Bacterial class A acid phosphatases as versatile tools in organic synthesis
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The research described in this thesis evaluates the possible applications of bacterial non-specific acid phosphatases (NSAPs) in organic chemistry. The project was carried out using the recombinant NSAPs from *Shigella flexneri* (PhoN-Sf) and *Salmonella enterica* ser. *typhimurium* LT2. It had been reported that these enzymes show transphosphorylation activity using cheap pyrophosphate (PPi) as phosphate donor and inosine or D-glucose as acceptor to produce 5’-inosine monophosphate or D-glucose-6-phosphate, respectively. This raised the question: Is it possible to apply this class of enzymes more generally in phosphorylation and dephosphorylation reactions that may have an industrial application? The work carried out in this project showed that the phosphatases are indeed valuable tools in various phosphorylation and dephosphorylation reactions.

In Chapter 1 a general introduction on chemical and enzymatic phosphorylation methods to prepare phosphomonoesters is given. Furthermore, the properties of the NSAPs are reviewed, with a section devoted to phosphorylation reactions carried out in the past by these enzymes. Some background information concerning directed evolution and stereoselectivity, two subjects important in this thesis, are also presented in this chapter.

Chapter 2 describes the phosphorylation of a wide variety of substrates by PhoN-Sf using PPi as phosphate donor. The acceptors consisted of carbohydrates, linear polyols, simple linear, branched, cyclic and aromatic alcohols. It was shown that the substrate specificity of PhoN-Sf is very relaxed, although D-glucose is above all the best substrate up to now. The phosphorylation of various substrates revealed subtle structural determinants that affect the phosphorylation. For instance, primary alcohols are phosphorylated to a higher extent than secondary alcohols and phosphorylation of aromatic alcohols results in lower yield of phosphomonoester compared to phosphorylation of non-aromatic equivalents.

Chapter 3 deals with the phosphorylation of dihydroxyacetone (DHA) to dihydroxyacetone phosphate (DHAP), a very interesting and valuable compound in the aldolase-catalyzed condensation reaction which stereoselectively produce new C-C bonds. After optimization of pH, PPi and DHA concentrations, 52 mM of DHAP was produced from 500 mM DHA and 240 mM PPi, with 2 μM of PhoN-Sf. Addition of a second portion of PPi after 100 minutes pushed the yield to 104 mM of DHAP. To show the practicality of this reaction, the production of DHAP by PhoN-Sf was combined in one-pot with rabbit muscle aldolase and propionaldehyde resulting in the dephosphorylated aldol adduct 5,6-dideoxy-5-threo-2-hexulose. Also 6-azido-6-deoxy-d-fructose was synthesized by this method using (RS)-3-azido-2-hydroxypropanal as aldehyde. This compound is the precursor for 1,5-dideoxy-1,5-imino-d-mannitol (1-deoxymannojirimicin) which is a glycosidase inhibitor. After isolation and hydrogenation of the precursor, the desired compound was produced.

A surprising phenomenon was found: phosphate cycling. It turned out that the enzymatically hydrolyzed phosphate from the aldol adduct remains bound in an activated state and is used again by the phosphatase to phosphorylate a new molecule of DHA, thereby reducing the amount of PPi necessary to complete the reaction.

In Chapter 4, a directed evolution project is discussed. The aim of this study was to increase the affinity of PhoN-Se towards DHA, in order to improve the yield and to decrease the amount of DHA needed. Four variants have been found, which all contained an R173G substitution. Three of these variants also contained a V78L mutation. The best variant found, V78L/R173G, produced 40% more DHAP at pH 5.0 within less than half the time compared to the wild type enzyme.
Chapter 5 focusses on the geometric- and stereoselectivity of PhoN-Sf and PhoN-Se in phosphorylations and dephosphorylations. There was a slight selectivity in dephosphorylation of cis- and trans-2-methylcyclohexanol phosphate by PhoN-Sf showing 2 times better selectivity towards the cis-isomer. In contrast, the dephosphorylation of O-phospho-DL-threonine by PhoN-Se proceeds with excellent stereoselectivity. An E-value of >200 in favour of the L-enantiomer was found. Surprisingly, the selectivity of PhoN-Se towards O-phospho-DL-serine was much less and reversed.

Chapter 6 elaborates on the improvement of the stereoselective dephosphorylation of O-phospho-DL-serine by PhoN-Se using directed evolution. Two mutations were found, N151D and V78L, showing E-values of 18.1 and 4.1 respectively, compared to 3.4 for the wild type enzyme. Furthermore, both variants show a slightly higher rate of hydrolysis compared to the wild type enzyme.