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Class I fructose-1,6-bisphosphate aldolases as catalysts for asymmetric aldol reactions

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Abstract

The activity of two commercially available bacterial class I fructose-1,6-bisphosphate aldolases (FruA) towards a number of aldehydes has been compared with rabbit muscle aldolase (RAMA), which is the most widely used enzyme for aldol reactions with dihydroxyacetone phosphate. The kinetic properties of the three aldolases are very similar, but the bacterial aldolases were much more stable than RAMA. Reaction of butanal and dihydroxyacetone phosphate catalyzed by FruA from *Staphylococcus carnosus* was performed on a 5 mmol scale in 53% isolated yield. Enantiomeric and diastereomeric purity of the major reaction product [90% (3*S*,4*R*)] was determined by chiral gas chromatography.

1. Introduction

The aldol reaction, which creates a C–C bond as well as two contiguous stereogenic centers, is of vital interest for organic synthesis.[1] The potential advantages of enzyme-catalyzed aldol reactions — mild reaction conditions, enantioselectivity and reduction of waste — have stimulated research in this field. The dihydroxyacetone monophosphate (DHAP) dependent aldolases, which in their natural role perform a retro-aldol reaction, have the advantage of nearly absolute control over the newly created stereogenic centers.[2] These enzymes have been used mainly to synthesize a wide variety of modified carbohydrates.[3–6] The best studied DHAP-depending aldolase, RAMA, accepts a number of unnatural aldehydes[7] as substrates, but its practical application is hampered by a low operational stability.[8] The substrate spectrum of the more stable bacterial aldolases suggests that they have a similarly relaxed acceptor specificity,[8,9] but this has not been studied in any depth. The bacterial aldolases we chose are the commercially available class I FruA from *S. carnosus*[9–11] and *S. aureus*,[12] which are known to be very heat- and pH-stable.[13] The former organism is a safer production host than the pathogenic *S. aureus* and, hence, its aldolase has been studied more intensively.

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We have compared the activities and stabilities of the above-mentioned bacterial aldolases with RAMA in the aldol reaction of DHAP with a wide variety of aldehydes to demonstrate their synthetic utility and scope. Stereoselectivity of the reaction of butanal with DHAP was investigated in detail. The four possible product stereoisomers were analyzed using chiral GC in combination with epimerization experiments, a method not previously employed to identify stereoisomers formed in these reactions.

2. Results and discussion

2.1. Specific activity, initial rates and conversion

The D-fructose-1,6-bisphosphate (fru-1,6-P_2) aldolase from rabbit muscle (RAMA), *S. carnosus* and *S. aureus* were compared for activity of the aldol reaction as well as retro-aldol reactions. The cleavage of the natural reactant –fru-1,6-P_2– was used to assay the retro-aldol activity. Catalytic activity in the synthetic direction was measured with DHAP as donor; D-glyceraldehyde-3-phosphate (GAP) and propanal were used as acceptors. Reactions were monitored by a coupled enzymatic assay[14] for DHAP. In this assay DHAP is reduced to glyceraldehyde-3-phosphate with triose-1-phosphate isomerase and NADH using D-glyceraldehyde-3-phosphate dehydrogenase. The decrease in absorption at 340 nm is proportional to the amount of DHAP present.

The results of the assays are tabulated in Table 1. All three aldolases converted GAP and propanal at similar rates. Under the reaction conditions used, the specific synthesis activity of the bacterial aldolases was approximately 50% of their cleavage activity, whereas RAMA had the same activity in both reactions.

Table 1
Specific activities of fructose-1,6-bisphosphate aldolases from different sources

aldolase	splitting Fruc-1,6-bisphosphate U/mg enzyme	synthesis propanal + DHAP U/mg enzyme	synthesis GAP + DHAP U/mg enzyme
S. carnosus	26.7	12.2	12.2
S. aureus	16.8	7.2	8.5
RAMA	22	24	22.4

The acceptor specificity of the three aldolases was investigated by incubating DHAP and a 100% excess of acceptor aldehyde with 1 PAU† of aldolase. Propanal was chosen instead of the conventionally used glyceraldehyde-3-phosphate because of better reproducibility, comparable kinetic properties and ease of use.

