From vanadium haloperoxidases to acid phospatases
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Citation for published version (APA):

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# CHAPTER 1

## Introduction

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4. **Outline of This Thesis**

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1. Vanadium Haloperoxidases

1.1 General Introduction of Vanadium Haloperoxidases

Vanadium haloperoxidases are enzymes that catalyse the oxidation of a halide \( (X') \) by hydrogen peroxide to the corresponding hypohalous acids according to Eq. (1).

\[
H_2O_2 + H^+ + X' \rightarrow H_2O + HOX
\]  

(1)

The enzymes are named after the most electronegative halide ion they are able to oxidize, therefore chloroperoxidase (CPO) oxidizes \( Cl^- \), \( Br^- \), \( I^- \) and bromoperoxidase (BPO) oxidizes \( Br^- \) and \( I^- \). The formed hypohalous acid may chemically react with either an organic compound to form the corresponding halogenated compound (Eq. 2) or in the absence of an organic compound with hydrogen peroxide to form singlet oxygen (Eq. 3).

\[
HOX + RH \rightarrow RX + H_2O
\]  

(2)

\[
HOX + H_2O_2 \rightarrow ^1O_2 + X' + H^+ + H_2O
\]  

(3)

This class of enzymes binds vanadate \( (HVO_4^{2-}) \) as a prosthetic group [1-3]. Vanadium-containing bromoperoxidases were found in the marine brown algae \textit{Ascophyllum nodosum} [4], \textit{Fucus distichus}, \textit{Macrocystis pyrifera} [5], the red seaweed \textit{Coralina pilulifera} [6] and \textit{Cor. officinalis} [7]. Vanadium chloroperoxidases were found in the fungus \textit{Curvularia inaequalis} [2], \textit{Drechslera bisepata}, and \textit{Embellisia didymospora} [8]. More recently vanadium iodoperoxidases were found in \textit{Pelvetia canaliculata} [9] and Laminariaceae family [10]. The prosthetic group vanadate can be removed by dialysis against 1mM EDTA in 100 mM citrate/phosphate (pH 3.8) [4] or by incubation in the presence of phosphate [11]. The loss of vanadate leads to the inactivation of enzyme, resulting in formation of an apo-enzyme. The apo-enzymes are easily reconstituted as a holo form by addition of orthovanadate at neutral pH [1]. However, the reconstitution by vanadate is inhibited competitively by phosphate, molybdate and arsenate, which are tetrahedral compounds and structural analogues of vanadate, with inhibition constants \( K_i \) of 60 \( \mu M \), 70\( \mu M \) and 120 \( \mu M \), respectively [1,12,13]. The structural similarity between vanadate and phosphate results in a striking and interesting connection between the vanadium-containing haloperoxidases and several phosphatases, which will be discussed later in this introduction.
1.2 Structures of Vanadium Chloroperoxidases (VCPO) and Bromoperoxidase (VBPO)

The first X-ray crystal structure of a vanadium haloperoxidase was determined by Messerschmidt and Wever [14] in 1996 (Fig. 1). The structural data of vanadium chloroperoxidase (VCPO) from Curvularia inaequalis yielded an enormous amount of information not only to the field of vanadium-containing enzymes but also to the field of many phosphatases. Following this structure determination, the crystal structures of the peroxide form of VCPO [15], vanadium bromoperoxidase (VBPO) from the brown seaweed Ascophyllum nodosum [16] and the red algae Corallina officinalis [17] were determined. These studies show that vanadate in these enzymes is covalently attached to a histidine (i.e. $N^\alpha$ of His496 in VCPO) while five residues (i.e. Arg360, Arg490, Lys353, Ser402 and Gly403 in VCPO) donate hydrogen bonds to the non-protein oxygens. The resulting structure is that of a

![Fig. 1 Ribbon-type representation of the VCPO molecule from C. inaequalis (PDB ID: 1IDQ) co-crystallized with vanadate. White part represents the active site residues and vanadate as a prosthetic group. The figure was prepared using PyMOL.](image)

![Fig. 2 Superposition VCPO from C. inaequalis (PDB ID: 1IDQ) and VBPO from A. nodosum (PDB ID: 1QI9) active sites. The main difference is found at the Phe397 residue in VCPO and the His411 residue in VBPO. The figure was prepared using the Swiss PDB viewer.](image)
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Fig. 3 Active site structure of the VCPO peroxo intermediate from C. inaquais (PDB ID: 1IDU). The figure was prepared using Swiss PDB Viewer.

trigonal bipyramid with three non-protein oxygens in the equatorial plane (Fig. 2). The fourth oxygen (hydroxide group) and the nitrogen atom from a histidine residue are at the apical positions. Steady-state kinetics of both VCPO and VBPO supported a model where a vanadium peroxo intermediate is formed during catalysis prior to oxidation of the halide. Indeed the crystal structure of the peroxide intermediate has been obtained in which the peroxide is bound side-on [15] (Fig. 3). This peroxo vanadate intermediate plays an important role during vanadium haloperoxidase catalysis. Steady-state kinetics of VCPO suggests [18-20] that protonation of the peroxo-intermediate is required for chloride oxidation whereas protonation is not required for bromide oxidation. This is based on the observation on that the $K_m$ for chloride is highly pH dependent but that of bromide is not significantly affected by pH. Fig.4 depicts the current proposal to explain the difference between VCPO and VBPO. In the proposed mechanism for chloride oxidation, the side-on bound peroxide is protonated to form a strong oxidizing state (Fig. 4, left). On the other hand bromide oxidation will already occur with the less reactive unprotonated side-on bound peroxide (Fig. 4, right).

Another very important feature of the peroxo intermediate is its large affinity to the active site. Peroxide and vanadate form peroxo vanadate that is bound much more strongly to the enzyme than vanadate alone [21]. It has also been reported that imidazole (such as histidine) binds to peroxo vanadate more strongly than vanadate [22]. Furthermore, the affinity for peroxo vanadate which has a $K_d$ value of less than 5 nM at pH 5.0 is much higher than that of vanadate. The estimated $K_d$ value of the binding of vanadate to apo-enzyme is larger than 1 μM at pH 5.0 [18]. Physiologically the very small $K_d$ value for peroxo vanadate may be relevant since much lower concentrations of vanadate can be used effectively by the enzyme [21]. Soedjak et al. have reported [11] that inactivation of VBPO from A. nodosum by phosphate was prevented by addition of H$_2$O$_2$. This is also in line with the fact that vanadate binds to enzyme more strongly in the presence of H$_2$O$_2$. 
1.3 Mutagenesis of VCPO and VBPO

Hemrika et al. have studied the role of the cofactor binding residues of VCPO by site-directed mutagenesis [19], showing the importance of His496, a residue that forms a covalent bond to vanadate, and the role of the positively charged residues. This is illustrated in Fig. 2 and 3. Mutation of the histidine residue to alanine resulted in production of an inactive enzyme because of loss of ability to bind vanadate [19]. The authors also proposed that the positively charged residues Arg360 and Arg490 enhance the withdrawal of electron density from the bound peroxide and that Lys353 polarises the bound peroxide. When these residues are changed into alanines, the mutant enzymes lose the ability to oxidize chloride, but still function as a bromoperoxidase [23]. Another histidine residue in the active site of VCPO, His404 seems to play a role as acid-base catalyst in the binding of H$_2$O$_2$ by deprotonation of the peroxide [15,20]. Data from steady-state kinetics suggested earlier [18] that vanadate coordinated water or a histidine residue may act as an acid-base catalyst with a $pK_a$ value in the range pH 5.6 – 6.5. The mutation of His404 to Ala (H404A) [20] resulted in the loss of chlorinating activity, although brominating activity of H404A was clearly present. However, partial inactivation of the enzyme was observed during turnover. The results of site-directed mutagenesis on the kinetic properties of VCPO studies are summarised in Table 1.

Table 1$^a$ Summary of mutagenesis study of VCPO from C. inaequalis.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Property</th>
<th>Mutants</th>
<th>Chlorination</th>
<th>Bromination</th>
</tr>
</thead>
<tbody>
<tr>
<td>His 496</td>
<td>Covalently bond to vanadate</td>
<td>H496A</td>
<td>inactive</td>
<td>inactive</td>
</tr>
<tr>
<td>His 404</td>
<td>Acid-base catalyst in the binding of H$_2$O$_2$</td>
<td>H404A</td>
<td>no activity</td>
<td>partial inactivation</td>
</tr>
<tr>
<td>Arg 360</td>
<td>Enhancement of electron density withdrawal</td>
<td>R360A</td>
<td>14 %</td>
<td>22 % (pH 4.2)</td>
</tr>
<tr>
<td>Arg 490</td>
<td>Enhancement of electron density withdrawal</td>
<td>R490A</td>
<td>&lt; 1.5 %</td>
<td>900 % (pH 6.3)</td>
</tr>
<tr>
<td>Lys 353</td>
<td>Positively charged residue directly linked to the bound peroxide</td>
<td>K353A</td>
<td>&lt; 1.5 %</td>
<td>43 % (pH 4.2)</td>
</tr>
</tbody>
</table>

$^a$ For details, see [19] and [20].
$^b$ Specific activity compared to recombinant VCPO.

