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Explaining Operational Instability of Amine Transaminases: Substrate-Induced Inactivation Mechanism and Influence of Quaternary Structure on Enzyme–Cofactor Intermediate Stability

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Supporting Information

ABSTRACT: The insufficient operational stability of amine transaminases (ATA) constitutes a limiting factor for high productivity in chiral amine synthesis. In this work, we investigated the operational stability of a tetrameric ATA with 92% sequence identity to a *Pseudomonas* sp. transaminase and compared it to the two commonly used dimeric ATAs from *Chromobacterium violaceum* and *Vibrio fluvialis*. In the presence of substrate, all three ATAs featured reduced stability in comparison to their resting stability, but the tetramer showed slower inactivation rates than the dimeric ATAs. Kinetic and thermodynamic analysis revealed an amine donor induced inactivation mechanism involving accumulation of the less stable aminated enzyme–cofactor intermediate. Dissociation of the enzyme–PMP complex forms the unstable apoenzyme, which can rapidly unfold. Crystal structure analysis shed light on the structure–function relationship suggesting that the cofactor–ring binding element is stabilized in the quaternary structure conferring higher operational stability by minimizing PMP leakage and apoenzyme formation. In contrast to the common practice, increasing the amine acceptor content improved the stability and substrate turnover of dimeric ATAs. An extra supply of the pyridoxal cofactor (PLP) enhanced the stability of dimeric and tetrameric ATAs but reduced the transamination activity. The ATA inactivation mechanism described here provides valuable aspects for both process development and protein engineering.

KEYWORDS: amine transaminase, stability, substrate-induced inactivation, pyridoxamine 5'-phosphate, unfolding, aggregation

1. INTRODUCTION

Biocatalysis, the use of enzymes for organic synthesis of fine chemicals and pharmaceuticals, has become a powerful alternative to chemical catalysts.¹ Replacing organic/metal catalysts by biocatalysts has several advantages, the most important being excellent regio- and stereoselectivity, fewer side products, and reduction in environmental impact.²

Amine transaminases (ATAs) are highly attractive biocatalysts, because they allow the direct, asymmetric amination of ketone substrates, affording optically pure primary and secondary amines.³ Depending on their enantioselectivity, ATAs belong to either the protein fold type I (*S* selective) or IV (*R* selective) of the pyridoxal 5'-phosphate (PLP) dependent enzyme superfamily.⁴ ATAs fall within the subclass of ω -transaminases that accept amines and substrates with a distal carboxylic acid group.⁵ For exertion of their trans-

amination activity, they are highly dependent on establishing an internal aldimine with the cofactor, which constitutes the holoenzyme (E-PLP). During catalysis, PLP serves as a molecular shuttle to transfer the nitrogen atom from the amine donor (amino acid or amine) to the amine acceptor (keto acid or ketone).⁶ Briefly, upon amine donor binding, the internal aldimine is replaced by the external aldimine, which is the intermediate complex between the enzyme and the cofactor–substrate conjugate. Final hydrolysis of the external aldimine yields the keto or ketone product together with the aminated cofactor pyridoxamine 5'-phosphate (PMP)–enzyme complex (E:PMP), which marks the end of the first half-

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reaction. Essentially the reverse reaction occurs upon amine acceptor binding, regenerating the internal aldimine form (E-PLP) and releasing the chiral amine product.

For 20 years, ATAs have been researched to enable their feasible application in the fine chemical and pharmaceutical industry.⁷ However, a major limitation for successful application constitutes their insufficient operational stability at high substrate concentrations, denaturing solvents, and elevated temperatures. Operational stability is a term that describes the ability of an enzyme to resist irreversible changes of its protein structure during catalysis.⁸ Such permanent loss of activity usually involves a reversible unfolding and an irreversible kinetic inactivation step.⁹ For industrial applications, the resting stability is relevant for enzyme storage (thus also referred to as storage stability), whereas the operational stability constitutes an important parameter for process productivity and economy.

Different stabilization methods have been investigated to counteract ATA inactivation. Immobilization of free enzymes or whole cells containing ATAs in aqueous and nonaqueous media was explored in several studies,¹⁰ but the residual activities of the stabilized ATAs varied significantly among the different formulations and were frequently poor. Recently, the use of surfactants as additives in organic media showed a stabilizing effect,¹¹ but the underlying mechanism and scalability remains unknown. Protein engineering constitutes a more directed approach to improve intrinsic stability. As of today, two mutagenesis studies yielded ATAs with improved operational stability: The R-selective ATA from *Arthrobacter citreus* was engineered toward stability and substrate specificity via directed evolution, yielding improved activity toward production of substituted 2-aminotetralines.¹² In a pioneering joint venture between the companies Merck and Codexis, a similar ATA was successfully modified by semirational design. Thereby, the ineffective chemical amination step in the synthesis of the antidiabetic drug sitagliptin could be substituted by the more efficient and environmentally friendly bioamination process.¹³

The most well-known and employed ATAs for chiral amine synthesis are arranged either in the form of a homodimer (PDB ID: 4A6T, 4E3R, 3WWH) or homotetramer (PDB ID: 4B9B, 3A8U, 3NSM). The active site is at the interface of the small and large domains of one monomer. Amino acid residues of the neighboring monomer complete the active site. In all PLP-dependent enzymes, the cofactor is firmly anchored in the “phosphate group binding cup”, which is composed of conserved amino acid residues from both monomers.¹⁴ However, at least in the dimeric ATAs from *Chromobacterium violaceum* (CvATA) and *Vibrio fluvialis* (VfATA), the E:PMP complex can dissociate once it is formed.¹⁵ Diffusion of the noncovalently bound PMP out of the active site was exploited in the production of PMP from PLP and amine donor, performing the first half of the transamination reaction only.¹⁵ However, VfATA quickly loses activity under such conditions.^{16,17} The loss of cofactor is assumed to play an important role in enzyme stability.^{11,18,19} Moreover, contradictory effects on activity and stability have been reported when using different PLP concentrations in complete and half-transamination reactions.^{20–24} So far, investigations were focused on dimeric ATAs, and it remains unexplored whether tetrameric ATAs behave differently.

