Stability and inactivation mechanisms of two transaminases

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To Yi and Elsa
Abstract

In the past decades, more and more enzymes are employed as biocatalysts in industrial processes because of their advantages, such as high efficiency, substrate selectivity and stereoselectivity. Among them, amine transaminases (ATAs) are pyridoxal 5'-phosphate (PLP) dependent enzymes. ATAs have gained attention for their excellent performance in chiral amine synthesis, and their broad substrate acceptance. However, the low operational stability of amine transaminases still limits their application in industry.

The amine transaminase from Chromobacterium violaceum (Cv-ATA) has been selected for further investigation for its relatively low operational stability. Co-solvents and various additives have been added to the enzyme storage solution to improve its storage stability at various temperatures. Co-lyophilization of Cv-ATA with surfactants has been applied to improve its enzymatic activity in neat organic solvents.

As a PLP-dependent dimeric enzyme, the Cv-ATA is not primarily inactivated due to tertiary structural changes. Instead, both dimer dissociation and PLP release may affect the enzyme stability. Therefore, the inactivation pathway of the Cv-ATA during operational conditions was explored. The unfolding of the enzyme was detected by several methods, and the detection of fluorescence intensity spectrum of tryptophan is extensively applied for its high sensitivity. The phosphate group of PLP can be coordinated into the phosphate group binding cup, which may influence the enzyme structural stability. Therefore, the effect of both PLP and inorganic phosphate ions (present in phosphate buffer) on the enzyme stability was explored.

The amine transaminase from Vibrio fluvialis (Vf-ATA) is another amine transaminase, which catalyses the same biocatalytic reaction and has a similar substrate scope as Cv-ATA. However, there is still a lack of data on the stability of Vf-ATA. Consequently, the operational stability of Vf-ATA in various environments was studied.

Keywords: Amine Transaminase, Operational Stability, Inactivation Pathway, Enzyme Unfolding, Phosphate Group Binding Cup
Sammanfattning

Under de senaste decennierna används fler och fler enzymer som biokatalysatorer i industriella processer på grund av fördelar som hög effektivitet, hög substratselectivitet och hög stereoselectivitet. Bland dessa enzymer finns amintransaminaser (ATA), som är pyridoxal 5'-fosfat (PLP) -beroende enzymer. ATA har fått uppmärksamhet för sin utmärkta prestanda vid kiral aminsyntes och sin breda substrataceptans. Den låga operativa stabiliteten hos amintransaminaser begränsar dock fortfarande deras användning i industriella tillämpningar.

Amintransaminas från Chromobacterium violaceum (Cv-ATA) har studerats i detta arbete på grund av sin relativt låga operativa stabilitet. Lösningsmedel och andra substanser har tillsats för att förbättra lagringsstabiliteten vid olika temperaturer. Frystorkning av Cv-ATA tillsammans med ytaktiva medel har applicerats för att förbättra enzymaktiviteten i organiska lösningsmedel.

Ett PLP-beroende dimert enzym, som Cv-ATA, är inte primärt inaktiverat på grund av tertiära strukturförändringar. I stället kan både dimerdissociation och PLP-frisättning påverka enzymstabiliteten. Därför undersöktes inaktivering av Cv-ATA under reella reaktionsbetingelser. Denatureringen av enzymet detekterades med flera metoder, och detektion av fluorescensintensitet av tryptofan användes i stor utsträckning för sin höga känslighet. Fosfatgruppen i PLP kan koordineras i fosfatgrupp-bindningsfickan, vilket kan påverka enzymstrukturens stabilitet. Därför undersökte effekten av både PLP och oorganiska fosfatjoner (närvarande i fosfatbuffert) på enzymstabiliteten.

Amintransaminas från Vibrio fluvialis (Vf-ATA) är ett annat amintransaminas som katalyserar samma reaktion och har ett liknande substratomfång som Cv-ATA. Det finns dock fortfarande brist på data om stabiliteten av Vf-ATA. Följaktligen studerades den operativa stabiliteten hos Vf-ATA i olika miljöer.
Public defense of dissertation

This thesis will be defended April 11\textsuperscript{th}, 2018 at 10:00 a.m. in Kollegiesalen, Brinellvägen 8, Stockholm, for the degree of “Teknologie doktor” (Doctor of Philosophy, PhD) in Biotechnology.

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List of appended papers

Paper I

Paper II

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Paper IV
Chen S., Berglund P. & Humble M. S. Inactivation pathway underlying the operational instability of an amine transaminase from *Chromobacterium violaceum*. Submitted.

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Paper III
Shan Chen contributed to the experimental design, executed the experiments and wrote the first draft of the paper.

Paper IV
Shan Chen contributed to the experimental design, executed the experiments and wrote the paper together with Maria Svedendahl Humble.
List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACP</td>
<td>Acetophenone</td>
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<tr>
<td>ATA</td>
<td>Amine transaminase</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BN-PAGE</td>
<td>Blue native PAGE, poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Cv-ATA</td>
<td>Amine transaminase from <em>Chromobacterium violaceum</em></td>
</tr>
<tr>
<td>D-PLP</td>
<td>Holo dimer enzyme.</td>
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<tr>
<td>DSF</td>
<td>Differential scanning fluorimetry</td>
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<tr>
<td>E</td>
<td>Enzyme</td>
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<tr>
<td>Em</td>
<td>Emission</td>
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<tr>
<td>Ex</td>
<td>Extinction</td>
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<tr>
<td>FI</td>
<td>Fluorescence intensity</td>
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<tr>
<td>GC</td>
<td>Gas Chromatography</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IUBMB</td>
<td>International Union of Biochemistry and Molecular Biology</td>
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<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>Dissociation constant</td>
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<tr>
<td>M</td>
<td>Monomer enzyme</td>
</tr>
<tr>
<td>MD equilibrium</td>
<td>Monomer dimer equilibrium</td>
</tr>
<tr>
<td>M-M</td>
<td>Dimer enzyme</td>
</tr>
<tr>
<td>M-PLP</td>
<td>Holo monomer enzyme</td>
</tr>
<tr>
<td>MTBE</td>
<td>Methyl tert-butyl ether</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>PGBC</td>
<td>Phosphate group binding cup</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal-5´-phosphate</td>
</tr>
<tr>
<td>PMP</td>
<td>Pyridoxamine-5´-phosphate</td>
</tr>
<tr>
<td>Pyr</td>
<td>Pyruvate</td>
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<tr>
<td>S-PEA</td>
<td>(S)-1-phenylethylamine</td>
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<tr>
<td>Vf-ATA</td>
<td>Amine transaminase from <em>Vibrio fluvialis</em></td>
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<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Melting temperature</td>
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<tr>
<td>Trp</td>
<td>Tryptophan</td>
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1. Enzymes

1.1 Enzymes

After million years of evolution, organisms have produced outstanding biocatalysts including proteins (enzymes) and nucleic acids. Among them enzymes are crucial, irreplaceable substances in life cycles. Enzymes could rapidly, selectively and efficiently catalyse reactions for supporting the survival requirements of the life cycle. Compared with using chemical catalysts, there are more advantages to utilize enzymes in chemical reaction and industrial processes. They have high catalytic efficiency and substrate specificity without byproduct formation and they work in mild reaction conditions. As enzymes have chemo-, regio- and stereoselectivity, they could catalyse the formation of chiral nonracemic chemicals, while common chemical reactions, without chiral species and in symmetrical environments, always yield racemic mixtures.

From the first enzyme employed in alcoholic drinks by the Chinese, 9000 years have past. Until now, more and more enzymes have been discovered and investigated. They can catalyze countless reactions both in vivo and in an artificial environment. They have been applied in various industrial areas, such as food processing, pharmaceutical formation and chemical production.

In spite of these advantages, the disadvantages of enzymes are obvious. Most enzymes are only active in conditions such as low (room) temperature, limited pH values, in aqueous media and normal pressure. In addition to that, the biological activity of enzymes can in some cases be inhibited by metal ions, substrates, products, or even substrate analogues. This can limit the industrial application of enzymes. Consequently, the enzyme knowledge has been enriched by various investigations on their evolution, synthesis, and catalytic features in recent decades. Lyophilization and immobilization techniques has been applied to improve enzyme stability in vitro; enzyme engineering has been done for improving enzyme properties. The exploration of enzyme folding and unfolding behavior has been assisting the understanding of enzyme inactivation, function and regulation. With the
increasing number of studies, more and more enzymes have been employed in laboratory and industrial processes.

Enzymes are proteins consisting of amino acids in special sequences which are determined by the encoding gene. The formed amino acids sequence will fold into local structural elements (α-helices and β-sheets), which is named secondary structure. These local structural elements will further fold into a tertiary structure by various interactions such as hydrophobic interactions, salt bridges, disulfide bonds, hydrogen bonds and van der Waals forces. Among them, the hydrophobic effects of amino acids side chains assist that the nonpolar groups gather and are folded into the internal of the protein to avoid contact with water\textsuperscript{12}. This effect is recognized as the main contribution to enzyme stability\textsuperscript{13, 14}. Pace and coworkers proved that hydrophobic interactions contribute more to protein stability with the increase of protein size\textsuperscript{14}. In addition to that, the hydrophobic effect also proved to contribute to oligomer formation by different peptide chains. The three-dimensional arrangement and subunits interrelation of oligomeric protein is defined as quaternary structure. In nature, lots of enzymes have been discovered that only show activity in oligomeric form.

All enzymes have been named systematically and are divided into six main classes based on their reaction mechanism by the Enzyme Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB): oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases\textsuperscript{5}. Among them, transferases are named for catalyzing the transfer of various molecular groups from one compound to another. The transferases have been widely found from various kinds of organisms. Some of them have already been used in commercial applications in industry or laboratory scale.