According to the crystal structure, of VCPO from C. inaequalis [14,15] and VBPO from Ascophyllum nodosum and Corallina officinalis [16,17], VBPOs contain a histidine residue (i.e. His411 of A. nodosum VBPO and His478 [17] or His480 [24] of Cor. officinalis VBPO, respectively) instead of Phe397 in VCPO as shown in Fig. 2. It has been proposed [16,19,20] that this histidine residue in VBPO may also play a role in protonation or deprotonation of the peroxide intermediate in the active site because His411 of A. nodosum VCPO is within hydrogen bonding distance to a modelled peroxy vanadate [16]. In recombinant VBPO from Cor. officinalis the effect of mutating the histidine residue...
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into alanine has been examined [24], showing the loss of the bromide oxidation ability of the enzyme, and conversion into an iodoperoxidase [24]. Actually this phenomenon is not surprising considering the mutagenesis studies of VCPO, which showed that nearly all mutations resulted in loss of the activity to oxidize chloride whereas the mutants still showed bromoperoxidase activity. The factors that determine why VCPO is a chloroperoxidase and VBPO is a bromoperoxidase are still not clear, despite the fact that the active site residues are identical. However, it is clearly not a single factor that is responsible for the difference in reactivity between VCPO and VBPO.

1.4 Conserved Active Site

In 1997 the striking similarity in the active site residues of vanadium-containing haloperoxidases and three families of acid phosphatases was published [25]. About that time Stukey and Carman [26] also identified the conserved phosphatase sequence motif, and Neuwald [27] reported an unexpected structural relationship between integral membrane phosphatases, such as type 2 phosphatidylic acid phosphatase, and soluble vanadium haloperoxidases. The above mentioned vanadate-binding amino acids in VCPO and VBPO were shown to be conserved in several acid phosphatases among others the large group of soluble bacterial non-specific class A acid phosphatases, mammalian glucose-6-phosphatases (G6Pase) and lipid phosphatases [14,25-30].

The conserved amino acid residues in sequence motifs of domain I: KX_6RP, domain II: PSGH and domain III: SRX_4HX_3D play very important roles in catalysis. The active site residues participate in the binding of vanadate or phosphate, act as a nucleophile, stabilize the penta-coordinated transition state and play a role in leaving group protonation [19]. As mentioned before, vanadium-containing haloperoxidases are inactivated to an apo-form by incubation in or dialysis against phosphate buffers. Furthermore phosphate competitively inhibits the reconstitution of VBPO by vanadate [12]. The cofactor vanadate is the biologically active form of vanadium and it is structurally similar to phosphate. Vanadate is also an inhibitor of many enzymes that function via a phospho-enzyme intermediate, including some phosphatases that bind ATP or other high-energy phosphate compounds. Thus, information on the binding of vanadate to vanadium haloperoxidases can be useful in modelling the phosphatase active site as found in many phosphatases (Table 2).
### Table 2: Amino acid sequence comparison of three domains conserved in vanadium haloperoxidases and phosphatases

<table>
<thead>
<tr>
<th>Source</th>
<th>Enzyme</th>
<th>Accession number</th>
<th>Domain I</th>
<th>Domain II</th>
<th>Domain III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curvularia maragalis</td>
<td>VCPO</td>
<td>X85369</td>
<td>353-462-KWEF-EFWRP-37-AYPSGHA-78-FEANISRFLGLVVRDFDAAD417DIL-508</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drechslera biseptata</td>
<td>VCPO</td>
<td>Y11123</td>
<td>1-250-EFWRP-37-AIFPSGHA-78-FEANISRFLGLVVRDFDAAD417DIL-152</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embellisia didymospora</td>
<td>VCPO</td>
<td>Y11620</td>
<td>349-457-KWEF-EFWRP-44-AYPSGHA-78-FEANISRFLGLVVRDFDAAD417DIL-511</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascophyllum nodosum</td>
<td>VBO</td>
<td>P81701</td>
<td>341-449-KHUVHRFARP-62-AYPSGHA-54-NVFAFGQRLGMIYRFQDG1Q6LILG-498</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corallina officinalis</td>
<td>VBO</td>
<td>AF218810</td>
<td>400-507-KPHNHRRNLRP-72-AYPSGHA-58-DNIAIGRNYACVYFSDQSFGSL-563</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corallina pilulaefera</td>
<td>VBO</td>
<td>D87657</td>
<td>400-507-KPHNHRRNLRP-72-AYPSGHA-58-DNIAIGRNYACVYFSDQSFGSL-563</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fucus distichus</td>
<td>VBO</td>
<td>AF053411</td>
<td>460-569-KWQVHRFARP-62-AYPSGHA-54-NVFAFGQRLGMIYRFQDG1Q6LILG-563</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia blattae</td>
<td>EB-NSAP</td>
<td>AB020481</td>
<td>133-141-KHLY-MKIRP-21-AYPSGHT-25-YELQSSRCVGCYTHWQSFDVARV-219</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morganeilla morganii</td>
<td>PboC-M</td>
<td>X64444</td>
<td>133-139-KHLY-MKIRP-21-AYPSGHT-25-YELQSSRCVGCYTHWQSFDVARV-219</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prevotella intermedia</td>
<td>PiACP</td>
<td>AB017537</td>
<td>135-141-KHLY-MKIRP-21-AYPSGHT-25-YQMQGSRCVGCYTHWQSFDVARV-221</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Providencia stuartii</td>
<td>PboN-Ps</td>
<td>X64820</td>
<td>133-139-KHLY-MKIRP-21-AYPSGHT-25-YELQSSRCVGCYTHWQSFDVARV-219</td>
<td></td>
<td></td>
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<tr>
<td>Salmonella enterica</td>
<td>PboN-Se</td>
<td>X59036</td>
<td>123-131-KHYY-MRIRP-21-AYPSGHT-25-YELQSSRCVGCYTHWQSFDVARV-219</td>
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<tr>
<td>Shigella flexner</td>
<td>PboN-Sf</td>
<td>D82966</td>
<td>133-139-KHLY-MKIRP-21-AYPSGHT-25-YELQSSRCVGCYTHWQSFDVARV-219</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zymomonas mobilis</td>
<td>PboC-Zm</td>
<td>M24141</td>
<td>132-138-KHNY-MKIRP-21-AYPSGHT-25-YQPTSSRCVGCYTHWQSFDVARV-219</td>
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<tr>
<td>Astotattilapia nubila</td>
<td>G6Pasc</td>
<td>AF008945</td>
<td>72-126-KWVL-FGWRP-19-GSPGPA-43-LVLLCISRYMAAFPHQ146VIG-184</td>
<td></td>
<td></td>
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<tr>
<td>Canis familiaris</td>
<td>G6Pasc</td>
<td>O19133</td>
<td>76-126-KWIL-FGQR-29-GSPGPA-43-LNVCLSRILYAFPHQ146VIG-184</td>
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<td></td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>G6Pasc</td>
<td>P35575</td>
<td>76-126-KWIL-FGQR-29-GSPGPA-43-LNVCLSRILYAFPHQ146VIG-184</td>
<td></td>
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</tr>
<tr>
<td>Mus musculus</td>
<td>G6Pasc</td>
<td>P35575</td>
<td>76-126-KWIL-FGQR-29-GSPGPA-43-LNVCLSRILYAFPHQ146VIG-184</td>
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<td></td>
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<tr>
<td>Rattus norvegicus</td>
<td>G6Pasc</td>
<td>L37333</td>
<td>72-126-KWIL-FGQR-29-GSPGPA-43-LNVCLSRILYAFPHQ146VIG-184</td>
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<td></td>
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<tr>
<td>Escherichia coli</td>
<td>PGPass</td>
<td>P18201</td>
<td>97-143-KWIL-FGQR-29-GSPGPA-43-LNVCLSRILYAFPHQ146VIG-184</td>
<td></td>
<td></td>
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<tr>
<td>Haemophilus influenzae</td>
<td>PGPass</td>
<td>P44570</td>
<td>94-143-KWIL-FGQR-29-GSPGPA-43-LNVCLSRILYAFPHQ146VIG-184</td>
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<tr>
<td>Rattus norvegicus</td>
<td>PAP</td>
<td>U90556</td>
<td>120-160-KYSI-GERLP-37-SFPSGHS-38-LFVGSSSRVSDKYTQHWSLDGILQ-235</td>
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<tr>
<td>Homo sapiens</td>
<td>PAP2a</td>
<td>AB000888</td>
<td>120-160-KYSI-GERLP-37-SFPSGHS-38-LFVGSSSRVSDKYTQHWSLDGILQ-235</td>
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<tr>
<td>Homo sapiens</td>
<td>PAP2a</td>
<td>AF043329</td>
<td>148-190-KYSI-GERLP-37-SFPSGHS-38-LFVGSSSRVSDKYTQHWSLDGILQ-235</td>
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<tr>
<td>Homo sapiens</td>
<td>PAP2b</td>
<td>AB000889</td>
<td>148-190-KYSI-GERLP-37-SFPSGHS-38-LFVGSSSRVSDKYTQHWSLDGILQ-235</td>
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<tr>
<td>Mus musculus</td>
<td>LPP-1</td>
<td>D84376</td>
<td>130-180-KVL-GEPL-37-SFPSGHS-38-LFVGSSSRVSDKYTQHWSLDGILQ-235</td>
<td></td>
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<tr>
<td>Saccharomycetes cerevisiae</td>
<td>LPP-1</td>
<td>U33057</td>
<td>136-192-KVL-GEPL-37-SFPSGHS-38-LFVGSSSRVSDKYTQHWSLDGILQ-235</td>
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<td>Saccharomycetes cerevisiae</td>
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<td>Saccharomycetes cerevisiae</td>
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<td>Rattus norvegicus</td>
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<td>Y07778</td>
<td>149-192-KVL-GEPL-37-SFPSGHS-38-LFVGSSSRVSDKYTQHWSLDGILQ-235</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Consensus**

