Stability and stabilization of \(\alpha\)-amino acid oxidase from the yeast *Trigonopsis variabilis*

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**Abstract**

The use of DAO (\(\alpha\)-amino acid oxidase) for the conversion of cephalosporin C has provided a significant case for the successful implementation of an \(\text{O}_2\)-dependent biocatalyst on an industrial scale. Improvement of the operational stability of the immobilized oxidase is, however, an important goal of ongoing process optimization. We have examined DAO from the yeast *Trigonopsis variabilis* with the aim of developing a rational basis for the stabilization of the enzyme activity at elevated temperature and under conditions of substrate turnover. Loss of activity in the resting enzyme can occur via different paths of denaturation. Partial thermal unfolding and release of the FAD cofactor, kinetically coupled with aggregation, contribute to the overall inactivation rate of the oxidase at 50°C. Oxidation of Cys108 into a stable cysteine sulfinic acid causes both decreased activity and stability of the enzyme. Strategies to counteract each of the denaturation steps in DAO are discussed. Fusion to a pull-down domain is a novel approach to produce DAO as protein-based insoluble particles that display high enzymatic activity per unit mass of catalyst.

**Introduction**

DAOs (\(\alpha\)-amino acid oxidases; EC 1.4.3.3) are well-characterized and technologically useful flavoenzymes that catalyse the \(\text{O}_2\)-dependent transformation of an \(\alpha\)-amino acid substrate into the corresponding \(\alpha\)-keto acid, ammonia and \(\text{H}_2\text{O}_2\) [1–3]. DAOs have been fruitful models with which to elucidate the catalytic mechanism of substrate dehydrogenation by enzyme-bound FAD that is now widely believed to take place via hydride transfer [4–10]. The enzymes display exquisite \(R\) compared with \(S\) enantioselectivity with respect to the reactive \(\alpha\)-carbon of the substrate while, at the same time, they can accommodate a wide range of side chain structures [1–3]. It is this combination of enzyme properties that provides considerable scope for application of DAOs in biocatalytic synthesis and for analytical purposes [2,3]. Crystallographic evidence and results from site-directed mutagenesis studies have revealed structural determinants of stereoselectivity and substrate specificity in DAOs [5–8,11]. Currently, the most important use of DAO is the conversion of cephalosporin C into 7-(5-oxoadipoamido) cephalosporanic acid that is further transformed chemoenzymatically into 7-ACA (7-aminocephalosporanic acid), the precursor of numerous cephalosporin C is typically performed in a batchwise operation by using an aerated stirred-tank reactor in which the biocatalytic particles are kept in suspension [12a]. Immobilization facilitates continuous reuse of the enzyme and improves the persistence of \(Tv\)DAO against inactivation under the conditions of the industrial process (for a general review, see [15]; [12a,14,16,17]). However, because the costs of enzyme production are relatively high, further enhancement of the operational stability of \(Tv\)DAO would lead to a significant economic benefit. A number of papers report results of experiments designed to develop a more stable \(Tv\)DAO [2,3,18–20]. Unfortunately, elucidation of relationships between the structure and stability of \(Tv\)DAO is currently not well advanced and, therefore, rational stabilization of the enzyme activity for improved process performance remains a difficult task. Likewise, planning the immobilization of \(Tv\)DAO such that activity is optimally retained and stability is optimally enhanced in the carrier-bound enzyme is a distant project (for a general review, see [21]). We have recently performed a detailed and systematic analysis of the kinetic mechanism underlying inactivation of \(Tv\)DAO under conditions of temperature stress (50°C) and during the reaction, and salient findings are reviewed in this paper. New approaches of mechanism-based stabilization of the enzyme activity are suggested.
Microheterogeneity of *TvDAO* resulting from the partial oxidation of Cys\textsuperscript{108}

