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## Exploring chemical versatility within the tautomerase superfamily

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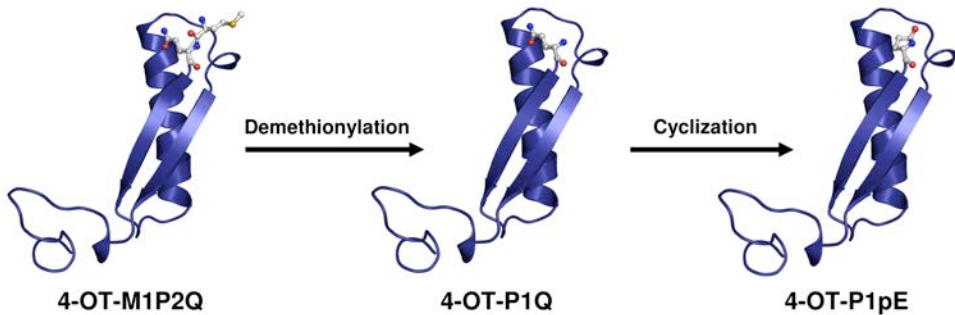
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# Chapter 6



**Demethionylation of Pro-1 variants of 4-oxalocrotonate tautomerase in *Escherichia coli* by co-expression with an engineered methionine aminopeptidase.**

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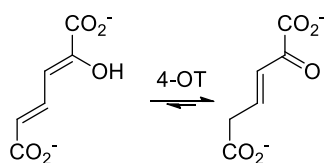
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## Abstract

4-Oxalocrotonate tautomerase (4-OT) catalyzes the enol-keto tautomerization of 2-hydroxymuconate, utilizing its N-terminal proline (Pro-1) as general base catalyst. Substituting Pro-1 with bulky or charged residues will result in poor or no post-translational removal of the translation-initiating methionine by the methionine aminopeptidase (MetAP) of the *E. coli* expression host. Here, we set out to investigate whether co-expression with previously engineered aminopeptidase MetAP-\*TG can be used to produce the P1S, P1H and P1Q variants of 4-OT in a demethionylated form. The P1S variant, which carries a small residue at the penultimate position (the first position after the initiating methionine), was found to be fully processed by wild-type MetAP. The P1S variant has low-level 2-hydroxymuconate tautomerase and promiscuous oxaloacetate decarboxylase activity. The P1Q and P1H variants of 4-OT, which carry bulky residues at the penultimate position, could only be obtained in a demethionylated form (a minor fraction of the purified protein is still composed of methionylated enzyme) by co-expression with MetAP-\*TG. Interestingly, the Gln-1 residue of the demethionylated P1Q variant undergoes intramolecular cyclization to form pyroglutamate (pE), yielding variant P1pE. Whereas the P1H/M1P2H mixture has low-level tautomerase activity, the P1pE/M1P2Q mixture has robust tautomerase activity. The substitution of Pro-1 by Gln, followed by removal of the initiating Met and cyclization of Gln-1 to form pE, is a unique way to obtain a structural analogue of proline on the N-terminus of 4-OT. This opens up new possibilities to study the importance of Pro-1 in recently discovered C-C bond-forming activities of this highly promiscuous tautomerase.

## Introduction

The enzyme 4-oxalocrotonate tautomerase (EC 5.3.2.6; 4-OT) from the soil bacterium *Pseudomonas putida* mt-2 catalyzes the enol-keto tautomerization of 2-hydroxyhexa-2,4-dienedioate to yield 2-oxohex-3-enedioate (Figure 1) as part of a degradative pathway for aromatic hydrocarbons.<sup>[1-3]</sup> 4-OT is a member of the tautomerase superfamily, a group of homologous proteins characterized by a structural  $\beta$ - $\alpha$ - $\beta$ -fold and a catalytic amino-terminal proline residue (Pro-1).<sup>[4,5,6]</sup> Pro-1 was found to be crucial for both the natural and various promiscuous activities of the enzyme, which include the hydrolytic dehalogenation of *trans*-3-chloroacrylic acid,<sup>[7,8,9]</sup> the aldol condensation of acetaldehyde with benzaldehyde,<sup>[10,11]</sup> the Michael-type addition of linear aliphatic aldehydes to a variety of aliphatic and aromatic nitroolefins,<sup>[12,13,14]</sup> and the *cis-trans* isomerization of nitrostyrene.<sup>[15]</sup> The chemical versatility of the amino-terminal proline is the core of the catalytic diversity displayed by 4-OT; Pro-1 has been proposed to act as a general base (tautomerase activity), a general acid (dehalogenase activity), or as a nucleophile (aldol condensation, *cis-trans* isomerization, and Michael-type additions).<sup>[7-16]</sup>

