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Chemical and enzymatic methodologies for the synthesis of enantiomerically pure glyceraldehyde 3-phosphates

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ABSTRACT

Glyceraldehyde 3-phosphates are important intermediates of many central metabolic pathways in a large number of living organisms. D-Glyceraldehyde 3-phosphate (D-GAP) is a key intermediate during glycolysis and can as well be found in a variety of other metabolic pathways. The opposite enantiomer, L-glyceraldehyde 3-phosphate (L-GAP), has been found in a few exciting new pathways. Here, improved syntheses of enantiomerically pure glyceraldehyde 3-phosphates are reported. While D-GAP was synthesized by periodate cleavage of D-fructose 6-phosphate, L-GAP was obtained by enzymatic phosphorylation of L-glyceraldehyde. ^{1}H- and ^{31}P NMR spectroscopy was applied in order to examine pH-dependent behavior of GAP over time and to identify potential degradation products. It was found that GAP is stable in acidic aqueous solution below pH 4. At pH 7, methylglyoxal is formed, whereas under alkaline conditions, the formation of lactic acid could be observed.

1. Introduction

The development of biochemistry is very much related to key discoveries made in the enzymatic reactions with D-glucose in yeast extract by Hans and Eduard Buchner,[1] Arthur Harden, and William Young[2] as well as in muscle extracts by the classical work of Gustav Embden,[3] Otto Meyerhof,[4] Carl Neuberg,[5] Jacob Parnas,[6] Otto Warburg,[7] Gerty and Carl Cori.[8] The sequence of enzymatic reaction steps for the complete anaerobic D-glucose catabolic pathway in eukaryotes, with the corresponding chemical structure of the metabolites, has been established with the Embden–Meyerhof–Parnas pathway (EMP). The natural variety of alternative D-glucose metabolic pathways[9] is of continuing interest in prokaryotes, with the Entner–Doudoroff pathway (ED) as the most common, as well as in archaea, since in a phylogenetic analysis of genomes the genes of 30% of the organisms could not be annotated to either the EMP- or the ED-pathway.[10] In order to elucidate the reaction topologies and protein functions under different conditions, especially when branchpoints are involved, the preparation of the corresponding enzyme substrates is important. The availability of all relevant metabolites from the different glycolytic pathways in pure and stable form as well as comprehensive knowledge about their stabilities has been a challenge up to the present. The central metabolite D-glyceraldehyde 3-phosphate (D-GAP), shared by the EMP and ED pathways in lower glycolysis, plays a role not only in glycolysis and gluconeogenesis, but in a variety of additional metabolic pathways like for example the methylerythritol phosphate (MEP) pathway,[11,12] photosynthetic carbon fixation,[13,14] the shikimate pathway,[15,16] clavulanic acid biosynthesis,[17] and thiamine metabolism.[18] In contrast, the L-glyceraldehyde 3-phosphate (L-GAP) has not been found to play the corresponding role in a L-glucose catabolic pathway in *Paracoccus* sp.[19] and the discovery of YghZ in *Escherichia coli* catalyzing the stereospecific reduction of L-GAP to L-Glycerol 3-phosphate[20] has provided the first evidence of a metabolic bypass for the removal of toxic L-GAP.

Otto Warburg observed already in 1924 that cancer cells metabolize glucose into lactate even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation,[21–23] a finding which has obtained renewed attention.[24,25]

The first synthesis of racemic glyceraldehyde 3-phosphate, later to be known as Fischer–Baer ester, had a considerable effect on the development of the Embden–Meyerhof scheme and enabled studies of its properties.[26–28] Since D- and L-glyceraldehyde 3-phosphates have different biological properties, the synthesis of pure enantiomers is highly important.[29–31] Whereas there are numerous papers dealing with syntheses of racemic GAP, the number of published protocols leading to enantiomerically pure or enriched D- or L-GAP is small and all of them have drawbacks which make them unattractive for lab scale production purposes.

The oldest published preparative formation of D-GAP is a lengthy multistep synthesis using mercury salts in one of the deprotection steps.[29] An attractive one step synthesis[31] starting with D-fructose 6-phosphate does not consider the pH lability of the product and

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gave in our hands varying yields and product purities. Another oxidative degradation of D-fructose 6-phosphate uses toxic and teratogenic lead-tetraacetate and requires extensive and non-scaleable purification at near neutral pH implying partial product degradation.[40,43] Alternatively the product was not purified at all but used directly in a next reaction step.[35] A disadvantage of the enzymatic synthesis of D-GAP using an aldolase-catalyzed cleavage of D-fructose 1,6-diphosphate is the often incomplete conversion due to the thermodynamic equilibrium of this reaction.[42] Therefore a considerable purification effort is necessary in order to obtain a pure product. The degree of purity of the isolated D-GAP as well as the type of aldolase used was not specified.[42]

In the L-GAP synthesis, the disadvantages of using racemic glyceraldehyde as starting material for the glycerokinase-catalyzed phosphorylation with the phospho-enolpyruvate/pyruvate kinase system for ATP recycling[35] will be outlined later in this paper. Scale-up of this procedure resulted in diminished product yields. A multi-enzyme procedure directed toward kinetic resolution of racemic GAP with D-glyceraldehyde-3-phosphate dehydrogenase suffers from the same drawback.[32]