Susceptibility to protein oxidative modification by O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2} is thought to be an important source of instability of oxidases. However, in only very few enzymes has a defined chemical conversion of the critical side chain that is sensitive to oxidation been correlated with consequences at the level of enzyme function \[22,23\]. We have shown recently that *TvDAO*, as isolated from its natural producer to be employed in the conversion of cephalosporin C, is composed of two protein forms that appear to differ solely in the oxidation state of Cys\textsuperscript{108} \[24\]. High-resolution anion-exchange chromatography on a MonoQ column was used to separate the reduced (native) and oxidized protein forms, which constituted, in a molar ratio of approx. 3:1, the active biocatalyst obtained from *T. variabilis*. Analysis of tryptic peptides by electrospray tandem MS allowed assignment of the modification as the oxidation of Cys\textsuperscript{108} into a cysteine sulfinic acid. The oxidized form of the enzyme displayed much lower specific activity than the natural enzyme, most probably because a substantial fraction of the protein was recovered in the inactive apo form \[26,29\]. We have shown that *TvDAO* accounts for approx. 35% of *E. coli* protein when added d-methionine, or limited supply of dioxygen suppressed the toxic activity of the enzyme \[30\]. Purified recombinant oxidase lacked the microheterogeneity of the natural oxidase preparation. The biochemical properties of recombinant *TvDAO* and the native enzyme where Cys\textsuperscript{108} was not modified were identical within limits of experimental error \[30\].

Dissection of denaturation steps that contribute to thermal inactivation of *TvDAO*

A detailed kinetic analysis was performed for inactivation of purified native and oxidized protein forms of *TvDAO* at 50°C in 10 mM Tris buffer (pH 7.5) \[31\]. Dissociation of FAD, protein conformational changes that resulted in an increased exposure of hydrophobic surface, and aggregation were denaturation processes that accompanied the inactivation. They were also monitored as a function of the incubation time at 50°C. Although the time courses for loss of activity in both enzymes were fairly well described by simple single-exponential decays, the underlying denaturation mechanisms were shown by experiments and kinetic modelling to be complex (Figure 2) \[31\]. Inactivation of both enzymes was proposed to occur via three parallel paths of irreversible denaturation. One main path leading to inactivation was FAD release, a process whose net rate constant was governed by the bimolecular rate constant of FAD binding to the apo enzyme (k\textsubscript{−b}). The estimated value of k\textsubscript{−b} was 25-fold lower in oxidized *TvDAO* than the native enzyme (Figure 2). Therefore the observed rate of cofactor dissociation was 3.5-fold faster from the oxidized enzyme than from native *TvDAO*, and this explains the 2-fold difference in the inactivation rates that was detected with an assortment of probes and affected the protein environment of the FAD cofactor. Oxidatively modified *TvDAO* was catalytically only approx. one-fourth as active as the native enzyme, reflecting a catalytic efficiency for reduction of O\textsubscript{2} lowered to approximately the same extent \[24\].

Control of protein quality through recombinant production in *Escherichia coli*

Production of a recombinant *TvDAO* in a host organism with a reducing cellular environment was expected to prevent cysteine oxidation such that, in an ideal case, structural microheterogeneity of the resulting oxidase preparation should be eliminated completely. Although *E. coli* would seem to be a suitable candidate for heterologous protein production, there appears to be a major problem with toxicity of *TvDAO* for this bacterium \[25,26\]. Furthermore, *TvDAO* produced in *E. coli* was often precipitated in inclusion bodies \[27,28\], or the soluble portion of it displayed much lower specific activity than the natural enzyme, most probably because a substantial fraction of the protein was recovered in the inactive apo form \[26,29\]. We have shown that *TvDAO* accounts for approx. 35% of *E. coli* protein when added d-methionine, or limited supply of dioxygen suppressed the toxic activity of the enzyme \[30\]. Purified recombinant oxidase lacked the microheterogeneity of the natural oxidase preparation. The biochemical properties of recombinant *TvDAO* and the native enzyme where Cys\textsuperscript{108} was not modified were identical within limits of experimental error \[30\].
of the two enzymes. Dissociation of FAD was kinetically coupled with aggregation, measured by static light scattering, and could be prevented almost completely by the addition of free FAD. The kinetic constants in Figure 2 were used to calculate the thermodynamic dissociation constant for FAD ($K_d = k_{b-a}/k_b$) in native and oxidized TeDAOs, and values of 0.5 and 20 $\mu M$ were obtained, respectively. By way of comparison, Arroyo et al. [32] reported a $K_d$ of 0.15 $\mu M$ for recombinant histidine-tagged TeDAO. Aggregation was markedly attenuated in the less stable Cys108-SO$_2$H-containing oxidase, indicating that it was a step accompanying but not causing the inactivation.

