel. Kristalline Kaninchenmuskelaldolase hat eine viel grössere Affinität zu Fruktosediphosphat ($K_M = 5 \cdot 10^{-5}$ *M*) als zu Fruktose-1-phosphat ($K_M = 3.4 \cdot 10^{-3}$ *M*).

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