In the present pool of known enzymes, around 30% are cofactor-dependent, which means that they need to coordinate the cofactor in order to be able to fulfill their biological activity\textsuperscript{15}. Generally, without the cofactor the enzyme is defined as apoenzyme, which is an enzymatically inactive form of the enzyme. Cofactors include inorganic or organic molecules, and hereby could be divided into prosthetic groups and coenzymes\textsuperscript{5}. The prosthetic groups include metal ions and other non-protein inorganic molecules. Coenzymes are organic molecules of low
molecular weight. Some of them are dynamically associated and dissociated with the enzyme during the storage such as NADH and ATP. Hereby, the enzyme affinity to the coenzyme may highly influence its stability, which will be discussed later. Other coenzymes may bind tightly to the enzyme, such as flavin adenine dinucleotide and flavin mononucleotide.

1.2 Oligomeric enzymes

Aggregation of homo- or hetero- monomers into oligomers is a common phenomenon in different classes of enzymes. The smallest structural unit for an oligomer is generally defined as monomer. Homo-oligomers consist of two or several monomers (subunits) having the same protein sequence. Hetero-oligomers are enzymes consisting of two or several subunits that differ in protein sequence. From the protein data bank\(^\text{16}\) it can be found that a high proportion of the discovered enzymes are biologically active as oligomers. Theoretically, one reason for this is that active sites of most oligomers are located at the interface of subunits.\(^\text{17}\) Association of subunits could assist the active site formation and assembly. Another reason is that the enzyme activity is subunit conformation-dependent. Regulation of the subunit association could modulate the enzyme catalytic activity\(^\text{18}\). Therefore, the oligomerization usually has the role to mediate and regulate the gene expression and enzyme function in physiological pathways of organisms\(^\text{17, 19, 20}\).

In nature, this self-association can happen either by covalent bonds between subunits or by a network of weak bonds. In the first case, the oligomerization formed by covalent bonds is an irreversible process, such as the formation of disulfide bonds of glutamate receptor 1\(^\text{21}\). In the second case, monomers aggregation to oligomers is mediated by hydrophobic interactions, hydrogen bonds and electrostatic interactions. However, this association can also occur by artificial assistance, such as changing of medium environments, immobilization, cross linking, or even protein engineering\(^\text{19}\).

Generally, the oligomerization process is advantageous for enzymes. Association of subunits could reduce the surface area and therefore
Enzymes improve the oligomer stability\textsuperscript{22-24} and enzymes could economically form large structures with small gene size by the oligomerization. However, the disadvantage of an oligomeric enzyme is also reflected in its complex stability behaviors, for instance, some oligomers may easily dissociate into monomers and therefore become inactive. This will be discussed in section 2.

1.3 Vitamin B\textsubscript{6} and PLP dependent enzymes

Vitamin B\textsubscript{6} displays a crucial role as a common cofactor in the metabolism of living organisms. Generally, vitamin B\textsubscript{6} has six chemical forms, which may transform with changing environment. The six forms include pyridoxine (PN), Pyridoxal (PL), Pyridoxamine (PM), and their related 5’-phosphate forms: Pyridoxine-5’-phosphate (PNP), Pyridoxal-5’-phosphate (PLP), pyridoxamine-5’-phosphate (PMP). PN is the form recognized as a nutritional supplement. While PLP (Figure 1) is the only biological active form of vitamin B\textsubscript{6}, which always functions as cofactor in PLP dependent enzymes to assist them to accomplish their function in organism\textsuperscript{25}. PMP is the intermediate during the catalytic process of amine transferases.

![Figure 1. The chemical structure of pyridoxal-5´-phosphate (PLP).](image)

PLP has an absorption peak at 390 nm. Generally, PLP will be irreversibly degraded when incubated in light. This photo degradation behavior has been known for a long time\textsuperscript{26}. The absorption of PLP at 390 nm will decrease when it is exposed to light, and this decreasing will stop when kept in the dark. Utilizing light to change enzyme catalytic behaviors is not a rare phenomenon\textsuperscript{27}. DNA photolyase, protochlorophyllide and oxidoreductase (POR) are enzymes that need light to be active, whereas some enzymes with hemes, flavins and metal centers as cofactors also
need to be activated by light\textsuperscript{27-30}. In this thesis, PLP photo degradation has been utilized to investigate the PLP reversible dissociation with the phosphate group binding cup (PGBC), as well as the inorganic phosphate ions competition with PLP about the same binding site of two amine transaminases.

With the isolation and investigation of the different forms of vitamin B\textsubscript{6}, the PLP dependent enzymes have been discovered, explored and studied. Until now, more and more PLP dependent enzymes have been found, which are all active in dimeric or higher oligomeric forms\textsuperscript{19}. Until now, the database showed they could catalyse 238 chemical reactions \textsuperscript{31,32} and are distributed in five of the six enzyme main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases\textsuperscript{25-33}. During the past decades, researchers have payed attention to the relationship between the protein structure and the enzymatic activity of PLP dependent enzymes\textsuperscript{34-37}. Generally, PLP will bind covalently to the enzyme by a Schiff base (a covalent internal aldimine) linkage. The Schiff base can be detected by UV-absorbance at 410-420 nm and by fluorescence intensity at extinction 415 nm and emission 560 nm\textsuperscript{38}.

The PLP-dependent enzymes have been divided in five fold types I to V\textsuperscript{32,39,40}. The aspartate aminotransferase family, the tryptophan synthase family, the alanine racemase family, the D-amino acid fold family and the glycogen phosphorylase family. Among them, most enzymes belong to the fold type I. They are active as dimers or higher oligomers with PLP covalently binding to the PGBC located at the interface between the two associated subunits.

The role of PLP in enzyme folding and inactivation has been recognized as an interesting area in the past decades. In nature, coenzymes are always synthesized and bind with enzymes in a cellular environment, and this situation prevents researchers to further study how and when the PLP molecules bind with the enzymes during the folding\textsuperscript{5}. In section 2, the PLP effect on enzyme stability has been discussed. Its effect on enzyme folding has also been investigated. Generally, a cofactor effects enzyme folding in two ways: 1) speeds up the folding process and guides the correct formation of the polypeptide chains, 2) only bind to the enzyme either partially folded or in native forms\textsuperscript{41,42}. PLP has been shown to speed up the refolding of an aspartate transferase\textsuperscript{43}. However,
PLP displayed different releasing behavior when the aminotransferase was incubated with different unfolding chemicals. This result indicates that the PLP’s roles in enzyme unfolding/refolding are very complex and that it may be influenced by the PLP binding affinity, stability of the PLP binding domain, the unfolding chemicals or the reaction environments.
2. Enzyme stability

2.1 Characterization of enzyme stability

Enzymes are expressed in vivo by various organisms. The use of enzymes in Biocatalysis requires the enzymes to be structurally stable in order to retain enzymatic activity in in vitro environments. Therefore, investigating and enhancing enzyme stability in various conditions is the primary and principal step before enzyme commercial application.

Generally, the enzyme stability reflects the enzyme tolerance to various in vitro environments and the resistance to inactivation. Enzyme stability can be divided into two types, related to the unfolding types, thermodynamic (conformational) stability and kinetic (long-term) stability\textsuperscript{46, 47}. In the first case, the thermodynamic stability is related to reversible conformational change of the enzyme. Theoretically, it is reflected by parameters such as free energy of unfolding, melting temperature, or the unfolding equilibrium constant\textsuperscript{46}. In the other case, the enzyme kinetic stability is the irreversible denaturation of the enzyme, which always can be detected by the optimum operating temperature, half-life temperature of half-inactivation\textsuperscript{46}.

![Free-energy diagram of enzyme unfolding](image)

**Figure 2.** Free-energy diagram of enzyme unfolding. N to U is related to thermodynamic stability; N to I is related to kinetic stability.
The inactivation pathway of an active enzyme (N) is shown in Figure 2, which is suggested to be a two-step process\textsuperscript{47}. The partly unfolded enzyme (U) is only enzymatically inactive and it will undergo irreversible unfolding to inactive enzyme (I)\textsuperscript{47}. The enzyme operational stability concerns resistance of the enzyme from inactivation at various operational situations in laboratory or industrial scale, such as high/low temperature, pressure, pH, organic solvents/co-solvents, or in presence of chemicals or salts. Therefore, the enzyme inactivation during an operational process is always related to both reversible and irreversible inactivation, and hereby, may be influenced by several factors, which will be discussed separately.

Usually, an increase of the temperature in a chemical process from 25 to 75 °C could improve the reaction rate 100-fold\textsuperscript{48}. In food and pharmaceutical industry, a high temperature is essential for eliminating bacterial growth\textsuperscript{47}. Moreover, a high temperature could decrease the medium viscosity and shift thermodynamic equilibrium. These advantages lead to the widely utilization of high temperatures in industrial scale, and hereby increasing enzyme thermal stability becomes crucial for applying them in industry.

An enzyme only exhibits its optimal activity and stability within a specific pH range. Different kinds of buffer salts may have different effect on the enzyme conformation, and hereby change its catalytic behavior\textsuperscript{49}. In addition to that, an enzyme may have varying freezing points in different salts, and some salts may lead to inactivation during enzyme lyophilization\textsuperscript{50,51}. Different salt concentrations have also been proven to influence enzyme stability. With a 20 times increased acetate or phosphate concentration, a 3- or 10-fold thermal stability enhancement of a \textit{P. amagasakiense} glucose oxidase has been obtained\textsuperscript{52}. Moreover, pH values also influence the oligomer dissociation and association\textsuperscript{53}. Especially for dimeric enzymes, monomerization will occur with reduced pH, and this is always recognized as the first step of multimeric enzyme inactivation\textsuperscript{53,54}. Therefore, it is important to investigate and select the optimal buffer and pH value for each enzyme.

In some cases, enzymes are inactivated at high pressure, but this phenomenon is not common for oligomers\textsuperscript{55,56}. In contrast, oligomeric enzymes may get enhanced stability by 1-2 kbar higher pressure\textsuperscript{57}. There
are also evidence of thermophilic and hyperthermophilic oligomer gaining higher thermal stability by increasing the pressure\textsuperscript{58, 59}.

