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Phosphorylation by Alkaline Phosphatase: Immobilization and Synthetic Potential

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Abstract

Phosphatases (AP, E.C. 3.1.3.1) are hydrolytic enzymes that naturally hydrolyse phosphomonoesters but in a so-called transphosphorylation reaction these enzymes are also able to transfer a phosphate group from phosphorylated compounds to alcoholic functions. This transphosphorylation catalysed by acid phosphatases using pyrophosphate as a phosphate donor has been studied in some detail. However, the acidic pH optimum of these enzymes limits some of their applications. The catalytic features of alkaline phosphatase are similar to the acid phosphatases and its alkaline pH optimum suggests a possible application of this enzyme in phosphorylation reactions which need to be carried out at higher pH. Here we explore the synthetic potential of bovine intestine alkaline phosphatase (AP) in the phosphorylation of dihydroxyacetone (DHA) and glycerol using pyrophosphate (PP_i) as phosphate donor. The phosphorylated compounds are intermediates in two multi-enzymatic cascade reactions for the synthesis of carbohydrates. The yields of dihydroxyacetone phosphate (DHAP) and glycerol-1-phosphate at pH 8 (2.6 mM and 2.2 mM, respectively) were comparable to the results obtained with the acid phosphatases at pH 4. Nevertheless, when the cascade reactions were carried out at pH 8, very low conversions were measured due to inactivation of the alkaline phosphatase by the product phosphate. To circumvent this inhibition, the alkaline phosphatase was immobilized on aldehyde-activated beads (Sepabeads EC-HA). The immobilization greatly diminished the inhibition by phosphate, and the immobilized alkaline phosphatase at pH 8 gave the same conversions in the cascade reaction starting from DHA as obtained with the acid phosphatase at pH 6. However, the immobilized enzyme was active for only one catalytic cycle and the beads could not be reused.

Keywords: alkaline phosphatase, phosphorylation, immobilization, dihydroxyacetone phosphate, glycerol-1-phosphate, cascade reaction, pyrophosphate

1. Introduction

The importance of phosphate esters as prodrugs, taste enhancers, nutritional supplements, and cosmetic ingredients has drawn the attention of chemists and prompted the development of efficient phosphorylation methods (Auriol et al., 2008; Heimbach et al., 2003; Scudder, Dwek, Rademacher, & Jacob, 1991; Westheimer, 1987). In particular the mild reaction conditions and reduced production of waste encouraged the development of enzyme-based technologies. Moreover, in contrast to chemical procedures, enzymes carry out phosphorylations with high regio and stereoselectivity without the need of group protection (Crans & Whitesides, 1985a; Crans & Whitesides, 1985b; Gross, Abril, Lewis, Geresh, & Whitesides, 1983; Li, Enomoto, Hayashi, Zhao, & Aoki, 2010). Kinases are well-known phosphorylating enzymes which transfers a phosphate unit from ATP to a variety of acceptors but the large-scale application is impeded by the need of regenerating ATP and in addition these enzymes are specific for the substrate to be phosphorylated (Faber, 2004). However, some hydrolytic enzymes can circumvent these issues: the non-specific alkaline and acid phosphatases (Sträter, Lipscomb, Klabunde, & Krebs, 1996).

These phosphatases catalyse in vivo the hydrolysis of phosphomonoesters to inorganic phosphate (P_i) and the corresponding free alcohol. However, phosphatases are also able to carry out transphosphorylation reactions in which a phosphate unit is transferred from a donor (phosphomonoesters or pyrophosphate PP_i) to an acceptor alcohol. The transphosphorylation reaction is thought to be a reversible two-step reaction in which the affinity for PP_i, alcohol, or water determines whether hydrolysis, transphosphorylation, or dephosphorylation occur (Asano,

Mihara, & Yamada, 1999b; Pradines, Klaebe, Perie, Paul, & Monsan, 1988, 1991; Reid & Wilson, 1971; Tanaka, Hasan, Hartog, van Herk, & Wever, 2003). The transphosphorylation reaction is essentially reversible and the equilibrium position depends on the conditions and the amount of reagents and products present in the reaction mixture. Thus, phosphatases are able to hydrolyze PP_i, transfer a phosphate to an acceptor alcohol, or hydrolyze phosphate esters.

The well-known acid phosphatases from *Shigella flexneri* (PhoN-Sf), *Shigella enterica ser. typhimurium* (PhoN-Se), and *Morganella morganii* have been widely used in the regioselectively phosphorylation of nucleosides such as inosine and guanosine to the corresponding 5'-phosphate derivatives (5'-IMP and 5'-GMP) used as taste enhancers (umami) (Asano, Mihara, & Yamada, 1999a; Asano et al., 1999b; Low & Saltiel, 1988; Mihara, Utagawa, Yamada, & Asano, 2000; Mihara, Utagawa, Yamada, & Asano, 2001; Tanaka et al., 2003), of glucose to glucose-6-phosphate, and of many other primary alcohols, such as glycerol and DHA (Babich et al., 2011; Tanaka et al., 2003; van Herk, Hartog, van der Burg et al., 2005; van Herk, Hartog, Schoemaker et al., 2006; van Herk, Hartog, Babich et al., 2009).