A lengthy approach for the preparation of both enantiomers of GAP was the enzymatic resolution of racemic glycidaldehyde diethylacetal via a series of reaction steps. As the resulting (*R*)- or (*S*)-glycidaldehyde diethylacetal respectively was subjected to a nucleophilic phosphorylation in a pH range which is unfavorable for the stability of GAP, no further purification was attempted and the material was used directly for the following reaction step instead.[38,39]

2. Results and discussion

2.1. Stability of DL-GAP

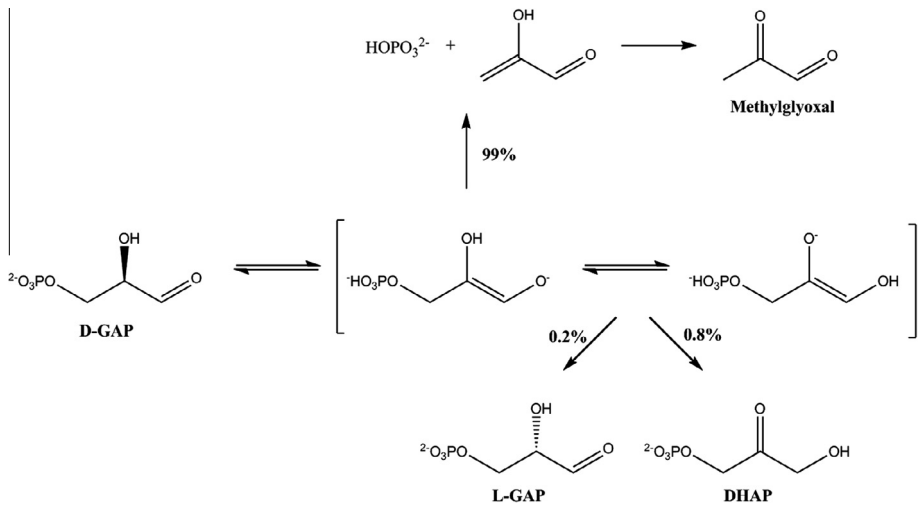
The instability of GAP under alkaline or neutral pH-conditions has been known for a very long time. Nevertheless, aqueous conditions in the neutral pH-range are used in nearly all published procedures dealing with synthesis, purification, or application of the compound. Here, ^{1}H and ^{31}P NMR experiments were used in order to identify degradation products and to examine pH-dependent behavior of GAP over time. Furthermore, these results could provide a closer insight in terms of stability and degradation mechanisms of GAP.

GAP is transformed to methylglyoxal via an enediolate phosphate transition product in water at pH 7 (Scheme 1).[32,33,41] In alkaline conditions, lactic acid is formed.[26,28,34] Figure 1 shows the comparison of ^{1}H NMR spectra of lactic acid and DL-GAP at strongly alkaline conditions in D_2O. The chemical shift values are exactly matching confirming the formation of lactic acid which has not been described in a previous kinetic analysis.[33] However, the signal at 4.1 ppm, which can be assigned to the C*H*OH proton, shows a different coupling pattern. While the signal is split up into a quartet in case of lactic acid resulting from coupling to the adjacent methyl group, it appears as a triplet in the spectrum of the DL-GAP degradation product under alkaline conditions.

Although the mechanism of the conversion from GAP to lactic acid is not definitely known, a possible explanation for the different coupling patterns is the deuteration of the newly formed methyl group by the deuterated solvent. This assumption is confirmed by the ratio of the observed integral values (only two protons in the case of the deuterated methyl group) and the weak H/D-coupling of the deuterated lactic acid methyl group (upper left in Fig. 1). There are two more interesting observations worth to note regarding the degradation of DL-GAP: (1) if the compound is left in neutral, aqueous solution for several days, methylglyoxal signals are appearing in the ^{1}H NMR spectrum, but no formation of lactic acid can be observed and (2) methylglyoxal itself does not show any formation of lactic acid even after 15 h under alkaline conditions. Therefore, the decomposition mechanism of GAP under alkaline conditions is different from the one under neutral conditions and methylglyoxal does not seem to be an intermediate in the non-enzymatic degradation of GAP at high pH-values.

In order to identify the pH-range where GAP is stable and to get first insights into degradation velocities, NMR spectra of aqueous solutions of DL-GAP at different pH values were recorded. To quantify the amount of remaining DL-GAP, the percentage of the integrals in the ^{31}P NMR spectra were plotted against time in solution (Fig. 2). As discussed earlier, in both routes of non-enzymatic GAP-degradation, the compound is dephosphorylated and inorganic phosphate is released.

It can be concluded that the product is stable at least for days under strongly acidic conditions. After 4 days at pH 4.2, degradation can be observed at a low level. Former experiments indicated full stability at pH 3.5. At pH 5.6 more than 50% of the compound is



Scheme 1. Non-enzymatic degradation of D-GAP to methylglyoxal and inorganic phosphate in water at pH 7.[33] The formation mechanism of the enediolate phosphate intermediate is analogous to L-GAP.