Sometimes reactions are run in organic solvents (co-solvents and water mixture or neat/pure organic solvents)\textsuperscript{60, 61}. There are many advantages of using organic solvents, such as increasing substrate solubility, decreasing side reactions taken place in water, reducing microbial infection in the media etc.\textsuperscript{47}. However, enzymes often have poor stability and activity in organic solvents, which limits their utilization in such systems. Theoretically, the mechanism of enzyme inactivation in co-solvents is that the co-solvents may strip water away from the enzyme, thereby change the enzyme conformation and which finally leads to denaturation\textsuperscript{62, 63}. However, for neat hydrophobic organic solvents, it is another situation. As an enzyme is insoluble in almost all hydrophobic organic solvents, it needs to be lyophilized into a powder state, and stirring and shaking are required during the reactions\textsuperscript{64}. The lyophilization of enzymes has also proved to be a step that may lead to enzyme inactivation\textsuperscript{65}. Therefore, the reasons for enzymes having much lower activity in neat organic solvents compared to water are intricate. Various additives (such as sugars, inorganic salts, surfactants)\textsuperscript{66, 67} have been added into the lyophilizing mixture to minimize the caused denaturation.

A cofactor dependent enzyme needs to bind with the cofactor to be active, thereby, the cofactor becomes another important factor influencing enzyme stability. Generally, the cofactor affects the enzyme stability in two ways. In one way, the cofactor affinity with the enzyme (related to its association/dissociation during storage) has great influence on the enzyme stability, which could be measured by $K_D$. The cofactor release from the holo enzyme may result in enzyme inactivation and the formation of apo enzyme, which is prone to irreversible unfolding\textsuperscript{68, 69}. On the other hand, some cofactors located at the interface between the subunits may influence the oligomer formation and stabilization\textsuperscript{70}.

\subsection*{2.2 Multimeric enzyme stability}

Generally, oligomers have higher stability because of their more rigid construction compared with monomers. However, the stability behavior of oligomers is more complex\textsuperscript{46, 57}. The inactivation of oligomers is related
to not only the tertiary structure changes\textsuperscript{48, 57, 71} but also to the subunits dissociation.

In most case, monomers are reversibly associated to dimer following a dynamic equilibrium (monomer-dimer (MD) equilibrium). The MD equilibrium is dynamically shifting between dimer and monomer depending on the enzyme storage environment, such as pH, ion strength, pressure or even enzyme concentrations\textsuperscript{59}. Previously, an amino butyrate aminotransferase was proven to be 100\% dissociated to monomer when the pH value was decreased to 5\textsuperscript{53}. Moreover, the mitochondrial aspartate aminotransferase which is active in dimeric form, formed monomers at pH 5.3 for the apo enzyme, while the holo enzyme dissociated to monomers at pH 3.6\textsuperscript{69}. In this thesis, the reversible dissociation of \textit{Cv}ATA to monomer at low pH has been explored with the aim to determine the affinity between PLP and one subunit of the dimeric \textit{Cv}ATA.

\section*{2.3 Methods to improve enzyme stability}

Generally, a characterization of optimal reaction conditions for an enzyme should be done before it is further investigated and utilized. However, sometimes enzymes need to be employed in unsatisfying conditions. Then, the enzyme operational stability needs to be improved. In the past decades, more and more methods have been employed to improve enzyme stability, and some of them will be discussed in this part.

**Immobilization.** Enzyme immobilization is a very common method, which could improve enzyme thermal stability, activity and especially prevent oligomer dissociation\textsuperscript{72-76}. Theoretically, immobilization of enzymes could be distinguished into four types: entrapment, adsorption, membrane confinement, and covalent binding. Multipoint covalent binding aims at immobilizing enzyme on the support by covalent bonds. This method is recognized as the most efficient methods to improve the enzyme thermal stability, and especially for multimeric enzymes\textsuperscript{77}.

**Crosslinking.** Crosslinking is another common method employed to improve multimeric enzyme stability. Sometimes, it can also be recognized as a foundation of immobilization of multimeric enzymes\textsuperscript{57, 78}. The crosslinking can be divided into two types. On one hand, using a poly-ionic polymer to cover the enzyme surface like a coat is extensively
recognized as a physical method. This method is good at preventing subunits dissociation, but incapable to improve enzyme rigidification. Hereby, this method is applied in some special conditions such as extreme pH or high ionic strength. On the other hand, the chemical method is to use a crosslinker to react with amino acid side chains to form bonds within or between the subunits, which could prevent the enzyme both from irreversible unfolding and subunits dissociation. Consequently, it could improve both thermal stability and conformational stability of the enzyme. Both physical and chemical crosslinking methods are highly efficient in enhancing oligomer stability.

**Protein engineering.** In recent decades, protein engineering is an important technique to improve enzyme characteristics, such as stability. Generally, the common methods are divided into 3 different strategies; rational design, semi-rational design and directed evolution. Among them, rational design is engineering the enzyme to improve its property with a rational approach, which is supported by a theoretical foundation. To apply this strategy, it is necessary to know the three-dimensional structure of the enzyme, or even the active site. For enhancing the enzyme activity, selectivity or enantioselectivity, the strategy usually focuses on the substrate binding pocket. In 2010, rational design of an (R)-selective transaminase (ATA-117, a homolog of an enzyme from *Arthrobacter sp.*) improved its activity towards prositegliptin ketone by designing mutations aiming at extending the substrate binding pocket. Deepankumar and co-workers have incorporated 3-fluorotyrosine into an amino transaminase. The mutation resulted in improved thermal stability and tolerance of organic solvents. Introducing disulfide bonds inside subunits has also been shown to enhance thermostability. The Lipase B from *Candida antarctica* got 8.5 °C increased $T_{50}$-value after 60 minutes of incubation. However, mutations committed to improve the subunit-subunit interaction are also recognized as a strategy to prevent oligomer dissociation and inactivation. Introducing disulfide bonds between subunits have displayed significant improvement of the multimeric enzyme stability.
3. Amine transaminases

3.1 Mechanism and structure

Amine transaminases (ATAs) belong to the enzyme class of transferases (E.C. 2.6.1.18), which catalyze the amino transfer from amino donors to amino acceptors (Scheme 1). The product amines can be chiral and are then very interesting in the pharmaceutical industry. Previously, ATA’s reaction mechanism and substrate specificity have been explored for their utilization in various cascade and one-pot reactions. With more and more three-dimensional structures of ATAs being solved and their active sites identified, rational design of ATAs has been utilized to improve their stability and activity towards various substrates.

Scheme 1. The general reaction of ATAs. The amino group is transferred from the amino donor to the amino acceptor.

ATAs obey a ping-pong bi bi catalytic reaction mechanism. As shown in Scheme 2, the amino donor will bind to the enzyme active site and forms the first product. After the first product leaves, the amino acceptor binds to the active site and then the second product forms. Therefore, the reaction could also be recognized as two half-transaminations, which has been used to quantify the active site of the amine transaminase from Chromobacterium violaceum.

Until now, all known ATAs are active as homo dimers or as higher oligomers. Each monomer has one active site located at the interface between two subunits. In addition, amino acid residues from both monomers complete the active site architecture. Each active site contains a cofactor-binding region (phosphate group binding cup) and the substrate-binding region. While the phosphate group of PLP coordinated into the phosphate group binding cup (PGBC), there are lots of investigations focusing on the PGBC construction in different
enzyme fold types and their role in PLP coordination and recognition\textsuperscript{32, 117, 118}. Generally, the substrate-binding region of ATAs consists of two binding pockets which differ in size. In most cases, the large pocket could accept large substituents, while the small pocket only accepts a methyl group. For that reason, ATAs show high enantioselectivity and rational design of aiming at specific substrates are generally focusing on the substrate binding pocket alteration.

\textbf{Scheme 2.} The ping-pong transaminase reaction mechanism\textsuperscript{54, 119, 120}. The Lys in the scheme is the catalytic lysine in the ATA active site.

Generally, ATAs need PLP and, hence, they belong to the PLP-dependent enzyme family (Section 1.3)\textsuperscript{32, 36, 121}. All known (S)-selective ATAs belong to fold type I and the (R)-selective ATAs belong to fold type IV\textsuperscript{104}. The fact that PLP effects enzyme activity, stability and unfolding has been discussed in section 3.2, and its effect on ATAs activity and stability will be further discussed in section 4.
3.2 Cv-ATA and Vf-ATA

The amine transaminase from *Chromobacterium violaceum* (Cv-ATA) is biologically active in holo dimeric form. It has broad substrate specificity, high stereoselectivity and enantioselectivity, which make the enzyme of interest for industrial applications\textsuperscript{100, 122-125}. Our research group have reported several important findings of this enzyme; the crystal structure of Cv-ATA in with and without PLP, an active site titration method, improved as well as reversed enantiomeric preference obtained by protein engineering and other general biochemical characteristics of Cv-ATA\textsuperscript{54, 103, 126, 127}. However, the use of Cv-ATA is still limited by its poor operational stability. Some substrates of Cv-ATA have low solubility in water and adding co-solvents (such as DMSO) in the reaction mixture or performing the reaction in neat organic solvents are needed for improving the substrate solubility and shifting reaction equilibrium\textsuperscript{61, 128, 129}. Hence, some methods have been explored to improve ATAs thermal stability and organic solvents tolerance\textsuperscript{60, 83, 88, 130, 131}. For instance, researchers have utilized the global incorporation of fluorotyrosine and multiple noncanonical amino acids incorporation to improve ATAs stability\textsuperscript{83, 131}.

As in other PLP-dependent enzymes, the active site of Cv-ATA is located at the interface between two monomers (Figure 3a), where the PLP covalently binds with a catalytic lysine and forms an internal aldimine (Schiff base), and hydrogen bonds are formed with both monomers\textsuperscript{82, 86, 113, 132}. Five years ago, the first crystal structures of Cv-ATA in both apo and holo form (PDB: 4A6R and 4A6T) were solved\textsuperscript{103}. The structures with and without PLP showed that the coordination of PLP to Cv-ATA caused a structural rearrangement of the apo enzyme to form a more rigid holo enzyme\textsuperscript{103}. The PLP binding affinity with the enzyme is an essential factor influencing the holo ATA’s activity, stability and unfolding\textsuperscript{43, 44, 133, 134}. The phosphate group binding cup, where the phosphate group of PLP binds, are therefore suggested to be important for Cv-ATA’s stability. Strategies to improve the PLP-binding in another ATA has also been explored by a single-point mutation\textsuperscript{135}.

