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The effect of phosphate group binding cup coordination on the stability of the amine transaminase from *Chromobacterium violaceum*

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ABSTRACT

The amine transaminase from *Chromobacterium violaceum* (*Cv*-ATA) is a pyridoxal-5'-phosphate (PLP) dependent enzyme. The biological activity of this enzyme requires the formation of a holo homo dimer. The operational stability of *Cv*-ATA is, however, low due to dimer dissociation. At the enzyme dimeric interface, two phosphate group binding cups (PGBC) are located. Each cup coordinates the phosphate group of PLP by hydrogen bonds originating from both subunits. Hypothetically, molecular coordination of phosphate groups (PLP or free inorganic phosphate) into the PGBC can affect both dimer stabilization and enzyme activity. To test this assumption, the influence of phosphate (as a functional group in PLP or as free inorganic anions) on the stability and activity of *Cv*-ATA was explored by various biophysical techniques. The results show that *Cv*-ATA has a relatively low affinity towards PLP, which results in an excess of apo dimeric enzyme after enzyme purification. Incubation of the apo dimer in buffer solution supplemented with PLP restored the active holo dimer. The addition of PLP or inorganic phosphate into the enzyme storage solutions protected *Cv*-ATA from both chemical and long term storage unfolding. The use of phosphate buffer leads to faster inactivation of the holo enzyme, compared to the use of HEPES buffer. These results open up for new perspectives on how to improve the stability of PLP-dependent enzymes.

1. Introduction

Amine transaminases (ATAs) constitute a group of PLP-dependent enzymes of industrial interest for stereoselective preparation of chiral amines from pro-chiral ketones [1–12]. Despite the great potential of using ATAs as biocatalysts for chiral amine production, the industrial large-scale applications involving amine transaminases are few. A general limitation to the application of native ATAs in larger scales is the insufficient enzyme stability [13,14]. However, there are ATAs that have been successfully modified by protein engineering and further applied for large-scale or industrial production of: the anti-diabetic compound Sitagliptin [8] or substituted *(S)*-2-aminotetralins [9–12].

The ATA from *Chromobacterium violaceum* (*Cv*-ATA, CV2025) is a well-known enzyme having a broad substrate scope towards various amino donors [6,15–17]. Several crystal structures of *Cv*-ATA [18,19] have been solved and its reaction mechanism has been exploited by quantum chemical methods [20,21]. Also, important enzyme characteristics of *Cv*-ATA, such as pH optimum [22,23], operational stability [13,14,18] and enantioselectivity [24] have been explored. To make it possible to compare different enzyme batches and ATA variants, an active-site titration method has been established [22]. More recently, *Cv*-ATA has been explored for whole-cell transamination in metabolically active *Saccharomyces cerevisiae* [25,26].

Further, all known PLP-dependent enzymes are active as multimeric molecules [27]. The known ATAs are biologically active as homo-oligomers, commonly dimers or tetramers [28]. *Cv*-ATA is active as a homo dimer of about 100 kDa, which is associated by non-covalent bonds. Each monomer contains one active site, which includes one PLP-binding pocket and one substrate-binding site. The two active sites are located at the dimer interface and contain amino acid residues from both monomers [18]. The phos-

Abbreviations: *Cv*-ATA, amine transaminase from *Chromobacterium violaceum*; CD, circular dichroism; DSF, Differential Scanning Fluorimetry; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid); K_D, dissociation constant PGBC Phosphate Group Binding Cup; PLP, pyridoxal-5'-phosphate; BN-PAGE, Blue Native Polyacrylamide Gel Electrophoresis.

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phate group of PLP (the non-ester phosphate oxygen atoms) is coordinated into "the phosphate group binding cup" [29] (PGBC). The PGBC is formed by amino acid residues originating from both monomers. The enzyme affinity towards PLP, i.e. how tight PLP binds into the PLP-binding pocket, has been shown to depend on the coordination of its phosphate group into the PGBC [30]. The active site of *Cv*-ATA is flexible and the formation of the holo enzyme causes major structural rearrangements [18]. Active site flexibility has previously been reported as a key factor leading to low PLP affinity [31].

The activity and stability of ATAs has been shown to depend on various storage and reaction conditions [11,13,14,18,32–34]. It has previously been shown that; the enzyme concentration, PLP- or co-solvent supplementation, improves both the activity and the dimeric structure stability of *Cv*-ATA [13,18,32]. Generally, for multimeric enzymes, the dissociation of subunits is the first step in the inactivation process [35–39]. Therefore, retained monomer association is an important and decisive factor for their stability. Previously, we showed that the low stability of *Cv*-ATA depends on dimer dissociation [13]. Therefore, methods to improve the dimer association are called for.

Previous studies have shown that *Cv*-ATA has higher transamination activity in HEPES buffer compared to phosphate buffer [15,22]. This may be due to coordination of free inorganic phosphate into the PGBC. Inorganic phosphate originating from phosphate buffer can coordinate into the PGBC of PLP-dependent enzymes [40,42]. We hypothesize that molecular coordination of phosphate groups into the PGBC promotes subunit association. Inorganic phosphate may compete with PLP about the position in the PGBC and cause enzyme inhibition. Due to the common use of phosphate buffer in ATA catalyzed transamination reactions, an extensive exploration of its impact on both activity and stability of *Cv*-ATA was performed to test this hypothesis.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich.