The relative initial rates and conversions of the reaction of DHAP with the different aldehydes are displayed in Table 2. The kinetic parameters of RAMA are quite comparable with the FruA of *S. carnosus* or *S. aureus*, as might be expected on the basis of the similarity in structure of the active sites. Conjugated aldehydes such as benzaldehyde and acrolein (V_{ini}=0) are not substrates, with the exception of pyridinecarboxaldehydes.[8] Longer alkyl chains result in a lower (relative) V_{ini} and conversion. Aldehydes bearing an electron withdrawing group such as chloroacetaldehyde have an activated carbonyl group, which increases the initial rate. Bulky and aromatic groups result in lower rates and conversions.

† 1 PAU (propanal aldolase unit) converts propanal at an initial rate of 1 μmol/min.

Table 2
Relative initial reaction rates and conversions for aldol reactions with DHAP

Aldehyde	Relative V_{ini} (%)			Conversion (% (t))		
	carnosus	aureus	RAMA	carnosus	aureus	RAMA
formaldehyde	133	57	43	81 (2h)	16 (2h)	31 (3h)
acetaldehyde	108	141	103	84 (2.5h)	64 (3h)	87 (2h)
propionaldehyde	100	100	100	67 (4h)	68 (2h)	67 (3h)
butanal	37	72	23	70 (4h)	53 (4h)	69 (3h)
pentanal	36	27	20	64 (3h)	50 (4h)	67 (4h)
hexanal	32	22	22	62 (5h)	56 (4h)	63 (3h)
heptanal	8	11	9	a		
octanal	2	<1	<1			
isobutyraldehyde	8	16	10			
isovaleraldehyde	7	9	8			
glyoxylic acid	17	13	14	94 (4h)	96 (6h)	97 (5h)
glyoxal	65	21	16	98 (5h)	83 (6h)	89 (6h)
methylglyoxal	35	32	9	86 (6h)	64 (5h)	69 (6h)
glycolaldehyde	253	354	145	96 (0.5h)	95 (3h)	94 (0.7h)
chloroacetaldehyde	308	282	275	76 (1.5h)	85 (4h)	87 (0.5h)
phenylacetaldehyde	33	93	28	66 (2h)	37 (1h)	22 (1h)
2-pyridine carboxyaldehyde	6	9	2			
3-pyridine carboxyaldehyde	7	2	3			
4-pyridine carboxyaldehyde	6	3	<1			

[a] Undisplayed conversions could not be determined because reaction rates were slower then hydrolysis of DHAP

Hence, we concluded that the generalizations made for the substrate spectrum of RAMA[7] also applied to bacterial type I aldolases.

2.2. *Comparison of aldolase stability under reaction conditions*

The reaction of DHAP with butanal was used to compare the stability of RAMA (tetramer, 160 kD) and *S. carnosus* FruA (monomer, 40 kD). After the maximum conversion was reached, the biocatalysts were concentrated and washed with buffer in Amicon centripreps (cut-off 10 kD). The aldolase from *S. carnosus* proved to be the most stable enzyme with a recovery of 45% compared to 0.1% for RAMA. With glyoxylic acid as acceptor aldehyde 100% of the bacterial FruA was recovered and 91% RAMA. This yield of RAMA is consistent with its reported[9] rate of deactivation under these conditions of 2.47% per h, which corresponds to 89% residual activity after 4.5 h under reaction conditions. The washed and concentrated aldolase from *S. carnosus* could readily be reused.