In contrast to acid phosphatase, which operates at pH values below 7, alkaline phosphatases are only active between pH 7 and 10. This enzyme contains four metal sites occupied by Zn^{2+} and Mg^{2+} (Le Du, Stigbrand, Taussig, Menez, & Stura, 2001; Millan, 2006; Stec, Holtz, & Kantrowitz, 2000) and is a dimer of two identical subunits with a molecular weight of approximately 160 kDa (Fernley, 1971). This enzyme shows hydrolytic activity towards many phosphomonoesters (Portmann, 1957; Stadtman, 1961) such as polyprenol phosphates (Koyama et al., 1990), sphingoid base 1-phosphate (Min, Yoo, E. Lee, Y. Lee, & W. Lee, 2002), nucleotides (Billich, Stockhove, & Witze, 1983), nucleotides (Billich, Stockhove, & Witze, 1983), and aromatic phosphate esters (Breslow & Katz, 1968; Edwards et al., 1990). The potential of alkaline phosphatase for synthetic enzymatic phosphorylation has been explored a long time ago by Pradines and co-workers (Pradines, Klaebe, Perie, Paul, & Monsan, 1988, 1991). In 1988, they reported the phosphorylation of primary alcohols and other substrates using different phosphate donors (Pradines et al., 1988). Diols and polyols were selectively monophosphorylated with good yields, whereas simple aliphatic primary alcohols were not accepted as well as amino- and sulfur-containing alcohols. Good yields were obtained only at very high alcohol concentrations (> 7 M). Only regioselectivity but no stereoselectivity was observed. Interestingly, the pH optima in the transphosphorylation reaction and in the hydrolysis differ, being pH 8.5 and 5.8, respectively. The great potential of AP in the large-scale production of glycerol-1-phosphate starting from very high glycerol concentrations (up to 11 M) and phosphate or pyrophosphate was also demonstrated (Pradines et al., 1991). The AP was immobilized on corn grits (EURA-MA 60-100 mesh, a cellulose-based carrier) and it was shown that the immobilized enzyme was more resistant against inhibition by glycerol-1-phosphate and P_i. In a batch reactor with embedded enzyme or in a continuous packed-bed reactor yields with respect to pyrophosphate were obtained of 41.3 % and only 18 % glycerol-1-phosphate, respectively.

Given the similar catalytic features of alkaline and acid phosphatase, we investigated the potential of alkaline phosphatase in the phosphorylation of DHA and glycerol as already studied for the acid phosphatases PhoN-Se and PhoN-Sf. These phosphorylated alcohols are key intermediates in two enzymatic cascade reactions leading to the synthesis of carbohydrates (Figure 1) (Babich et al., 2011; van Herk, Hartog, Schoemaker et al., 2006; van Herk, Hartog, Babich et al., 2009).

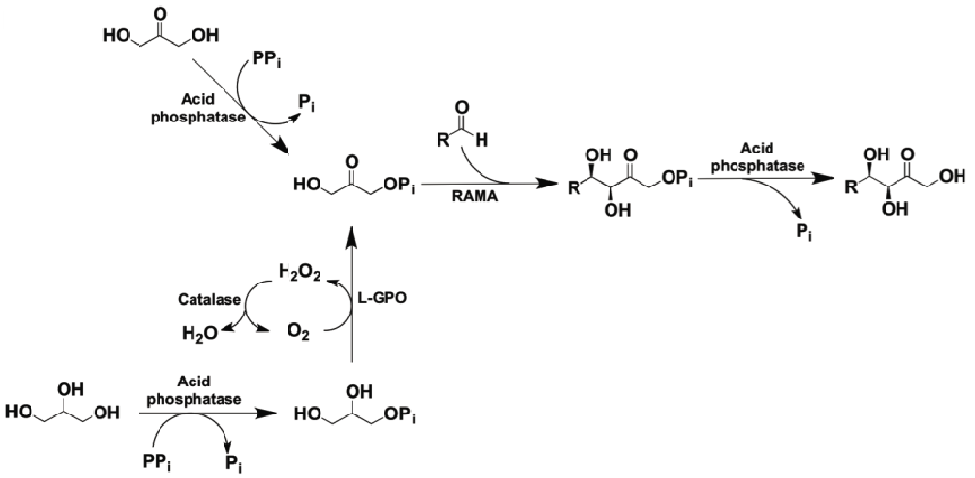

Figure 1. Scheme of the one-pot cascade reactions starting from DHA or glycerol at pH 6

The acid phosphatase-mediated phosphorylation of glycerol with the phosphate donor PP_i produces glycerol-1-phosphate. The L-enantiomer is then oxidized by glycerol phosphate oxidase in the presence of oxygen to produce DHAP. The oxidation takes place with concomitant formation of hydrogen peroxide, which is converted by catalase. In the next step of the cascade, DHAP reacts with the aldehyde catalyzed by rabbit muscle aldolase (RAMA) to provide the phosphorylated aldol product. This aldol product is ultimately dephosphorylated by PhoN-Sf leading to the enantio- and diasteromerically pure carbohydrate. This essentially irreversible step shifts the thermodynamic equilibrium of the cascade to aldol product once PP_i becomes exhausted. It is also possible to start a cascade from DHA which is phosphorylated to DHAP and then converted into the final product.