2.2. Expression of Cv-ATA

One colony, of *E. coli* BL21(DE3) containing the gene of *Cv*-ATA (CV2025) in pET28a(+) with a N-terminal His_6-tag, was incubated in 10 mL LB (Luria-Bertani) medium (1% tryptone, 1% NaCl and 0.5% yeast extract) supplemented with kanamycin (50 mg L^{-1}) at 37 °C and 220 rpm overnight. The overnight culture was transferred into 1 L of TB (Terrific Broth) medium (1.2% peptone, 2.4% yeast extract, 72 mM K_2HPO_4, 17 mM KH_2PO_4 and 0.4% glycerol) supplemented with kanamycin (50 mg L^{-1}). The culture was incubated at 220 rpm and 37 °C until OD_{600} reached 0.7-0.9. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) was added in order to induce enzyme expression at 220 rpm and 20 °C for 24 h. The cells were harvested by centrifugation (Beckman coulter Avanti J-26XP) at 8000 rpm and 4 °C for 30 min.

2.3. Purification of Cv-ATA

The cell pellet was re-suspended in Ni-NTA lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) prior to cell disruption by sonication (Branson Sonifier 250) and cell debris removal by centrifugation (20,000 rpm, 30 min, 4 °C). The obtained supernatant was sterile filtered (0.45 μm) before addition to a Ni-NTA Sepharose column. The Ni-NTA Agarose (Cat no. IB 2-3201-010) was purchased from IBA. The purification procedure was performed according to manufacturerís manual. The enzyme was eluted using Ni-NTA elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Buffer exchange was performed on PD10 column (Cat no. 17-0851-01, GE Healthcare) to HEPES or sodium phosphate (Na_2HPO_4) buffer (50 mM, pH 8.2), according to the manufacturer's manual. The obtained enzyme solution was almost colorless.

2.4. Obtaining the apparent apo (apo_{app}) and saturated holo ($holo_{sat}$) form of Cv-ATA

The almost colorless enzyme solution obtained after purification and desalting on PD10 column were in apo_{app} form. To obtain the $holo_{sat}$ form, apo_{app} *Cv*-ATA was incubated with 5 mM PLP (Sigma, P9255) at 4 °C overnight followed by 1 h at 37 °C the next day. Then, a second desalting step over a PD10 column to HEPES or sodium phosphate buffer (50 mM, pH 8.2) was made with the aim to remove the excess of unbound PLP in solution. All fresh enzyme samples were stocked in HEPES or sodium phosphate buffer (50 mM, pH 8.2) and stored at 4 °C or appointed condition. The total enzyme concentration was measured at 280 nm on a NanoDropTM1000 Spectrophotometer. The fraction of holo enzyme in solution was determined by dividing the absorption measured at 415 nm (Schiff base) by the absorption at 280 nm (total protein) on a Cary® 50 UV–vis Spectrophotometer.

2.5. Melting temperature

Melting temperatures (T_m) were measured using DSF [44] on a CFX96™ Real-Time PCR Detection System and a C1000™ Thermal Cycler. All enzyme samples (20 μl) contained; 1 mg mL^{-1} *Cv*-ATA (or specified concentration) and (3.75×) SYPRO® Orange protein gel stain diluted in HEPES or sodium phosphate buffer (50 mM, pH 8.2). The temperature range was set (15–95 °C) with a constant temperature rate of 1 °C min^{-1}. The obtained data was fitted to the Boltzmann equation in order to calculate the melting temperatures.

2.6. Dissociation constant K_D measurement

Differential Scanning Fluorimetry (DSF) were used to determine the K_D value of PLP and inorganic phosphate to apo *Cv*-ATA at 20 °C. The following dilution series were prepared for PLP (4.8 μM to 10 mM), HEPES and sodium phosphate (15.2 μM to 500 mM). Each sample (50 μl) contained; 0.11 mg mL^{-1} enzyme, (16×) SYPRO® Orange protein gel stain (Sigma, S5692), 5 μl of prepared ligand (PLP, sodium phosphate or HEPES) and buffer (50 mM, pH 8.2). The thermal denaturation data was fitted to the Boltzmann equation to obtain the melting temperatures. These values were then used to calculate the K_D values using a single site ligand binding model [43].

2.7. Enzyme activity assay

The *Cv*-ATA-catalyzed reactions (1 mL) were prepared as follows in 1 mL UV-cuvettes: 1 μg *Cv*-ATA, 2.5 mM pyruvate and 2.5 mM (*S*)-1-phenylethylamine in HEPES or sodium phosphate buffer (50 mM, pH 8.2). The reactions were mixed by (3×) gentle inversion of the cuvette containing the reaction solution. Then, the initial rate of acetophenone formation at 245 nm was measured on a Varian Cary® 50 UV–vis Spectrophotometer.

For the $holo_{sat}$ *Cv*-ATA stored in buffer supplemented with PLP experiments. $Holo_{sat}$ *Cv*-ATA (0.1 mg mL^{-1}) was prepared with PLP (0.5–5 mM) or without PLP supplementation in either HEPES or sodium phosphate buffer (50 mM, pH 8.2) and stored at 23 °C in dark. The activity assay was taken at selected time point in 72 h.