| XXX-XXXXP | XXXXXXH | XXXXXXXXXXxxxxxx | xxxxxxxxxxxxxxxxxxxxxxxxxx |

---

*Thirty-five proteins that contain a novel haloperoxidase-phosphatase motif were used to generate the proposed consensus domains. Numbers shown the outside of domains I and III refer to the numbering of the first and last amino acid in those domains from primary sequence, respectively. The intervening numbers refer to the number of amino acids between the domains. Bold letters indicate amino acids that are conserved in the consensus sequence. The table was modified from [31].*
1.5 From Vanadium Haloperoxidases to Acid Phosphatases

Based on sequence similarity it has been proposed [25-30] that the architecture of the active site in the two classes of enzymes is very similar. In fact, it has been shown that apo-CPO possesses phosphatase activity and is able to hydrolyse para-nitrophenyl phosphate (pNPP). The turnover with pNPP as a substrate is only 1.7 min⁻¹ [25]. When the crystal structure of VCPO [14] was published and the phosphatase activity of apo-CPO was discovered by Hemrika et al., no structural data for the related phosphatases were available. A common architecture of the active site has important implications for research in the field of non-metal phosphatases. The fact that vanadium haloperoxidases and above mentioned phosphatases share the conserved active site sequence and apo-CPO has phosphatase activity gave significant clues to the understanding of the catalytic mechanism of the phosphatases. One of the most interesting enzymes in Table 2, which shows significant alignment with the vanadium haloperoxidases, is mammalian G6Pases, the key enzyme in gluconeogenesis and glycogenolysis. Despite the intensive research on G6Pase, there is no crystal structure available up to now because of the difficulty to purify this membrane-bound enzyme in a homogeneous state and to crystallize it. Thus it is possible to predict which amino acid residues of these phosphatases are involved in catalytic activity using VCPO as a model for a starting point. This is because the trigonal pyramidal coordination of the vanadate in the vanadium haloperoxidases is so similar to that described for the nucleophilic attack of a histidine to the orthophosphate in the phosphorylated substrate. In fact the new membrane topology suggested for G6Pase was based on the similarity of the active site sequence between VCPO and G6Pase [23,32].

In 2000, X-ray structures of a nonspecific acid phosphatase from Escherichia blattae (EB-NSAP) co-crystallised with the transition-state analogue sulfate and molybdate were determined by Ishikawa et al [30] (see next section for details). The similarity of the residues involved in binding oxyanions in VCPO from C. inaequalis and EB-NSAP is remarkable, confirming that these families are indeed evolutionary related and share the same ancestor [25].

Since apo-CPO was found to function as a phosphatase on pNPP hydrolysis, the next question arises. “Do the soluble and membrane-bound phosphatases exhibit peroxidase activity when vanadate is bound to their active site?” This was one of the main questions when this project was started and also to evaluate more closely the degree to which catalytic activities for the different class of enzymes have been retained. This will be discussed later in the thesis.
2. Acid Phosphatases

2.1 Classification of Phosphatases

Phosphatases are the enzymes that catalyse the hydrolysis of phosphate monoesters and polyphosphates (EC 3.1.). Classification of phosphatases was initially based on the biochemical and biophysical properties of these enzymes such as pH optimum (acid, neutral or alkaline), substrate profile (nonspecific or specific for certain substrates) and molecular size (high or low molecular weight) [33]. However, it is recognized now that phosphatases can be grouped into different molecular family according to similarity at the level of primary structure [33]. In cell physiology, these enzymes play a very important role in phosphoryl group transfer.

2.2 Bacterial Nonspecific Acid Phosphatases (NSAP)

Bacterial nonspecific acid phosphatases (NSAP) are non-metal soluble periplasmic proteins or membrane-bound lipoproteins. They are found in members of Enterobacteriaceae and are able to hydrolyse a broad range of unrelated phosphate monoesters. The optimal pH for this class of enzyme is at acidic to neutral pH values. NSAPs are monomeric or oligomeric proteins containing polypeptide components with an $M_r$ of 25–30 kDa. When cloning of some NSAP genes became possible, three the different molecular families of NSAPs were classified in molecular class A, B and C NSAPs. Although both class A and B of enzymes include a group of secreted phosphatases with an $M_r$ of approximately 25 kDa, class A NSAPs and class B NSAPs are completely unrelated at the sequence level [33]. After the discovery of class A and B molecular class C acid phosphatases have been identified as secreted bacterial lipoproteins with an $M_r$ of approximately 30 kDa. Since vanadium haloperoxidases share conserved active site residues only with a number of bacterial class A NSAPs [25,34] and not with class B and C NSAPs, further introduction is focused on class A NSAPs.

2.2.1 Bacterial class A1 acid phosphatases

The class A NSAPs possess a conserved sequence motif, \( KX_8RP-(X_{12-54})-PSGH-(X_{31-54})-SRX_8HX_3D \) [33]. The same motif is shared by several lipid phosphatases, mammalian glucose-6-phosphatases and vanadium haloperoxidases as mentioned previously [23,25,35]. The class A NSAPs are further classified into class A1, A2 and A3 NSAPs dependent on the whole amino acid sequences, substrate specificities and inhibition effects. For instance, fluoride inhibits the phosphatase activity of class A2 and A3 NSAPs but not that of class A1. Several metal ions (see section 2.2.2 and 2.2.3 for details) inhibit only class A3 NSAPs. Class A1
## INTRODUCTION

### Table 3 Bacterial class A nonspecific acid phosphatases.

<table>
<thead>
<tr>
<th>Bacteria (strain)</th>
<th>Enzyme</th>
<th>Classification</th>
<th>Genes (EMBL accession No.)</th>
<th>PDB code</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cedecea davisea (CIP 8034)</td>
<td>Cedecea davisea (ATCC 33855)</td>
<td>Enterobacter aerogenes (CIP 6086)</td>
<td>Escherichia blattae (JCM 1650)</td>
<td>Hafnia alvei (ATCC 29926)</td>
<td>Klebsiella oxytoca (CIP 666)</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------</td>
<td>----------------</td>
<td>-----------------------------</td>
<td>----------</td>
<td>------------</td>
</tr>
</tbody>
</table>

*Acid phosphatases (EC 3.1.3.2).

**Collection of the Institut Pasteur; ATCC, American Type Culture Collection; JCM, Japan Collection of Microorganisms.**

The GenBank accession number.

NSAPs show higher phosphatase activity on 5'-nucleotide monophosphates (NMPs) rather than 3'-NMPs whereas class A2 NSAPs are able to hydrolyse both 5' and 3' NMPs well. Class A3 NSAPs preferably catalyse the hydrolysis of nucleotide triphosphates (NTPs), but they hardly hydrolyse NMPs.

Although *Zymomonas mobilis* NSAP (PhoC-Zm) was the first sequenced class A enzyme followed by those from *Salmonella enterica* ser. *typhimurium* (PhoN-Se), *Morganella morganii* (PhoC-Mm) *Shigella flexneri* (PhoN-Sf), PhoC-Zm has not been purified and further characterized [33]. Among these class A NSAPs in Table 3, *Escherichia blattae* nonspecific acid phosphatase (EB-NSAP) was the first enzyme for which the crystal structure was determined [30]. As shown in Fig 6, the active site structure of EB-NSAP shows the striking similarity to that of VCPO. Table 3 shows several class A NSAPs from bacteria (modified from [33]) and their classification.