Despite the tremendous importance of operational stability for industrial applications, the precise mechanism of ATA inactivation is still largely unknown.¹¹ Detailed mechanistic knowledge about the thermodynamics and kinetics of ATA

inactivation, as well as the factors that influence this process, is highly needed for targeting this Achilles' heel of ATA-catalyzed biosynthesis.

In this work, we investigated the mechanistic reasons for the poor operational stability of ATAs and the influence of quaternary structure, cofactor, and substrates. By means of thermodynamic and kinetic experiments, we found that accumulation of the first half-reaction intermediate (E:PMP) promotes ATA inactivation. The stability of this intermediate is greater in the tetrameric ATA, which was found to be more robust and exhibited improved operational stability in comparison to the dimeric CvATA and VfATA. Our analysis identified a structural domain that appears to confer stability. Furthermore, we present new insight into the effect of substrate ratio and PLP concentrations on stability and transamination activity. Altogether, this study opens new opportunities for successful protein engineering and application of ATAs in chiral amine synthesis.

2. MATERIALS AND METHODS

2.1. Materials. All chemicals such as (S)-1-phenylethylamine (PEA), 4-phenyl-2-butanone (PB), (S)-(+)-4-phenyl-2-butanamine (PBA), isopropylamine (IPA), acetone (ACE), L-alanine (ALA), sodium pyruvate (PYR), pyridoxal 5'-phosphate (PLP), pyridoxamine 5'-phosphate (PMP) and all solvents were purchased from Sigma-Aldrich Corp. in Sweden. The tetrameric wild-type amine transaminase (wt-ATA), obtained from a metagenomic library, ATA from *Chromobacterium violaceum* and *Vibrio fluvialis* were supplied by c-LEcta GmbH, Leipzig, Germany, as a crude enzyme powder. Wt-ATA, CvATA, and VfATA catalyze the conversion between 40 mM PEA and PYR (pH 8, 30 °C) with 4.5, 2.5, and 3.2 U/mg, respectively. Purified wt-ATA (N-His₆-wt-ATA, 48 U/mg) was provided by Manchester University. Standard 1.5 mL glass HPLC vials and 4.5 mL glass vials were purchased from VWR of Sweden and were (screw) capped with a silicon septum (1.8 mm thick) coated with a PTFE (Teflon) layer facing the solution.

2.2. Enzyme Stability Measurements: Operational and Resting Stability. To access enzyme (ATA) stability, two methods were employed: (a) activity-based measurements and (b) thermodynamic analysis measuring the change in melting point (T_m). The former quantifies the loss in active enzyme using a standardized activity assay, whereas the latter approach accesses thermodynamic stability by measuring protein unfolding via the temperature at which half of the enzyme amount is unfolded. The *operational stability* refers to the stability in the presence of substrate (operating respectively catalyzing conditions), whereas the *resting stability* (E-PLP) is without substrate (e.g., only in buffer). Both stability states can be investigated under different conditions, varying for example temperature and PLP content.

2.2.1. Activity-Based Measurements. For quantification of the activity (initial rates, $\mu\text{mol min}^{-1} \text{mg}^{-1}$), the enzyme (typically 5–10 mg/mL) was incubated in 4.5 mL glass vials under different conditions (*incubation solution*: e.g. 2 mL with IPA and PB, increased temperatures, etc.). Samples (50 μL) were taken at distinct time intervals (with a 100 μL Hamilton syringe), diluted in 50 μL of 20 mM phosphate buffer pH 8 including 0.1 mM PLP, and centrifuged (15 s, 10⁴g), and subsequently 60 μL was transferred into an HPLC vial with a 100 μL glass inset and placed in the HPLC autosampler (30 °C). Then, to determine the residual activity, 10–15 μL

(0.025–0.075 mg enzyme) of the 60 μ L was automatically injected and mixed into the *activity assay* solution (1 mL, 40/40 mM PEA/PYR, 0.1 mM PLP in 20 mM phosphate buffer pH 8). The formation of ACP was followed for 15 min, with automatic sampling every 3 min. All initial rate measurements at 25–45 $^{\circ}$ C were conducted in the HPLC autosampler with automated mixing and reaction monitoring, as described elsewhere.²⁵ For initial rate determination above 45 $^{\circ}$ C, reactions (typically 0.1 mg/mL enzyme) were performed in 4.5 mL glass vials for 15 min in a thermoshaker and ACP formation was followed by transferring 10 μ L samples into 40 μ L of 1 M NaOH solution (to quench the reaction), centrifuged (15 s, 10⁴g), and analyzed by HPLC. To ensure that the observed activity loss was not reversible within a time frame of at least 1 h, we diluted (1:1) the samples in 0.1 mM PLP (20 mM buffer, pH 8) and incubated them for 1 h at room temperature. No significant difference in residual activity was noticed in comparison to samples analyzed immediately (data not shown). Cofactor inhibition experiments were conducted in 40 mM PEA/PYR, 20 mM phosphate buffer pH 8 at 30 $^{\circ}$ C, with varying PLP content (0.1–10 mM). For the ATA productivity comparison, the reaction between PEA and PB was chosen in order to be able to follow the change in all reaction components (PEA, PB, ACP, PBA) by HPLC.

Free cofactor (PLP and PMP) and enzyme internal cofactor analysis was performed using high-performance size exclusion chromatography (SEC) with 10 mg/mL enzyme as described in ref 25. For first half of the reaction experiments either SEC or reverse phase HPLC analysis was used. Using PLP (typically 0.5 mM) and amine donor (20–50 mM PEA, IPA or ALA) resulted in the formation of the corresponding ketone (or keto acid) and PMP. The formation of ACP is proportional to PMP release, and the former was quantified by reverse phase HPLC. A 40 mM sodium phosphate buffer (pH 8) was used to ensure higher buffer capacity (because PMP constitutes a stronger base than ALA).