In the two-enzyme three-steps cascade reaction the (acid) phosphatase using PP_i phosphorylates DHA to DHAP, which is coupled by the fructose-1,6-bisphosphate aldolase (RAMA) to an aldehyde yielding a phosphorylated sugar. The third and last step is the dephosphorylation of the sugar by the phosphatase already present in the reaction (Figure 1) (van Herk et al., 2006). The other cascade reaction starts from glycerol and involves four enzymes and four steps (Babich et al., 2011). Glycerol is phosphorylated to glycerol-1-phosphate which is then oxidized by glycerol-1-phosphate oxidase (GPO) and molecular oxygen to DHAP and catalase removes the hydrogen peroxide formed. DHAP then undergoes the already described aldol condensation reaction by RAMA and the final dephoshorylation step yields the sugar. Both pathways gave very high yields of sugar, 60 % in the two-enzyme cascade with DHA and 100 mM propanal (van Herk et al., 2006) and complete conversion of 100 mM propanal in the glycerol cascade reaction (Babich et al., 2011). Very high conversions were obtained with a genetically engineered mutant of PhoN-Se: the mutant V78L yielded 100 % product in only 2 hours in the cascade reaction starting from DHA (van Herk et al., 2009).

These cascade reactions were carried out in one pot at pH 6, which is neither the optimal condition for the acid phosphatase, nor for the aldolase, nor for the oxidase. PhoN-Sf and PhoN-Se are mostly active at pH 4-4.5, whereas RAMA and GPO have a more alkaline pH optimum, between 7 and 8. However, all the enzymes show activity at pH 6. At higher pH values, the activity of RAMA (and also GPO) is enhanced, but PhoN-Sf becomes inactive. Thus, alkaline phosphatase, which has a pH optimum around 8-9, may be a good substitute for the acid phosphatase since it would be possible to carry out reactions at higher pH, maximizing the rate and activity of the aldolase and also of the oxidase, in case of the glycerol cascade reaction, resulting in higher yields or shorter reaction times. This work describes the phosphorylation of dihydroxyacetone and glycerol by alkaline phosphatase using PP_i, its immobilization on solid beads, and its use in cascade reactions.

2. Method

2.1 Hydrolysis of PP_i by Alkaline Phosphatase and Inhibition Studies

Alkaline phosphatase (AP) from bovine intestine was supplied by Sigma Aldrich and stored at 4 °C in 5 mM Tris buffer pH 7, containing 5 mM $MgCl_2$ and 0.1 mM $ZnCl_2$. AP activity was determined by a spectrophotometric assay using *p*-nitrophenol phosphate (*p*NPP) as substrate. The hydrolysis produces *p*-nitrophenol (*p*NP), which absorbs at 405 nm. The assay mixture contained 100 mM of *p*NPP in 1 M diethanolamine (DEA, pH 9.8), 0.5 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. 10 μL of a proper enzyme dilution was added to 1 mL of the assay mixture and

allowed to react for 5 minutes at 20 °C. The absorbance was then monitored at 405 nm with a UV-Vis Cary-50 spectrophotometer. The activity was calculated using an extinction coefficient of 18.5 mM^{-1} cm^{-1}. One unit will hydrolyze 1 μmol of *p*NPP per minute at pH 9.8 at 20 °C. The activity test was carried out also in present of different concentrations of inorganic phosphate to verify whether the enzyme suffered from phosphate inhibition. Reactions were carried out in 1 M DEA pH 8, 100 mM *p*NPP, 0.1 mM $ZnCl_2$, 0.5 mM $MgCl_2$, 6 U/mL AP and 10, 25, and 100 mM of sodium phosphate at 20 °C. After 5 minutes the absorbance was recorded at 405 nm and activity calculated as described above.

The time course of PP_i hydrolysis was determined at pH 7 and 8 with different concentrations of PP_i (50, 100, and 250 mM) using 6 U/mL of AP, 0.1 mM $ZnCl_2$, at 30 °C. The time course of the disappearance of PP_i and formation of P_i was determined every 30 minutes by HPLC analysis after 10-fold dilution of samples in water and after calibration with analytical grade standard solution of PP_i and P_i. HPLC analysis was performed using an Alltech OA1000 organic acid column (0.65 x 30 cm) equipped with a Dionex 580LPG pump and Dionex UVD-340 UV detector and Shodex RI-101 detector. The column was eluted with 25 mM H_2SO_4 at 0.4 mL/min at room temperature. Chromeleon software was used for the acquisition and evaluation of the data. By adding $MgCl_2$ the effect of Mg^{2+} on the rate of hydrolysis of *p*NPP was tested and the best ratio Mg^{2+}/PP_i was determined. The reactions were carried out with 100 mM PP_i, 0.1 mM $ZnCl_2$, 6 U/mL AP at pH 8 for 1 day at 30 °C with different concentrations of $MgCl_2$: 100 mM (Mg^{2+}/PP_i = 1:1), 66 mM (Mg^{2+}/PP_i = 2:3), and 50 mM (Mg^{2+}/PP_i = 1:2). Every hour, samples were taken, diluted 10-fold in water and analyzed by HPLC.

2.2 Phosphorylation by Alkaline Phosphatase

The phosphorylation of DHA to DHAP by AP was tested using PP_i as phosphate donor. The time course and the pH dependency of the phosphorylation reaction were determined. Typical reaction mixtures contained 100 mM DHA, 50 mM PP_i, 0.1 mM $ZnCl_2$, 2,4, or 6 U/mL of AP in a pH range between 7 and 10 at 30 °C. The pH was set by addition of HCl or NaOH to the PP_i/DHA mixture until the desired value was reached. DHAP was determined spectrophotometrically using a coupled assay with L-glycerol-1-phosphate dehydrogenase (G3PDH), which reduces DHAP to L-glycerol-1-phosphate with concomitant oxidation of NADH to NAD^+. The assay mixture contained 100 mM Tris/acetate pH 7.5, 1 U/mL G3PDH, and 0.16 mM NADH. Every 10 minutes samples of 20 μL of the phosphorylation reaction mixture were added to 980 μL of assay mixture and incubated for 3 minutes. The absorbance was recorded at 340 nm and the amount of DHAP was calculated using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