The class A1 enzymes exhibit broad substrate specificity (Table 4). They are able to hydrolyse 5'- and 3'- nucleoside monophosphates, glucose 6-phosphate and aryl phosphates, such as *para* nitrophenyl phosphate (pNPP) and phenolphthalein phosphate (PDP), but not diesters [33]. EB-NSAP catalyses hydrolysis of pNPP, phenyl phosphate, glucose 6-phosphate and ATP. Although the activity is low (4.5 % of pNPP hydrolysis), the enzyme also uses glucose 1-phosphate as a substrate [30]. PhoN-Mm reveals the highest activity rates with 5'-nucleotides, glucose 6-phosphate and aryl-phosphates, and
has an optimum pH around 6 (pNPP as substrate) [36]. PhoN-Sf also appears to be more active on 5'-nucleosides than on 3'-nucleosides and the optimum pH is 6.6 with pNPP as substrate [42]. The phenomenon that 5'-nucleotides are preferred above the 3'-nucleosides upon hydrolysis seems to be the common feature of this class of enzymes. The activity of class A1 NSAPs is not inhibited by EDTA, tartrate or fluoride, but is slightly inhibited by high (100 mM) Pi concentrations.

The amino acid sequence of PhoN-Sf shows significant homology to that of class A1 NSAPs such as PhoN-Ps (83.2 %) and PhoC-Mm (80.6 %), and less homology to that of class A2 NSAPs including PhoN-Se (47.8 %) and PhoC-Zm (34.8 %) [42].

2.2.2 Bacterial class A2 acid phosphatases

The prototype of class A2 NSAPs is the nonspecific acid phosphatase from Salmonella enterica ser typhimurium (PhoN-Se) [33]. NSAP from Salmonella enterica ser. typhimurium (PhoN-Se) was the first class A enzyme purified and characterized in detail [33]. Later this enzyme was classified as class A2 phosphatase. It has a wider substrate specificity compared to that of class A1 enzymes (Table 4). Unlike class A1 enzymes, the reaction rates of PhoN-Se are similar for the various substrates with $K_m$ values in the range of 1 – 2 mM [33,39]. The substrate preference for 5'NMPs over 3'NMPs, which is seen for class A1 NSAPs, is not a property for class A2 NSAPs. Although PhoN-Se activity is not inhibited by EDTA or various divalent cations such as Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ca$^{2+}$, Ba$^{2+}$, Ni$^{2+}$ and Zn$^{2+}$, it is inhibited by fluoride and mercuric ions. High concentrations (>100 mM) of Pi partially inhibit enzyme activity. The difference in fluoride resistance is a clear key point to distinguish class A1 and A2 enzymes.
**INTRODUCTION**

### Table 4  Substrates for class A nonspecific acid phosphatases.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Class</th>
<th>Enzyme</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>p-</strong>-nitrophenyl phosphate (pNPP)</td>
<td>A1 A2</td>
<td>A3</td>
<td>[30,33,36,42]</td>
</tr>
<tr>
<td>Pyrophosphate (PPi)</td>
<td>A1 A2</td>
<td>A3</td>
<td>[30,33,36,43,44]</td>
</tr>
<tr>
<td>5'-nucleoside monophosphates</td>
<td>A1 A2</td>
<td>A3</td>
<td>[30,33,36,42,44]</td>
</tr>
<tr>
<td>3'-nucleoside monophosphates</td>
<td>A1 A2</td>
<td>A3</td>
<td>[30,33,36,42,44]</td>
</tr>
<tr>
<td>Nucleoside diphosphates</td>
<td>A2 A3</td>
<td>A3</td>
<td>[33,42]</td>
</tr>
<tr>
<td>Nucleoside triphosphates</td>
<td>A3</td>
<td>A3</td>
<td>[33,43]</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>A1 A2</td>
<td>A3</td>
<td>[30,33,36,42]</td>
</tr>
<tr>
<td>Glucose 2-phosphate</td>
<td>A1</td>
<td>A3</td>
<td>[33,36]</td>
</tr>
<tr>
<td>Glucose 1-phosphate</td>
<td>A1</td>
<td>A3</td>
<td>[30]</td>
</tr>
<tr>
<td>Phenolphthalein diphosphate (PDP)</td>
<td>A1</td>
<td>A3</td>
<td>[33,36]</td>
</tr>
<tr>
<td>Phenyl phosphate</td>
<td>A1</td>
<td>A3</td>
<td>[30]</td>
</tr>
<tr>
<td>Carbamoylphosphate</td>
<td>A1</td>
<td>A3</td>
<td>[30]</td>
</tr>
<tr>
<td>Hexose phosphate</td>
<td>A2</td>
<td>A3</td>
<td>[42]</td>
</tr>
<tr>
<td>Pentose phosphatase</td>
<td>A2</td>
<td>A3</td>
<td>[42]</td>
</tr>
<tr>
<td>a-glycerophosphate</td>
<td>A2</td>
<td>A3</td>
<td>[42]</td>
</tr>
<tr>
<td>b-glycerophosphate</td>
<td>A1 A2</td>
<td>A3</td>
<td>[33,36,42]</td>
</tr>
</tbody>
</table>

*Bold letters indicate high activity of each class of enzymes.*

#### 2.2.1  Bacterial class A3 acid phosphatases

Among the phosphatases in Table 3, only the *Shigella flexneri* apyrase (Apy-Sf) belongs to class A3 group. The enzyme shows a distinctive activity on nucleoside triphosphates (NTPs), which are hydrolysed to corresponding nucleoside diphosphates (NDPs) [33]. It is active on pyrophosphate (PPi), but has low activity on pNPP. The hydrolysis reaction does not require metal ions and the activity is not inhibited by EDTA similarly to other class A NSAPs. However, like class A2 enzymes, its activity is inhibited by fluoride. Unlike class A2 enzymes various cations including Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ca$^{2+}$, Ba$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ inhibit Apy-Sf. Sodium azide and orthovanadate also inhibit its activity. Because of its specificity towards substrates and its optimum pH (between 7 to 7.5), Apy-Sf can be considered as an ATP dephosphorydrolase or apyrase (EC 3.6.1.5.). Upon sequence comparison, in spite of functional dissimilarity with other NSAPs, it shows the striking similarity with other class A enzymes [33].

Because of the significant active site sequence similarity between *Shigella* apyrase, class A NSAPs and vanadium haloperoxidases, Babu et al. [43] assayed crude and purified apyrase for haloperoxidase, peroxidase and catalase activities. None of these activities were found even though up to 1 mM of orthovanadate was added into the assay mixture. Moreover, the presence of H$_2$O$_2$ and NaCl or NaI did not affect apyrase or pyrophosphatase activities. However, the assay for peroxidase activity was carried out using chloride as a substrate. Bromide or iodide should have been used because it is very likely that if the phosphatases were able to function as a peroxidase, they would not be able to oxidize chloride. In addition, considering the turnover of apo chloroperoxidase phosphatase activity (approximately 1 min$^{-1}$) [25], the peroxidase activity of phosphatase would require a long incubation time, therefore incubation of the assay mixture for 15 min as carried out in their work [43] is likely to be
too short to detect any peroxidase activity. Further the experiments in which the effect of H$_2$O$_2$ and chloride or iodide on apyrase activity was studied were performed without vanadate. Under this condition the apyrase will function as an “apo” haloperoxidase and the affinity for substrates of apyrase (NTPs or PPi) is probably much higher than that of halide.

1.3 Glucose 6-phosphatase (G6Pase)

As glucose is the major energy source for many mammalian cells, glucose 6-phosphatase (G6Pase) (EC 3.1.3.9.) plays a very important role in glucose homeostasis because the enzyme catalyses the last step in gluconeogenesis and glycogenolysis (Fig. 7). Unlike other soluble phosphatases it is tightly associated with the endoplasmatic reticulum (ER) and nuclear membranes of liver and kidney cells [23,35,45,46]. The liver plays a major role in blood glucose homeostasis by maintaining a balance between the uptake and storage of glucose via glycogenesis and the release of glucose via glycogenolysis and gluconeogenesis [45]. Glucose is formed from gluconeogenic precursors in both liver and kidney tissues, and in the liver also from glycogen. Both gluconeogenesis and glycogenolysis result in the formation of G6P, which has to be hydrolysed by G6Pase before being liberated as glucose into the circulation [46]. Glucose is mostly stored as glycogen in the liver. A deficiency of G6Pase causes a serious disease called glycogen storage disease type 1 (von Gierke disease), which is an autosomal recessive disorder with an incidence of about 1:100 000 in humans [23].