2.2.2. Melting Point Measurements. Melting points (T_m) were measured using differential scanning fluorimetry (DSF).²⁶ Enzyme solution (6.25 mg/mL of purified N-His₆-tag wt-ATA, dialyzed against 50 mM sodium phosphate buffer, 0.1 mM PLP, pH 8) was mixed with (0.5 μ L/1 mL enzyme solution) SYPRO Orange protein gel stain (Sigma–Aldrich, S5692). Substrate solution was then added (20 mM stock of IPA, ALA, PEA, and PEA/PYR all in 50 mM buffer pH 8 and 0.1 mM PLP or 1 mM PLP) to finally yield 0.25 mg of enzyme and 10 mM substrate in 200 μ L (96-well PCR plate, BIO-RAD). To determine melting points, a CFX96 Real-Time PCR Detection System was used together with the C1000 Thermal Cycler. The temperature was set to 25 $^{\circ}$ C and increased at a rate of 1 $^{\circ}$ C/min until 95 $^{\circ}$ C was reached. In order to form enzyme intermediates (E:PMP), the reaction solutions were incubated for 30 min at room temperature before the measurements. Fluorimetric data were exported, and MS Excel (in-house template for data processing) was used to perform a Boltzmann fit of the data to determine melting points. Values are given as the mean of three individual samples for each set of reaction conditions.

2.3. HPLC Analysis. A Dionex UltiMate 3000 RSLC system (Thermo Fisher Scientific, Sweden) equipped with a UV/vis detector was used. The amines and ketones were analyzed (1 μ L injection sample) by reverse phase chromatography using a pH-stable Gemini-NX 3u C18 110 Å column (dimensions 100 $\times$ 2 mm) (Phenomenex, Denmark). Amine separation was

conducted at high pH (11), creating uncharged species. Reaction compounds were separated in isocratic mode (35% acetonitrile and 65% water at pH 11, adjusted with HPLC grade NaOH) at a flow rate of 0.45 mL/min and detected at 260 nm with the UV/vis detector (column and detector compartment were kept at 30 $^{\circ}$ C). Under such conditions PEA (1.6 min) and ACP (2.4 min) eluted within a total analysis time of 3 min. For PBA (2.7 min) and PB (3.8 min) a total analysis time of 4.3 min was required. For size exclusion chromatography (SEC) experiments a Yarra 3u SEC-2000 column (300 $\times$ 4.6 mm, Phenomenex, Denmark) was used with 100 mM sodium phosphate as elution buffer (pH 6.8, containing 0.3 M NaCl) in an isocratic manner. The column compartment was tempered (20 $^{\circ}$ C), and the protein content of each eluting peak was measured at 280 nm. Similarly, protein-bound and free cofactor were measured at three different wavelengths simultaneously (PMP at 325 nm, PLP at 389 nm, external and internal aldimine at 410 nm). For more details see ref 25.

2.4. Crystallization and Structure Determination of wt-ATA. Purified protein (N-His₆-wt-ATA) was concentrated to 15 mg/mL in 50 mM Tris-HCl pH 8.0, 0.3 M NaCl, and 0.5 mM PLP prior to setting up crystallization screens. Initial crystallization screening was carried out in 96-well sitting-drop plates at 293 K with the help of a mosquito robot (TTP Labtech) and using various commercial screens. Tiny yellow crystals appeared after roughly 16–18 h with a condition from the polyethylene glycol (PEG)/ion screen (Hampton Research), containing 100 mM sodium malonate, pH 5.0, and 12% (w/v) PEG 3350. Addition of 4% (v/v) formamide to the crystallization solution was essential to increase the crystals to a typical size of 120 $\times$ 100 $\times$ 50 μ m³. For cryoprotection, crystals were briefly rinsed in crystallization solution supplemented with 20% (v/v) PEG 400 and flash-cooled with liquid nitrogen. A similar procedure was used to obtain PMP-bound crystals, by briefly soaking crystals in cryoprotectant solution supplemented with 100 mM L-alanine. X-ray diffraction data were collected at 100 K at beamline P14 of the EMBL Outstation at DESY in Hamburg (PLP-bound wt-ATA, PDB ID 5LH9) and at beamline ID29 of the ESRF in Grenoble (PMP-bound wt-ATA, PDB ID 5LHA), using PILATUS 6 M detectors (Dectris, Switzerland). Reflections were indexed and integrated using XDS,²⁷ and the merging of the data was done with AIMLESS²⁸ from the CCP4 software suite²⁹ (see Table S2 in the Supporting Information for data collection and processing statistics). Phaser³⁰ was used for molecular replacement with a mixed input model prepared from two homologous structures (PDB entries 3FCR and 3I4J). The quality of the molecular replacement phases and the resulting electron density map was high enough to allow automatic model building of PLP-bound wt-ATA with ARP/wARP.³¹ The resulting model was further improved by several manual model-building cycles using Coot³² alternated with coordinate and temperature factor refinement using Phenix_refine.³³ Water molecules were added during the last cycles of model building and refinement. The PMP-bound structure was determined by molecular replacement using a dimer of the PLP-bound structure as a search model. Model building and refinement was performed in the same way as for PLP-bound wt-ATA. All models were validated using Molprobit.³⁴

2.5. Accession Numbers and Structural Analysis. The atomic coordinates and structure factors for PLP-bound wt-ATA (entry 5LH9) and PMP-bound wt-ATA (entry 5LHA) have been deposited in the PDB, Research Collaboratory for

Structural Bioinformatics, Rutgers University.³⁵ Protein structures were visualized and analyzed with the PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC.

3. RESULTS AND DISCUSSION

3.1. Identification and Structural Characterization of wt-ATA. A new wild-type amine transaminase (wt-ATA) with 92% sequence identity to an uncharacterized enzyme from a *Pseudomonas* sp. (Table S1 in the Supporting Information) was identified by metagenomic library screening. The cloned enzyme showed promising catalytic properties in utilizing the industrially attractive amine donor isopropylamine (IPA) to aminate 4-phenyl-2-butanone (PB), yielding the pharmaceutically relevant 4-phenyl-2-butanamine (PBA).³⁶ Dynamic light scattering and size exclusion chromatography (SEC) revealed that purified wt-ATA forms homotetramers in solution (Figure S1 in the Supporting Information). The enzyme was crystallized and its crystal structure determined at 1.95 Å resolution (Table S2 in the Supporting Information). Except for the N-terminal His-tag, which appears to be disordered, all residues of wt-ATA showed interpretable electron density and were included in the model (residues 1–449). Inspection of the electron density further revealed that in all four active sites PLP had formed a Schiff base (internal aldimine) with the active site lysine (Lys284). The overall subunit structure and oligomeric organization of PLP-bound wt-ATA is closely similar to the fold-type I tetrameric ATAs from *Pseudomonas aeruginosa* (PDB entry 4B9B)¹⁹ and *Arthrobacter aurescens* (PDB entry 4ATP),³⁷ despite low sequence identities of 30–34%. In addition to the PLP-bound wt-ATA structure, a crystal structure of PMP-bound wt-ATA was determined at 1.89 Å resolution (Table S2) by soaking a PLP-bound crystal with L-alanine (ALA). Soaking PLP-bound crystals in an ALA solution resulted in a color change of the crystals from pale yellow to colorless, consistent with the conversion of the internal aldimine to PMP. Except for an adjustment in the side chain conformation of Lys284, and breakage of the covalent bond with the cofactor, no significant structural rearrangements were observed in the wt-ATA tetramer upon conversion from the PLP internal aldimine state to PMP (Figure S1).