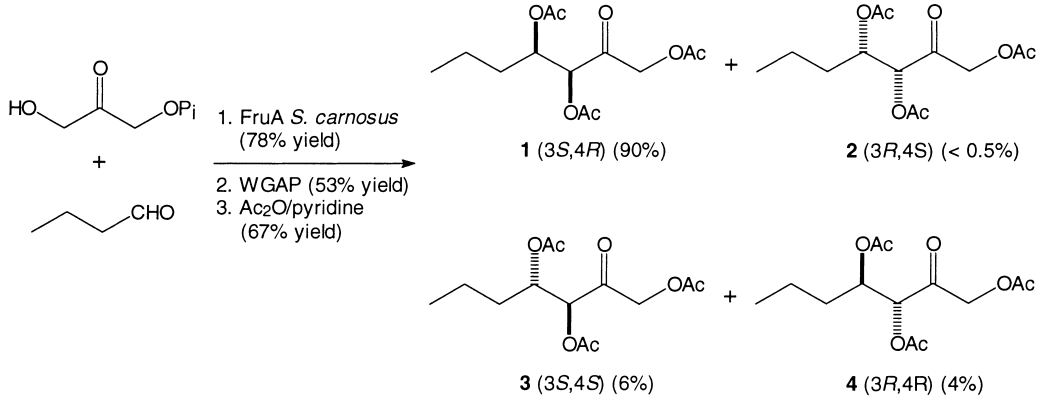
2.3. Reaction on a 5 mmol scale

In order to test the validity of the figures from Table 2 for larger scale reactions the reaction of butanal and DHAP was conducted on a 5 mmol scale. The concentration of DHAP was increased from 20 to 50 mM. Conversion of DHAP was 78%. Treatment with wheatgerm acid phosphatase (WGAP) followed by acetylation gave 1,3,4-tri-*O*-acetyl-5-deoxy-5-ethyl-D-xylulose, in 53% overall yield. The values from Table 2 thus form a good basis for performing larger scale reactions.

2.4. Stereoselectivity

In one example — the reaction of butanal with DHAP — we investigated the steric course of the reaction in detail. This was done by chiral GC analysis of the reaction products after dephosphorylation and acetylation. Previously,[15] diastereomers had been identified by NMR, but chiral GC has the advantage that enantiomers can be discriminated.

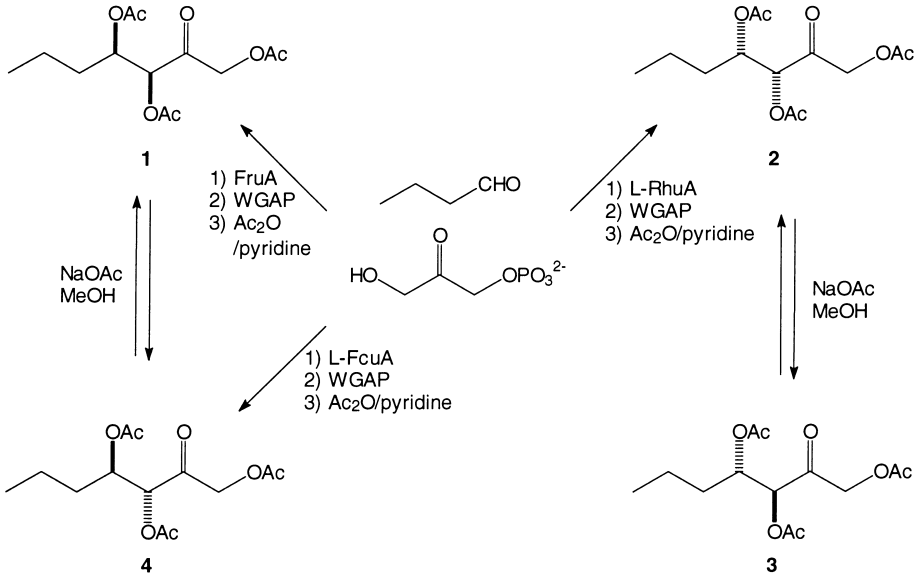
The reaction of butanal and DHAP, catalyzed by FruA from *S. carnosus* afforded a major (90% selectivity) product to which we assigned the (3*S*,4*R*) configuration **1** (see Scheme 1) on the basis of the known steric preferences of the catalyst.[9] Two minor, presumably stereoisomeric, products were formed with 6% and 4% selectivity. In order to elucidate their structures we synthesized two of the remaining three possible stereoisomers of the aldol adduct independently. Reaction of butanal and DHAP catalyzed by L-rhamnulose-1-phosphate aldolase[15] (L-RhuA) afforded the (3*R*,4*S*) product **2**; L-fuculose-1-phosphate aldolase[15,16] (L-FcuA) afforded the (3*R*,4*R*) stereoisomer **4** (Scheme 2). The (3*S*,4*S*) stereoisomer **3** could not be synthesized directly, because no D-tagatose-1,6-bisphosphate aldolase was available. However, epimerization of **2** with sodium acetate in methanol at room temperature‡ afforded, after 4 h, an equilibrium mixture of 45% **2** and 55% of its 3-epimer **3**. Hence, all four aldol adducts of butanal and DHAP could be resolved by chiral GC. To reconfirm the structural assignments, **1** was similarly subjected to epimerization; after 2 h at room temperature an equilibrium mixture of 60% **1** and 40% of its 3-epimer **4** was obtained.