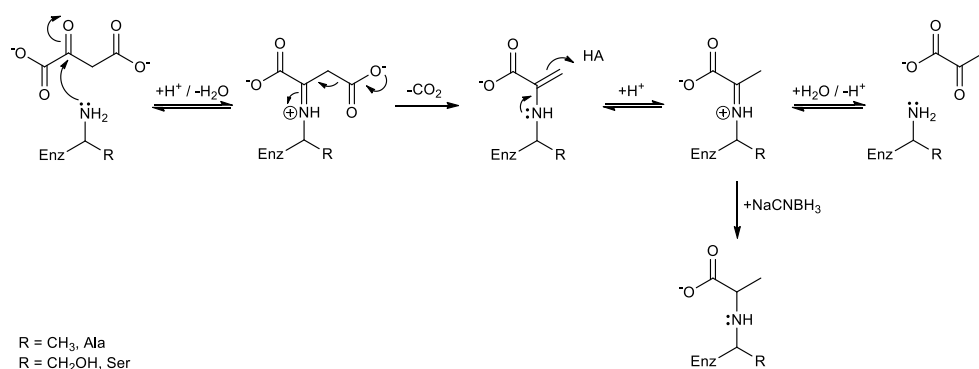


**Figure 1.** The 4-OT-catalyzed tautomerization of 2-hydroxyhexa-2,4-dienedioate to 2-oxo-hex-3-enedioate.

In order to gain insight into the structural and mechanistic roles of Pro-1 in the native tautomerase activity of 4-OT, Whitman and coworkers have studied mutants in which Pro-1 has been replaced by an alanine, glycine, leucine or valine.<sup>[17]</sup> From this work it was concluded that the stronger basicity of the secondary amine functionality of proline compared to the primary amine functionality of the introduced residue, and the rigidity of the pyrrolidine-ring, are key determinants for the catalytic efficiency of the enzyme towards its native substrate. During this study, mass spectral analysis of the P1L and P1V variants revealed that the translation-initiating methionine was either not or incompletely removed by the expression host *E. coli*. As a result, the P1L variant was obtained as the M1P2L protein showing that no demethylation had occurred. The P1V variant was obtained as a mixture of P1V and M1P2V proteins, of which 55% was constituted by demethylated protein. It was found that the presence of the initiating methionine had a pronounced effect on the  $k_{\text{cat}}$  of these variants towards the native tautomerase activity of 4-OT.<sup>[17]</sup> Overall the  $k_{\text{cat}}$  dropped by more than three orders of magnitude, which is likely caused by the structural effects the methionine imposes on the active site resulting in unfavorable positioning of the amino-terminus to act as a general base. These findings clearly show that the removal of the initiating methionine is a prerequisite in the study of Pro-1 variants of 4-OT.

One way of obtaining Pro-1 variants of 4-OT in a pure and fully demethionylated form is by peptide synthesis.<sup>[18]</sup> Drawbacks of this method are the high costs involved and the fact that the synthetic protein needs to be reconstituted in buffer in a properly folded and active form. While this is an experimental challenge, it has been demonstrated to work for 4-OT.<sup>[10,11,18]</sup> Dawson and coworkers chemically synthesized the P1G and P1A variants of 4-OT, thereby introducing a primary amine functionality at the amino-terminus of the enzyme replacing the secondary amine functionality of Pro-1.<sup>[18]</sup> Interestingly, both the P1G and P1A variants were found to promiscuously catalyze the decarboxylation of oxaloacetate through a covalent imine-mechanism, yielding pyruvate and carbon dioxide as the products (Figure 2).

In order to develop an alternative manner of producing demethionylated Pro-1 variants of 4-OT and to avoid possible problems with respect to reconstitution, we explored the possibility to demethionylate new Pro-1 variants of 4-OT by biological means. The enzyme in *E. coli* responsible for the removal of the initiating methionine from newly synthesized proteins is methionine aminopeptidase (MetAP).<sup>[19]</sup> A key determinant in the substrate specificity of MetAP is the nature of the penultimate residue (i.e., the residue following the initiating methionine) of the substrate protein.<sup>[20,21]</sup> MetAP only accepts polypeptides with an amino-terminal methionine as substrate when the side chain of the penultimate residue is small (e.g. Gly, Ala or Pro). As a result, about 40% of the endogenous cytosolic proteins of *E. coli* are not demethionylated and likewise the size of the side chain of the penultimate residue of recombinant proteins produced by *E. coli* will determine whether demethionylation occurs or not.<sup>[19]</sup> As demethionylation for recombinant proteins is often required, Liao and coworkers have engineered MetAP towards a larger substrate scope by increasing the size of the substrate binding pocket of the enzyme.<sup>[22]</sup> This work has resulted in an engineered MetAP (termed MetAP-\*TG) that is capable of removing the initiating methionine from recombinant proteins with bulkier or charged penultimate residues.



**Figure 2.** Proposed mechanism of the 4-OT P1A and 4-OT P1S catalyzed decarboxylation of oxaloacetate involving a covalent imine intermediate, which can be trapped using NaCNBH<sub>3</sub>.

Inspired by the work of Liao and coworkers, we set out to investigate whether the engineered variant MetAP-\*TG can be used to demethylate Pro-1 variants of 4-OT that could not be studied in the past due to the reason described above. We have chosen to construct the 4-OT variants P1S, P1H and P1Q. The serine substitution was selected because of the relatively small side chain of serine, which may allow demethylation of the P1S protein by wild-type MetAP.<sup>[22]</sup> In addition, the mutation to serine may introduce a new functional group at the amino-terminus of 4-OT. The variants P1H and P1Q were selected because wild-type MetAP does not accept peptide substrates with a histidine or glutamine at the penultimate position.<sup>[22]</sup> Additionally, the side chains of these residues offer interesting features. The imidazole functionality of the side chain of histidine could act as a catalytic base, possibly mimicking the role of Pro-1 of wild-type 4-OT. Following demethylation of the P1Q variant, the amino-terminal glutamine could undergo an intramolecular cyclization reaction (deamination) yielding pyroglutamate (pE), which is an interesting proline analogue (5-oxoproline, Figure 3).<sup>[23]</sup> The formation of amino-terminal pyroglutamate, either from a glutamine or glutamate residue, is a known post-translational modification that is important for the stability or activity of a variety of proteins, e.g. in human immunoglobulin gamma antibodies.<sup>[24,25]</sup> Proteins harboring an amino-terminal pyroglutamate are less susceptible to proteolytic degradation.<sup>[23, 26]</sup> Examples where an amino-terminal pyroglutamate is essential for catalysis in enzymes are rare, a notable example being a family of amphibian ribonucleases.<sup>[27,28]</sup> In bullfrog (*Rana catesbeiana*) ribonuclease 3, an amino-terminal pyroglutamate assists in catalysis by perturbing the  $pK_a$  of two catalytic histidine residues.<sup>[27,28]</sup> Given that the amide-nitrogen of the  $\gamma$ -lactam ring of pyroglutamate is not likely to be able to function as a general base catalyst, it would be interesting to investigate whether a P1pE variant of 4-OT is catalytically active.

Herein, we describe the expression, purification and characterization of the P1S, P1H and P1pE variants of 4-OT. The purified proteins were subjected to ESI-MS analysis to establish their level of demethylation and characterized for their 2-hydroxyisovalerate tautomerase activities. Additionally, the P1S variant was subjected to labeling studies with oxaloacetate and characterized towards its oxaloacetate decarboxylase activity, parallel to the work of Dawson and coworkers on the P1A and P1G variants of 4-OT (Figure 2).