3.2. Effect of Substrate on ATA Stability. For activity-based stability measurements, an incubation solution was used to expose ATA to different reaction conditions (e.g., varying or adding substrate, solvent, cofactor, temperature, etc.), and the residual activity was determined by transferring the enzyme samples at specific time intervals into the assay solution (optimal conditions: 40/40 mM PEA/PYR, 30 °C). The activities in the assay reaction (PEA/PYR) of directly transferred samples ($t = 0$ h) for wt-ATA, CvATA, and VfATA (5 mg/mL) prepared in buffer were comparable, amounting to 4.5, 2.5, and 3.2 U/mg, respectively. All ATAs exhibited about 100-fold lower activities for the model reaction (IPA/PB). More details are given in section 2.2.

The thermostability of tetrameric wt-ATA differed significantly on incubation in the presence or absence of substrate, which corresponds to its resting and operational stability, respectively. To simulate industrial-like conditions^{13,38} during incubation, the amine donor (IPA, 1 M) was supplied in excess to the poorly soluble amine acceptor (PB, solubility 10 mM at 25 °C). Comparing the change in activity after 4 h of incubation at 45 °C under resting and operating conditions, as shown in Figure 1A, revealed that wt-ATA is significantly more stable in the resting state in comparison to its operating state.

Figure 1. Influence of temperature and substrate on amine transaminase (ATA) stability: (A) operational and resting stability of tetrameric wt-ATA at different temperatures taken after 4 h incubation; (B) comparison of wt-ATA stability with two dimeric ATAs (VfATA and CvATA) under resting (buffer, empty bars) and operational conditions (filled bars) using 1 M isopropylamine (IPA) and 10 mM PB; (C) varying the IPA content at constant 10 mM PB for all three ATAs shown as a semilogarithmic plot, where residual activities are given relative to the respective resting activity of each individual enzyme taken at the same time interval (4 h). Reaction conditions: 5 mg/mL of enzyme powder and 20 mM Na-phosphate buffer, 0.1 mM PLP, and final reaction pH 8 was used in all experiments. Further explanations are given in section 3.2.

The residual activity of wt-ATA in the resting state had hardly changed after 4 h, whereas about 50% loss of activity was observed in the presence of substrate (1 M IPA and 10 mM PB). In other words, the temperature at which the residual activity diminished by 50% (T_{50}) decreased for the operational state by about 9 °C in comparison to the resting state. Conclusively, such a difference in stability under resting and

operational conditions suggests a substrate-induced inactivation mechanism.

High amine donor concentrations have earlier been recognized as inefficient synthesis conditions for amine production employing dimeric VfATA,^{17,39,40} while in other studies the presence or absence of substrate as well as different substrate concentrations were noticed to influence ATA stability.^{11,16,20} To generalize the observed substrate-induced inactivation mechanism, we compared the inactivation kinetics of wt-ATA with the two most frequently employed ATAs: CvATA and VfATA from *Chromobacterium violaceum* and *Vibrio fluvialis*, respectively. The last two enzymes are catalytically active as homodimers,^{16,41,42} and all three ATAs show relatively low similarity in their amino acid sequence (Table S1 in the Supporting Information). The time-dependent change in residual activity of all three ATAs presented in Figure 1B shows that CvATA and VfATA have lower operational and resting stabilities than wt-ATA. The resting stabilities of both dimers were about half that of the tetrameric wt-ATA, and their operational stabilities diminished almost completely within 4 h at 45 °C (Figure 1B). For all three ATAs, loss of activity was accompanied by strong protein precipitation that correlated with the decrease in ATA (protein) content and was particularly pronounced in the presence of substrate (Figure S2 in the Supporting Information).

The pronounced instability of three ATAs under operating conditions was found to strongly depend on the substrate ratio (amine donor to acceptor). As shown in Figure 1C, the residual activity after 4 h of incubation at 45 °C inversely correlated with the IPA content, when the acceptor PB was kept saturated (ca. 10 mM). The active enzyme fractions of the two dimers CvATA and VfATA rapidly diminished at substrate ratios greater than 1, whereas the activity losses for tetrameric wt-ATA were less drastic, indicating a higher tolerance against amine donor (IPA) inactivation. Further experiments (Figure S3 in the Supporting Information) supported the observation that rather the amine donor to acceptor ratio affects the degree of inactivation (thus operational stability) and not the presence of both substrates per se. Indeed, incubating ATAs in a solution with low substrate ratio seemed to have an activating effect. As shown in Figure 1C (and Figure S3), the residual activity of wt-ATA after 4 h at 45 °C exceeded its reference activity on incubation in buffer (resting stability) by about 10% and 14% at a 1:1 and 1:2 ratio between IPA and PB, respectively. This apparent activation effect at lower substrate ratios was even more pronounced for dimeric CvATA and VfATA and yielded up 40% residual activity (stability) improvement (Figure 1C). Notably, for wt-ATA the yellow color of the reaction medium produced by PLP did not change much after 4 h, at all substrate ratios tested, whereas the reaction medium of the two dimers became pale at higher substrate ratios (5:1, 50:1 and 100:1, Figure 1C). Decolorization of the reaction medium indicates the transformation of (here 0.1 mM) PLP into PMP. Depletion of PLP and accumulation of PMP is correlated with decreased stability, as we discuss in more detail in section 3.3.2. Although a certain solvent effect of high IPA content on ATA's stability cannot be excluded, our results indicate that the substrate ratio is the dominating factor. Practically, high substrate ratios are common,⁴³ because of poor amine acceptor (ketone) solubility as well as a means to shift the unfavorable reaction equilibrium. In the following, a detailed investigation on amine donor induced inactivation and the role of the quaternary structure is

given to explain the results demonstrated in Figure 1 and discussed above.

