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Dihydroxyacetone kinase-catalyzed Phosphorylation

Dominik Gauss,[a] Israel Sánchez-Moreno,[b] Isabel Oroz-Guinea,[b] Eduardo García-Junceda,[b] and Roland Wohlgemuth*[a]

Abstract: Site- and enantioselective kinases have been very useful catalysts for biocatalytic phosphorylations in straightforward syntheses of phosphorylated metabolites. Biocatalytic phosphorylations catalyzed by recombinant dihydroxyacetone-kinase beyond the dihydroxyacetone substrate have been investigated with quantitative ^{31}P-NMR spectroscopy using pyruvate-kinase-catalyzed ATP-regeneration. A nearly 100% conversion of D-glyceraldehyde to D-glyceraldehyde 3-phosphate has been found. Interestingly, with pure L-glyceraldehyde as substrate, practically no formation of L-glyceraldehyde 3-phosphate was observed.

Catalytic asymmetric phosphorylations without the use of protecting groups for the phosphoryl group donors as well as for the substrates and the development of suitable catalysts are of major synthetic interest for the introduction of phosphoryl groups in a selective and sustainable way. As a large number of catalytic phosphorylation reactions play key roles in biological cells and are performed with excellent selectivity, it is no surprise that enzymes have also proven to be very suitable and versatile catalysts for asymmetric phosphorylation reactions from laboratory to industrial large-scale [1]. In contrast to chiral small molecular weight organocatalysts, where the two enantiomers can be used to catalyze the formation of the two enantiomeric products, the search for enantiocomplementary biocatalysts requires various different strategies [2]. For certain enzyme classes like hydrolases, alcohol dehydrogenases or transaminases both enantioselective enzymes have been found. Glycerol kinases have all led to products with the L-configuration and glycerol kinases with opposite enantioselectivity have so far not been discovered. Our aim was to extend the kinase-catalyzed asymmetric synthesis of L-glyceraldehyde 3-phosphate (L-GAP) [3], which is toxic to cells, to the other enantiomer and major metabolite D-glyceraldehyde 3-phosphate (D-GAP). D-GAP was so far obtained by chemical synthesis or a one-pot enzymatic reaction sequence using the three enzymes fructose-bisphosphate aldolase, *sn*-glycerol 3-phosphate dehydrogenase and formate dehydrogenase [3]. In addition, *in situ* D-GAP synthesis has also been performed in different enzyme cascade reactions, such as acid phosphatase/DERA aldolases [4] and triosephosphate dehydrogenase/fructose-1,6-biphosphate aldolase/FSA aldolase [5].Therefore we looked for suitable kinase candidates. Among the different kinases ATP-dependent dihydroxyacetone kinases are of interest because they are able to differentiate between hydroxy- and keto-groups. Dihydroxyacetone kinases (DHAK) are highly conserved and specific enzymes of microbial glycerol utilization pathways are occurring widely in plants, animals and some microorganisms. In an artificial pathway they catalyze the detoxification of dihydroxyacetone by phosphorylation [6] to the glycolytic intermediate dihydroxyacetone phosphate [7]. The roles of the active site residues of dihydroxyacetone kinases have been investigated by structural and mechanistic studies [8].

A number of dihydroxyacetone kinases have been applied in the straightforward preparation of dihydroxyacetone phosphate by catalyzing the phosphorylation of dihydroxyacetone (Figure 1) and using ATP as phosphoryl donor [9]. ATP regeneration in large scale reactions has been successfully solved by using two different cofactor recycling systems: (i) the enzyme acetate kinase (AK) with acetyl phosphate as final donor [10] and (ii) the enzyme pyruvate kinase and the substrate phosphoenol pyruvate [5].