## Results and discussion

*Purification and characterization of 4-OT variants co-expressed with MetAP-\*TG.* The genes encoding the 4-OT variants (P1S, P1Q and P1H) were expressed separately, or co-expressed with the gene encoding MetAP-\*TG, in *E. coli* BL21 (DE3). All genes are under transcriptional control of the T7 promoter and the enzymes were produced either constitutively (pET-20b(+)) based expression vectors) or under control of the *lac* operator (pET-26 and pET-29 based expression vectors). The 4-OT variants were purified to near homogeneity by a two-step column chromatography protocol. The typical yield of the 4-OT variants was 10 to 25 mg per liter of cell culture. MetAP-\*TG was used only as an *in-vivo* catalyst in the co-production experiments and as such was not purified from the

expression host. The over-production of MetAP-\*TG was evaluated by analyzing the total soluble protein fraction on SDS-PAGE gels, and the band corresponding to MetAP-\*TG is observed around 30 kDa. The level of over-production of MetAP-\*TG that has been observed was assumed to be sufficient for the removal of the amino-terminal methionine residue of the 4-OT variants.

The demethionylation efficiency of the 4-OT variants was determined by analyzing the purified proteins by electrospray ionization mass spectrometry (ESI-MS). The ratio of the peak area corresponding to the processed and non-processed (+131 Da) fraction was used to calculate the percentage of processed (demethionylated) 4-OT variant in each batch (Table 1). The 4-OT P1S variant was found to be fully processed by wild-type MetAP from the expression host, thus co-expression with the engineered MetAP-\*TG was not necessary. The variants 4-OT P1H and 4-OT P1Q, however, were found not to be processed by wild-type MetAP. Co-expression of these variants with the engineered MetAP-\*TG showed that post-translational processing can be achieved *in-vivo*; however, the efficiency of methionine removal differed significantly between purified protein samples from different growth experiments.

**Table 1.** The monomer masses of the 4-OT P1S, 4-OT P1Q, and 4-OT P1H variants with and without the initiating methionine, as determined by ESI-MS, and the calculated relative amounts of methionylated and demethionylated monomers.

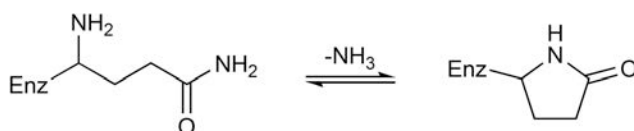
Enzyme	M <sub>w</sub> +Met1 (Da) <sup>a</sup>	Relative amount (%) <sup>b</sup>	M <sub>w</sub> -Met1 (Da) <sup>a</sup>	Relative amount (%) <sup>b</sup>
4-OT P1S	N.D.	0	6802	100
4-OT P1Q	6973	32	6825 <sup>c</sup>	68
4-OT P1H	6982	13	6851	87

<sup>a</sup>The reported values are accurate to  $\pm 1$  Da. <sup>b</sup>Calculated from the integration of the peaks in the reconstructed mass spectrum. <sup>c</sup>Mass corresponding to P1pE instead of P1Q due to (spontaneous) intramolecular cyclization of glutamine-1 forming pyroglutamate. N.D., not detected.

For the 4-OT P1H variant, 20-80% methionine removal was generally observed. The highest percentage of processing (87%, Table 1) was obtained by expressing 4-OT P1H constitutively (pET-20b(+)-based expression vector) and MetAP-\*TG under control of the *lac* operator (pET-29b(+)-based expression vector). IPTG was added to the growth medium prior to the inoculation of the medium, to ensure expression of MetAP-\*TG from the start as the 4-OT variant to be processed is expressed constitutively. For the 4-OT P1Q variant, the highest percentage of processing (68%, Table 1) was achieved by expressing MetAP-\*TG constitutively (pET-20b(+)-based expression vector) and the 4-OT P1Q variant under control of the *lac* operator (pET-26b(+)-based expression vector). This alternative way of co-expressing the 4-OT P1Q variant with MetAP-\*TG with respect to the system used for the P1H variant was developed because of low demethionylation efficiencies (<20%) for the P1Q variant using the original method. Reasoning that an ample amount of MetAP-\*TG needs to be present at the start of production of the 4-OT P1Q variant, the gene of 4-OT P1Q was cloned into an alternative vector under control

of the *lac* operator. Instead, cloning the gene of MetAP-\*TG in the pET-20b(+) vector ensured constitutive expression of the demethylation catalyst. Using this expression system, the production of MetAP-\*TG prior to that of the 4-OT P1Q variant can be guaranteed and up to 68% methionine removal was achieved.

An interesting observation made from the ESI-MS analysis of the P1Q variant is that the demethylated protein was found to have a mass of  $6825 \pm 1$  Da, instead of 6842 Da, which is the calculated mass of the demethylated P1Q variant. The mass difference of 17 Da can be explained by the expulsion of ammonia upon intramolecular cyclization of the amino-terminal glutamine (deamination) to form the proline analogue pyroglutamate (Figure 3). Since a peak corresponding to a mass of 6842 Da (amino-terminal glutamine) is absent in the ESI-MS spectrum of this purified batch, the demethylated fraction of this protein appears to harbor exclusively a pyroglutamate at the amino-terminus.



**Figure 3.** Formation of pyroglutamate (i.e., 5-oxoproline) by deamination of an amino-terminal glutamine.

To investigate whether the loss of 17 Da indeed occurs at the amino-terminal position and not somewhere else in the protein, a new purified batch of the P1Q variant was produced, which by ESI-MS analysis was shown to consist of a mixture of three proteins with masses of  $6825 \pm 1$  Da,  $6842 \pm 1$  Da and  $6973 \pm 1$  Da, which correspond to the P1pE, P1Q and M1P2Q variants, respectively. This protein mixture was digested with endoproteinase GluC and the resulting peptides were analyzed by nano-LC-MS (Table 2). The results clearly demonstrate that the modification that leads to a loss of 17 Da was exclusively associated with the peptide corresponding to the amino-terminus of the protein (note the 17 kDa difference in the masses of the QIAQIHILE and pEIAQIHILE peptides). Subsequent nano-LC-MS/MS analysis of the QIAQIHILE and pEIAQIHILE peptides gave characteristic sets of b-ions, which revealed that the net loss of 17 Da occurs at the N-terminal residue (Table 2). Thus, the protein species with a mass of 6825 Da observed in reconstructed ESI mass spectra of the 4-OT P1Q variant most likely corresponds to a protein harboring an amino-terminal pyroglutamate.