3.3. Amine Donor Induced Inactivation Mechanism and Role of the Quaternary Structure. 3.3.1. *The Less Stable Enzyme–Cofactor Intermediate E:PMP.* An amine donor induced inactivation mechanism, depicted in Scheme 1,

Scheme 1. Postulated Inactivation Mechanism of Amine Transaminases via PMP Dissociation Leading to the Structurally Altered Apoenzyme (E)^a

^aE first unfolds reversibly (E^U) and then aggregates irreversibly (E^I). Reactivation of E occurs upon PLP binding. The first (I) half-reaction yields the ketone product (P_K) and aminated cofactor–enzyme complex (E:PMP) through deamination of the amine donor (S_A). In the second (II) half-reaction amine product (P_A) is formed upon amine acceptor (S_K) binding, regenerating E-PLP.

was postulated to mechanistically describe the observed inactivation behavior and consumption of PLP (Figure 1). We hypothesized that the stability problem is related to the transfer from the stable holoenzyme (E-PLP) to a less stable intermediate form: i.e. the aminated enzyme–cofactor form (E:PMP). The noncovalent E:PMP complex can dissociate and the formed cofactor-devoid apoenzyme (E) features an increased tendency for unfolding and irreversible inactivation through aggregation (Figure S2 in the Supporting Information). An excess of amine donor with respect to the acceptor promotes the first half-reaction I and therefore less stable E:PMP forms accumulate. Equal or higher amine acceptor content will do quite the opposite, promoting the second half-reaction II, restoring the stable E-PLP form from E:PMP.

We sought to test this hypothesis of a thermodynamically less stable E:PMP form by measuring apparent melting points (*T*_m), and the results are shown in Figure 2. In the resting state (E-PLP), wt-ATA displayed a *T*_m value of 63.8 °C (Figure 2). On addition of different amine donors, wt-ATA performed only the I half-reaction, generating E:PMP (Figure S4 in the Supporting Information). Under I half-reaction conditions, the *T*_m value of wt-ATA decreased by about 6 °C for all amine donors tested (Figure 2). When amine acceptor was added, E-PLP was continuously recovered from E:PMP as the transamination cycle (I + II half-reaction) could proceed, yet the measured *T*_m value was still about 4 °C lower than that of the resting state (Figure 2). These results support our hypothesis that converting E-PLP into E:PMP decreases the overall thermodynamic stability of the enzyme.

When the full transamination reaction was catalyzed, an equal ratio between donor and acceptor had a positive effect in

Figure 2. Change in thermal stability (melting point, T_m) of different intermediate states of tetrameric wt-ATA formed at different reaction conditions in the absence (–) or presence of substrate(s) (10 mM). The reference T_m value represents E-PLP (63.8 °C), which was set to 0. The E-PMP was generated through I half-reaction catalysis in the presence of amine donor (S_A). Amine acceptor (S_K) was added to allow restoration of the E-PLP form through the full transamination cycle, I + II half-reaction. The plus sign indicates an extra PLP supply of 1 mM; otherwise 0.1 mM PLP was used. Error bars represent the deviation among three experiments.

comparison to the situation when only amine donor was present, recovering about 2 °C in T_m (Figure 2). This thermodynamic improvement induced by the amine acceptor correlated with the gain in operational stability at reduced amine donor to acceptor ratios (Figure 1C). Amine acceptor binding enables re-formation of E-PLP through the second half-reaction II (Scheme 1), thereby lowering the abundance and residence time of the unstable E:PMP form. Similar behavior was reported for dimeric CvATA.¹¹ In this study, the T_m value of CvATA decreased by about 10 °C, on exposure to the same amine donors (ALA, PEA) as used together with wt-ATA. However, the positive effect of amine acceptor on the stability was stronger for CvATA than for wt-ATA determined in this study. In the work of Chen et al.,¹¹ the T_m value of CvATA exceeded its resting state value in the presence of an acceptor, which was not the case for wt-ATA used in this study (Figure 2 and Figure S5 in the Supporting Information). The differing T_m results between dimeric and tetrameric ATA coincide with the pronounced stabilization effect on dimeric CvATA and VtATA in comparison to wt-ATA at increased amine donor to acceptor ratios (as presented in Figure 1C). For tetrameric wt-ATA, the stability gain above the resting state was only minor and the sole presence of acceptor hardly showed any influence on E-PLP stability (Figure S5). Another stabilizing effect was seen when the PLP concentration in the reaction medium was increased (Figure 2), and the possible reasons for this are discussed in section 3.3.4.

3.3.2. Cofactor Leakage and Binding Properties of Tetramer and Dimer Differ. In the full transamination reaction, both substrates compete for the active site, but high amine donor to acceptor ratios accumulate E:PMP through increased I half-reaction catalysis (Scheme 1). Medium decolorization at high amine donor to acceptor ratios (Figure 1C) supports our hypothesis depicted in Scheme 1. However, in order for substantial PLP conversion to occur, PMP must diffuse out of the active site before new PLP can bind. The tendency for the

exchange between the E:PMP and E-PLP forms was found to be dependent on the quaternary structure. Note that because both dimeric ATAs showed very similar behaviors (Figures S6–S8 in the Supporting Information), the following comparison is mainly given for CvATA and wt-ATA.