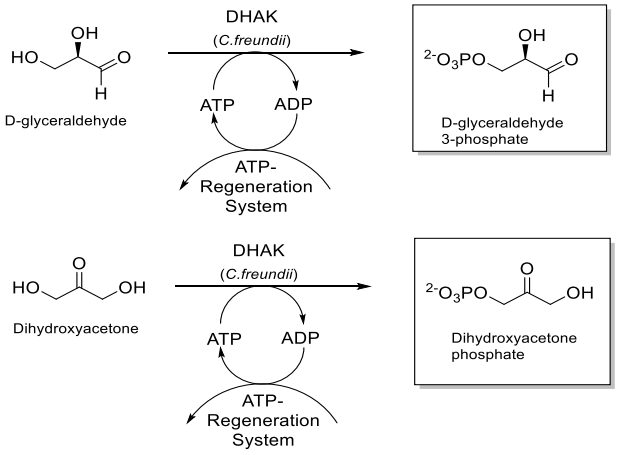

Figure 1. Dihydroxyacetone kinase-catalyzed phosphorylations of dihydroxyacetone and D-glyceraldehyde using ATP as phosphoryl group donor and an enzymatic regeneration system for the ATP cofactor

The *dha* operon expression is not only induced by dihydroxyacetone, but also by glyceraldehyde [11], which also fits into the active site of the ATP-dependent dihydroxyacetone kinase. As several ATP-dependent dihydroxyacetone kinases have been found to take racemic glyceraldehyde as substrate [12] and enantiomerically pure D- and L-glyceraldehyde became accessible [13], we investigated racemic glyceraldehyde as well as the enantiomerically pure D- and L-glyceraldehyde as substrates of the recombinant *Citrobacter freundii* dihydroxyacetone kinase (Figure 1), which was expressed in *E. coli*.

Using a coupled enzymatic assay for measuring the DHAK activity (see experimental section) a value of 1.71 U/ml was obtained for the racemic glyceraldehyde, while with pure D-

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glyceraldehyde a value of 1.93 U/ml was obtained under the same conditions. However, when L-glyceraldehyde was used as substrate, no activity was detected even upon increasing enzyme concentration significantly. In addition, the DHAK activity was studied as a function of increasing concentrations of racemic and D-glyceraldehyde and was found to level off at millimolar concentrations (Figure 2).

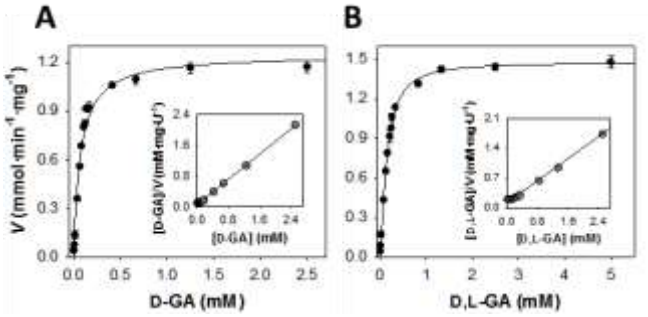

Figure 2: DHAK activity at increasing concentrations of (A) D-glyceraldehyde and (B) D,L-glyceraldehyde. The insert shows the Hanes-Woolf plot.

The K_M of 0.15 mM for the racemic D,L-glyceraldehyde was almost two times than the one for the enantiopure D-glyceraldehyde (0.072 mM), while the k_{cat} values were almost coincident (see table below). These results are coherent with the fact that only the D-glyceraldehyde in the racemic mixture is being phosphorylated by the DHAK. From these experiments we can conclude that DHAK is completely stereoselective for D-glyceraldehyde and that L-glyceraldehyde did not interfere significantly with the phosphorylation reaction.

Subs	V_{max} (U·mg^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}·s^{-1}$)
D,L-GA	1.48 ±0.01	0.15 ±0.01	3.13 ±0.03	20870 ±1405
D-GA	1.25 ±0.03	0.072 ±0.005	2.65 ±0.06	36805 ±2690

Stereoselective phosphorylation can be used for the kinetic resolution of racemic glyceraldehyde. Experiments with racemic glyceraldehyde have shown a conversion to the 3-phosphorylated compound corresponding to about 50% of the amount observed with the pure D-enantiomer, thus resolving racemic DL-glyceraldehyde by phosphorylation (figure 3).

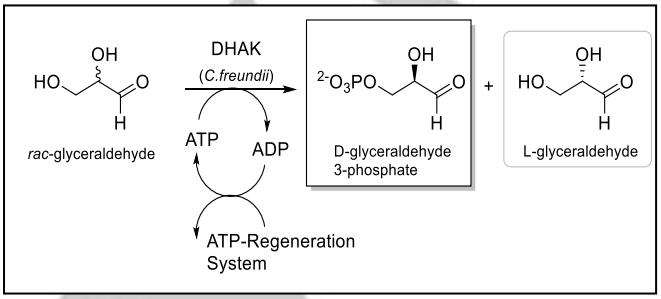

Figure 3. Resolution of DL-glyceraldehyde by dihydroxyacetone kinase-catalyzed phosphorylation of D-glyceraldehyde to D-glyceraldehyde 3-phosphate.