The (S)-selective amino transaminase from *Vibrio fluvialis* (Vf-ATA) is another PLP dependent enzyme, which has similar substrate specificity compared with Cv-ATA\textsuperscript{136-138}. Substrate characterization and kinetic resolution of Vf-ATA have been done to excavate the enzyme’s
commercial value\textsuperscript{139, 140}, while immobilization, homology modeling and directed evolution have been employed to improve the enzyme\textsuperscript{139, 141, 142}. The crystal structure of Vf-ATA (PDB: 4E3Q) has been solved\textsuperscript{139}. The enzyme is bioactive in holo dimeric form with a similar PLP location compared to Cv-ATA (Figure 3b).

![Crystal structure of ATAs](image)

**Figure 3.** Ribbon representation of the crystal structure of two ATAs. PLP (Cv-ATA) or PMP (Vf-ATA) molecule is shown as balls in element colors in one of two active sites. (a) holo Cv-ATA (PDB ID: 4A6T), One subunit in pink and one subunit in gray. (b) holo Vf-ATA (PDB ID: 4E3Q), One subunit in blue and one subunit in gray. Molecular graphics created by YASARA (http://www.yasara.org) and PovRay (http://www.povray.org).

The prospect to employ ATAs in industrial scale is promising and optimistic. The work in this thesis will be important for further employment of ATAs’ in more efficient processes for amine synthesis.
4. Present investigation

4.1 The effect of organic solvents and additives on the stability of Cv-ATA

In a previous study, the \( T_m \) value of \( \text{Cv-ATA} \) was measured in different buffer conditions\textsuperscript{103}. In that study, \( \text{Cv-ATA} \) incubated in HEPES buffer (100 mM, pH 7.4, 100 mM NaCl) displayed a \( T_m \) value of 78 °C. The enzyme was therefore considered to be thermostable, due to the high \( T_m \) value. But in fact, \( \text{Cv-ATA} \) is inactive at that temperature and shows poor activity at elevated temperatures (\textbf{Paper I}). The low operational stability of \( \text{Cv-ATA} \) has limited its application in large scale. Consequently, we decided to investigate the parameters that may affect the stability of \( \text{Cv-ATA} \). In our previous study, additives and co-solvents showed positive stabilization effects during enzyme storage and were therefore used as a starting point in this investigation.

Firstly, the melting temperature of holo \( \text{Cv-ATA} \) with different additives, substrates and co-solvents were evaluated (Figure 4). The results showed that the amine substrates (S-alanine and S-PEA) had a negative effect on the enzyme thermal stability by decreasing the \( T_m \) value 10 °C. As amine substrates react with the enzyme, PLP is converted into PMP, which is not covalently bound with the enzyme and can therefore be released from the enzyme active site. This may affect the enzyme stability and is further discussed in section 4.2 (\textbf{paper IV}).

Both, DMSO and methanol displayed a negative effect on the thermal stability of holo \( \text{Cv-ATA} \) (Figure 4). With the increase of DMSO/methanol concentration, a decrease in enzyme thermal stability was shown. It should be noted that the \( T_m \) value could not be determined as the concentration of DMSO and methanol was over 20%. Addition of glycerol and sucrose showed a positive effect on the enzyme thermal stability. The \( T_m \) value of holo \( \text{Cv-ATA} \) increased with increasing glycerol and sucrose concentrations.
Figure 4. The melting temperature ($\Delta T_m$) of Cv-ATA supplemented with different additives or co-solvents. (Paper I)
Figure 5. The storage stability of Cv-ATA at room temperature (23 °C, dark); (A) in HEPES buffer (50 mM, pH 8.2); (B) in HEPES buffer with DMSO; (C) in HEPES buffer with methanol; (D) in HEPES buffer with glycerol; (E) in HEPES buffer with sucrose; (F) in HEPES buffer with PLP (1 mM), S-PEA (5 mM) or surfactants (Brij C10 and octyl β-D-glycopyranoside, 2.5 mg/ml of each). (Paper I)

After that, the storage stability of the holo enzyme was explored by storing holo enzyme in HEPES buffer supplemented with co-solvents (5-50%) or different concentrations of additives at 23 °C in darkness (Figure 5). Surfactant was also included in the latter experiments for their stabilization effect. The results in Figure 5 show that enzyme stored in
HEPES buffer (50 mM, pH 8.2) was inactive after 5 days of storage. In contrast with this, samples showed higher storage stability when stored with addition of co-solvents. An addition of 5% DMSO or methanol showed a slight enhancement of the storage stability, while enzymes stored in 10-40% of DMSO or methanol maintained most of their residual activity after 24 days of incubation at 23 °C in darkness. Adding 50% of methanol in the enzyme stock solution will lead to enzyme denaturation, although it still has a positive effect on the enzyme storage stability. However, a higher glycerol proportion is needed to retain the enzyme stability. Using 20-50% glycerol in the enzyme stock solution could assist the enzyme to maintain 100% of activity after 24 days. Sucrose showed an incapacity to enhance enzyme stability while PLP and the mixed surfactants displayed a slight positive influence on enzyme storage stability.

As an indicator of enzyme degradation, a protein precipitate should be displayed in the solution. However, this phenomenon was only displayed when holo Cu-ATA was stored in 40-50% methanol. Samples stored in HEPES buffer lost the enzyme activity without any visible protein precipitation. Therefore, to explore the oligomerization states of the enzyme, BN-PAGE was performed. The BN-PAGE showed that all
samples containing inactive enzyme showed monomeric form, while all samples with retained activity appeared in dimeric form (Figure 6). Hereby, instead of irreversible unfolding, dimer dissociation was suggested to be the reason for holo Cv-ATA inactivation.

**Figure 7.** Residual activity of Cv-ATA after storage at 4 °C for 21 days in HEPES buffer with or without co-solvent, (A) methanol or (B) glycerol. Samples for activity determination were taken before or after samples were reactivated at 37 °C. (Paper I)

**Figure 8.** (a) Residual activity (%) of Cv-ATA stored at 65 °C in HEPES buffer with or without co-solvents (methanol, DMSO or glycerol) or sucrose. (b) Residual activity of Cv-ATA in various concentrations of co-solvents at 23 °C. (Paper I)

After purification, the fresh Cv-ATA solution was stored at 4 °C before use. Only around 10% of the original activity could be detected when the enzyme was directly employed to catalyze a reaction after storage. Despite this, storing the enzyme at 4 °C is commonly used since it could predominantly prevent irreversible denaturation of the enzyme. An additional Cv-ATA reactivation process at 37 °C is necessary to recover 100% of enzyme activity after storage at 4 °C. Storing Cv-ATA with different co-solvents at 4 °C was also explored (Figure 7). After 21 days of
storage at 4 °C, enzyme samples were reactivated at 37 °C and the enzyme activity was measured at selected time points. The samples stored in HEPES buffer only maintained 7% residual activity after storage in the fridge. Samples with 20-50% glycerol kept their full activity. The enzyme obtained an increased activity with the raise of the methanol concentration in the storage solution. After 4 hours of incubation at 37 °C, all samples recovered 100% of residual activity. In addition, the enzyme could gain 114% residual activity after being stored in 50% glycerol at -20 °C for six months, while the corresponding samples stored at 23 °C retained 77% of residual activity.

The storage behaviour of Cv-ATA at a high temperature (65 °C) was also explored. Co-solvents and additives in selected concentrations were used in the experiments (Figure 8). Figure 8a shows that Cv-ATA is inactive in half an hour when incubated at 65 °C and addition of methanol, DMSO and sucrose did not improve the enzyme stability. The only sample showing a positive result contained 50% glycerol. After 24 hours of storage, Cv-ATA stored in 50% glycerol still retained around 20% of residual activity. This result proved that glycerol could enhance Cv-ATA thermal stability. In addition, the holo enzyme-catalyzed reaction in co-solvents has also been investigated (Figure 8b). The result showed that Cv-ATA had reduced initial activity with the increase of the co-solvents proportion in the reaction mixture. Among them, glycerol displayed the lowest negative influence on the enzyme activity.

Furthermore, reactions catalysed with lyophilized holo Cv-ATA were explored. The reactions were run with 20 mg lyophilized enzyme in 1 ml of dry organic solvents (MTBE, isooctane or toluene) at 23 °C (Figure 9). The conversions of the biocatalytic reactions were analyzed using GC after 24 or 48 hours of reaction. Holo Cv-ATA co-lyophilized with Brij®C10 and octyl β-D-glycopyranoside (1:1) showed a 5-fold enhanced conversion compared with samples lyophilized without surfactant.
Figure 9. The lyophilization of Cv-ATA with surfactant and the effect on the reaction in organic solvents. The reactions were run in MTBE, isooctane or toluene with (+) or without (-) surfactants. The conversion (%) was calculated after 24 hours (□) or 48 hours (■) of reaction. (Paper I) Reactions (1 mL) consisted of 20 mg lyophilized Cv-ATA with or without surfactants, S-PEA (50 mM), methoxyacetone (150 mM) and decane (20 mM). Reactions were incubated on a shaker at 23 °C. The surfactants were Brij® C10 and octyl β-D-glycopyranoside (1:1).