When the I half-reaction was performed with 50 mM amine donor (PEA), CvATA aminated all available PLP (2 mM) into PMP (Figure 3A) within 3 h. In contrast, wt-ATA converted

Figure 3. Cofactor release and binding properties of dimeric CvATA (left, gray box) and tetrameric wt-ATA (right, blue box). The free cofactor species (A and B) and enzyme-bound cofactors (C and D) were monitored simultaneously during the first half-reaction catalysis. Free PLP and PMP were detected at 325 and 389 nm, respectively, while the enzyme-bound cofactor is given as the absorption ratio between the specific absorption of cofactor over protein to normalize changes in protein content (E:PMP at 325/280 nm, internal aldimine (E-PLP) and external aldimine absorb both at 410 nm and only their sum is measured at 410/280 nm). E denotes the apoenzyme. To measure all cofactor species (free and bound), SEC was employed to separate and analyze the different compounds simultaneously.²⁵ The reaction solution contained 10 mg/mL of enzyme, 50 mM PEA (S_A), 2 mM PLP, and 40 mM Na phosphate buffer, pH 8, 30 °C.

only about one-third of the available PLP within the same time (Figure 3B). It is worth mentioning that the I half-reaction equilibrium lies on the product side (ACP and PMP), due to water as the second substrate²³ and excess amine donor. In addition, PLP was in about 50–100-fold excess in comparison to the enzyme concentration (approximately 0.02–0.1 mM). Thus, the altered PLP conversion profiles between wt-ATA and CvATA (Figure 3A,B) were caused by their differing PMP/PLP exchange properties. On measurement of the average amount of enzyme-bound PMP, the quaternary structure affected the propensity for PMP diffusion/release, thus conferring altered E:PMP stability between CvATA and wt-ATA. The first measurement point at time zero in Figure 3 was done in the

absence of amine donor, representing the resting state (E-PLP) and, thus, providing a reference ($[E:PMP] \approx 0$). As is evident from Figure 3C,D, the E-PLP forms of both ATAs are rapidly converted into E:PMP upon amine donor addition, but only wt-ATA maintained its E:PMP complex (Figure 3D), whereas CvATA released PMP, returning to its initial value (no E:PMP) (Figure 3C). The high tendency for E:PMP dissociation into the apo form of CvATA and VfATA may be due to a weak binding affinity. Apo forms of other aminotransferases were reported to bind PLP about 100-fold more tightly than PMP.⁴⁴ However, the tetrameric wt-ATA seems to feature a lower E:PMP dissociation tendency in comparison to the dimeric enzymes. Overall, the quaternary structure of ATAs not only determines the stability of the E:PMP complex and the PMP/PLP exchange properties but also influences how rapidly the cofactor-devoid apoenzyme is formed. It is the apoenzyme that is the least stable form and prone to aggregation. Avoiding apoenzyme formation or rapid recovery of the E-PLP form minimizes irreversible inactivation (Scheme 1).

3.3.3. PLP Supply Prevents Irreversible Enzyme Inactivation but Reduces Activity. Our results together with other literature reports^{11,45} suggest that the irreversible inactivation step proceeds once the E:PMP complex dissociates into free PMP and apoenzyme (Scheme 1). We found the dimeric apoenzyme exhibits rapid, temperature-dependent inactivation after E:PMP dissociation occurred (Figure S7 in the Supporting Information). Cellini and co-workers demonstrated for dimeric alanine:glyoxylate transaminase that enhanced PMP dissociation reduces thermal stability and drastically increases the formation of insoluble higher aggregates.⁴⁵ Alanine:glyoxylate transaminase is categorized into the same PLP-enzyme fold type I, but of a different class (V) in comparison to CvATA, VfATA, and wt-ATA (class III).⁴ A recent study on ATA stability by Chen et al. revealed that dimeric CvATA slowly dissociates (within days) into its monomers at low temperatures (4 °C and room temperature) and that this subunit dissociation process is suppressed at higher PLP concentrations.¹¹

We investigated the feasibility of extra PLP supply as stabilization agent to improve ATA's operational stability at higher temperatures (50 °C) by rescuing the apoenzyme from aggregation through E-PLP re-formation. As shown in Figure 4, the operational stability of wt-ATA (Figure 4A) and CvATA (Figure 4B) indeed improved with increasing PLP concentrations (0.1–10 mM) at an amine donor to acceptor ratio of 50 (0.5 M IPA, 10 mM PB). However, the net effect on ATA productivity was about 0 for both tetramer and dimer, because what was gained in stability was lost through PLP inhibition. In the case of tetrameric wt-ATA, higher PLP content inhibited formation of E:PMP and consequently apoenzyme accumulation. The even more pronounced stabilization effect of PLP on CvATA seen in Figure 4B can be explained by the rapid PMP/PLP exchange rate of the dimer, which, on the other hand, consequently results in a suppressed II half-reaction and amine product formation rate (see Figures S8 and S9 in the Supporting Information). However, once the PLP concentration was depleted with reaction progress, the dimeric apoenzyme rapidly inactivated. For further explanations see Figures S7–S9 in the Supporting Information. As a conclusion, the PLP supply does not help to enhance ATA productivity and these findings are thus highly relevant for industrial process development and costs. Moreover, this is the first report showing a shift between ketone and amine product formation

Figure 4. Effect of PLP supply on operational stability for tetrameric wt-ATA (A) and dimeric CvATA (B) at 50 °C. Both ATAs catalyzed a full transamination reaction (0.5 M IPA, 10 mM BA, 10 mg/mL of ATA) with increasing PLP content (0.1–10 mM). The overall inactivation rate constant (k_{obs}) and half-life ($t_{1/2}$) were estimated by data fitting using a single-exponential decay function (A) or polynomial function (B).

for dimeric ATAs with excess PLP, despite the presence of amine acceptor. While the activity improvement of CvATA upon extra PLP supply was found in this study and that by Kaulmann et al.,²¹ Cassimjee and co-workers reported that the dimeric ATA of *Arthrobacter citreus* and CvATA required an optimal ratio of PLP to enzyme for maximum performance; otherwise, strong inhibition occurred.^{22–24} These contradicting results may originate from measuring only one of the reaction products, and the I half-reaction product (ketone) alone will not give a proper value for the formation of the target amine (Figure S9).