Fig. 7 Circulation pathways of gluconeogenesis and glycogenolysis. Glucose 6-phosphatase catalyses the hydrolysis of glucose 6-phosphate to glucose. The system pathway forming glucose is stimulated when phosphoenolpyruvate carboxykinase is increased by glucagon or glucocorticoid. On the other hand the addition of insulin withholds the formation of glucose.
INTRODUCTION

2.3.1 Kinetic property — substrates and inhibitors

The specificity of G6Pase seems rather narrow compared to that of NSAPs. In intact microsomes, it catalyses almost specifically the hydrolysis of glucose 6-phosphate (G6P) [46]. The $K_m$ value for G6P is approximately 2 – 3 mM, which is higher than the intracellular concentration of G6P (0.05 – 1 mM). Thus the physiological activity of the enzyme is regulated by substrate concentration [46]. Treatment with detergents, whether anionic, cationic or neutral significantly increases its activity on mannose 6-phosphate, glucosamine 6-phosphate and 2-deoxy-D-glucose-6-phosphate [46,57]. In addition to the above mentioned substrates, inorganic pyrophosphate (PPi) [57], carbamoyl phosphate [58] and adenosine triphosphate (ATP) [59] are known as substrates of G6Pase. G6Pase is able to catalyse not only the hydrolysis of G6P but also the synthesis of G6P from glucose and various phosphate donors such as PPi, mannose 6-phosphate and carbamoyl phosphate via phosphotransferase activity [46,57,60-62]. These activities are more apparent in microsomes treated with detergents [46,63].

Phosphate (Pi) inhibits G6Pase [64] as well as the NSAPs. Inhibition by Pi is non-competitive in intact microsomes, but competitive in the presence of detergents [46,65]. Glucose is a non-competitive inhibitor of the enzyme, irrespective of the presence of detergents with a $K_i$ of 50 – 200 mM [46,66]. Vanadate, a structural analogue of phosphate, is a competitive inhibitor of the phosphohydrolase and phosphotransferase activity of G6Pase [46,48,67]. The vanadate inhibition is greater for the

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**Insulin and the insulin-mimetic effect of vanadate**

Overproduction of hepatic glucose is the major cause of fasting hyperglycemia in all forms of diabetes [47]. Hepatic glucose production is the balance between the fluxes through glucokinase (GK) and G6Pase [47] (see Fig. 2). Insulin, a hormone produced and secreted by the β-cells of the pancreas, influences in vivo hepatic glucose fluxes by changing $V_{max}$ or $K_m$ values of hepatic enzymes, and by changing hepatic substrate (glucose or G6P) concentrations. The activity and the $V_{max}$ value of G6Pase are decreased by high insulin concentrations [47], and insulin has a dominant negative effect on glucocorticoid stimulation of G6Pase mRNA levels as well [45]. Vanadate, an essential prosthetic group for vanadium-containing haloperoxidases and a strong inhibitor for phosphatases including G6Pase [48], and vanadium compounds, such as peroxyvanadium compounds, are able to mimic most of the biological effects of insulin [49,50]. The activating effect of vanadate and insulin on glycogenesis and on glycogen synthase, resulting in lowering of the glucose level, resembles each other [49]. However the action of vanadium salts is mediated through insulin-receptor independent alternative pathways [50]. Vanadate treatment resulted in a decrease in insulin-receptor mRNA level, but insulin does not have this effect [51]. It has been reported that vanadate normalizes the activities of hepatic enzymes and liver mRNA content of such enzymes including GK and phosphoenolpyruvate carboxykinase [52,53]. Interestingly tungstate [54], selenate [55] and molybdate [56] (only in vitro) reveal similar insulin mimetic effect as vanadate. They are chemically similar to vanadate and phosphate since they have similar tetrahedral configuration. However, it is important to note that vanadate and pervanadate compounds are potential inhibitors of G6Pase (see next page for details). This could directly explain an insulin mimetic effect of vanadate compounds. G6Pase, which is evolutionally related to vanadium chloroperoxidase, is likely to have higher affinity for pervanadate than vanadate. This could also be one of the reasons the enhanced insulin mimetic effect of pervanadate as compared to vanadate [21]. Although vanadium compounds are seriously considered as a possible treatment for diabetes [50], their toxicity in clinical use should not be ignored.
phosphotransferase \( (K_i = 0.79 \, \mu M \) and \( 1.3 \, \mu M \) for detergent-treated microsomes and intact microsomes, respectively) than the hydrolase activity \( (K_i = 2.2 \, \mu M \) and \( 5.6 \, \mu M \) detergent-treated microsomes and intact microsomes, respectively) of the enzyme [46,48]. Similarly, peroxovanadium compounds inhibit the G6Pase activity [68]. Oxodiperoxovanadium(1,10-phenanthroline) vanadate (V) and oxodiperoxovanadium(pyridine-2-carboxylate) vanadate (V) are competitive inhibitors of G6Pase with \( K_i \) values of 0.96 \( \mu M \) and 0.42 \( \mu M \) (intact microsomes) and 0.50 and 0.21 \( \mu M \) (detergent-treated microsomes), respectively [68]. Tungstate, another structural analogue of phosphate and vanadate, is also known as a potent competitive inhibitor of G6Pase, with a \( K_i \) in the \( 10 - 25 \, \mu M \) range (intact microsomes) and in the \( 1 - 7 \, \mu M \) range (detergent-treated microsomes) [46,69].

Interestingly, chloride ion competitively inhibits both G6P hydrolase activity and phosphotransferase activities [45,70]. However, with intact microsomes, chloride ion inhibits carbamoyl phosphate/glucose phosphotransferase activity \( (K_i = 19 \, \text{mM}) \) more extensively than G6P hydrolase activity \( (K_i = 117 \, \text{mM}) \) [45,70]. When chloride ion concentration reduces from 35 down to 15 mM, which occurs in cells following the regulatory volume decrease, it leads to lessened inhibition of biosynthetic activity of G6Pase (phosphotransferase activity for phosphorylation of glucose), but it has little effect on the G6P hydrolase activity. The difference in chloride concentration affects significantly the phosphotransferase activity \( (K_i = 19 \, \text{mM}) \) but not the hydrolase activity \( (K_i = 117 \, \text{mM}) \). This will result in an increase in the cellular concentration of G6P, in other words, shifting the flux toward G6P production [70]. G6P is an important glycogenic intermediate, and the net result is an increase in glycogen synthesis [45].

2.3.2 Membrane topology

Despite decades of researches and the presence of enormous amount of experimental data, one of the most difficult problems in G6Pase research is its purification because of the instability of the enzyme after the extraction from membranes [46,71,72]. As a consequence the enzyme has not been crystallized and structural data are not available. However, at the beginning of the 1990s, the cloning of cDNA encoding the murine G6Pase [73] was successful, followed by the cloning of \( G6Pase \) genes from rat and human [74,75]. This has led to the deduction of the primary amino-acid sequence of the catalytic subunit. The first membrane topology model was proposed by Lei et al. [75] with six transmembrane \( \alpha \)-helices (Fig. 8, top). Later the X-ray crystal structure of VCP0 from \( C. \ inaequalis \) was determined [14] and the striking similarity of active site residues between VCP0, mammalian G6Pases and several classes of phosphatases was discovered [25]. Based on the tertiary structure of VCP0, Hemrika et al. [23,32] proposed a new topology model for G6Pase with a nine transmembrane \( \alpha \)-helices and its active site oriented towards the luminal side of the ER (Fig. 8, bottom). As illustrated in Fig. 8, in the latter
model the three domains are placed in close proximity forming a compact active site on the ER luminal side, whereas in the former model the active site residues are scattered on the ER luminal side and cytoplasmic side. Considering the similarity of active site structure of VCPO and NSAP from *E. blattae* (Fig. 6), it is now very likely that G6Pase has indeed a structure as proposed in the lower part of Fig. 8.

This new proposal was soon confirmed by Pan *et al.* using immunodetection of a chimeric protein containing the expression tag FLAG and using partial proteolysis [35,62]. Their experiments using N- and C-terminal tagged G6Pase showed that in intact microsomes the N terminus was resistant to protease digestion, whereas C terminus was sensitive to such treatment. This confirms that G6Pase possesses an odd number of transmembrane helices with its N and C termini facing the ER lumen and the cytoplasm, respectively [35], as illustrated in the new topology (Fig. 8, bottom) with 9 helices, whereas the old topology (Fig. 8, top) shows only 6 helices. Moreover the mutagenesis study of lipid phosphate phosphatase-1 (LPP-1), whose active site sequence is homologous to G6Pase (Table 2), supports the new membrane topology of G6Pase. It was shown that the active site of LPP-1 is located on the outer surface of plasma membrane [29].