To provide further evidence for the formation of an amino-terminal pyroglutamate, we have used the mixture of P1pE, P1Q and M1P2Q variants in labeling studies with 3-bromopyruvate (3BP). This compound is a well-studied affinity probe for 4-OT, with the amino-terminal proline (Pro-1) being the sole target of modification.<sup>[33,34]</sup> Incubation of wild-type 4-OT with 3BP leads to alkylation of the prolyl-nitrogen with a pyruvoyl-group, which is the result of nucleophilic attack of Pro-1 on the C3-carbon of 3BP, leading to the expulsion of bromide and alkylation of Pro-1.<sup>[33,34]</sup> Interestingly,



incubation of the protein mixture with 3BP did not result in labeling of the protein fraction with an observed mass of 6825 Da, whereas the fractions with a mass of 6842 Da and 6973 Da were partially labeled (Table 3). In a separate control reaction using identical conditions, wild-type 4-OT was found to be completely labeled (Table 3). The partial labeling of the P1Q (6842 Da) and M1P2Q (6973 Da) proteins harboring a primary amine functionality at their amino-terminus, follows the trend in reduced nucleophilicity of a primary amine versus a secondary amine (i.e, proline). Moreover, the complete loss of reactivity towards 3BP of the protein species with a mass of 6825 Da is indicative of an amino-terminal residue with poor nucleophilicity, which is consistent with the expected low reactivity of pyroglutamate. Taken together, the results provide convincing evidence that the substitution of Pro-1 by Gln, followed by removal of the initiating Met, results in cyclization of Gln-1 to form pyroglutamate at the N-terminus.

**Table 2.** Identification, by nano-LC-MS and MS/MS, of pyroglutamate (pE) as the amino-terminal residue of the demethylated 4-OT P1Q variant.

Peptide fragment	Mass <sub>calcd</sub> (Da) <sup>a</sup>	Mass <sub>obs</sub> (Da) <sup>b</sup>
MQIAQIHILE	1194.64 <sup>c</sup>	1194.64 <sup>c</sup>
QIAQIHILE	1063.60 <sup>c</sup>	1063.60 <sup>c</sup>
QIAQIH		691.3
QIAQ	441.2	441.1
QI	242.2	242.1
pEIAQIHILE	1046.58 <sup>c</sup>	1046.58 <sup>c</sup>
pEIAQIH	674.4	674.5
pEIAQ	424.2	424.2
pEI	225.1	225.2

<sup>a</sup>These values are calculated using the average molecular mass. <sup>b</sup>The reported masses correspond to the b-ions. <sup>c</sup>These values correspond to the total mass of the parent ion.

**Table 3.** The monomer masses as determined by ESI-MS of wild-type 4-OT and a batch of the 4-OT P1Q variant composed of a mixture of P1pE, P1Q and M1P2Q, untreated and treated with 3BP. The samples were treated with NaBH<sub>4</sub> prior to ESI-MS analysis to ensure reduction of the pyruvoyl-adduct to its lactyl-derivative, resulting in irreversible alkylation and the net addition of 87 Da to the total mass of the protein.

Protein	Mass (Da) <sup>a</sup> , untreated with 3BP	Mass (Da) <sup>a</sup> , treated with 3BP
4-OT	6812	6900
P1pE	6825	6825
P1Q	6842	6843, 6930
M1P2Q	6973	6974, 7061

<sup>a</sup>The reported values are accurate to  $\pm 1$  Da.

The demethylation efficiencies of the 4-OT P1Q and 4-OT P1H variants are slightly lower than those reported by Liao and coworkers for glutamine and histidine substitutions at the penultimate position of their target proteins.<sup>[22]</sup> SDS-PAGE analysis of the *E. coli* cell lysate showed that the level of expression of MetAP-\*TG when co-expressed with the variants 4-OT P1Q and 4-OT P1H is similar to that reported by Liao *et al.*<sup>[22]</sup> The slightly lower efficiency of methionine removal for the 4-OT variants is therefore unlikely to be due to an insufficient amount of demethylation catalyst. As MetAP-\*TG utilizes a pair of Co<sup>++</sup> ions as a cofactor, the amount of MetAP-\*TG produced by overexpression might exceed the trace amount of Co<sup>++</sup> ions that is present in the growth medium.<sup>[22]</sup> To eliminate this concern, the 4-OT P1Q and 4-OT P1H variants were co-expressed with MetAP-\*TG under conditions that were found to result in the best achievable methionine removal, and the growth medium was supplemented with 100  $\mu$ M CoCl<sub>2</sub>. However, the addition of additional Co<sup>++</sup> ions to the growth medium did not result in an increased efficiency of methionine removal from either the 4-OT P1Q or 4-OT P1H variant.

*Tautomerase activities of the 4-OT variants.* The tautomerase activities of the P1S, P1Q and P1H variants were assayed using 2-hydroxyhexa-2,4-dienedioate as the substrate (Figure 1). All three variants were found to catalyze the tautomerization reaction, albeit with markedly different catalytic efficiencies (Table 4). Before comparing the kinetic parameters, the issue of incomplete removal of the initiating methionine in the case of the 4-OT P1Q and 4-OT P1H variants needs to be considered. Neither the structural consequences of the presence of the initiating methionine in the active site of 4-OT nor the effect it has on the manner in which the monomers assemble into homohexameric oligomers are known. The kinetic parameters of the 4-OT P1Q and 4-OT P1H variants reported in Table 4 likely represent those observed for a mixture of active sites with and without the amino-terminal methionine. The purified batches of each variant that showed the highest percentage of methionine removal (Table 1) were used for the activity assay. The 4-OT P1S variant was found to be fully demethylated, thus the kinetic parameters reported in Table 4 for this variant may be directly compared to those of the wild-type enzyme.