Glycerol was also phosphorylated by AP into glycerol-1-phosphate using PP_i as phosphate donor. Reaction mixtures contains 100 mM glycerol, 50 mM PP_i, 0.1 mM $ZnCl_2$, and 6 U/mL AP, at pH 8, 30 °C. The product glycerol-1-phosphate was detected in a coupled enzymatic assay with G3PDH. The dehydrogenase oxidizes only L-glycerol-1-phosphate using NAD^+ and hydrazine. The formation of NADH can be detected spectrophotometrically at 340 nm. Typical assay mixture contains 450 mM glycine pH 9.5, 274 mM hydrazine, 2.4 mM EDTA, 2.5 mM NAD^+, and 20 U/mL G3PDH. Time points (20 μL) were taken from the reaction of AP with glycerol and PP_i and incubated for 5 minutes with 980 μL of assay mixture at room temperature. Absorbance was recorded at 340 nm and the concentration of L-glycerol-1-phosphate calculated using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$. This value was then multiplied by a factor of 2 in order to take into account also the amount of D-glycerol-1-phosphate not detected by the assay.

2.3 Immobilization of Alkaline Phosphatase

Alkaline phosphatase was immobilized on three different epoxy-functionalized supports: Immobeads-150, purchased from Sigma-Aldrich, Sepabeads EC-EP and Sepabeads EC-HA, both purchased from Resindion. The latter beads posses a linker functionalized with an amino group, which has to be activated with glutardialdehyde. The activation was carried out in 100 mM phosphate buffer, pH 8 and 1.5 % glutardialdehyde for 2 hours under rotation of the beads at room temperature. The beads were then washed three times with potassium phosphate buffer 20 mM, pH 7. The immobilization was performed on a 0.5 mL scale with 30 U/mL AP, 30 mM potassium phosphate buffer pH 8, 0.5 mM $MgCl_2$, and 10 mg (dry weight) of Immobeads, or 25 mg (wet weight) of Sepabeads EC-EP or EC-HA glutardialdehyde activated beads. The Eppendorf tubes were then slowly rotated for 24 h at 20 °C. Afterwards, the beads were washed three times with 100 mM potassium phosphate, pH 7. By testing the remaining activity in the supernatant using *p*NPP the time course of the binding process was monitored.

2.4 Cascade Reaction with Alkaline Phosphatase

The two-enzyme cascade reaction was carried out with 500 mM DHA, 100 mM PP_i, 100 mM propanal, 0.1 mM $ZnCl_2$, 6 U/mL AP, 6 U/mL RAMA, at pH 7 or 8 at 30 °C. At selected time samples were taken and diluted

10-fold in water before the HPLC analysis with the same method described above. The cascade reaction was repeated under the same conditions with AP immobilized on Sepabeads EC-HA (50 µL of settled beads, 20 U) at pH 8 in 1 mL scale. The time course of the product formation was measured and was compared to the reaction performed with immobilized acid phosphatase (PhoN-Sf, 25 µL settled beads, 1 U) at pH 6 (Babich et al., 2012a). To investigate the reusability of the immobilized catalyst, fed-batch cascade reactions were carried out with both immobilized AP and PhoN-Sf under the conditions described above. The beads were incubated with the substrates and allowed to react for 24 h. At the end of this first cycle, the supernatant containing the product and unreacted substrates was removed, the beads washed three times with 20 mM potassium phosphate pH 8 and incubated with fresh reaction mixture for another cycle.

A four-enzyme cascade reaction was performed with AP at pH 8 in presence of 1 mL of 100 mM PP_i, 500 mM glycerol, 100 mM propanal, 0.1 mM $ZnCl_2$, 10 U/mL catalase, 6 U/mL RAMA, 50 U/mL L-glycerol-3-phosphate oxidase (GPO), and 50 µL of settled Sepabeads EC-HA with AP (approximately 20 U) at 30 °C. The time course of the reaction was determined as described above for the DHA cascade reaction. A parallel reaction was performed with 1 U/mL PhoN-Sf at pH 6 at the same conditions (Babich et al., 2011).

3. Results

3.1 Hydrolysis of PP_i by Alkaline Phosphatase and Inhibition Studies

Since AP is a hydrolytic enzyme, the rate of the hydrolysis of the phosphate donor PP_i was determined at pH 7 and 8. Table 1 shows the amount of PP_i and P_i present in the reactions after 4 hours incubation at 30 °C with different starting concentration of PP_i.

Table 1. Hydrolysis of PP_i by alkaline phosphatase

PP_i (mM)	Remaining PP_i (mM)		Formed P_i (mM)	
	pH 7	pH 8	pH 7	pH 8
50	0.8	0.5	98	100
100	67	35	66	130
250	230	200	40	100

Hydrolysis of various concentrations of PP_i by AP (6 U/mL) at pH 7 and 8 and 30 °C after 4 hours incubation. In the absence of the enzyme no hydrolysis of PP_i occurs.