Currently there are two concepts of structure function relationship for G6Pase, the “substrate transport-catalytic unit” hypothesis [65,76] and “combined conformational flexibility-subunit” hypothesis [77,78]. In the former hypothesis, the catalytic unit of G6Pase is sequestered on the luminal side of the ER, and at least four additional membrane-spanning translocases allow substrates access to the catalytically active site. In this case G6Pase is depicted as a multicomponent system. On the other hand “combined conformational flexibility-subunit” hypothesis proposes that the enzyme is deeply embedded within the ER membrane, and it possesses both catalytic and substrate/product transport activities. This hypothesis views G6Pase as a multifunctional enzyme. The new membrane topology model alone does not lead to any conclusion which hypothesis is more adequately correct [32]. However, at least the proposed location of active site towards the luminal side of ER membrane is not agreement with the latter hypothesis [32]. The former proposal, the substrate transport-catalytic unit model, is widely accepted at this moment [62].

2.3.3 Catalytic centre

The phosphohydrolytic reaction involves the formation of a phosphoryl enzyme complex (E-P) in which a covalent bond is present between the imidazolium group of a histidine residue (N-3) on the enzyme and the phosphate moiety of G6P [62,64,79]. The phosphohydrolase component of the microsomal G6Pase system has thus been identified as a 36.5 kDa polypeptide by $^{32}$P-labeling of E-P complex during steady-state hydrolysis [46,62,80].
Fig. 8 Membrane topology models for G6Pase. Top: First proposed 6 transmembrane-helix topology model (adopted from [75]). Bottom: Newly proposed & current 9 transmembrane-helix topology model (adopted from [23,35]). The current model presents all residues aligning active site residues of VCPO facing the luminal side of ER membrane.
Based on the crystal structure of VCPO, the amino acids predicted to participate in G6Pase catalysis include Lys76, Arg83, His119, Arg170, and His176 (Fig. 8 bottom, highlighted) corresponding to Lys353, Arg360, His404, Arg490, and His496 in VCPO (see also Fig. 6 left). In VCPO His496 covalently binds vanadate. When this vanadate-binding residue is changed into alanine, the mutant enzyme is inactivated due to loss of the ability to bind the prosthetic group [19]. Thus it was predicted that His176 of G6Pase would form a covalent bond with phosphate, forming a phosphoryl enzyme complex. Later Ghosh et al. [81] confirmed that His176 of G6Pase was indeed the phosphoryl acceptor using a [32P]G6Pase intermediate. As shown in Fig. 8, human G6Pase contains five methionine residues (positions 1, 5, 121, 130 and 279). These methionine residues can be cleaved by cyanogen bromide. Therefore after cleavage His119 is predicted to be within a 13.5 kDa peptide with isoelectric point of 5.3 (residues 6 – 121) and His176 is predicted to be within a 16.8 kDa peptide with isoelectric point of 9.3 (residues 131 – 279). [32P]phosphate remains bound to a 17 kDa peptide with an isoelectric point above 9, showing His176 is the phosphate acceptor in the enzyme [81].

Glycogen storage disease type 1a (GSD-1a) is caused by a deficiency in G6Pase, and 75 G6Pase mutations have been identified up to date [82]. The following active site mutations have been found in the G6Pase gene of GSD-1a patients, K76N, R83C, R83H, H119L, R170Q and H176P. These mutations resulted in abolishment of G6Pase activity [73,82,83]. Furthermore, the mutagenesis study by Ghosh et al. also shows that alanine mutagenesis of proposed active site residues Lys76, Arg83, His119, Arg170 or His176 in G6Pase completely abolishes phosphatase activity, demonstrating the importance of these residues [81]. In case of VCPO, mutations of Lys353, Arg360 or Arg490 resulted in decrease of enzymatic activity but not abolishment. Because of these differences it has been claimed that the structural requirements for the active site residues in G6Pase and VCPO differ [81]. However, the difference of enzymatic properties between G6Pase (membrane-bound) and VCPO (soluble) should also be taken account. Because mutations of membrane protein may cause improper folding of the protein or it may be more sensitive to mutations compared to soluble proteins.
3. Vanadium Haloperoxidases and Acid Phosphatases in Biocatalysis

3.1 Enantioselective Sulfoxidation Catalysed by Vanadium Bromoperoxidase

Optically pure compounds are highly wanted in the synthesis of pharmaceuticals, foods and fragrances. High enantioselectivity and purity of the chiral products is very important. One enantiomeric form may have the required effect whereas the other is ineffective and even may be toxic. Although nowadays there are many homogeneous catalysts available in stereoselective conversions, biocatalysts are also popular because of their high degree of stereoselectivity. In general it is very difficult to change from a chemical process to a biocatalysis process. However, the advantages of biocatalytic procedures should be appreciated for being more environmentally friendly, simpler or milder.

Ten Brink et al. have reported [85] that vanadium bromoperoxidases (VBPOs) are able to catalyse the enantioselective sulfoxidation of organic compounds. The reaction rate of enantioselective sulfoxidation of methyl phenyl sulfide catalysed by VBPOs is rather slow (approximately 1 min⁻¹). However, the enantiomeric excess (ee) of VBPO from A. nodosum catalysed reaction has been improved up to 96% ee for the (R)-enantiomer dependent on the enzyme concentration [84]. VBPO from Cor. pilulifera shows 55% ee for the (S)-enantiomer [85]. In contrast, VCPO from C. inaequalis catalyses the production of a racemic mixture even though its yield is 55%, which is higher than that of VBPO from Cor. pilulifera (18%) [85]. This seems to be an intrinsic characteristic of VCPO, which is also related to the difference between VCPO and VBPO. According to ten Brink et al. [84], VBPO from A. nodosum promotes the direct transfer of oxygen from the vanadium bound peroxide to the sulfide in a selective manner. This suggests that the aromatic sulfide binds near/in the active site with a relatively low affinity (Fig. 9, left). However, VCPO from C. inaequalis is not able to mediate the direct and

![Scheme 2](image-url) Enantioselective sulfoxidation of methylphenyl sulfide (thioanisole) catalysed by VBPO.

![Fig. 9](image-url) Schematic models for the sulfoxidation mechanism of VBPO (left) and VCPO (right). The sulfide is shown as R₁S₄. Adopted from [84].
selective transfer of peroxide oxygen to methyl phenyl sulphide. Therefore a different oxidation pathway in VCPO has been suggested. The peroxo vanadate intermediate of VCPO is able to oxidize the aromatic sulphide by a 1-electron transfer step, resulting in the formation of a positively charged sulphur radical, which migrates from the enzyme and is subsequently converted into the product via chemical steps (Fig. 9, right). Apparently the VCPO is too strongly oxidizing.

Unfortunately, despite many attempts, an expression system for VBPO from \textit{A. nodosum} is not available up to now. If a large-scale production of VBPO becomes available, useful applications could be envisaged. Also the possibility to carry out directed evolution on VCPO towards an enantioselective oxidation catalyst may be of interest.

3.2 \textit{Regioselective Phosphorylation of Nucleosides by Class A Acid Phosphatases}

Food additives such as glutaminic acid or inosine 5'-monophosphate (5'IMP) are widely used in Asian countries. In Japan these flavours are called "umami", a fifth sense of taste following saltiness, sweetness, bitterness and sourness. \textit{Umami} means depth, tastiness or deliciousness in Japanese, when one finds a food or drink appetizing. These \textit{umami} flavours are found in seaweeds such as \textit{kombu} (Laminaria japonica, giant kelp) containing glutaminic acid, and in fish such as bonito or anchovy, containing 5'IMP. To extract the \textit{umami} flavour, it is important to use bones or juice of meats, fish, mushrooms or seaweeds. Nowadays these flavours are synthesized chemically.

Especially nucleotides are often used not only as food additives but also as pharmaceutical intermediates. Their biological activity is related to the position of the phosphate group. 5'IMP or guanosine 5'-monophosphate (5'GMP) are used as a flavour potentiator in various foods whereas inosine 2'-monophosphate (2'IMP) and inosine 3'-monophosphate (3'IMP) are tasteless [86-90]. The phosphorylation of nucleosides in the C5'-position can be achieved both by chemical and enzymatic procedures. The chemical method requires phosphoryl chloride (POCl$_3$) during synthesis. However, the use of POCl$_3$ is not desirable because of its toxicity and its problem in handling. Moreover the chemical
method is rather complex. The enzymatic method is based upon the use of inosine kinase from *Escherichia coli* as a phosphorylating enzyme. Although the enzymatic process is simpler, inosine kinase requires ATP as a phosphate donor, which is a rather expensive reactant and therefore it is necessary to regenerate ATP, making the process more complex.

3.2.1 A new enzymatic phosphorylation

Asano *et al.* discovered a new enzymatic method of regioselective phosphorylation of nucleosides by enterobacteria [86] and purified acid phosphatases from these bacteria [87,89] using pyrophosphate (PPI) as a phosphate donor. The advantage of this new method is its simplicity, low cost and mild reaction conditions compared to the chemical or existing enzymatic processes.