3.3.4. Cofactor Binding Motifs in Dimers Are Stabilized in Tetramers. To explain the structure–function relationship for the altered PMP leaking properties and lowered operational stability of dimeric structures, we analyzed the ATA quaternary structures. Comparing the cofactor binding regions of several ATA crystal structures, we focused our attention on a tertiary structure element that shields the cofactor from the bulk solvent and shapes the bottom of the active site entrance (locations are shown in Figure 5). We refer to it as the cofactor-ring motif, as it interacts in particular with the pyridine ring of the cofactor, detailed in Figure 5. In the tetrameric wt-ATA, the cofactor-ring motif stretches from residue 145 to 168, from 147

Figure 5. Location (top) and structure (bottom) of the cofactor-ring binding motif for tetrameric wt-ATA and the two dimeric species CvATA and VfATA. The essential elements for operational stability and cofactor binding are highlighted as spheres. The location (top) of the cofactor binding motif is shown in red, and its detailed tertiary structure (bottom) is given for each ATA. The phosphate-group loops of wt-ATA, CvATA, VfATA are shown in light purple, black, and green, respectively. The N-terminal region sits above the phosphate-group loop shown in dark blue, gray, and dark green for wt-ATA, CvATA, and VfATA, respectively. The internal aldimine (E-PLP, top) and PLP (bottom) are shown as yellow sticks. The black arrow indicates the active site entrance.

to 169 in dimeric VfATA, and from 151 to 173 in dimeric CvATA. The structures are very similar for all three ATAs, and they can be described as a double loop-helix motif. The first loop contains the highly conserved tyrosine residue (e.g., Tyr148/150/153 in wt-ATA/VfATA/CvATA) that packs in an edge-on orientation from the *re* face against the pyridine ring of PLP or PMP. A highly conserved valine is positioned on the opposite side (*si* face), and together both residues tightly sandwich the cofactor.

Importantly, in the unstable apoenzyme the cofactor-ring motif becomes increasingly dynamic or even disordered when no cofactor is bound. This is particularly evident for VfATA, where density for this motif is missing in the apoenzyme crystal structure, as illustrated in Figure 6. For CvATA, there are two apoenzyme structures reported.^{19,41} In one of the apoenzyme structures (4A6R), the motif becomes disordered, as judged by loss of density in the more C-terminal helix (Figure S10 in the Supporting Information) and increased *B* factors in the more N-terminal helix in comparison to the holo form (4A6T).⁴¹ The other apo-CvATA structure (4BA4) shows density even in the absence of cofactor.¹⁹ However, similar to the case for 4A6R, the N-terminal helix remains rather unchanged, while the C-terminal helix shows high *B* factors, which suggests increased dynamics even in this apo structure in comparison to the holo structure of the same study (4AH3).¹⁹

In addition to its role in cofactor binding, the cofactor-ring motif is also part of the dimer–dimer interface in the tetrameric structure. This is important, because it offers an explanation for the differences in PMP retention observed between dimers and tetramer. Our hypothesis is that the missing stabilizing interactions from the second dimer allows for higher dynamics within the cofactor-ring motif of dimeric ATAs. From the structures in Figure 6 and Figure S10 in the Supporting Information, it is apparent that major structural changes within

Figure 6. Structural changes of VfATA at the active site region shown as alignment between two holo forms (E-PLP and E:PMP) and its cofactor-devoid apo form (E). Essential amino acid residues are depicted as sticks and are labeled. The cofactors PLP and PMP are depicted as yellow sticks, oxygen is shown in red, and nitrogen is shown in blue. PDB ID: E, 3NUI; E-PLP, 4E3R; E:PMP, 4E3Q.⁴⁶

the motif are required for PMP to diffuse out. The rearrangement seen in the VfATA crystal structure includes displacement of Vf-Tyr165 (Tyr168 in CvATA, Figure S10), which provides more flexibility for Vf-Tyr150 (Cv-Tyr153) to rotate/move away from the cofactor binding region (Figure 6). Loss of the sandwiching interactions makes cofactor dissociation, particularly for PMP, more probable.

Furthermore, the E:PMP structure of wt-ATA (Figure S1 in the Supporting Information, 5LHA) and VfATA (Figure 6,

4E3Q) show no significant differences from the E:PLP structure. On the one hand, the lack of changes in dimeric VfATA suggests that PMP also stabilizes the cofactor-ring motif and it is not until PMP is released into the solution that this motif becomes more dynamic. On the other hand, such a diffusion-driven mechanism would not explain the drastically reduced PMP dissociation tendency for tetrameric wt-ATA. It should also be emphasized here that the cofactor's phosphate group is firmly anchored in the "phosphate-group binding cup", significantly contributing to cofactor fixation.¹⁴ In contrast to the covalently bound PLP (E-PLP), retention of PMP only depends on noncovalent interactions. Importantly, these phosphate group interactions are partially lost in dimeric VfATA and CvATA as the phosphate-group loop becomes displaced (Figure S10 in the Supporting Information), and this may play an important role in PMP dissociation.^{19,41} As shown in Figure 6, the phosphate-group loop is relocated in the apo structure and the interaction between cofactor and Thr322' is lost. Moreover, in the apo form, density for the N-terminus region (residue 3-32) is completely missing for VfATA (for CvATA see Figure S10). The phosphate-group loop, part of monomer 2, is located between the N-terminus region and the cofactor-ring motif, both belonging to monomer 1 (Figure 6). It is difficult to judge from crystal structures in which order the structural rearrangements occur and if PMP dissociation is triggering these events. However, the cofactor-ring motif is exclusively stabilized by the second dimer in the tetrameric wt-ATA crystal structure (Figure 5). Neither the N-terminus nor the phosphate-group loop establishes significant interactions with the second dimer unit. This specific structural feature of wt-ATA seems to confer improved PMP retention in comparison to the dimeric ATAs, where the cofactor-ring motif is not stabilized.

The stabilization of the cofactor-ring motif by the neighboring dimer is a feature not unique to wt-ATA but is also coherent with other tetrameric ATAs of the same fold type I and class III (Figure S11 in the Supporting Information), e.g., from *Pseudomonas aeruginosa* (4B9B),¹⁹ *Bacillus anthracis* (3N5M), and *Arthrobacter aurescens* (4ATP).³⁷ In all of these cases the three structural elements discussed above are structured (Figure 5 and Figure S11). Instead of the cofactor, the *Bacillus* crystal 3N5M contains a sulfate ion at the phosphate group binding location, revealing that lack of PLP does not compromise structural integrity as observed for the dimers. However, most tetrameric ATA crystal structures deposited in the Protein Data Bank³⁵ contain the cofactor. The difficulty in obtaining any apo crystal form of tetrameric ATAs¹⁹ strongly indicates that low PMP diffusion is a feature of tetrameric ATAs in general. Thus, the absence of the second dimer unit translates into increased dynamics of the partially solvent exposed cofactor-ring motif, and this seems to correlate with increased PMP dissociation. Reduced motif stability and increased probability of forming the apoenzyme provide excellent explanations for the lower operational stability of dimeric ATAs in comparison to the tetrameric wt-ATA. To our knowledge, this connection among quaternary structure, cofactor dissociation, and low operational stability has not been discussed before.