All three Pro-1 variants of 4-OT show a significant decrease in  $k_{cat}$ , whereas the  $K_m$  values are little affected, confirming that Pro-1 is a critical residue for catalysis. The 4-OT P1Q variant, purified as the mixture P1pE/M1P2Q, was found to be the most efficient tautomerase of the three 4-OT mutants with a  $k_{cat}$  of 133 s<sup>-1</sup>. The overall catalytic efficiency (in terms of  $k_{cat}/K_m$ ) is only ~25-fold lower than that of wild-type 4-OT. Of all Pro-1 variants of 4-OT that have been characterized to date, the P1pE/M1P2Q protein exhibits the highest catalytic efficiency towards the native tautomerase substrate.<sup>[17,18]</sup> To address how much M1P2Q contributes to the tautomerase activity of the P1pE/M1P2Q mixture, we produced the P1Q variant without co-expression of MetAP-\*TG. ESI-MS analysis of the purified protein revealed a single peak in the reconstructed mass spectrum corresponding to 6973  $\pm$  1 Da, which shows that the protein was isolated solely as the M1P2Q species (calculated mass: 6973 Da). This allowed us to characterize the tautomerase activity of the non-demethylated M1P2Q protein,

which catalyzed the tautomerization reaction with a  $k_{\text{cat}}$  of  $45 \text{ s}^{-1}$  and a  $K_{\text{m}}$  of  $130 \text{ }\mu\text{M}$  (Table 4). Interestingly, when comparing these kinetic parameters to those obtained for the same reaction catalyzed by the P1pE/M1P2Q mixture, no change in  $K_{\text{m}}$  was observed but the  $k_{\text{cat}}$  was found to be lowered about 3-fold. As the M1P2Q fraction only constitutes about 30% of the total of the P1pE/M1P2Q mixture, these kinetic data suggest that the P1pE-fraction makes a contribution to the observed  $k_{\text{cat}}$  of the P1pE/M1P2Q mixture. However, it is important to emphasize that the electron-withdrawing property of the carbonyl functionality of pyroglutamate results in a much

**Table 4.** Kinetic Parameters for the 4-OT P1S, 4-OT P1Q, and 4-OT P1H catalyzed enol-keto tautomerization of 2-hydroxyhexa-2,4-dienedioate and for the 4-OT P1S, 4-OT P1A, and 4-OT P1G catalyzed decarboxylation of oxaloacetate.

Enzyme	Substrate	$k_{\text{cat}} (\text{s}^{-1})$	$K_{\text{m}} (\mu\text{M})$	$k_{\text{cat}}/K_{\text{m}} (\text{M}^{-1} \text{s}^{-1})$
wt4-OT <sup>a</sup>		$1330 \pm 36$	$50 \pm 4$	$2.66 \times 10^7$
4-OT P1S <sup>a</sup>		$15.3 \pm 0.4$	$47 \pm 4$	$3.25 \times 10^5$
4-OT P1pE/M1P2Q <sup>a</sup>		$133 \pm 2$	$127 \pm 7$	$1.05 \times 10^6$
4-OT M1P2Q <sup>a</sup>		$45 \pm 1$	$130 \pm 9$	$3.46 \times 10^5$
4-OT P1H/M1P2H <sup>a</sup>		$0.07 \pm 0.003$	$73 \pm 9$	959
4-OT P1S <sup>b</sup>		$8.1 \pm 0.7 \times 10^{-3}$	$3.0 \pm 0.7 \times 10^3$	2.7
4-OT P1A <sup>c</sup>		0.08	700	114
4-OT P1G <sup>c</sup>		0.02	$2.0 \times 10^3$	10

<sup>a</sup> These steady-state kinetic parameters were measured in 10 mM  $\text{NaH}_2\text{PO}_4$  buffer (pH 7.3) at 22 °C. <sup>b</sup> These steady-state kinetic parameters were measured in 50 mM  $\text{NaH}_2\text{PO}_4$  buffer (pH 7.3) at 22 °C. <sup>c</sup> These kinetic data were obtained from Brik *et al.*<sup>18</sup> Errors are standard deviations. For all enzymes, including the protein mixtures P1pE/M1P2Q and P1H/M1P2H, the  $k_{\text{cat}}$  values are calculated on basis of the total protein monomer concentration.

lowered basicity of its  $\gamma$ -lactam nitrogen with respect to the secondary amine functionality of proline, making it highly unlikely that pyroglutamate plays a direct role in catalysis.

The kinetic parameters determined for the P1S variant show that it functions as a tautomerase with a  $k_{\text{cat}}$  of  $15 \text{ s}^{-1}$ . As the  $K_{\text{m}}$  was found to be unaffected by the P1S substitution and the 4-OT P1S mutant was found to be fully demethionylated, the detrimental effect on  $k_{\text{cat}}$  with respect to wild-type is likely due to either poor positioning and/or activation of the serine-1 hydroxyl as a catalytic base or the weaker basicity of the primary amine functionality of this residue. The significant decrease in  $k_{\text{cat}}$  and unaffected  $K_{\text{m}}$  are consistent with the trends observed for the P1G and P1A variants analyzed by Whitman and coworkers.<sup>[17]</sup> Thus, the weaker basicity of the primary amine functionality of Ser-1 as compared to the secondary amine functionality of Pro-1 is likely to be the cause for the observed decrease in  $k_{\text{cat}}$ .

The 4-OT P1H variant, purified as the mixture P1H/M1P2H, has very low-level activity with a  $k_{\text{cat}}$  of  $0.07 \text{ s}^{-1}$ . The  $K_{\text{m}}$  is little affected by the histidine substitution, as it is increased only  $\sim 1.5$  fold with respect to that of the wild-type enzyme. As the batch of enzyme used in this kinetic assay consists for 87% of demethionylated enzyme, the large decrease in  $k_{\text{cat}}$  cannot be explained by the presence of the initiating methionine on the remaining 13% of enzyme. Therefore, the large decrease in  $k_{\text{cat}}$  is likely due to the presence of the histidine residue at the amino terminus. Hence, either the secondary amine functionality of His-1 functions as a poor catalytic base, or the relatively large side chain of His-1 causes a poor positioning of the amino-terminal primary amine functionality, thereby impairing its ability to function as the catalytic base.

*Oxaloacetate decarboxylase activity of 4-OT P1S.* Dawson and coworkers have previously shown that the P1A and P1G mutants of 4-OT exhibit promiscuous decarboxylase activity using oxaloacetate as the substrate.<sup>[18]</sup> This prompted us to test whether the P1S mutant of 4-OT, the variant that is fully processed, has oxaloacetate decarboxylase activity (Table 4). Indeed, the P1S mutant possesses decarboxylase activity, but its catalytic efficiency is  $\sim 40$ - and  $\sim 4$ -fold lower than that reported for the P1A and P1G variants, respectively.