Table 5 shows several microorganisms and purified recombinant acid phosphatases screened for phosphotransferase activity and that are able to use inosine as a phosphate acceptor and PPI as a phosphate donor. Asano *et al.* [86] used 18 strains of bacteria and 11 strains of yeasts in the screening process that had phosphotransferase activity. High regioselective phosphotransferase activity for phosphorylation of inosine to synthesize 5'IMP was found in bacteria that contain class A1 acid phosphatases (Table 5).

PPI is a safe compound and also is used as a food additive. PPI can be simply synthesized from phosphate at low costs. However, PPI has a chelating effect and binds multivalent metals such as Ca^{2+}, Mg^{2+}, and Fe^{2+}. Therefore the use of PPI is not suitable for phosphatases that require metal ions [91] because PPI will inhibit the activity. Class A acid phosphatases do not require metal ions, and most of

<table>
<thead>
<tr>
<th>Bacteria (strain)</th>
<th>Ratio of IMP 5':3':2'</th>
<th>Recombinant enzyme</th>
<th>Classification</th>
<th>$K_m$ (mM)$^c$</th>
<th>$V_{max}$ (U/mg)$^c$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cedecea davisea</em> (JCM 1685)</td>
<td>100:3.9:1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[86]</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em> (IFO 14930)</td>
<td>100:2.0:0.6</td>
<td>AP/TP-Ea</td>
<td>Class A1</td>
<td>218</td>
<td>3.75</td>
<td>[86,89]</td>
</tr>
<tr>
<td><em>Escherichia blattae</em> (JCM 1650)</td>
<td>100:0.2:0</td>
<td>AP/TP-Eb</td>
<td>Class A1</td>
<td>200</td>
<td>2.75</td>
<td>[86,89]</td>
</tr>
<tr>
<td><em>Hafnia alvei</em> (IFO 3731)</td>
<td>100:2.4:0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[86]</td>
</tr>
<tr>
<td><em>Klebsiella planicola</em> (IFO14939)</td>
<td>100:1.0:0.2</td>
<td>AP/TP-Kp</td>
<td>Class A1</td>
<td>231</td>
<td>2.65</td>
<td>[86,89]</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (IFO3318)</td>
<td>100:2:0:0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[86]</td>
</tr>
<tr>
<td><em>Morganella morganii</em> (NCIMB10466)</td>
<td>100:3.4:1.4</td>
<td>AP/TP-Mm</td>
<td>Class A1</td>
<td>117</td>
<td>6.09</td>
<td>[86,87,89]</td>
</tr>
<tr>
<td><em>Providencia stuartii</em> (ATCC 29851)</td>
<td></td>
<td>AP/TP-Ps</td>
<td>Class A1</td>
<td>156</td>
<td>6.21</td>
<td>[89]</td>
</tr>
</tbody>
</table>

$^a$Phosphorylation of inosine using PPI as a phosphate donor.

$^b$JCM, Japan Collection of Microorganisms; IFO, Institute for Fermenation, Osaka; NCIMB, National Collections of Industrial Food and Marine Bacteria; ATCC, American Type Culture Collection

$^c$The 1 ml reaction mixture contained 50 mg (wet weight) of cells.

$^d$Acid phosphatase with regioselective phosphotransferase activity (AP/TPase), named by Mihara *et al.*[89]. AP/TP-Eb; acid phosphatase EB-NSAP, AP/TP-Mm; acid phosphatase PhoC-Mm.

$^e$The reaction mixture contained a purified recombinant phosphatase.
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enzymes in this class are able to hydrolyse PPi to form phosphoryl intermediate (Table 4).

Phosphorylation of nucleosides or polyhydroxycompounds is thought to be a two-step reaction (for example, see also Scheme 1 in G6Pase section and Scheme 4 on the next page). First the enzyme binds to phosphate donor compounds to form a phosphoryl intermediate. In the second step the phosphoryl intermediate is either attacked by water (hydrolysis) or by a nucleoside/polyhydroxy compound resulting in phosphorylation. The $K_m$ for a substrate, therefore, is a very important factor that determines whether an effective reaction occurs.

When the affinity for the nucleoside/polyhydroxy compound is low the reaction prefers to proceed via hydrolysis of phosphate monoesters. Under physiological conditions the phosphoryl group transfer is carried out by a coupling reaction using ATP (or less frequently using other NTPs) as phosphate donor. The ability of a phosphorylated compound to transfer its phosphoryl group(s) to another compound is termed its phosphoryl-group-transfer potential. This is determined by the standard free energies of hydrolysis (Table 6). Each compound is capable of driving the phosphorylation of compounds lower on the scale of Table 6, provided that suitable coupling mechanisms (enzymes) are available. For example, as written below phosphorylation of adenosine to AMP or glucose to glucose 6-phosphate (G6P) using PPi is in principal possible.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$\Delta G^\circ$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoenolpyruvate</td>
<td>-62</td>
</tr>
<tr>
<td>1,3-Bisphosphoglycerate</td>
<td>-49</td>
</tr>
<tr>
<td>Phosphocreatine</td>
<td>-43</td>
</tr>
<tr>
<td>Pyrophosphate (PPi)</td>
<td>-33</td>
</tr>
<tr>
<td>Phosphoarginine</td>
<td>-32</td>
</tr>
<tr>
<td>ATP $\rightarrow$ AMP $\rightarrow$ PPi</td>
<td>-32</td>
</tr>
<tr>
<td>Acetyl CoA</td>
<td>-32</td>
</tr>
<tr>
<td>ATP $\rightarrow$ ADP $\rightarrow$ Pi</td>
<td>-30</td>
</tr>
<tr>
<td>Glucose 1-phosphate</td>
<td>-21</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>-14</td>
</tr>
<tr>
<td>Glycerol 3-phosphate</td>
<td>-9</td>
</tr>
<tr>
<td>AMP $\rightarrow$ Adenosine + Pi</td>
<td>-3</td>
</tr>
</tbody>
</table>

Therefore high-energy phosphate compounds such as ATP, PPi, phosphoenolpyruvate or carbamoyl phosphate (not shown in Table 6) are useful phosphate donors and they are capable of forming the phosphoryl enzyme intermediate described in Scheme 3. As mentioned, under physiological conditions ATP is used as a phosphate donor to play a major role as free energy currency. However in
applications PPI is an ideal phosphate donor in phosphorylation of nucleoside/polyhydroxycompunds. The broad substrate specificity of class A NSAPs is another very useful property that allows coupled phosphorylation reactions. As long as high-energy phosphate compounds are substrates for class A NSAPs, they can be used as a phosphate donor. At the same time, to make transphosphorylation possible, the phosphate acceptors have to be substrates for this class of enzymes as well. The regiospecificity of nucleosides phosphorylation by class A1 NSAPs C5' on positions reported by Mihara et al. [89] is likely to originate from preference of 5'NTPs over 3'NTPs (see also Table 4). The basis for this is the low affinity and activity for 3'NTPs of this class of enzymes. As a consequence these compounds are hardly phosphorylated. This phenomenon and phosphorylation mechanism is described in Chapter 5 [44] of this thesis. The difference between class A1 NSAP (PhoN-Sf) and class A2 NSAP (PhoN-Se) in phosphorylation and dephosphorylation is discussed, and the steady-state study confirms that the regiospecificity of phosphorylation of nucleoside is related to the specificity of substrates used in dephosphorylation.

### Scheme 4

Overall mechanism of phosphorylation and dephosphorylation catalysed by acid phosphatases. Step (1) and (2), formation of phosphorylated enzyme intermediate. Reaction (3), hydrolysis of the intermediate "EP". Equilibrium (4), forward reaction; reaction of "EP" to yield a binary enzyme-phosphorylated substrate (E-R-O-Pi), backward; dissociation of the dephosphorylated substrate. Equilibrium (5), forward; dissociation into the phosphorylated substrate and free enzyme, backward; hydrolysis of the phosphorylated substrate. Reproduced with permission [44].

#### 3.2.2 Random mutagenesis of acid phosphatases from M. morganii and E. blattae for the enhancement of nucleoside phosphorylation activity

The novel enzymatic method of nucleoside phosphorylation using class A NSAPs is very simple and has great potential in biocatalysis. However, there are a number of problems to be solved. Firstly, the solubility of nucleosides is often limited. In case of inosine, the solubility is approximately 80 mM at room temperature. According to Mihara et al. [88], the $K_m$ value for inosine of the wild-type PhoN from Morganella morganii (PhoN-Mm) is 117 mM, which is higher than the solubility of inosine at the conditions they used. Secondly, all of the synthesized 5' IMP is rehydrolysed to inosine as the reaction
time is prolonged during inosine phosphorylation (Fig. 10). To solve these problems and in order to suppress the dephosphorylation reaction and to increase the efficiency of the transphosphorylation reaction, Mihara et al. carried out a random mutagenesis on the phoC-Mm acid phosphatase gene by error prone PCR [88]. About 2000 transformants that over-expressed mutated phoC-Mm were screened for increased yield of the phosphotransferase reaction. An improved variant PhoC-Mm I171T was obtained in the first round, and was chosen as the parent for the second generation. About 3000 transformants were screened in the second round, and a more improved mutant PhoC-Mm I171T-G92D was obtained. The time course of 5' IMP synthesis using E. coli over-producing the wild-type and mutated phoC gene products was measured (Fig. 11), showing a significant enhancement of inosine phosphorylation. The productivity of mutant I171T/G92D is superior to that of wild-type or other mutants. Furthermore, dephosphorylation of the synthesized 5' IMP was considerably depressed. Therefore the mutated enzyme phosphorylates nucleosides to a useful extent at a practical level.