Scheme 1. Aldol reaction of butanal and DHAP catalyzed by *S. carnosus* FruA. Selectivities are in parentheses

On the basis of chiral GC the minor products of FruA from *S. carnosus* were identified as the *anti* stereoisomers **3** (6% selectivity) and **4** (4% selectivity). Because the stereogenic center at C-4 is not readily susceptible to epimerization, the formation of **3** must reflect a lack of complete stereoselectivity at C-4. In contrast, epimerization of **1**, either during the reaction of the work-up, could contribute to

‡ Epimerization presumably proceeds via enolization and is also observed under acidic conditions (*p*-TosOH was added).



Scheme 2. Structural assignments of the four possible aldol adducts

the observed formation of **4**. NMR analysis of the (phosphorylated) reaction products suggests that *S. carnosus* aldolase forms 8.5% of the *anti* stereoisomers **3** and **4**. Hence, the remaining 1.5% presumably results from epimerization during the work-up.

2.5. Conclusion

More than 20 aldehydes were found to be substrates for the aldolases from rabbit muscle, *S. carnosus* and *S. aureus*. Reaction of butanal with DHAP catalyzed by FruA from *S. carnosus* afforded the (3*S*,4*R*) stereoisomer **1** as the major product (90%) together with 6% of the (3*S*,4*S*) isomer **3** and 4% of the (3*R*,4*R*) isomer **2**. Stereoselectivity of the aldolases must be looked at for each acceptor substrate, since isomers are formed in different proportions. Bacterial aldolases are more stable than RAMA, especially when the aldehydes are more apolar. Reaction rates and chemical yields are comparable. Isolation of enzymes from the reaction mixture is feasible with high recovery and makes *S. carnosus* aldolase more efficient than RAMA.

3. Experimental

3.1. General

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 300 MHz on an Oxford NMR 300. UV spectroscopy was performed with a Varian Cary 3 Bio equipped with a Cary temperature controller. Chiral gas chromatography was performed with a diacetyl-*tert*-butylsilyl-β-cyclodextrin (50 m×0.25 mm, df 0.25 μm) column. DHAP was prepared from its ethyl hemiacetal dimer barium salt (Fluka).

3.2. FruA activity assay

The reaction products, DHAP and GAP were assayed with a coupled enzyme system. To 1.95 ml 50 mM Tris-buffer pH 7.6 containing 0.16 mM NADH in a 2 ml cuvette, were added 20 μl 190 mM fructose-

1,6-bisphosphate, 20 μl of a mixture containing 1.25 unit D-glyceraldehyde-3-phosphate dehydrogenase and 12.5 unit triose-1-phosphate isomerase. Then 50 μl diluted aldolase was added and absorption monitored at 25°C. 1 unit (U) aldolase converts 1 μmol fructose-1,6-bisphosphate per minute.

3.3. DHAP-assay

DHAP was assayed with a coupled enzyme system: reduction of DHAP with NADH-consuming glycerol-3-phosphate dehydrogenase.[14] From a diluted DHAP solution, 50 μl was added in a quartz cuvette containing 1.95 ml 50 mM Tris pH 7.6, 0.16 mM NADH, 1.25 U glyceraldehyde-3-phosphate and 12.5 U triose-1-phosphate isomerase. The absorption was monitored at 340 nm at 20°C. Blank DHAP: 0.0047 mM/min. The molar adsorption coefficient taken was 6.22 $l.mmol^{-1}$ cm^{-1}. Reaction rates were calculated with the method of least squares. Relative rates were calculated by dividing $V_{propanal}$ by $V_{aldehyde}$ and multiplying by 100.