In conclusion, the storage stability of holo Cv-ATA could be improved by adding co-solvents into the stock solution. An addition of 5-20% methanol or DMSO showed a significant improvement of enzyme storage stability at 4-23 °C. Addition of 20-50% glycerol in the stock solution could also significantly improve enzyme storage stability at -20 to 65 °C. Adding co-solvents in the stock solution in certain proportions could assist the holo enzyme to maintain 100% of residual activity after 24 days of storage at 23 °C while the sample stored in HEPES buffer lost its activity in 5 days. The enzyme thermostability at 65 °C could be enhanced by adding 50% of glycerol in the stock solution, whereas one sample gained (114%) activity after 6 months storage in -20 °C in the same solution. Both, methanol and glycerol can enhance substrate solubility in water solution. However, methanol is easier to remove from the enzyme stock or the reaction solution. Both solvents can be applied to enhance the operational stability of holo Cv-ATA. Regarding reactions in neat organic solvents, the co-lyophilization of the holo enzyme with surfactant could clearly enhance the product conversion.
4.2 Unfolding of Cv-ATA

As previously shown, Cv-ATA was inactive after 3-7 days of storage at 23 °C. The potential enzyme unfolding during storage or during an operational process required to be analyzed. Consequently, the unfolding behavior of Cv-ATA was studied by tryptophan fluorescence intensity (FI). In each Cv-ATA monomer, there are 11 tryptophan amino acid residues. Among them, Trp60 is the only residue located close to the PLP binding site.

Firstly, the unfolding of apo_{app} and holo_{sat} Cv-ATA was monitored. The tryptophan FI was monitored every minute (1000 cycles in total) at 23 °C (extinction at 280 nm and emission at 355 nm). The apo_{app} enzyme incubated in HEPES buffer displayed a higher unfolding rate compared with enzyme incubated in phosphate buffer (Figure 10a). Simultaneously, the Schiff base degradation in holo_{sat} Cv-ATA was faster in phosphate buffer compared to in HEPES buffer (Figure 10b). This result indicated that the phosphate ions may have a stabilizing effect on apo enzyme, but can compete with the PLP about the PGBC (see part 4.3). The holo_{sat} enzyme incubated in phosphate showed a steady FI during the first 200 min followed by an increased FI (Figure 10a). At the same time, the Schiff base of the holo_{sat} enzyme in phosphate buffer decreased faster compared to the sample in HEPES buffer (Figure 10b). This result indicates that phosphate ions can compete with PLP about the position in the PGBC.

In figure 10a, the apo_{app} Cv-ATA showed a high initial FI compared to the holo_{sat} enzyme. Others have also detected this phenomenon for other cofactor dependent enzymes^{43, 144}. PLP had low FI at extinction 280 nm and emission 355 nm, which suggests that the free PLP released from the holo enzyme is not causing the increase of FI. Therefore, the rearrangement of Cv-ATA induced by PLP coordination may be the reason that leads to low FI. However, experimental support is still needed for this hypothesis. Compared to apo_{app} Cv-ATA, the FI decrease rate of holo_{sat} Cv-ATA was low during enzyme storage. Two reasons for this have been suggested: 1) the higher stability of the holo enzyme; and 2) the PLP release during storage leads to that the holo enzyme turns to apo and results in increasing FI, which would balance a decreased FI induced by unfolding.
Figure 10. Unfolding of the apo_{app} and holo_{sat} forms of Cv-ATA incubated in HEPES (H) and phosphate buffers (P) (50 mM, pH 8.2) at 23 °C. FI of (a) tryptophan with extinction at 280 nm and emission at 355 nm and (b) Schiff base with extinction at 415 nm and emission at 560 nm was monitored. (Paper IV)

Figure 11. Urea-induced unfolding of apo_{app} and holo_{sat} Cv-ATA in HEPES buffer (50 mM, pH 8.2) (a) monitored by measuring tryptophan FI (4 M urea) and (b) Schiff base FI (0-8 M urea). (Paper IV)

The relation between the release of PLP and the steady holo_{sat} enzyme unfolding (Figure 11a) was illustrated by increasing the enzyme unfolding rate by utilizing urea. In Figure 11a, holo_{sat} enzyme supplemented with 4 M urea showed a rapid initial FI increase. At the same time, the corresponding apo_{app} enzyme preparation showed decreased FI with different rate at different periods of time. Schiff base measurements showed that the holo_{sat} enzyme decreased fast in the beginning of the incubation (Figure 11b). Higher concentrations of urea resulted in increased unfolding rates. For samples incubated in 4 M urea, the Schiff base FI decreased during the first 50 min (Figure 11b). At the same time, the tryptophan FI of the holo_{sat} enzyme increased (Figure 11a). Hypothetically, the initial increased FI of holo_{sat} enzyme could be due to the presence of apo enzyme after PLP release. The steady unfolding curve
of the holo\textsubscript{sat} enzyme is partly influenced by the PLP release. This means it may be too complex to study holo \textit{Cv}-ATA unfolding by only tryptophan FI measurements.

Therefore, the unfolding process of \textit{Cv}-ATA during incubation at varying pH was also evaluated by measuring the tryptophan FI (Figure 12). The apo\textsubscript{app} enzyme showed a similar unfolding rate at the different pH, which indicates that the pH had a minor effect on the enzyme tertiary structure. Different pH will lead to shifts in the monomer dimer equilibrium (MD equilibrium). A low pH resulted in enzyme monomerization and increased PLP release from the holo\textsubscript{sat} enzyme. Therefore, the pH affected the tryptophan FI of the holo enzyme.

![Figure 12](image-url)

**Figure 12.** Unfolding of the apo\textsubscript{app} and holo\textsubscript{sat} \textit{Cv}-ATA incubated in phosphate buffer (50 mM, pH 3, 5, 8) at 23 °C.

The effect of substrate binding on the unfolding rate of \textit{Cv}-ATA was evaluated by monitoring tryptophan FI of the apo\textsubscript{app} \textit{Cv}-ATA incubated in HEPES buffer supplemented with different substrates (acetophenone, pyruvate, \textit{S}-alanine or \textit{S}-PEA) (Table 1). Lower concentrations (<5 mM) of substrates displayed minor effects on the \textit{Cv}-ATA unfolding. But, as the concentration was higher (>10 mM), the unfolding rate increased by 20%. Samples incubated with amines (\textit{S}-PEA and \textit{S}-alanine) showed a higher unfolding rate compared with samples incubated with ketones (acetophenone or pyruvate).

To sum up, the low tryptophan FI of the holo enzyme compared to that of the apo enzyme could be caused by the presence of PLP. Hereby, apo enzyme was suggested for the unfolding measurements. A lower buffer
pH did not influence the enzyme tertiary structure, while the tested substrates accelerated the enzyme unfolding.

**Table 1.** Unfolding rate of the apo<sub>cvs</sub> Cv-ATA incubated in HEPES buffer (50 mM, pH 8.2) with four kinds of substrates at 23 °C.

<table>
<thead>
<tr>
<th>(a.u./min)</th>
<th>ACP</th>
<th>S-Alanine</th>
<th>Pyruvate</th>
<th>S-PEA</th>
</tr>
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<tbody>
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<td>mM</td>
<td></td>
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<td>146</td>
<td></td>
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</tr>
<tr>
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<td>25</td>
<td>-185</td>
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</tbody>
</table>

ACP, acetophenone; S-PEA, (S)-1-phenylethyl amine; a. u. arbitrary unit
4.3 The effect of molecular coordination into the phosphate group binding cup on the stability of ATAs

Cv-ATA is known to be active in its holo dimeric form. The two active sites are located at the interface of the two subunits\textsuperscript{103}. Both subunits are necessary to provide amino acid residues to fulfill the catalytic reaction. There are several reported crystal structures of the apo and holo forms\textsuperscript{103}. PLP is covalently bound with one catalytic lysine as an internal aldimine, the phosphate group of PLP coordinates into PGBC with hydrogen bonds from both subunits\textsuperscript{103}. In section 4.1, it was shown that the inactive Cv-ATA was in monomeric form (Figure 6), and therefore the dimer dissociation was suggested to be the principal reason for Cv-ATA inactivation. As it was showed in figure 5f, the addition of 1 mM PLP resulted in prolonged Cv-ATA storage stability. Thus, phosphate ions (both from PLP and from phosphate buffer) coordinated to the PGBC may influence both dimer stabilization and enzyme activity. To test this assumption, the binding affinity of PLP and phosphate ions to the PGBC of ATAs was compared and their effect on ATAs stability was explored in several ways.

Firstly, the dissociation constant (\(K_D\)) between PLP and apo Cv-ATA was measured by differential scanning fluorimetry (DSF)\textsuperscript{145}. The \(K_D\) of PLP binding to the PGBC of Cv-ATA and Vf-ATA has been measured to 78 \(\mu\)M and 8 \(\mu\)M, respectively (\textbf{Paper II and III}). The \(K_D\) of PLP to Cv-ATA is relatively high compared with Vf-ATA and other transaminases\textsuperscript{146, 147}. This indicates that Cv-ATA has a low PLP binding affinity.

Normally, the freshly purified Cv-ATA or Vf-ATA is incubated with 10 mM PLP in 4 °C overnight and then kept at 37 °C during one hour, followed by a desalting process to remove the unbound PLP. After that, the obtained enzyme was defined as holo\textsubscript{sat} enzyme (considered to be 100% holo enzyme, one monomer covalently bound with one PLP). Then, the apo enzyme ratio in the freshly purified enzyme was determined by the \(\text{Abs}_{415}/\text{Abs}_{280}\) method. After purification, around 95% Cv-ATA was determined to be in apo form (here called apo\textsubscript{app} Cv-ATA), while Vf-ATA had 64% in apo from after purification (\textbf{Paper II and III}). The relatively low PLP binding affinity to the PGBC of Cv-ATA was supposed to be the
reason for the high proportion of the apo form in freshly purified Cv-ATA solution. When a re-purification of holo enzyme was performed, 50-70% holo Cv-ATA and around 23-31% holo Vf-ATA lost their PLP during this process. This result illustrates that PLP release during purification is one possible reason for the high amount of apo enzyme. Compared to the Cv-ATA, Vf-ATA showed a higher binding affinity with PLP, and PLP release may therefore have a lower effect on its operational stability.