This enhancement is due to the change in $K_m$ for substrates of PhoC-Mm by mutation. As summarised in Table 7, the kinetic constants are significantly changed in the I171T-G92D mutant. The affinity for inosine became much higher, whereas that of 5' IMP was decreased. This is why suppression of rehydrolysis of the synthesized 5' IMP is observed. At the condition described in the legend of Fig. 11, the mutant I171T/G92D is able to phosphorylate inosine very effectively because the $K_m$ value of 43 mM is lower than the soluble inosine concentration. In addition, the mutant has a much lower $V_{max}$ in the dephosphorylation of 5' IMP than the wild-type. The $K_m$ value for the phosphate acceptor (inosine, in this case) is a very
Table 7: Summary of kinetic constants for transphosphorylation and dephosphorylation reactions.

<table>
<thead>
<tr>
<th></th>
<th>Activity</th>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morganella morganii PhoC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>Phosphorylation</td>
<td>Inosine</td>
<td>117</td>
<td>6.09</td>
</tr>
<tr>
<td>I171T/G92D</td>
<td></td>
<td></td>
<td>42.6</td>
<td>2.67</td>
</tr>
<tr>
<td>G92D</td>
<td></td>
<td></td>
<td>114</td>
<td>0.983</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Dephosphorylation</td>
<td>5'IMP</td>
<td>0.836</td>
<td>30.3</td>
</tr>
<tr>
<td>I171T/G92D</td>
<td></td>
<td></td>
<td>1.35</td>
<td>5.67</td>
</tr>
<tr>
<td>G92D</td>
<td></td>
<td></td>
<td>1.49</td>
<td>4.70</td>
</tr>
<tr>
<td><strong>Escherichia blattae NSAP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>Phosphorylation</td>
<td>Inosine</td>
<td>202</td>
<td>1.83</td>
</tr>
<tr>
<td>G74D/I153T</td>
<td></td>
<td></td>
<td>109</td>
<td>1.39</td>
</tr>
<tr>
<td>S72F/G74D/I153T</td>
<td></td>
<td></td>
<td>37.0</td>
<td>3.46</td>
</tr>
</tbody>
</table>

For details, see [88] and [90].

Important factor. Transfer of the phosphoryl group to a phosphate acceptor leads to the production of a binary enzyme-phosphate acceptor-phosphate complex that ultimately dissociates to yield a phosphorylated acceptor and free enzyme [88]. However, the phosphate acceptor always competes with water, and a rather high concentration of acceptor is required for the transphosphorylation reaction. Further the affinity for the phosphate acceptor should be sufficiently high to be able to compete with $\text{H}_2\text{O}$ (Scheme 4) [44].

Based on the discovery by Mihara et al., Ishikawa et al. have made corresponding mutations into nonspecific acid phosphatase from *Escherichia blattae* (EB-NSAP). Interestingly the variant G74D/I153T in EB-NSAP, corresponding to G92D/I171T in PhoC-Mm, shows also the decrease in the $K_m$ value for inosine, resulting in the increase of productivity of 5' IMP [90]. Furthermore several mutations were introduced into the EB-NSAP G74D/I153T. The strategy produced a S72F/G74D/I153T mutant with a 5.4-fold lower $K_m$ value. Unlike PhoC-Mm mutants, the EB-NSAP S72F/G74D/I153T mutant shows an enhanced $V_{max}$ value as compared to the wild-type enzyme [90]. It is very likely that these residues Gly74 and Ile153 in EB-NSAP (corresponding to Gly92 and Ile171 in PhoC-Mm) are responsible for the inosine binding. These residues are not the active site residues involved in binding to the phosphate group. However they are located very close to the active site, and are conserved in other class A1 NSAPs. Therefore it is likely that mutagenesis of these residues will also affect the kinetic properties of other class A1 NSAPs.

From an industrial point of view, it is important to have an engineering cycle of 1) a synthesis process from reactants to products, 2) a test of biocatalyst selection and characterization, 3) the modelling of biocatalyst for a suitable reaction, 4) the prediction and implementation of biocatalyst engineering, 5) the application of the biocatalyst and 6) optimisation of the reaction and reactor. For biocatalyst engineering, random mutagenesis is a great tool to create a number of potential variants that
are ideally suited for a specific reaction. Mihara et al. performed random mutagenesis [88] with screening, and Ishikawa et al. used the data from the random mutagenesis as a model and a prediction tool. Further they carried out site-directed mutagenesis [90] and created a mutant that was more effective. If structural data are available, this is of great advantage in the cycle and helps to predict where in the protein mutagenesis has to be carried out. In fact Ishikawa et al. published [30] the first X-ray structure of EB-NSAP nearly at the same time as the research on inosine phosphorylation started. These studies from the Japanese group show that the biocatalytic studies are multidisciplinary. Biocatalysis involves a broad range of sciences, such as Biochemistry, Microbiology, Molecular Biology, Bioinformatics, and last but not least Organic Chemistry. It is likely that biocatalytic procedures will find increasing applications in industry considering the environmental and economical restrictions required nowadays.

4. Outline of This Thesis

—from vanadium haloperoxidases to acid phosphatases—

The object of the research described in this thesis was to evaluate the relationship between vanadium-containing haloperoxidases and acid phosphatases that share a conserved active site. The project was carried out using the recombinant vanadium chloroperoxidase (VCPO) from Curvularia inaequalis, the native vanadium bromoperoxidase (VBPO) from Ascophyllum nodosum, the recombinant non-specific acid phosphatase from Salmonella enterica ser. typhimurium (PhoN-Se) and the recombinant non-specific acid phosphatase from Shigella flexneri (PhoN-Sf). When this project was started, there were no structural data of the related phosphatases available. Thus VCPO was used as a template and compared with phosphatases to devise catalytic mechanisms for hydrolysis and to identify important residues in catalysis in these enzymes. Indeed, it is known that the apo CPO is able to convert a phosphatase substrate. Therefore the main target was to investigate whether acid phosphatases were able to function as a peroxidase when substituted with vanadate in the active site. Furthermore the research was focused on understanding the mechanism of action of these enzymes in detail and the role of the various amino acids in catalysis. Overall, the project aims to provide insights into the evolutionary and mechanistic relationship between the vanadium haloperoxidases and the acid phosphatases.

In Chapter 2, a site-directed mutagenesis study of VCPO was carried out to investigate the roles of active site residues S402A and F397H. The former mutant was created to see the importance of the residue Ser 402 in the halogenation reaction. The F397H was made to compare the active site of VCPO
and VBPO. Analysis of the steady-state kinetic data point to an interesting difference in the protonation state of the peroxo-intermediate in chloride and bromide oxidation, respectively.

Chapter 3 describes inhibition studies of VCPO using hydroxylamine, hydrazine and sodium azide. These compounds were found to be catalytic inhibitors of VCPO, binding to the peroxo vanadate intermediate during turnover. Furthermore, the inactivation of VCPO by phosphate ion was studied, showing the rapid loss of co-factor vanadate. More importantly the inactivation was prevented in the presence of H₂O₂, confirming the strength of binding of the peroxo vanadate intermediate to the enzyme.

Chapter 4 gives an answer to the main question of this project. Class A acid phosphatases from Shigella flexneri (PhoN-Sf) and Salmonella enterica (PhoN-Se) show brominating activity when substituted with vanadate. Moreover these enzymes were able to catalyse the sulfoxidation of methyl phenyl sulfide.

In Chapter 5, the phosphorylation and dephosphorylation of polyhydroxy compounds by PhoN-Sf and PhoN-Se is reported. The characteristic difference in these reactions between class A1 NSAP PhoN-Sf and class A2 NSAP PhoN-Se is discussed. Also apo BPO showed phosphatase activity, although the turnover of dephosphorylation of pNPP and PPi was even slower than that of apo CPO.
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References


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[53] Bosch, F., Hatzoglou, M., Park, E., and Hanson, R. (1990) Vanadate inhibits expression of the gene for phosphoenolpyruvate carboxykinase (GTP) in rat hepatoma cells, J. Biol. Chem. 265, 13677-13682