Aldol reactions were performed in 1 ml 50 mM Tris buffer pH 7.6 containing 20 mM DHAP and 40 mM aldehyde, reactions were initiated by adding 1.0 U aldolase. At intervals of 0.5–5 min during 3–30 min 50 μl aliquots were taken and quenched with 15 μl 7% perchloric acid. After 30 min 10 μl 1 M NaOH was added followed by 175 μl of 50 mM Tris pH 7.6. This neutralized mixture was then assayed for DHAP.

3.4. Enzyme recovery

In a 25 ml round bottomed flask equipped with a magnetic stirrer which contained 10 ml 50 mM Tris-buffer pH 7.6, 50 mM DHAP and 100 mM aldehyde, 10 U FruA (lyophilized RAMA and *S. carnosus* FruA) were added and stirred for 3 h at RT. After this the reaction mixture was cooled to 4°C, poured into (10 or 30 kDa) Amicon centripreps, centrifuged, washed with 2×10 ml Tris-buffer and concentrated to 0.5 ml. The residual activity was determined by adding 10 μl concentrated recovered enzyme to 1.95 ml 50 mM Tris-buffer pH 7.6 and 0.16 mM NADH in a 2 ml cuvette. Subsequent addition of 20 μl mixture of 1.25 U glyceraldehyde-3-phosphate and 12.5 U triosephosphate isomerase exposed any residual DHAP or product if present. Then the reaction was started by addition of 20 μl 190 mM fructose-1,6-bisphosphate and absorption was monitored at 25°C.

3.5. Synthesis of 1,3,4-tri-O-acetyl-5-deoxy-5-ethyl-D-xylulose (**1**)

A 50 ml solution containing 50 mM Tris pH 7.6, 50 mM DHAP, 100 mM butanal and 48 U *S. carnosus* aldolase was stirred at room temperature. Conversion was 78% (based on consumption of DHAP) after 4 h. Treatment with wheat germ acid phosphatase (WGAP) overnight at pH 5.5 gave after extraction with ethyl acetate 214 mg (c.y. 53% yield) product. Acetylation with 20 ml pyridine and 10 ml acetic anhydride and subsequent column chromatography (EtOAc:hexane 1:3) yielded 250 mg (67%) of colorless oil. $[\alpha]_D^{25}$=25.0 (c 1.05, $CHCl_3$).

3.5.1. ^1H NMR (300 MHz, $CDCl_3$)

δ=4.75 (d, H-1a), 4.88 (d, H-1b); 5.31 (d, H-3); 5.37 (m, H-4); 1.55 (m, H-5a), 1.33 (m, H-5b); 1.55 (m, 2H-6); 0.94 (t, 3H-7); 2.22 (s, 3H-1′); 2.16 (s, 3H-3′); 2.078 (s, 3H-4′).

3.5.2. ^{13}C NMR (300 MHz, $CDCl_3$)

δ=66.79 (C-1), 198.41 (C-2), 77.15 (C-3), 71.52 (C-4), 32.38 (C-5), 18.51 (C-6), 13.71 (C-7), 170.07 (CO-1′), 20.76 (CH_3-1′), 170.19 (CO-3′), 20.42 (CH_3-3′), 169.81 (CO-4′), 20.36 (CH_3-4′).

3.6. Acetylation of GC samples

Acetylation prior to GC injection was conducted by solving 2 to 3 mg of analyte in 0.1 ml pyridine using a 2 ml sample bottle. After addition of 50 μl acetic anhydride the mixture was shaken for 10 s at room temperature. Immediately 1 ml diethyl ether and 0.3 ml 4 N HCl was added to neutralize the mixture and extract the acetylated compound. The water layer was removed and the organic layer dried with sodium sulfate. This solution was then ready for injection. Under these conditions no racemization occurred — only after subjecting the analyte for more than 5 min to the acetylation mixture was epimerization observed.

3.7. Preparation of product samples

Butanal and DHAP were reacted following the procedure of the preparation of 1,3,4-tri-*O*-acetyl-5-deoxy-5-ethyl-D-xylulose with 50 U L-fuculose-1-P aldolase or 50 U L-rhamnulose-1-P aldolase. After extraction with ethyl acetate the products were acetylated according to the acetylation of GC samples.

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