Figure 2 shows that a second path of denaturation took place in parallel with FAD dissociation. It was characterized by a rate constant $k_a$, which was identical within limits of error for native and oxidized TeDAOs and did not depend on the protein concentration used. We proposed that $k_a$ stands for partial thermal unfolding reactions, causing conversion of the active enzyme into a form that lacks activity but has FAD bound. Using changes in the CD signal at 222 nm as reporter of protein thermal unfolding, Arroyo et al. [32] carried out a temperature-ramp experiment in which a solution of histidine-tagged TeDAO [5 $\mu M$; 20 mM potassium phosphate buffer, pH 8.0, containing 20% (v/v) glycerol, 5 mM 2-mercaptoethanol and 5 mM EDTA] was heated from 10 to 70°C at a rate of 20°C·h$^{-1}$. Under these conditions, they determined a value of 41.4°C·h$^{-1}$ for the apparent midpoint of denaturation of the enzyme. Their results support the notion that partial unfolding should be a major cause of inactivation of TeDAO at 50°C.

Conversion of native TeDAO into the oxidized, Cys$^{108}$-SO$_2$H-containing enzyme could be a third denaturation process during thermal inactivation of TeDAO at 50°C. Dib et al. [31] took samples at several time points during the course of inactivation of highly purified, native TeDAO and analysed them using MonoQ chromatography. The results revealed gradual decay of the well-defined protein peak of the native enzyme as a function of the incubation time, concomitant with the appearance of three new protein peaks that probably represent denaturation and fragmentation products. The main peak of the three co-eluted with an authentic standard of oxidized TeDAO. The apparent transformation of Cys$^{108}$ took place at a low rate (k < 0.03 h$^{-1}$) and is of only minor importance for the loss of enzyme activity at 50°C. However, the situation could be completely different at a lower temperature and under reaction conditions when O$_2$ is present and H$_2$O$_2$ formed. Several authors described that loss of TeDAO activity was accelerated in an oxidizing environment containing H$_2$O$_2$ [18,20,32–35]. Among other oxidation-labile residues possibly involved in chemical inactivation of TeDAO, including Cys$^{298}$ [35], Met$^{156}$, Met$^{209}$ and Met$^{226}$ [20], Cys$^{108}$ could be a strong candidate to be modified under oxidative stress conditions of the biocatalytic reaction. Rapid loss of enzyme activity in soluble TeDAO under conditions of O$_2$ supply by aeration appears to be due to protein denaturation at the gas/liquid interface rather than oxidative modification [16,36].

The kinetic scenario of thermal denaturation of TeDAO (Figure 2) provides interpretation for observations regarding the stability of TeDAO that were previously not well accounted for. It also serves as a mechanism-based tool for the stabilization of the soluble enzyme and carrier-bound immobilized forms of it. The inactivation rate of the enzyme increased with increasing concentrations of the protein subunit in the range 1–20 $\mu M$ (10 mM Tris buffer, pH 7.5; 50°C) [31] and 5–100 $\mu M$ (50 mM potassium phosphate buffer, pH 8.0; 30°C) [37]. Stabilization by external FAD, added in 100-fold molar excess over native protein, was gradually lost upon raising the concentration of the TeDAO subunit from 1 to 20 $\mu M$. In the absence of exogenous FAD, the oxidized Cys$^{108}$-SO$_2$H-containing form of TeDAO was approx. 2-fold less stable than the native enzyme. However, differences in stability of the two TeDAO forms were completely eliminated upon addition of a high concentration of cofactor (1 mM) [31]. Slavica et al. [37] showed that the rate-determining step of inactivation of TeDAO changed from FAD dissociation to partial unfolding at high temperature. Therefore the relative stabilizing effect of exogenous FAD is expected to decrease as the incubation temperature increases. It will also be interesting to employ data shown in Figure 2 for the kinetic analysis of the inactivation of TeDAO immobilized on to an insoluble carrier. Betancor et al. [33] demonstrated that dissociation of FAD was a main contributor to the loss of activity in TeDAO covalently bound to chemically activated agarose. Interestingly, these authors also found that stabilization of the enzyme activity was optimal when using highly activated insoluble supports that promoted multipoint attachment of proteins. It is tempting to speculate that the extra stability brought about by multipoint covalent immobilization of TeDAO is the result of a lowered thermal unfolding rate constant that reflects reduced conformational flexibility in the carrier-bound enzyme. Figure 2 could therefore be a point of departure for improving the complementarity between carrier and TeDAO such that the process stability of the immobilized biocatalyst is optimized (for a general review, see [21]).
Selective chemical modification of \( \text{TvDAO} \)