The dissociation constant of inorganic phosphate and apo<sub>app</sub> Cv-ATA was determined to 14 mM. This number is 180 fold higher than that of the $K_D$ for PLP binding. During this project, the enzyme preparations were stored at higher phosphate buffer concentration (50-100 mM) than the determined $K_D$ value (14 mM). Inorganic phosphate ions present in the buffer solutions may therefore compete with PLP about the position in the PGBC during enzyme storage. In addition, it should be noted that no binding of HEPES to the apo enzyme could be measured using the same method.

The holo<sub>sat</sub> enzyme-catalyzed reactions were performed in varying buffer concentrations (25-500 mM, pH 8.2) (Figure 13a). The initial activity of holo<sub>sat</sub> Cv-ATA and Vf-ATA decreased with increasing buffer concentration, which indicates that an increased buffer concentration may inhibit the enzyme (Figure 13a). Importantly, the enzyme showed a higher initial activity in phosphate buffer, almost 2 fold, compared to the enzyme in HEPES buffer. However, less product formation of Cv-ATA was shown in phosphate buffer compared to in HEPES buffer at the same concentration (Figure 13c). Therefore, our conclusion is that phosphate buffer should not be recommended as reaction buffer for holo<sub>sat</sub> Cv-ATA. In contrast, holo Vf-ATA performed with almost the same product formation in both of the two buffers (Figure 13d), which is different compared to Cv-ATA. As Vf-ATA showed a higher binding affinity to PLP, the stability of holo Vf-ATA in phosphate buffer is less affected by inorganic phosphate ions. Since the enzyme has a higher initial rate in phosphate buffer, it should be highly recommended to use phosphate buffer as a Vf-ATA reaction buffer.

A possible competition between phosphate ions and PLP was explored by monitoring the FI of the Schiff base of holo<sub>sat</sub> enzyme during enzyme storage. The result showed that the degradation rate of FI decreased with
increased buffer concentration (Figure 13b). An increased buffer concentration might prevent PLP release from the enzyme, and hence enhance the structural holo \( \text{Cv-ATA} \) and \( \text{Vf-ATA} \) stability. But, an increased buffer concentration could result in decreased enzyme residual activity (Figure 13a). Also, incubating the holo enzyme in phosphate buffer resulted in an increased FI degradation rate, compared to when the enzyme was incubated in HEPES buffer. As a result, holo\( \text{sat} \) enzyme was inhibited. These results indicate that our hypothesis that the phosphate ions may compete with PLP about the position in the PGBC might be verified.

**Figure 13.** (a) Residual activity of holo\( \text{sat} \) enzyme in varying buffer concentrations (25-500 mM, pH 8.2, \( \triangle \) \( \text{Cv-ATA} \) in HEPES buffer, \( \Box \) \( \text{Vf-ATA} \) in HEPES buffer, \( \blacktriangle \) \( \text{Cv-ATA} \) in phosphate buffer, \( \blacksquare \) \( \text{Vf-ATA} \) in phosphate buffer); (b) The rate of the reduction of the FI (Schiff base) of holo\( \text{sat} \) enzyme incubated in varying buffer concentrations (25-500 mM, pH 8.2, \( \triangle \) \( \text{Cv-ATA} \) in HEPES buffer, \( \Box \) \( \text{Vf-ATA} \) in HEPES buffer, \( \blacktriangle \) \( \text{Cv-ATA} \) in phosphate buffer, \( \blacksquare \) \( \text{Vf-ATA} \) in phosphate buffer). (c) Product (acetophenone) formation using holo\( \text{sat} \) \( \text{Cv-ATA} \) catalysis in two different buffers (25 mM, pH 8.2, blue line for HEPES buffer, red line for phosphate buffer); (d) Product (acetophenone) formation using holo\( \text{sat} \) \( \text{Vf-ATA} \) catalysis in two different buffers (25 mM, pH 8.2, blue line for HEPES buffer, red line for phosphate buffer). (Paper II and III)
The behavior of holo$\text{sat}$ Cv-ATA in HEPES or phosphate buffer was compared by storing the enzyme in different buffer concentrations at 23 °C in light (to induce photo degradation of PLP) or in the dark (Figure 14a and 14b). Higher buffer concentrations may help the holo enzyme to retain PLP, although, this slightly decreases the enzyme activity. Samples stored in 500 mM HEPES both in light or in the dark maintain around 30% higher residual activity compared with samples stored in 25 mM HEPES after 48 h of storage. Enzyme stored in phosphate buffer showed a decreased initial residual activity with increased buffer concentration (25-200 mM), which may be caused by a competition between released PLP and free inorganic phosphate about the position in the PGBC.
The effect of inorganic phosphate on the enzyme thermal stability was explored by melting temperature measurements. After 3 days of storage, the $T_m$ values of holo_{sat} enzyme were higher than those of apo_{app} enzymes both for $Cv$-ATA and $Vf$-ATA. Apo_{app} $Cv$-ATA stored in phosphate buffer was 4 °C higher, than the corresponding sample stored in HEPES buffer (Figure 15a). In contrast with this, the phosphate ions showed less effect on the $T_m$ value of apo_{app} $Vf$-ATA. In addition, no significant effect could be detected for the holo enzyme in phosphate buffer (Figure 15a). The holo_{sat} enzyme displayed increased $T_m$ values with increased PLP concentration in both HEPES and phosphate buffer (Figure 15b). The $T_m$ value for both $Cv$-ATA and $Vf$-ATA could increase 5 or 8 °C with added PLP (0-5 mM). Additional PLP could stabilize $Cv$-ATA (apo and holo), while the inorganic phosphate may stabilize the apo enzyme, but have a negative effect on the holo enzyme stability.

As discussed previously, the PGBC is located at the interface between two subunits, and the coordination into the PGBC may influence the enzyme dimerization. BN-PAGE was applied to explore the impact of inorganic phosphate on the dimeric structure of apo $Cv$-ATA (Figure 16). Freshly purified apo_{app} $Cv$-ATA was stored in the dark for 3 days at 23 °C. As shown in Figure 16, all samples retained their dimeric structure after mixing with different buffers. However, after 3 days of storage, the samples stored in HEPES buffer (50 or 500 mM, pH 8.2) dissociated to monomer (Lane 1 or 2). The sample stored in phosphate buffer (500 mM, pH 8.2) showed only a minor fraction of dimer dissociation to monomer.
The sample stored in HEPES buffer (50 mM, pH 8.2) with a 10 mM PLP addition, maintained 100% dimeric structure (Lane 4). These results illustrate that both PLP and inorganic phosphate ions have stabilizing effects on the dimeric structure of apo Cv-ATA.

Figure 16. Fresh samples of apo_app Cv-ATA stored in different buffer solutions (pH 8.2). Samples was loaded on BN-PAGE directly after mixing in different buffer (Day 1) or after 3 days of storage (Day 3) at 23 °C in darkness. Lane 1: HEPES buffer (50 mM). Lane 2: HEPES buffer (500 mM). Lane 3: Phosphate buffer (500 mM). Lane 4: HEPES buffer (50 mM) supplemented with 10 mM PLP. (Paper II)

Figure 17. Urea (2 M) induced unfolding monitored by fluorescence intensity (a.u.). The Fluorescence emission spectra were recorded using SYPRO® orange dye. Samples in HEPES buffer are marked with solid lines, samples in phosphate buffer are marked with dotted lines.) (a) Cv-ATA; (b) Vf-ATA. (Paper II and III)

The impact of the phosphate group (from PLP or inorganic phosphate ions) on enzyme unfolding was also explored (Figure 17). All samples (apo_app or holo_sat) were prepared in either HEPES or phosphate buffer, with or without PLP and supplemented with 2 M urea. The apo_app enzyme
incubated in HEPES unfolded faster than the other samples. This illustrates that both PLP and inorganic phosphate may protect the apo enzyme from urea-induced unfolding. For the holo enzyme, the presence of inorganic phosphate speeds up the enzyme unfolding. The presence of PLP did not show an obvious protecting effect on Cv-ATA, but it showed significantly reduced unfolding of Vf-ATA.

**Figure 18.** Far-UV CD spectra of Cv-ATA in apo\textsubscript{app} or holo\textsubscript{sat} form. All samples were stored in HEPES (left) or phosphate (right) buffer (50 mM, pH 8.2) at 4 or 23 °C for 0 (fresh) or 5 days. The vertical black line is marking the ellipticity at 222 nm ([θ]222nm). (Paper II)

The effect on the Cv-ATA secondary structure by phosphate groups was also explored by circular dichroism measurements (Figure 18). The holo\textsubscript{sat} enzyme showed a higher content of α-helixes (the ellipticity at 222 nm)\textsuperscript{148} compared to the apo\textsubscript{app} enzyme, both before and after storage. After 5 days of storage at 4 or 23 °C, the secondary structure of the enzyme samples displayed different reduced levels of α-helix elements. The enzyme stored at 4 °C showed higher secondary structure compared to the enzyme stored at 23 °C, which means that a low temperature could prevent the enzyme from irreversible unfolding. Most of the samples stored in phosphate buffer showed a higher level of secondary structure compared to samples stored in HEPES buffer. This phenomenon might be explained by the ability of phosphate ions to coordinate into the PGBC. Inorganic phosphate may have the same stabilizing effect on the secondary structure of Cv-ATA, as PLP.
In conclusion, phosphate groups (originating either from phosphate buffer or from PLP) can coordinate into the PGBC and stabilize ATAs by promoting dimer association and preventing irreversible unfolding. The apo_{app} Cv-ATA showed a higher $T_m$ value when stored in phosphate buffer (68 °C), compared with when stored in HEPES buffer (64 °C) (Paper II). Also, a reduced unfolding rate was displayed in phosphate buffer, compared to in HEPES buffer.

The Cv-ATA enzyme displayed a relatively lower affinity to PLP compared to the values published for other ATAs ($K_D = 78 \ \mu$M). After purification, almost 95% of the enzyme was present in the apo form. One reason for this could be that PLP is released during the purification. This was evidenced by re-purification of the holo_{sat} enzyme. Inorganic phosphate could bind to the PGBC and the $K_D$ value was determined to 14 mM. Therefore, the competition between PLP and phosphate ions will lead to holo enzyme inactivation as shown in Figure 14.