Although chromatographic and enzymatic methods have been very useful for the analysis and development of new biocatalytic asymmetric phosphorylations, quantitative spectroscopic methods with rich information content like ^{31}P-NMR have greatly facilitated direct kinetic analyses of such reactions [14]. Therefore we have investigated the same phosphorylation reactions also by ^{31}P-NMR, which confirmed the enzymatic assays. While no phosphorylation reaction could be observed using enantiomerically pure L-glyceraldehyde as substrate, a nearly 100% formation of D-GAP has taken place after about two hours reaction time.

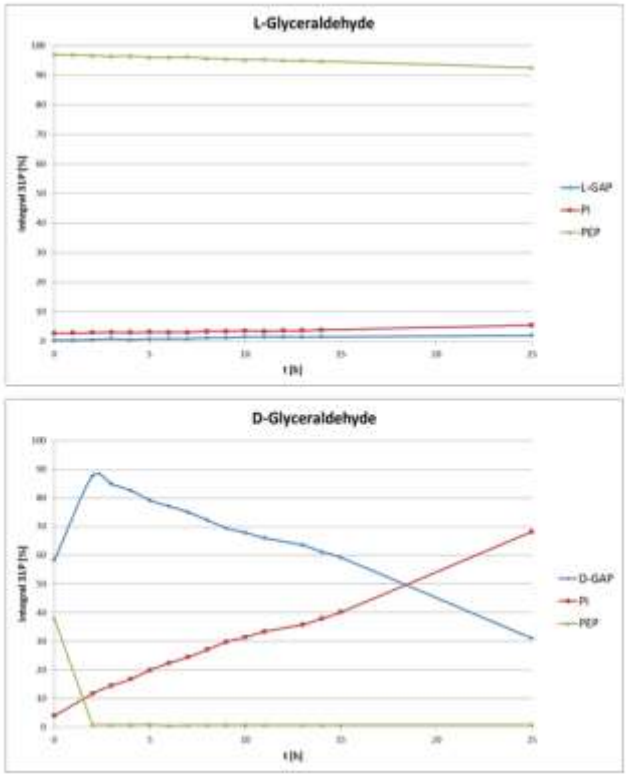

Figure 4: ^{31}P-NMR kinetics of dihydroxyacetone kinase-catalyzed phosphorylation of D-glyceraldehyde to D-glyceraldehyde 3-phosphate (below) and the analogous experiment by replacing D-glyceraldehyde by L-glyceraldehyde (above). Almost no conversion is observed using L-glyceraldehyde as substrate, whereas D-glyceraldehyde shows an almost 100% conversion after 2h. As D-glyceraldehyde 3-phosphate is not stable under neutral conditions [3], a decrease of the formed product and a simultaneous increase of newly formed inorganic phosphate (P_i) is observed.

Due to the low stability of D-GAP under neutral pH conditions [3], the phosphorylated glyceraldehyde product formed decomposes slowly, accompanied by the formation of inorganic phosphate (figure 4). This degradation can be controlled by using an adequate buffer and reaction pH, as previously reported [5] or by keeping a short reaction time and a rapid change to pH 4 after ending the reaction [3]. Since the D-GAP purification by precipitation as a Ba^{2+} salt intermediate requires acidic pH, it does not contribute to the D-GAP degradation and the Ba^{2+} salt intermediate is stabilized for further processing [3,5], which is being considered and optimized.

The discovery of dihydroxyacetone kinase as an enantio-complementary enzyme to glycerol kinase, which catalyzes only the phosphorylation of L-glyceraldehyde, is very valuable for a highly selective phosphorylation of D-glyceraldehyde. It is also interesting that the dihydroxyacetone kinase from *C. freundii* and the glycerol kinase from *E. coli* are both composed of subunits of similar size and contain the amino acid motif G-K-G as the central part of the putative ATP-binding site. Dihydroxyacetone kinase exists however as a dimer, while glycerol kinase forms a tetramer [15].