At both pH 7 and 8, 50 mM PP_i is completely hydrolysed by AP yielding 100 mM of free phosphate. 100 mM PP_i is hydrolyzed faster at pH 8 than at pH 7 but the hydrolysis is not complete after 4 hours since complete hydrolysis should result in 200 mM free phosphate. With 250 mM PP_i, the reaction is also faster at pH 8 but the reaction is very slow and reaches a steady level with no further hydrolysis (data not reported). This suggests a possible inhibition by the substrate PP_i, as several authors already reported (Butterworth, 1968; Fernley & Walker, 1967; Morton, 1955; Nayudu & Miles, 1969). This is in contrast to the acid phosphatases PhoN-Sf and PhoN-Se, which completely consume PP_i under similar conditions and which are not inhibited by PP_i (Tanaka et al., 2003; van Herk et al., 2005). The inhibition could be due to the fact that AP is a zinc-dependent enzyme and PP_i may chelate Zn^{2+} causing depletion of the metal from the active site and enzyme inactivation. Butterworth showed that for the pig kidney alkaline phosphatase the inhibitory concentration of PP_i depended on the concentration of Mg^{2+} ions present in the mixture (Butterworth, 1968). Maximum pyrophosphatase activity was measured at a 1:1 Mg^{2+}/PP_i ratio, but inhibition was reported when the concentration of Mg^{2+} exceeded the total PP_i concentration. Therefore, it was suggested that this alkaline phosphatase was most active towards the complex $MgPP_i^{2-}$ and just slightly active toward the free PP_i^{4-}. An excess of Mg^{2+} would also form the specie Mg_2PP_i, which is a strong inhibitor. The presence of both species in solution would result in a competition and finally inhibition (Butterworth, 1968).

However, the effect of Mg^{2+} varies in AP from different sources. The duodenal alkaline phosphatase is most active when the ratio Mg^{2+}/PP_i is 2:3 (Nayudu & Miles, 1969). In intestinal and liver AP other authors did not report the activity-enhancing effect of Mg^{2+} in 1: 1 ratio with PP_i or its inhibitory effect at higher concentrations (Eaton & Moss, 1967). To clarify this we measured the effect of different concentrations of Mg^{2+} ions on the PP_i hydrolysis by the bovine intestine alkaline phosphatase under our reaction conditions. Figure 2 shows the rate of hydrolysis of 100 mM PP_i in absence and in presence of different concentrations of $MgCl_2$.

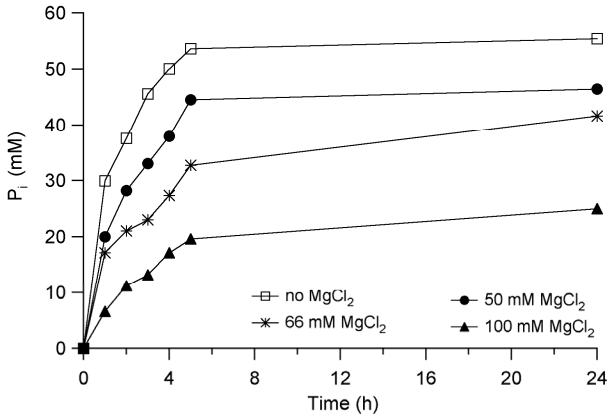

Figure 2. Rate of hydrolysis of PP_i in presence of various concentrations of $MgCl_2$. Reaction mixtures contain 100 mM PP_i, 0, 50, 66, or 100 mM $MgCl_2$, at pH 8, 30 °C. The PP_i/Mg^{2+} ratios are 2:1 for 50 mM $MgCl_2$, 3:2 for 66 mM $MgCl_2$, and 1:1 for 100 mM $MgCl_2$

Not only the initial rate of the reaction is decreased but also the extent of the hydrolysis is strongly affected by $MgCl_2$. This demonstrates that the pyrophosphatase activity of the alkaline phosphatase from bovine intestine is not positively affected by Mg^{2+}, but rather is inhibited. Thus the complex $MgPP_i^{2-}$ is not the true substrate for intestinal AP. This experiment also shows that the hydrolysis of PP_i is suppressed and after 24 hours incubation, no more phosphate is produced. This may be due to inhibition of AP by phosphate, the product of the reaction, but in presence of $MgCl_2$ less phosphate is formed than in its absence. This indicates that the enzyme is not only inhibited by phosphate but also by Mg^{2+}, whose inhibitory effect is additive to the inhibition by phosphate.

To investigate the inhibition of AP by the formed P_i the hydrolysis of PP_i was studied in presence of various concentrations of PP_i and P_i in order to determine the origin of the inhibition of the enzyme activity. When the hydrolysis of 100 mM *p*NPP was studied at different concentrations of P_i (10, 25, and 100 mM) partial inhibition (15 % in respect to the reaction without P_i) occurred already with 10 mM P_i, while the activity was only 40 % when 25 mM P_i was present. The enzyme was completely inactive in 100 mM P_i. This suggests that 100 mM PP_i will never be hydrolyzed completely, because P_i will inhibit the alkaline phosphatase already at low concentrations as has already been reported by many authors (Morton, 1955; Portmann, 1957).

3.2 Phosphorylation by Alkaline Phosphatase

Despite the inhibition of alkaline phosphatase by P_i which is formed during hydrolysis of PP_i, the enzyme was tested in transphosphorylation reaction of DHA and glycerol at pH 9 and the conversions were compared with the ones obtained with the acid phosphatases PhoN-Se and PhoN-Sf at pH 4. In order to optimize the DHAP formation two parameters were investigated: the AP concentration and the pH dependency. As Figure 3A shows the concentration of DHAP formed increased when the concentration of enzyme was increased from 2 to 4 U/mL, although 6 U/mL does not result in a further increase of product. The maximal concentration of DHAP formed was 2.2 mM and no dephosphorylation was observed within the first 140 minutes. That no hydrolysis of DHAP occurs is probably caused by inactivation of the AP by the phosphate formed. The results obtained from these experiments agree with previous studies performed with acid phosphatases PhoN-Se and PhoN-Sf in which respectively 1.6 and 3 mM of DHAP were obtained at pH 4, using the same concentrations of PP_i and DHA (van Herk, Hartog, Schoemaker et al., 2006; van Herk, Hartog, Babich et al., 2009). In contrast the acid phosphatases are not inhibited by phosphate formed and phosphorylation of DHA was rapidly followed by dephosphorylation of DHAP.