In contrast, Vf-ATA did not display all these phenomena (Paper III). It showed a higher binding affinity to PLP compared to Cv-ATA ($K_D = 8 \ \mu$M), and hereby, it is less affected by inorganic phosphate ions. The Schiff-base of Vf-ATA has a higher degradation rate in phosphate buffer than in HEPES, which is similar to Cv-ATA. The increased degradation rate in phosphate buffer for Vf-ATA confirms that PLP release from Cv-ATA is faster than from Vf-ATA (lower PLP affinity of Cv-ATA compared to Vf-ATA). The initial reaction rate of the holo Vf-ATA in phosphate buffer is higher than in HEPES buffer, moreover, the product amount was almost the same in the two buffers. These results suggest that phosphate buffer is a better reaction buffer for Vf-ATA. Both PLP and inorganic phosphate could improve the thermal stability of apo_{app} Vf-ATA, as well as prevent the enzyme from chemical or long-term storage unfolding.
4.4 Inactivation pathway of Cv-ATA

Previously, it has been discussed that Cv-ATA is active in holo dimeric form with two active sites located at the interface between the two subunits\(^{103}\). Generally, the reason for a dimeric enzyme to be inactivated during storage is dimer dissociation and structural assembly alteration\(^{44, 144, 149}\). However, it is a more special situation with holo Cv-ATA. The cofactor PLP is located at the subunit interface and therefore may affect the hydrophobic interaction between the two monomers. Phosphate ions from PLP or phosphate buffer coordination into the PGBBC was shown to affect the Cv-ATA dimerization and stabilization in section 4.3. Moreover, Cv-ATA has a lower binding affinity with PLP. Thus, the coordination of PLP leads to a more complex process of holo Cv-ATA degradation compared with some other ATAs.

Our proposed inactivation pathway of holo Cv-ATA (Scheme 4) is based on a monomer dimer equilibrium (MD equilibrium). The presence of 95% apo dimeric Cv-ATA after purification and the relatively lower PLP affinity (section 4.3) forms the basis for our hypothesis that PLP may be released before the holo dimer dissociates to monomer, which finally lead to inactive enzyme (Pathway (a)). The pathway starts from holo Cv-ATA in holo dimeric form with PLP covalently bound to the catalytic lysine through a Schiff base linkage (D-PLP). As shown in Scheme 4, PLP can be released from D-PLP before (pathway (a)) or after the dimer dissociation (pathway (b)). Both pathways finally lead to the folded monomer (M) and free PLP in the solution. Among them, the M-M, M-PLP and M are suggested to be inactive.

![Scheme 4](image)

**Scheme 4.** The proposed inactivation pathway of holo Cv-ATA. Abbreviations: D-PLP, folded holo-dimer-PLP complex; M-PLP, folded monomer-PLP complex; M-M-PLP, folded dimer with one monomer bound PLP and one monomer unbound with PLP; M-M, folded apo-dimer; M, folded monomer. (Paper IV)
Figure 19. (a) Melting temperatures ($T_m$) of holo$_{sat}$ enzyme in different enzyme concentrations (HEPES buffer, 50 mM, pH 8.2); (b) Relative activity (%) of holo$_{sat}$ enzyme prepared in various concentrations stored in HEPES buffer (50 mM, pH 8.2) at 23 °C in darkness. The enzyme concentrations were 0.1; 0.2; 0.5; 1 or 5 mg/ml, the measurements lasted up to 7 days. (Paper I and IV)

Figure 20. BN-PAGE of holo$_{sat}$ Cv-ATA stored in HEPES buffer (50 mM pH 8.2) at varying enzyme concentrations at 23 °C in darkness. The enzyme concentrations were 0.2; 0.5; 1 or 5 mg/ml, the measurements lasted up to 7 days. (Paper I)

Generally, the storage stability of dimeric enzymes is dependent on the enzyme concentration, which also influences the MD equilibrium57. Consequently, holo Cv-ATA stored in different enzyme concentrations has been explored by melting temperature measurements, activity assays (Figure 19) and combined with BN-PAGE analysis (Figure 20). The $T_m$ value of fresh holo Cv-ATA (0.1 mg/ml) was measured to 63.2 °C in HEPES buffer (50 mM, pH 8.2). The $T_m$ value of the holo enzyme showed enzyme concentration dependence and performed a 7 °C increase when the enzyme concentration was raised from 0.1 mg/ml to 5 mg/ml (Figure 19a). After 3-4 days of storage in darkness, enzyme stored in lower concentrations than 5 mg/ml only maintained around 40-60% residual activity. Also, a clear monomer lane appeared on the BN-PAGE gel. After 7 days of storage, samples stored in 5 mg/ml still retained 80% of residual activity and mostly displayed dimer. All the other samples were inactive.
and displayed monomer on the gel. This result illustrates that the stability behavior of \( \text{Cu-ATA} \) follows the MD equilibrium, and that holo \( \text{Cu-ATA} \) is inactive as it is dissociated to monomer. This forms the basis of the proposed inactivation pathway shown in Scheme 4.

To compare \( K_a \) and \( K_b \) in Scheme 4, the PLP release during the enzyme storage is a decisive factor to monitor. Hereby, the PLP degradation induced by light has been utilized to show the PLP release during the storage (Figure 21)\(^{26} \). The storage stability of the enzyme was detected in light or darkness, without or with PLP (0.5-5 mM). The sample without additional PLP was inactive after 72 hours when stored in light, while the same sample stored in the dark still maintained 64% of residual activity (Figure 21b). The residual activity of the sample with extra PLP decreased faster within the first 48 hours, but still retained 10-35% residual activity after 72 hours of storage (Figure 21a). Corresponding to this, samples with additional PLP appeared to have higher activity during the whole storage process in darkness, and maintained 80-100% residual activity after 72 hours of storage (Figure 21b). To sum up, \( \text{Cu-ATA} \) has a higher storage stability in darkness compared with in light. This phenomenon confirms that PLP is released from D-PLP during enzyme storage (pathway (a)). The D-PLP could be stabilized by additional PLP when stored in the dark, and this illustrates that the PLP release from the enzyme is reversible. In section 4.3, PLP was shown to have a low binding affinity to PLP compared with other ATAs, and its dissociation during the purification resulted in the high apo enzyme proportion present in the enzyme solution (section 4.3). Incubation of the freshly purified apo enzyme with additional PLP could produce holo enzyme again. This result supports the reversible PLP release during storage (pathway (a)). It also supports the hypothesis that the release of PLP from the holo enzyme is advantageous (\( K_a > K_b \)) in the holo dimer inactivation process, and that it happens during the whole storage time. Strategies for improvement of the enzyme’s capacity to preserve PLP may improve the operational stability of \( \text{Cu-ATA} \). Moreover, this also opens a new view to improve the operational stability of cofactor dependent enzymes.

To compare the \( K_b \) and \( K_c \) (Scheme 4), different preparations of apo\(_{\text{app}}\) and holo\(_{\text{sat}}\) \( \text{Cu-ATA} \) were analyzed by BN-PAGE (Figure 22). After 5 days of storage, both apo\(_{\text{app}}\) and holo\(_{\text{sat}}\) \( \text{Cu-ATA} \) preparations stored at 4 °C (Lane 4-7) maintained the dimeric form, whereas samples stored at 23 °C
(Lane 1-3) showed varying degrees of dimer dissociation (Figure 22). For samples stored at 23 °C, the apo\textsubscript{app} enzyme sample was mostly dissociated to monomer (Lane 3), while the holo\textsubscript{sat} enzyme (stored with or without additional PLP) in Lane 1 and 2 were only slightly dissociated. Therefore, the apo enzyme dissociating to monomer in Scheme 4 is suggested to be more advantageous than the holo enzyme ($K_c > K_b$). Simultaneously, this result also supports that PLP coordinated to the PGBC could improve the Cv-ATA dimerization and stabilization, as was discussed in section 4.3.

![Figure 21](image1.png)

**Figure 21.** Storage stability of holo\textsubscript{sat} Cv-ATA with pyridoxal-5'-phosphate (PLP) (▲0.5 mM PLP, ●1 mM PLP, △2 mM PLP, ○5 mM PLP) or without PLP (■0 mM PLP). Samples stored in HEPES buffer (50 mM, pH 8.2) at 23 °C in (a) light or (b) darkness. (Paper IV)

![Figure 22](image2.png)

**Figure 22.** A BN-PAGE of Cv-ATA in different enzyme preparations after 5 days of storage in darkness. Lane 1: Fresh holo\textsubscript{sat} Cv-ATA with 3 mM PLP suspension stored at 23 °C. Lane 2: Fresh holo\textsubscript{sat} Cv-ATA stored at 23 °C. Lane 3: Fresh apo\textsubscript{app} Cv-ATA stored at 23 °C. Lane 4: Fresh holo\textsubscript{sat} Cv-ATA with 3 mM PLP suspension stored at 4 °C. Well 5: Fresh holo\textsubscript{sat} Cv-ATA stored at 4 °C. Well 6: Fresh apo\textsubscript{app} Cv-ATA stored at 23 °C. Well 7: Supernatant before purification stored at 4 °C. (Paper I)

Others have shown that aspartate transaminase dissociates from dimer to monomer at low pH values\textsuperscript{53}. The active holo\textsubscript{sat} dimer of Cv-ATA has previously shown to be inactive at low pH (3-4)\textsuperscript{54}. Hereby, the ability of monomeric Cv-ATA to retain the PLP ($K_d$ in Scheme 4) was further
explored by utilizing the knowledge that low pH induces dimer dissociation\textsuperscript{69}. Buffers, HEPES and phosphate (50 mM), were prepared at various pH-values. The holo Cv-ATA initial activity, storage stability and Schiff base degradation in different buffer pH were investigated (Figure 23). The holo\textsubscript{sat} Cv-ATA reached the highest initial activity at pH 8.2 in both HEPES and phosphate buffers (Figure 23a). The initial activity decreased with reduced buffer pH, and was finally inactive at pH 3-5. Holo\textsubscript{sat} Cv-ATA showed a similar behavior when stored in buffers of different pH compared with reactions in the same buffers (Figure 23c). Enzyme stored at pH 8 showed the highest storage stability. After 120 min, the enzyme still maintained 70% residual activity, while it was inactivated after 15 min at pH 3-5 (Figure 23c).