Prior studies from this group [24] and others [35,38,39] have shown that cysteine residues play an important though not very well understood role for the catalytic function and stability of \( \text{TvDAO} \). Selective, thiol group-directed modification of the enzyme was therefore thought to hold promise for stabilization of the oxidase on condition that the method of derivatization discriminates against those cysteine side chains that are structurally and catalytically important. Covalent modification of \( \text{TvDAO} \) using maleimide-activated PEG [poly(ethylene glycol)] 5000 yielded a stable bionconjugate in which three exposed cysteine side chains were derivatized. The PEGylated enzyme showed approx. 3.3-fold slowed dissociation of the FAD cofactor at 50°C, and this caused a 2-fold thermostabilization of the enzyme activity [40]. The stability under reaction conditions at 30°C was also enhanced significantly in the PEG–oxidase conjugate. The PEGylation did not affect the enzyme activity for oxidative deamination of D-methionine when 2,6-dichloroindophenol replaced \( \text{O}_2 \) as the co-substrate. However, it causes a 9-fold decrease in substrate catalytic efficiency for the dioxygen-dependent reaction.

Physical entrapment of \( \text{TvDAO} \)

Dib et al. [30] used \( \text{E. coli} \) cells overproducing recombinant \( \text{TvDAO} \) to develop a novel whole-cell preparation of the enzyme that could be provided with only minimal downstream processing. A single freeze-and-thaw cycle was used to mildly permeabilize the cells. The physically entrapped \( \text{TvDAO} \) thus obtained was employed for the conversion of \( \alpha^- \)-alanine (5 mM) in an aerated stirred-tank reactor. The enzyme could be reused for at least three rounds of reaction with only small losses of activity (for details, see [30]). The free oxidase was, however, inactivated rapidly under the same reaction conditions, showing a half-life (\( t_{1/2} \)) of only approx. 1.2 h. Therefore entrapment of \( \text{TvDAO} \) yielded a stabilized insoluble enzyme preparation and facilitated continuous processing.

Fusion to a pull-down domain and production of enzymatically active protein aggregates of \( \text{TvDAO} \)

A main disadvantage of immobilization of enzymes on to insoluble, usually porous carriers is that a large portion of non-catalytic mass is introduced into the final preparation of the enzyme. Therefore, if one managed to precipitate the target enzyme into an insoluble particle with good retention of the specific activity of the soluble enzyme, a potentially very useful biocatalyst could be obtained. CLECs (cross-linked enzyme crystals) represented the first commercialized realization of this otherwise general concept [41,42]. Considering that preparation of a particular CLEC is limited by its requirement for a successful crystallization of the (purified) target enzyme, Sheldon et al. [43,44] introduced CLEAs (cross-linked enzyme aggregates) as a novel immobilization technology that has broader scope for biocatalysis than CLECs. CLEAs are made by a general procedure that involves salt- or solvent-induced (micro)aggregation of the enzyme or a desired combination of several enzymes, followed by cross-linking with glutaraldehyde to obtain a stable all-protein precipitate. While CLEAs eliminate the need to crystallize the enzyme of interest, there remains the inherent disadvantage that some purification of the crude protein is required prior to its precipitation. In addition, the exact protocol of aggregation and cross-linking must be established for each enzyme anew, implying that a substantial effort must be devoted to the optimization of conditions. A significant improvement of CLEA production could come from a molecular design of the aggregation process such that it now features elements of biological recognition and specificity. We reported recently on a novel concept of controlled protein precipitation \( \text{in vivo} \) in which the target enzyme was fused to the CBD (cellulose-binding domain) of \( \text{Clostridium cellulosivorans} \), and expression in \( \text{E. coli} \) was performed under conditions that induced selective pull-down of the folded chimaeric protein via intermolecular self-aggregation of the CBD [45]. The case of \( \text{TvDAO} \) showed that upon fusion of the CBD to its N-terminus, the otherwise mainly soluble recombinant enzyme was quantitatively precipitated in protein particles, which displayed 40% of the specific activity of the highly purified oxidase. The aggregated CBD retained the ability to bind microcrystalline cellulose. The cellulose-bound oxidase was stabilized approx. 36 times against inactivation of the soluble enzyme during conversion of D-methionine and bubble aeration. CBD–enzyme fusions could find more widespread application in biocatalysis.

References