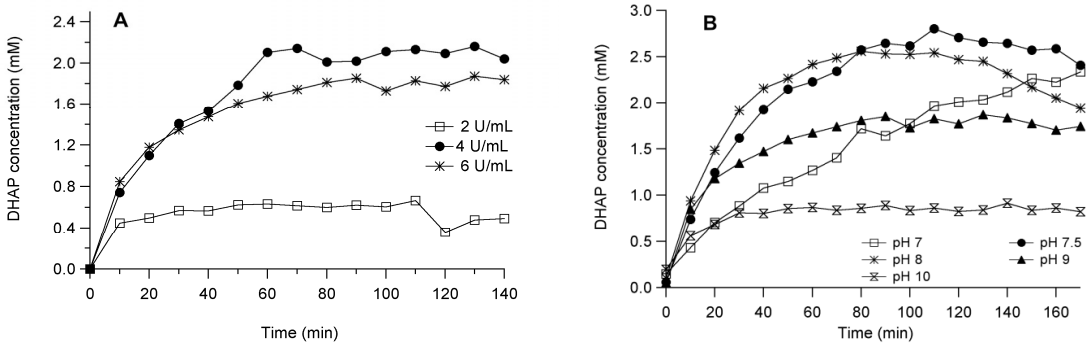

Figure 3. A) Time course of the phosphorylation of DHA by three different concentrations of AP (2, 4, and 6 U/mL). Reaction mixtures contains 100 mM DHA, 50 mM PP_i, at pH 9, at 30 °C. B) pH dependency of the phosphorylation of DHA. Reaction mixtures contains 100 mM DHA, 50 mM PP_i, 6 U/mL AP, at 30 °C, and pH values from 7 to 10

In Figure 3B the pH dependency of the phosphorylation reaction is depicted. The highest concentration of DHAP (2.8 mM) was obtained at pH 7.5 after 110 minutes incubation. Higher pH values resulted in a considerably lower concentration of DHAP. The combination of yield and reaction time (2.6 mM after 80 minutes) suggested that a pH of 8 would be a good starting point for further optimization.

The phosphorylation of glycerol to glycerol-1-phosphate by AP using PP_i as phosphate donor at the same conditions used for the phosphorylation of DHA was also studied. Pradines and coworkers already showed that AP was able to produce 82 mM glycerol-1-phosphate (55 % yield based on PP_i) using a very high concentration of glycerol (7.5 M) and 150 mM PP_i, with a 95 : 5 ratio of glycerol-1-phosphate vs. glycerol-2-phosphate. The highest glycerol-1-phosphate concentration, 0.2 M, was obtained after 500 hours incubation at 40 °C in a mixture of 11 M glycerol, 0.4 M phosphate, 500 U/mL AP, at pH 7.9. When we investigated the formation of glycerol-1-phosphate with 100 mM glycerol, 50 mM PP_i, 6 U/mL AP, at pH 8 and at 30 °C a maximal concentration of 2.2 mM DL-glycerol-1-phosphate was found in 4 hours and also in this case no dephosphorylation was observed. The same amount of glycerol-1-phosphate was obtained with PhoN-Sf at pH 6 (data not reported). These results suggest that the alkaline phosphatase behaves similarly to the acid phosphatase in the phosphorylation of DHA and glycerol, yielding comparable amounts of products. Although the AP suffers from phosphate inhibition, these results indicate that AP is potentially useful in the cascade reactions starting from DHA and glycerol in particular at higher pH values.

3.3 Immobilization of Alkaline Phosphatase

To allow the reuse of the catalyst and to improve its catalytic stability alkaline phosphatase was immobilized. Furthermore immobilization may also prevent inactivation by phosphate. Immobilization of AP was reported on glass beads, agarose (Sepharose), the epoxy carrier Eupergit-C (Taylor, 1985), corn grits (Pradines et al., 1991), and a macroporous chitosane based carrier earlier (Zubriene, Budriene, Lubiene, & Dienys, 2002). In this study AP was immobilized on methacrylic porous beads: Immobeads-150, Sepabeads EC-EP, and Sepabeads EC-HA. Immobeads-150 and Sepabeads EC-EP contain epoxy functions, which react with the amino groups of the lysine residues present on the surface of the enzyme. Sepabeads EC-HA contains a longer linker with an amino group which reacts with glutardialdehyde during the activation process. After the activation, the aldehyde on the linker will react with the lysine residues of the enzyme. When the enzyme was exposed to Immobeads-150 and Sepabeads EC-EP the activity in the supernatant did not decrease. This means that the enzyme was not immobilized on these beads. In contrast, with the Sepabeads EC-HA aldehyde activated beads the decrease in activity in the supernatant was very rapid. Complete immobilization was observed already after 4 hours. The difference in binding to the different types of beads can be explained by a low reactivity of the enzyme towards epoxy-functionalized beads and apparently a higher affinity for the longer spacer carrying aldehyde groups of the Sepabeads EC-HA. Length, flexibility, hydrophobicity/hydrophilicity, and charged/neutral character of spacers are known to have a strong influence on the outcome of the immobilization process, influencing not only the binding capability, but also retention of activity, stability, and catalytic performances.