The mechanism of oxaloacetate decarboxylation by 4-OT P1A was proposed to involve covalent catalysis.<sup>[18]</sup> A reductive amination performed on a reaction mixture containing oxaloacetate and the P1A variant resulted in alkylation of the amino-terminus of the protein by a species with a mass consistent with that of the covalent adduct of one pyruvate molecule.<sup>[18]</sup> This finding is consistent with a mechanism in which the amino-terminal primary amine functionality acts as a nucleophile and attacks the carbonyl carbon atom of the substrate, forming a covalent imine intermediate that acts as an electron sink, thereby facilitating decarboxylation of the C4-carboxylate group. The resulting product, pyruvate, can be liberated from the amino-terminus by hydrolysis.<sup>[18]</sup> A similar reductive amination experiment was performed with the P1S variant (and the P1A variant as a control), followed by ESI-MS analysis of the protein to reveal the presence of a covalent adduct. Incubation of the P1S variant (1 mg) with

oxaloacetate (10 mM) and subsequent reduction by NaCNBH<sub>3</sub> showed a major peak at 6874 ± 1 Da and a minor peak at 6802 ± 1 Da (P1S monomer) in the ESI-MS spectrum with a ratio of 5 to 1. The major peak corresponds to the monomer modified by a single adduct of 72 Da. The same modification of 72 Da was observed for the control experiment performed with the P1A mutant. Incubation of the P1S variant (1 mg) with pyruvate (10 mM), however, yielded an ESI-MS spectrum that showed a major peak at 6802 ± 1 Da (P1S monomer) and a minor peak at 6874 ± 1 Da (P1S monomer + 72 Da) in a ratio of 5 to 1. These findings are consistent with the observations reported by Dawson and coworkers, the 72 Da adduct corresponding to pyruvate.<sup>[18]</sup> The reversal of the ratio between the native and the alkylated monomer that was observed when the P1S variant was incubated with pyruvate (instead of oxaloacetate), strongly suggests that the pyruvate-adduct that is observed when the protein is incubated with oxaloacetate is an intermediate of the decarboxylation reaction, rather than the result of alkylation by pyruvate (as a product of decarboxylation) that is released into solution.

*Concluding remarks.* In conclusion, the P1S variant of 4-OT, which carries a relatively small residue at the penultimate position, was found to be fully demethylated by wild-type methionine aminopeptidase (MetAP) from the *E. coli* expression host. Like the previously studied P1G and P1A variants of 4-OT, the P1S variant has low-level 2-hydroxymuconate tautomerase and promiscuous oxaloacetate decarboxylase activity. The latter activity likely involves the formation of a covalent imine intermediate between the amino-terminal amine functionality of Ser-1 and the ketoacid substrate. The P1Q and P1H variants of 4-OT, which carry bulky penultimate residues, could only be obtained in a demethylated form (a minor fraction of the purified protein is composed of methylated enzyme) by co-expression with an engineered variant of MetAP, termed MetAP-\*TG. Whereas the P1H variant (i.e., the mixture of P1H/M1P2H) has very low-level tautomerase activity, both the methylated P1Q variant of 4-OT (M1P2Q;  $k_{\text{cat}} = 45 \text{ s}^{-1}$ ) and the demethylated P1Q variant of 4-OT, which by spontaneous intramolecular cyclization is converted into the P1pE variant, appear to function as 2-hydroxymuconate tomerases (P1pE/M1P2Q mixture;  $k_{\text{cat}} = 133 \text{ s}^{-1}$ ). The precise catalytic activity of the 4-OT P1pE variant, and which residue of 4-OT P1pE is responsible for deprotonating the enol functionality of the substrate, remains to be determined, which awaits the preparation of homogenous P1pE enzyme. In future work, we therefore aim to produce 4-OT P1pE in *E. coli* via intracellular cleavage of a N-terminal fusion tag with tobacco etch virus (TEV) protease, according to a recently published method for overproduction of N-terminal pE-modified proteins.<sup>[35]</sup>

The substitution of Pro-1 by a glutamine, followed by *in-vivo* removal of the initiating methionine and cyclization (i.e, deamination) of the glutamine, is a unique way to obtain a structural analogue of proline (5-oxoproline, Figure 3) on the amino-terminus of 4-OT. This opens up new possibilities to study the catalytic importance of Pro-1 in the recently discovered promiscuous C-C bond-forming aldolase and 'Michaelase' activities of 4-OT.<sup>[10-14]</sup> Labeling studies performed with the affinity probe 3BP demonstrate that pyroglutamate is less nucleophilic than proline in the active site of 4-OT. This makes the

P1pE variant an excellent negative control in the study of nucleophilic addition reactions catalyzed by 4-OT.

## Materials and methods

*Materials.* The sources of the chemicals, biochemicals, components of buffers and media, PCR purification, gel extraction and miniprep kits, DEAE- and phenylsepharose, pre-packed PD-10 Sephadex G-25 columns, and the oligonucleotides, enzymes and reagents used in the molecular biology procedures are reported elsewhere.<sup>[29,30]</sup> All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless stated otherwise. 2-Hydroxyumuconate was a kind gift of Professor dr. C.P. Whitman (University of Texas at Austin, TX). The plasmid pET-29(MetAP-\*TG) harboring the gene coding for the engineered *E. coli* methionine aminopeptidase was kindly supplied by dr. Y.-D. Liao (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan).

*General methods.* Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere<sup>[31]</sup> or as suggested by the manufacturer. The PCR was carried out in a DNA thermal cycler (model GS-1) obtained from Biolegio (Nijmegen, The Netherlands). DNA sequencing was performed by ServiceXS (Leiden, The Netherlands) or Macrogen (Seoul, Korea). Protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 10% polyacrylamide. The gels were stained with Coomassie brilliant blue. Protein concentrations were determined by the method of Waddell.<sup>[32]</sup> Kinetic data were obtained on a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus, Software Ltd., Horley, U.K.) obtained from Sigma Chemical Co. ESI-MS and MS/MS spectra were recorded using an LCQ electrospray mass spectrometer (Applied Biosystems, Foster City, CA).