The recent discovery of a glyceraldehyde-phosphorylating function to a human triokinase [16] shows the importance of this enzyme function in D-fructose utilization for human health and is of much interest to the molecular understanding of the Hers metabolic pathway [17].

Experimental Section

DHAK from *C. freundii* was overexpressed in *E. coli* strain BL21 (DE3), containing the plasmids pRSET-*dhak* as previously described [7c]. Recombinant protein was purified by size-exclusion chromatography using an AKTA-FPLC system (GE Healthcare Life Science). In each standard purification, 5 ml of cell free extract from induced bacteria cultures were loaded on a HiLoad 26/60 Superdex 200 PG column. Purification was carried out in 50 mM phosphate buffer pH 7.2 containing NaCl (0.15 M) at a constant flow rate of 1.0 ml/min. Only central fractions in the DHAK peak were taken in order to get the high-purity enzyme required for D-GAP synthesis. Fractions containing DHAK were pooled together in a dialysis membrane and incubated overnight at 4°C in 3 L of Tris-HCl buffer 5 mM (pH 7.2), under stirring. The dialyzed protein was freeze-dried in glass vials (15 ml) and stored at 4°C.

DHAK activity was measured by the detection of the ADP with a coupled enzymatic system, where the decrease of NADH absorbance at 340 nm is directly proportional to substrate phosphorylation. The activity was measured at 25 ºC for about 10 min in a final volume of 1 mL, containing Tris-HCl (50 mM, pH 8.0), NADH (0.2 µmoles), ATP (5.0 µmoles), PEP (5.0 µmoles), $MgSO_4$ (5.0 µmoles), substrate (DHA,; D,L-GA; D-GA or L-GA; 2.5 µmoles), pyruvate kinase (1.4 U), L-lactate dehydrogenase (5.0 U) and DHAK. The assay was validated by measuring ADP and DHAK activity using DHA as substrate as dihydroxyacetonephosphate formation can be measured also with glycerophosphate dehydrogenase. The activity value obtained with the two methods was quite similar.

NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer equipped with a BBO probe head with z-gradient using 243 MHz for ^{31}P. Each spectrum has been acquired with 64 scans at 298.2 K in H_2O/D_2O (9:1) using inverse gated decoupling with a flip angle of 30° and a delay of 10 s between each pulse.

NMR-samples for quantitative examination of the enzymatic phosphorylation reaction have been prepared mixing aqueous solutions of the appropriate enantiomer of glyceraldehyde (100 mM final concentration), phosphoenolpyruvic acid monopotassium salt (100 mM final concentration), adenosine 5'-triphosphate disodium salt hydrate (4.4 mM final concentration), $MgCl_2$ (15 mM final concentration) and D_2O (10% final concentration). The pH was adjusted to 6.7 and DHAK from *C. freundii* (10 U) and pyruvate kinase from rabbit muscle (2 U) were added.

^{31}P NMR-spectra were recorded in hourly intervals as described above for the first 14 hours and after 25 h. For quantification, the sum of the integrals of the arising glyceraldehyde 3-phosphate (D/L-GAP), phospho-enolpyruvic acid (PEP), and inorganic phosphate (Pi) were normalized to 100 and the particular values were plotted against the reaction time.

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Keywords: D-Glyceraldehyde 3-phosphate • Dihydroxyacetone kinase • ^{31}P-NMR • Biocatalysis • Enzymatic Phosphorylation

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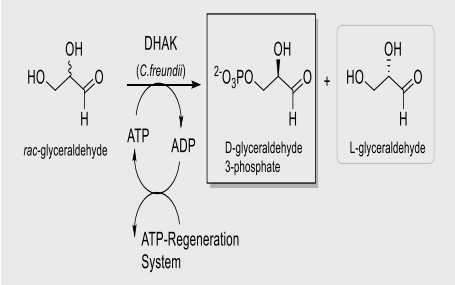
Layout 1:

COMMUNICATION

Biocatalytic phosphorylations catalyzed by recombinant dihydroxyacetone kinase have been investigated with quantitative ^{31}P-NMR and pyruvate-kinase-catalyzed ATP-regeneration starting with dihydroxyacetone as substrate. A nearly 100% conversion of D-glyceraldehyde to D-glyceraldehyde-3-phosphate has been found. Practically no formation of glyceraldehyde 3-phosphate was observed with L-glyceraldehyde as substrate.



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