3.4 Cascade Reaction Using Alkaline Phosphatase

In order to investigate the synthetic potential of the alkaline phosphatase, cascade reactions as illustrated in Figure 1 starting from DHA were carried out with the soluble and immobilized AP. Typically two-enzyme cascade reactions contain 100 mM PP_i, 500 mM DHA, 100 mM propanal, 6 U/mL AP, 6 U/mL RAMA, pH 8 at 30 °C. Figure 4 shows the time course of the formation of the aldol adduct 5,6-dideoxy-D-threo-2-hexulose, the concentrations of phosphate liberated and phosphorylated product formed using AP.

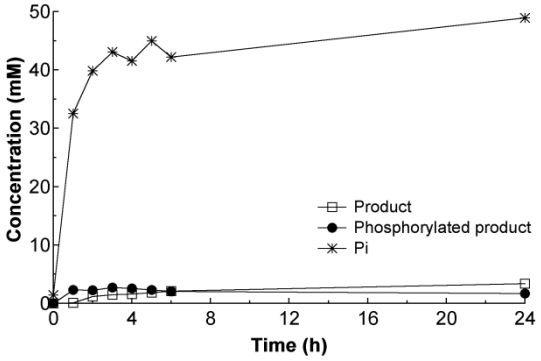

Figure 4. Time course of the two-enzyme cascade reaction using soluble AP. The reaction mixture contains 100 mM PP_i, 500 mM DHA, 100 mM propanal, 6 U/mL AP, 6 U/mL RAMA, pH 8 at 30 °C

A large amount of P_i is formed initially, however, very little product is synthesized, less than 3 mM. It is also clear that the reaction proceeds very fast in the first 3 hours of incubation, and then it slows down without completely hydrolyzing PP_i after 24 h. Also the carbohydrate is still phosphorylated even after 24 h. This is certainly due to AP inhibition by P_i.

The cascade reaction was repeated using the same conditions with the immobilized enzyme on Sepabeads EC-HA, to verify whether the immobilized enzyme was still active and whether the immobilization suppressed the inactivation by phosphate. The same reaction was performed also with acid phosphatase previously immobilized on Immobeads-150 (Babich et al., 2012a; van Herk et al., 2006). To limit the amount of free phosphate produced and thus limiting the inactivation of AP only 100 mM PP_i was added. For comparison in the experiment with PhoN-Sf the same concentration of PP_i was used. In Figure 5 the time course of the formation of the product and phosphorylated product is shown. Although the AP is slower than the PhoN-Sf, after 24 h the same conversion is reached (30–33 mM, as calculated from the initial concentration of propanal).

AP dephosphorylates the phosphorylated product with slower rate than PhoN-Sf, but the product is completely hydrolyzed at the end of the reaction after 24 h. Phosphate formation after 24 h (180 mM) was equal with both phosphatases. These data suggest that immobilized AP is less susceptible by inactivation by phosphate and that immobilization greatly improved the efficiency of this enzyme, opening the way for new applications.

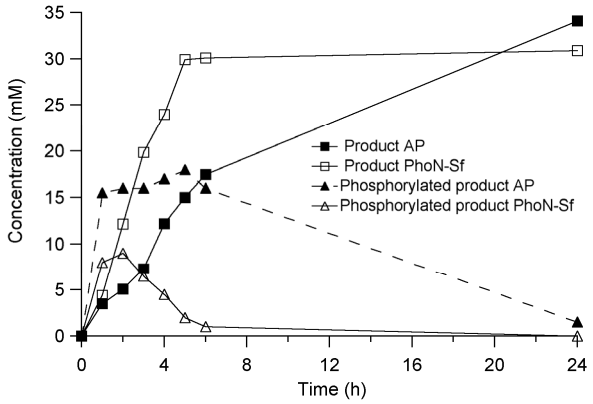

Figure 5. Time course of the formation of product and phosphorylated product in the two-enzyme cascade reaction using immobilized AP or immobilized PhoN-Sf. Reaction mixtures contain 500 mM DHA, 100 mM PP_i, 100 mM propanal, RAMA 6 U/mL, 20 U/mL immobilized AP at pH 8 or 1 U/mL immobilized PhoN-Sf at pH 6, at 30 °C

Once the suitability of immobilized AP in the cascade reaction was established, the reusability of the catalyst was checked. The cascade reaction was performed with immobilized AP and immobilized PhoN-Sf and at the end of every cycle of 24h, the beads were washed and incubated with fresh reaction mixture consisting of 500 mM DHA, 100 mM PP_i, 100 mM propanal and RAMA for another cycle. During the first cycle, 34 mM and 32 mM of product were obtained using immobilized PhoN-Sf and immobilized AP, respectively. The beads were then washed and incubated with fresh reaction mixture. After 24h, at the end of the second cycle, PhoN-Sf beads formed 25 mM of product whereas the AP beads lost most of their activity and gave only 6 mM of product. Also nearly no PP_i hydrolysis occurred. Therefore, it is clear that on one hand immobilization protects AP from inactivation by P_i yielding a reasonable amount of product in the cascade reaction, but on the other hand immobilization does not stabilize AP sufficiently during turnover and the catalyst cannot be used for more than one cycle.