*Construction of pET20b(+)-based expression vectors for the production of 4-OT P1S, 4-OT-P1Q, and 4-OT P1H.* The mutant genes of 4-OT were generated by PCR using plasmid pET-3b(4-OT) as the template.<sup>[8]</sup> The forward primers, which contain a *NdeI* restriction site (underlined) and the desired mutation (in bold) followed by 22 bases corresponding to the coding sequence of the 4-OT gene, were as follows: 5'-A TAG CAG GT C CAT ATG TCG ATT GCC CAG ATC CAC ATC CTT G-3' (P1S-F), 5'-A TAG CAG GTC CAT ATG CAG ATT GCC CAG ATC CAC ATC CTT G-3' (P1Q-F) and 5'-A TAG CAG GTC CAT ATG CAT ATT GCC CAG ATC CAC ATC CTT G-3' (P1H-F). In all cases, the following reverse primer was used: 5'-G TGA TGT TAT GGA TCC TCA GCG TCT GAC CTT GCT-3' (*Bam*HI restriction site underlined). The amplification mixture was made up in Phusion High-Fidelity buffer and contained 250 μM of each dNTP, 150 ng of each primer, 50 ng of pET-3b(4-OT), and 1 U of Phusion DNA polymerase. The cycling parameters were 95 °C for 5 min followed by 35 cycles of 98 °C for 30 s, 45 °C for 30 s and 72 °C for 20 s, with a final elongation step of 72 °C for 10 min. The resulting PCR product and the pET-20b(+) vector (Novagen) were digested with *NdeI* and *Bam*HI restriction enzymes, after which the vector was dephosphorylated with alkaline phosphatase. Following purification, the PCR product

and vector were ligated using T4 DNA ligase. An aliquot of the ligation mixture was transformed into competent *E. coli* DH10B cells. Transformants were selected at 37 °C on LB/ampicillin plates. Plasmid DNA was isolated from several colonies and analyzed by restriction analysis for the presence of the insert. The cloned gene was sequenced to verify that no mutations had been introduced during the amplification of the gene. The newly constructed expression vectors were named pET-20b(4-OT/P1S), pET-20b(4-OT/P1Q) and pET-20b(4-OT/P1H).

*Construction of the pET-26b(4-OT/P1Q), pET-26b(4-OT/P1H) and pET-20b(MetAP\**TG*) expression vectors.* In order to obtain the genes coding for the 4-OT P1Q and 4-OT P1H variants for the recloning procedure, sufficient amounts of the plasmids pET-20b(4-OT/P1Q) and pET-20b(4-OT/P1H) were digested with *Nde*I and *Bam*HI restriction enzymes (in separate experiments), and the mutant genes of 4-OT were subsequently purified from 1% agarose gels. The pET-26b vector was digested with *Nde*I and *Bam*HI restriction enzymes, dephosphorylated with alkaline phosphatase, and purified. The 4-OT P1Q and 4-OT P1H genes and the vector were ligated using T4 DNA ligase. The pET-20b(MetAP\**TG*) expression vector was constructed using the same procedure with the following modification. The MetAP\**TG* gene used for the recloning procedure was obtained by digesting a sufficient amount of the pET-29(MetAP\**TG*) plasmid with *Sac*I and *Hind*III restriction enzymes, followed by gel-based purification. The pET-20b(+) vector was digested with *Sac*I and *Hind*III restriction enzymes, dephosphorylated with alkaline phosphatase, and purified. The MetAP\**TG* gene and the vector were ligated using T4 DNA ligase. Of each ligation mixture an aliquot was transformed into competent *E. coli* DH10B cells. Transformants were selected at 37 °C on LB/ampicillin plates. Plasmid DNA was isolated from several colonies and analyzed by restriction analysis for the presence of the insert. The cloned gene was sequenced to verify that no mutations had been introduced during the recloning procedure.

*Expression and purification of mutant 4-OT proteins.* The mutant 4-OT proteins and MetAP\**TG* were co-produced in *E. coli* BL21(DE3) using the T7 expression system. The 4-OT P1S and 4-OT P1H mutants were expressed as follows. Fresh BL21(DE3) transformants containing the appropriate expression plasmid (or plasmids) were collected from an LB agar plate and used to inoculate LB medium (10 mL). After growth for 3-6 h at 37 °C, this culture was used to inoculate fresh LB medium (1 L) to a starting OD<sub>600</sub> of about 0.01. LB agar plates and LB medium were supplemented with ampicillin (100 µg/mL), or with ampicillin (100 µg/mL) in combination with kanamycin (30 µg/mL) and IPTG (100 µM) when the 4-OT variants were co-produced with MetAP\**TG*. After overnight growth at 37 °C, with vigorous shaking, cells were harvested by centrifugation (10 min at 2300g) and resuspended in 10 mM NaH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.3, to a total volume of about 10 mL.

The 4-OT P1Q variant was expressed using the same protocol with the following modification. When expressed using the vector pET-20(4-OT P1Q), the host organism was grown in LB medium as described above, yielding non-demethylated 4-OT P1Q (M1P2Q). When using the pET-26(4-OT P1Q) vector, auto-induction (ZYM) medium (10 g/L tryptone, 5 g/L yeast extract, 25 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM Na<sub>2</sub>SO<sub>4</sub>,

pH 6.7) was used, supplemented with 0.5% (v/v) glycerol, 0.05% (w/v) glucose, MgSO<sub>4</sub> (2 mM), kanamycin (30 µg/mL), and 0.2% (w/v) lactose. When co-produced with MetAP-\*TG using the vector pET-20b(MetAP-\*TG), the medium was further supplemented with ampicillin (100 µg/mL). Co-production with MetAP-\*TG resulted in the production of the 4-OT P1Q variant as a mixture of P1pE/P1Q/M1P2Q or P1pE/M1P2Q proteins.

The purification of the mutant 4-OT proteins is identical to that of wild-type 4-OT, which has been reported elsewhere.<sup>[10,11]</sup> Purified proteins were concentrated to ~10 mg/mL in 10 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3), frozen in liquid nitrogen, and stored at -80 °C until further use.