3.4. Strategies for Optimizing Reaction Conditions for Chiral Amine Synthesis. From the above discussion it is apparent that ATA stability is strongly influenced by several factors: i.e., the quaternary structure, cofactor (PLP), substrate ratio (amine donor/acceptor), and reaction temperature. The

drastic difference between ATA's resting and operational stability (see Figure 1A) is problematic for industrial application and process productivity. Despite their rather thermophilic character in the resting state (e.g., T_m value of 63 °C for wt-ATA; Figure 1A), the optimal temperature for activity in most situations is much lower (≤ 40 °C; Figure S12 in the Supporting Information). The structural instability of ATAs during catalysis thus prevents the use of higher temperatures for the purpose of increasing productivity: i.e., faster reaction rates and higher substrate/product solubility. Poor ketone substrate solubility constitutes a major obstacle to achieving high amine product concentrations that in turn would benefit process productivity. Performing ATA-catalyzed reactions at elevated temperatures (>40 °C) and in engineered media using water-miscible solvents is a frequent option to enable higher substrate/product concentrations.^{38,47} However, such conditions are poorly tolerated by ATAs without increasing their heat and solvent stability via protein engineering.^{12,13}

The mechanistic understanding of ATA inactivation obtained from this study enabled us to tune the enzyme performance via the substrate ratio. In Figure 7, the transamination efficiencies

Figure 7. Effect of substrate ratio on the productivity of ATA. Reaction conditions: conducted at 40 °C with 10 mM PB, 50 or 10 mM PEA, 0.5 mM PLP, 0.1 mg/mL wt-ATA, and 0.5 mg/mL CvATA and VfATA. Both products (ACP and PBA) were monitored, but the conversion is based on PBA formation. For more details see the text. An automated HPLC platform was used,²⁵ and each data point represents the average of two independent experiments; the deviation was $<3\%$.

of all three ATAs at high (5:1) and low (1:1) substrate ratio at 40 °C are compared. PEA was employed, as it serves as a generally well accepted amine substrate together with PB. Low enzyme concentrations (≤ 0.5 mg/mL) were used to observe the effect of inactivation and not to be limited by the reaction equilibrium due to fast reaction rates. Tetrameric wt-ATA

gained in conversion using the high (5:1) substrate ratio; about 80% conversion of PB was achieved with wt-ATA within 80 h. At equal PEA and PB concentrations, the reaction appears to be limited by its equilibrium and/or inhibited by the product, as a yield of only about 53% was obtained. In contrast, both CvATA and VfATA exhibited the highest turnovers at an equal substrate ratio. However, their poor operational stability only allowed for a maximum conversion of about 28%; thus, there was no dependence on the reaction equilibrium. As is evident from the crossing point of equal and high ratios for the VfATA-catalyzed reactions (7.5 h, inset in Figure 7), the kinetic advantage of the 5:1 ratio was lost due to enzyme inactivation, whereas the reaction with equal substrate ratio continued to convert PB into PBA, yielding almost doubled turnover. For CvATA, the equal substrate ratio enabled directly higher turnover in comparison to the 5:1 ratio; no crossing point was seen. The difference in initial conversion rates between CvATA and VfATA quite likely originated from their substrate specificity (toward donor and acceptor).

Our data suggest that the productivity of dimeric ATAs can be optimized by tuning the substrate ratio toward more stabilizing conditions. The substrate (donor/acceptor) ratio can be adjusted to rescue the apoenzyme from irreversible inactivation and thus constitutes an important parameter for process development. However, the often unfavorable reaction equilibrium in chiral amine synthesis has to be taken into consideration: for example, by employing appropriate in situ product removal and/or amine donor fed batch strategies. For instance, a very efficient method for achieving product removal using supported liquid membrane extraction was recently reported by our group.⁴⁸

4. CONCLUSIONS

Comparing a novel tetrameric wild-type amine transaminase (wt-ATA) with the two popular dimeric ATAs from *C. violaceum* and *V. fluvialis*, we discovered that these ATAs follow an amine donor induced inactivation mechanism and that low amine donor to acceptor ratios favor operational stability. However, literature reports commonly employ the amine donor in excess, thus promoting ATA inactivation according to our results. In addition, the quaternary structure has a strong influence on ATA's operational stability. In particular, the stability of the enzyme–PMP complex (E:PMP) was found to play a key role in the overall resistance to amine donor induced inactivation, because loss of cofactor (PLP or PMP) yields the aggregation-prone apoenzyme. Our results suggest that the tetramer structure specifically stabilizes the essential cofactor-ring binding motif. We postulate that, if this cofactor-ring binding motif unfolds, further rearrangements and loss of PMP are triggered, which ultimately leads to irreversible inactivation. In dimeric ATAs, the lacking neighboring dimer unit causes increased structural flexibility and exposure to the solvent that is assumed to accelerate diffusion of PMP out of the active site. Thus, this structural key element offers an excellent target for rational protein engineering aiming at improved operational stability. We are currently investigating the specific stabilization of the E:PMP state.

Further biochemical analysis of other tetrameric and dimeric ATAs would reveal whether the more rigid protein scaffold of tetramers generally confers superior stability in comparison to dimeric ATAs. The different kinetic behaviors of dimeric and tetrameric ATAs in terms of cofactor consumption and release

give valuable information for bioprocess development and rational selection of stable ATA candidates.

Although the tetrameric ATA exhibited superior stability at high substrate ratios, maximizing operational stability is absolutely critical, because minimizing enzyme inactivation would improve process economy. Thus, to yield an optimal amine synthesis process, both kinetic and stability aspects need to be accounted for.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.6b02100.

Additional information on X-ray diffraction data, crystal structures, structural comparison between different transaminases, protein sequence comparison, ATA inhibition, ATA inactivation (substrate ratio, half reaction), thermal stability vs activity, and high-performance size exclusion chromatography analysis of wt-ATA (PDF)

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Notes

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■ ABBREVIATIONS

ATA, amine transaminase; ACE, acetone; ACP, acetophenone; ALA, L-alanine; PB, 4-phenyl-2-butanone; PBA, 4-phenyl-2-butanamine; PEA, 1-phenylethylamine; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate

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