The alkaline phosphatase was also employed in the cascade reaction starting from glycerol (Figure 1) (Babich et al., 2011). In this cascade glycerol (500 mM) is phosphorylated by PP_i and the product DL-glycerol-1-phosphate is oxidized by L-glycerol-1-phosphate oxidase (GPO) using oxygen and generating hydrogen peroxide, which is eliminated by catalase. DHAP undergoes then aldol coupling with propanal as in the cascade reaction starting from DHA. The glycerol cascade was carried out in presence of immobilized AP at pH 8 (20 U/mL) and for comparison with immobilized PhoN-Sf at pH 6 (1 U/mL) with 0.5 M glycerol, 100 mM PP_i, 100 mM propanal, 10 U/ml catalase, 6 U/ml RAMA and 50 U/ml GPO, at 30 °C. After 24 h incubation, the reaction with AP yielded only 3 mM of product versus 50 mM of product given by PhoN-Sf. However, PP_i was completely hydrolyzed and thus the immobilized AP was not inhibited by phosphate, as seen before. The low yield may be explained by a slower rate of glycerol-1-phosphate formation that limits the overall performance of the cascade reaction. One way to study this is to have more AP present in the incubation or to increase the concentration of glycerol to 3 M. The cascade reaction was therefore carried out with 3 M glycerol and indeed a higher yield (30 mM) of product was obtained. The same reaction carried out with PhoN-Sf yielded complete conversion. This may relate to K_m value of AP for glycerol, which may be even higher than the K_m of PhoN-Sf for glycerol (0.7 M) (Babich et al., 2011).

4. Discussion

In this work the potential use of alkaline phosphatase in phosphorylation reactions has been investigated. This enzyme shares an overall reaction mechanism with the well-studied acid phosphatases PhoN-Sf and PhoN-Se, but it has a pH optimum at more alkaline values. This suggests the enzyme could substitute the acid phosphatase when more alkaline conditions are required. Most aldolases have a pH optimum in the range pH 7 to 8 and cascade reactions using phosphatase and aldolase could be more efficient when performed at higher pH. Furthermore the AP is commercially available in contrast to the bacterial acid phosphatases. As shown by us PP_i did not inhibit the alkaline phosphatase from bovine intestine, but the product of the reaction, P_i, had a strong inhibitory effect on the activity of AP as found by many authors (Butterworth, 1968; Fernley & Walker, 1967;

Morton, 1955; Nayudu & Miles, 1969). Already at 10 mM of P_i the hydrolysis of PP_i was inhibited for 10 % and at 25 mM P_i 40 % inhibition was found. Nevertheless, the phosphorylation of 100 mM DHA by AP at pH 8.0 yielded about 2.5 mM of DHAP which is about the same as that found for PhoN-Se and PhoN-Sf at pH 4 (1.6 and 3 mM of DHAP, respectively) at the same concentrations of PP_i and DHA (van Herk, Hartog, Schoemaker et al., 2006; van Herk, Hartog, Babich et al., 2009). Similarly, 100 mM glycerol was phosphorylated by AP and PP_i to same extent at pH 8 as found for PhoN-Se and PhoN-Sf at pH 6. However, AP in contrast to PhoN-Se and PhoN-Sf, did not dephosphorylate DHAP or glycerol-1-phosphate because of the inhibition by the P_i formed.

The inhibition by P_i is probably the reason why in the cascade reaction (Figure 4) starting from DHA using the soluble AP, a very low product concentration is found. Once an inhibitory concentration of Pi is formed, formation of DHAP will slow down and as a result the aldol reaction catalysed by the aldolase will slow down significantly. Thus the strong inhibition by phosphate prevents application of the soluble enzyme in one-pot cascade reactions. However, the alkaline phosphatase which was immobilized on polymeric porous beads carrying an aldehyde as reactive functionality (Sepabeads EC-HA) was much less sensitive to inhibition by P_i. As a result the DHA cascade reaction using immobilized AP was nearly as efficient as using the immobilized PhoN-Sf. The only difference is that AP only slowly hydrolyses the phosphorylated carbohydrate to the final product. Unfortunately, during turnover the immobilized AP looses it activity much faster that immobilised PhoN-Sf. This may be due to loss of Zn^{2+} and Mg^{2+} from the metal binding sites which are not present in PhoN-Sf.

In contrast to our findings with PhoN-Sf (Babich et al., 2011) very little product was formed in the cascade reaction starting from 0.5 M glycerol using immobilized AP. It was however possible to improve this by increasing the concentration of glycerol to 3 M. This shows that the glycerol cascade reaction using AP requires further fine-tuning of the conditions to optimize the product formation.

We conclude that alkaline phosphatase from bovine intestine may be a substitute for acid phosphatase in enzyme cascade reactions only under particular conditions, such as using the immobilized enzyme. Although immobilization decreased the inhibitory effect of P_i on AP, the use of this enzyme in the cascade reaction carried out at higher pH value did not result in larger product formation compared to PhoN-Sf. Thus in general the acid phosphatases have advantages. However, the use of immobilized AP in the phosphorylation of substrates that are unstable under acidic conditions is a viable option. Further the enzyme is present in many organisms and it may well be that AP from other sources are less prone to inhibition by phosphate. Another option would be to use triphosphate, PPP_i, instead of PP_i as phosphate donor since this yields in principle more phosphorylated product and upon hydrolysis less free phosphate. Finally we recently developed (Babich et al., 2012b) a new flow process with immobilized acid phosphatase and immobilized aldolase, to synthesize complex chiral carbohydrate analogues from achiral inexpensive building blocks in a three-step cascade reaction. Using immobilized alkaline phosphatase instead of acid phosphatase in such a flow system would allow the physical separation of immobilized AP from the Pi formed, preventing its inhibition by phosphate.

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