*Enzyme assays.* The tautomerization activity of wild-type 4-OT and the mutant 4-OT proteins were measured by monitoring the ketonization of 2-hydroxymuconate to 2-oxohex-3-enedioate in 10 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3) at 22 °C. Stock solutions of 2-hydroxymuconate were generated by dissolving the appropriate amount of 2-hydroxymuconate in absolute ethanol. The assay buffer was prepared by diluting the appropriate amount of enzyme (from a ~10 mg/mL stock solution) in 50 mL of 10 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3). The enzyme was allowed to equilibrate for one hour at 22 °C. The ketonization of 2-hydroxymuconate to 2-oxohex-3-enedioate was monitored by following the increase in absorbance at 236 nm ( $\epsilon = 6,58 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ).<sup>[2]</sup> The assay was initiated by the addition of a small aliquot of substrate (2-15 µL) to 1 mL of the assay buffer in a quartz cuvette. At all substrate concentrations, the nonenzymatic rate was subtracted from the enzymatic rate of ketonization.

The oxaloacetate decarboxylase activity of the 4-OT P1S and 4-OT P1A variants was measured by following the depletion of the enol-form of oxaloacetate (oxobutanedioic acid) in 50 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3) at 22 °C.<sup>[18]</sup> A stock solution (1 M) of oxaloacetate was made by dissolving the crystalline free acid in absolute ethanol directly before use. The assay buffer was prepared by diluting the appropriate amount of enzyme (from a ~10 mg/mL stock solution) in 30 mL of 50 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3) and allowed to equilibrate for one hour at 22 °C. The assay was initiated by adding a small aliquot of substrate (2-15 µL) to 1 mL of the assay buffer in a quartz cuvette. The decarboxylation of the keto-form of oxaloacetate was monitored by following the decrease of absorbance of the enol-form at 295 nm ( $\epsilon = 3,0 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$ ). Upon addition of the substrate to the assay buffer and the establishment of the enol-keto equilibrium, the rate of decarboxylation of the keto-form may be assumed to be equal to the rate of depletion (i.e., ketonization) of the enol-form, as the keto-form of the substrate exists in rapid equilibrium with the enol-form under the assay conditions. At all substrate concentrations, the nonenzymatic rate was subtracted from the enzymatic rate of decarboxylation.

*Mass spectral analysis of mutant 4-OT proteins and peptide mapping.* The protein samples for ESI-MS analysis were made up as follows. A protein sample stored in 10 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3) was exchanged against 5 mM NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 7.3) using a pre-packed PD-10 Sephadex G-25 gel filtration column. The protein was concentrated to a concentration of 0.5 - 1 mg/mL using a Vivaspin centrifugal



concentrator equipped with a 5000 Da molecular weight cut-off filtration membrane (Sartorius Stedim Biotech S.A., France). An appropriate amount of protein was subsequently diluted to a final concentration of 25  $\mu\text{M}$  in a sample volume of 100  $\mu\text{L}$ . Samples were frozen in liquid nitrogen and stored at  $-20\text{ }^{\circ}\text{C}$  prior to ESI-MS analysis.

For peptide mapping studies, samples were prepared as described elsewhere.<sup>[10]</sup> LC-MS/MS analysis and data processing were performed as follows. The obtained peptide fractions were introduced into an LC-MS/MS system using an Ultimate 3000 nano (Dionex, Amsterdam, The Netherlands) in-line connected to an LTQ Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany). The sample mixture was subsequently loaded on a trapping column (Acclaim PepMap C18, 5 mm length x 300  $\mu\text{m}$  I.D., 5  $\mu\text{m}$  particle size, 100  $\text{\AA}$  porosity, Dionex, Thermo Fisher Scientific Inc.). After back-flushing from the trapping column, the sample was loaded on a reverse-phase column (Acclaim PepMap C18, 15 cm length x 75  $\mu\text{m}$  I.D. x 3  $\mu\text{m}$  particle size, 100  $\text{\AA}$  porosity, Dionex, Thermo Fisher Scientific Inc.). Peptides were loaded in 0.1% formic acid, and were separated with a linear gradient from 1% solvent A (0.1% formic acid) to 90% solvent B (0.1% formic acid in acetonitrile) at a flow rate of 250 nL/min. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS/MS acquisition for the five most abundant doubly and triply charged ions with a minimal signal of 2500 in a given MS spectrum. Full scan MS spectra were acquired in the Orbitrap at a target value of  $1.0 \times 10^6$  with a resolution of 60,000. The five most intense ions which meet the set criteria were then isolated for fragmentation in the linear ion trap, with a dynamic exclusion of 10 seconds. Peptides were fragmented after filling the ion trap at a target value of  $1.0 \times 10^4$  ion counts. Data was analyzed using Peak6 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada).

*Irreversible alkylation of 4-OT and 4-OT P1Q with 3-bromopyruvate.* The irreversible alkylation of wild-type 4-OT and 4-OT variants with 3BP and subsequent sample preparation for ESI-MS analysis was performed as described elsewhere.<sup>[29]</sup>

*Reductive amination of 4-OT P1S and 4-OT P1A.* Imine formation of 4-OT mutants with oxaloacetate or pyruvate was determined as follows. Stock solutions of oxaloacetate (100 mM) and pyruvate (200 mM) were made up in 10 mM  $\text{NaH}_2\text{PO}_4$  buffer (pH adjusted to 7.3). The enzyme (1 mg, 147  $\mu\text{M}$ ) was incubated for 5 minutes at  $22\text{ }^{\circ}\text{C}$  in the presence of oxaloacetate (10 mM) or pyruvate (10 mM) in 1 mL of 10 mM  $\text{NaH}_2\text{PO}_4$  buffer (pH 7.3). The reaction mixture was subsequently made 4 mM in  $\text{NaCNBH}_3$  (from a 1 M stock solution in  $\text{H}_2\text{O}$ ) and incubated for 10 minutes at  $22\text{ }^{\circ}\text{C}$ , thus irreversibly trapping iminium ions by reduction to their respective amines. The reaction mixture was then exchanged to 5 mM  $\text{NH}_4\text{HCO}_2$  buffer (pH 7.3) for ESI-MS analysis of the modified proteins, as described above.

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