Corresponding Schiff base fluorescence intensity measurements was made to explore the Schiff base degradation during storage (Figure 23b and 23d)\textsuperscript{38,70}. After adding 0.04 mg holo\textsubscript{sat} Cv-ATA into the specified buffer followed by 5 s of mixing the enzyme with buffer and then the monitoring was started. In all samples, a fast decrease of the Schiff base during the initial 15 min was evident (Figure 23d). As the enzyme solution was added to the buffer, the enzyme concentration was diluted 10 fold, and the reduced enzyme concentration will shift the MD equilibrium from dimer to monomer. Consequently, the rapid FI decrease during the initial 15 min of the samples was suggested to be induced by shifting of MD equilibrium from dimer to monomer, and the monomer was therefore suggested to be uncapable of retaining PLP. For samples stored at pH 3-7, the buffer pH induced shifting of the MD equilibrium was suggested to be the reason for the resulting higher decrease rate of FI compared to at pH 8. In addition, at pH 3 the Schiff base was degradated 4 fold faster than at pH 8 (Figure 23b). Almost no Schiff base could be detected at pH 3 after 20 min of incubation (Figure 23d), while no activity could be detected after 20 min of incubation at pH 3 (Figure 23c). This indicates that PLP release resulted in inactivation of the holo enzyme incubated at pH 3. In the following BN-PAGE analysis, Cv-ATA was shown to dissociate to monomer after 20 min of incubation at pH 3. This result proved that the monomeric form of Cv-ATA cannot retain its coordination with PLP. Therefore, the PLP release from apo monomer in Scheme 4 is suggested to be advantageous in comparison to the other three steps (\(K_d \gg K_a, K_b, K_c\)). PLP could be released from the monomer as soon as it is formed.
Figure 23. The effect of pH on the activity and stability of holo\textsubscript{sat} Cv-ATA. (a) Initial activity of holo\textsubscript{sat} Cv-ATA in buffers of different pH (□ HEPES buffer, ■ phosphate buffer). (b) Schiff base (FI) decrease rate of holo\textsubscript{sat} Cv-ATA stored in phosphate buffer (pH 3-10). (c) Storage stability of holo\textsubscript{sat} Cv-ATA in phosphate buffer at varying pH. (d) Monitoring of the Schiff base (FI) of holo\textsubscript{sat} Cv-ATA stored in phosphate buffer (pH 3-10). (\textit{Paper IV})

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Figure 24. BN-PAGE and residual activities (%) of Cv-ATA before and after reactivation. Lanes 1-3: Holo\textsubscript{sat} Cv-ATA incubated in 50 mM phosphate buffer at pH 3, 5, and 8 for 20 min. Lanes 4-6: Holo\textsubscript{sat} Cv-ATA after reactivation. (\textit{Paper IV})
It is worth noting that the apo monomeric \textit{Cu}-ATA could reversibly form the holo dimer by the re-activation process. The holo \textit{Cu}-ATA only maintains 5, 76\%, and 84\%, respectively, of the residual activity after 20 min of incubation in phosphate buffer (pH 3, 5, and 8) (Figure 23). After this, the enzyme was reactivated by buffer exchange to HEPES buffer (50 mM, pH 8.2) supplemented with 10 mM PLP, followed by incubation at 4 °C overnight. The BN-PAGE and the residual activity measurements showed that the reactivated samples (pH 3, 5, and 8) could re-associate to dimer and recover 66\%, 100\%, and 114\%, respectively, of residual activity (Figure 24). These results illustrated that the inactivation pathway is reversible.

To conclude, the inactivation pathway of holo \textit{Cu}-ATA was studied by comparing each step. The holo enzyme with additional PLP showed increased stability when stored in darkness compared to in light (Figure 21). This result proved the PLP release and degradation during storage (Pathway (a)). Additional PLP in the enzyme storage solution could improve the enzyme storage stability (Figure 21), which indicates that the PLP is reversibly released from D-LLP. For Pathway (b), the inactive samples were shown to be in monomeric form (Figure 6 and 20 in Paper I); and the dissociation of dimer lead enzyme inactivation (Figure 23 and 24 in Paper IV). Both these results suggest that the M-PLP is inactive or even small probability to present in the equilibrium for the release of PLP from M-PLP is advantageous to all steps (discussed latter). If we consider the D-PLP could be stabilized by additional PLP (Figure 21), that behavior indicated that the PLP reversible release from D-LLP is advantageous than the dissociation of D-PLP to M-PLP ($K_a > K_b$). The BN-PAGE showed that the apo dimer enzyme (M-M) dissociating to monomer is more advantageous than the holo enzyme (D-PLP) ($K_c > K_b$) (Figure 22). By utilizing the low buffer pH 3, the M-PLP lost the PLP as soon as it is formed in 20 min. This phenomenon illustrates that the presence of M-PLP is ephemeral and hard to detected ($K_d \gg K_a, K_b, K_c$). Consequently, the relations of the equilibria for the dimer \textit{Cu}-ATA inactivation are summarized as $K_d \gg K_a, K_c > K_b$. It is suggested that pathway (a) is more advantageous than pathway (b), which consequently suggests that preventing PLP to be reversibly released from \textit{Cu}-ATA is fundamental for improving the operational stability of \textit{Cu}-ATA.
5. Concluding remarks and future outlook

Previously, ATAs have been explored and applied in cascades and one-pot reactions. For that, the enzyme operational stability is an important factor, which influences enzyme activity during the process. Therefore, several methods have been utilized to investigate the enzyme stability of ATAs in this thesis.

The substrate concentration is known to influence the biocatalytic reaction equilibrium. Also, many substrates for ATAs have a low solubility in aqueous media. Therefore, co-solvents are often employed to increase the substrate solubility during the transamination reaction process. However, addition of co-solvents may lead to enzyme denaturation. Paper I showed that the Cv-ATA was inhibited when used in reactions in aqueous solution with co-solvents (such as DMSO, methanol). Adding co-solvents (DMSO, methanol, and glycerol) in the Cv-ATA storage solution could significantly improve the enzyme storage stability and even improve the enzyme thermal stability. Co-lyophilization of Cv-ATA with surfactants also improved the enzyme stability in neat organic solvents. These results not only provide a better storage suggestion, but also demonstrate that a deep understanding of the Cv-ATA inactivation mechanism is necessary.

Theoretically, the enzyme operational stability is connected to both thermal stability (reversible) and long-term (irreversible) stability. Therefore, finding suitable strategies to improve enzyme operational stability becomes more complex. In Paper I, the inactive enzyme stocks were all shown to be in monomeric form while the active enzyme samples displayed dimeric construction on BN-PAGE. Hence, dimer dissociation was suggested to be the primary reason for Cv-ATA inactivation. As apo Cv-ATA showed a faster monomerization compared with holo enzyme, the role of PLP in enzyme inactivation has to be further explored.

The “phosphate group binding cup” in ATAs is located at the interface of two subunits. Since PLP has been shown to affect the Cv-ATA activity, stability and unfolding, it was meaningful to explore the role of the PGBC related to Cv-ATA stability. In paper II, phosphate ions from phosphate buffer and PLP both showed coordination with three amino acid residues of Cv-ATA in docking simulations. In addition to that, the phosphate
buffer showed competition with PLP about binding with Cv-ATA, and therefore, holo Cv-ATA showed a decrease of the storage stability in phosphate buffer. In contrast, additional inorganic phosphate ions or PLP could help apo Cv-ATA to resist dimer dissociation and irreversible unfolding.

In **Paper IV**, the inactivation pathway of Cv-ATA was studied. The PLP release from holo Cv-ATA was advantageous compared to the holo Cv-ATA monomerization. Therefore, the low PLP affinity to Cv-ATA has been shown to be the reason for the poor Cv-ATA operational stability. One solution is PLP supplementation. Recording the FI of tryptophan is a common method used for monitoring enzyme unfolding. The holo enzyme showed a markedly lower FI compared with the apo enzyme. Since a reasonable explanation for this phenomenon is lacking, the unfolding behavior of Cv-ATA incubated in various environments was explored by tryptophan FI in **paper IV**. The lower FI of holo Cv-ATA was supported to be caused by PLP binding. PLP release during enzyme storage will result in the increase of FI. Hereby, the apo enzyme was assumed to be a better choice for enzyme unfolding experiments.

Almost two decades have past since Vf-ATA was found. Its substrate scope has been studied and protein engineering has been made to improve its properties. However, there was still a lack of knowledge about the Vf-ATA operational stability. In **paper III**, various operational environmental effects on Vf-ATA activity, stability and unfolding were explored, which can guide further application of the Vf-ATA.

With more and more in-depth investigations of enzyme properties and reaction mechanisms, there is a rapidly increasing utilization of enzymes in chemical reactions for the synthesis of chemical products in industry. There are increasing awareness in companies of the trend of using biocatalytic processes to substitute chemical ones. The future of the commercialization of ATAs is promising and hopeful. There are lots of research efforts about ATAs during the last decades. Methods to improve ATAs stability and activity for special substrates and conditions have been investigated, and this will turn ATAs into a more attractive option in industrial application. Investigation of inactivation mechanisms of the enzymes and enhancing their stability in various operational environments are crucial for their large-scale application. In this study of
the holo Cu-ATA inactivation pathway, strategies aiming at improving dimer association and PLP affinity are highly recommended for improving their stability further. However, there is still a need for a lot of basic research for successfully utilizing these enzymes in large-scale industrial process.
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7. Bibliography


