Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3891–3897

BIOORGANIC & MEDICINAL CHEMISTRY

The Product of the Natural Reaction Catalyzed by 4-Oxalocrotonate Tautomerase Becomes an Affinity Label of Its Mutant

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Received 12 July 2002; accepted 24 July 2002

Abstract—4-Oxalocrotonate tautomerase (4-OT) catalyzes the isomerization of 4-oxalocrotonate, **1**, to 2-oxo-3E-hexenedioate, **3**, using a general acid/base mechanism that involves a conserved N-terminal proline residue. The P1A and P1G mutants have been shown to catalyze this isomerization but at reduced rates. Analysis of these mutants by mass spectrometry demonstrated that P1A is susceptible to a 1,4-addition of the N-terminal primary amine across the double bond of enone **3** to form a covalent adduct. Although slower than the isomerization reaction, the addition is fast, with 50% of the active sites being alkylated within 12 min. By contrast, the wt4-OT shows no detectable modification over 24 h. These results support the hypothesis that avoidance of nucleophilic reactions, such as the irreversible Michael addition to the product, could be a contributing factor in the evolutionary conservation of N-terminal proline residues in 4OT.

Introduction

4-Oxalocrotonate tautomerase (4-OT, EC 5.3.2), which was discovered in the soil bacterium *Pseudomonas putida* mt-2, is a homohexameric enzyme with 62 amino acids per monomer.[1] It catalyzes the isomerization of the unconjugated α-ketoacid, 2-oxo-4E-hexenedioate (4-oxalocrotonate, **1**) to its conjugated tautomer, 2-oxo-3E-hexenedioate, **3** (Scheme 1). Extensive structural and kinetic studies with this enzyme have established the catalytic mechanism[2] in which the essential secondary amine of the N-terminal Pro residue ($pK_a \sim 6$) acts as a general base, transferring a proton from C-3 to C-5 to produce **3** through the dienolate intermediate, **2**. It has been proposed on the basis of both mutagenesis studies[2] and calculations[3] that, in addition to the general base catalysis by Pro1, the enzyme uses Arg39 to stabilize the oxyanion in **2**.

Being the only natural amino acid with a secondary amine group, proline can present a unique chemical functionality at the N-terminus. Interestingly, the entire 4-OT superfamily, which includes 4-OT, macrophage migration inhibitory factor (MIF, also known as phenylpyruvate tautomerase, PPT),[4] and 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI),[5] are characterized by a hydrophobic active site that accommodates a catalytic N-terminal proline with a perturbed pK_a. Kinetic studies with 4-OT in which Pro1 was mutated to either Ala or Gly (P1A or P1G) showed that although the substrate binding did not change significantly, the k_{cat} values dropped by two orders of magnitude. These results were attributed, at least partially, to the decreased basicity and increased flexibility of the general base.[6]

Michael acceptors, such as α,β-unsaturated ketones, are potential affinity labels because they can covalently trap a nucleophilic active-site residue, for example primary and secondary amines. Accordingly, the enzymatic conversion of a substrate into a Michael acceptor could become part of a suicide inhibition mechanism.[7] Although the product **3** of the natural reaction catalyzed by 4-OT is an α,β-unsaturated ketone, we find that it does not alkylate the active-site proline over 24 h. In

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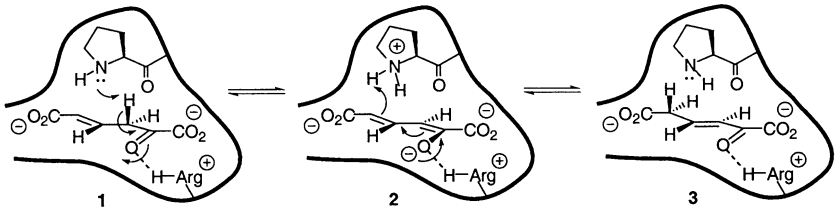
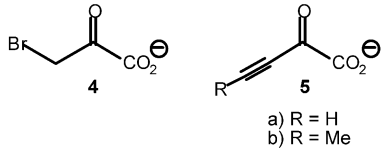
PII: S0968-0896(02)00385-1



Scheme 1.



Scheme 2.

contrast, several irreversible inhibitors, including 3-bromopyruvate,[8] **4** and highly reactive Michael acceptors such as 2-oxo-3-butynoate, **5a**, and 2-oxo-3-pentynoate, **5b** (Scheme 2), have been described.[7] The crystal structure of 4-OT that was irreversibly inactivated by **5b** confirmed previous results that the Pro1 amine group was indeed alkylated by this inhibitor.[7,9] Apparently, steric factors and rigidity of the pyrrolidine ring prevents the approach of the nucleophilic amine residue ($pK_a \sim 6$) to C-4 in **3** and thereby protect the wild-type enzyme from product poisoning.

We have recently described an example of catalytic promiscuity in the mutants of 4-OT in which a change in the catalytic activity and mechanism of this enzyme was observed in a designed single amino acid mutant. While the wild-type enzyme catalyzed only the tautomerization of 4-oxalocrotonate, the P1A and P1G mutants both catalyzed two mechanistically distinct reactions: the original tautomerization reaction (via a general acid/base mechanism) and the decarboxylation of oxaloacetate (via an nucleophilic mechanism).[10] Previous NMR studies on these mutants have indicated that the general protein structure of the P1A and P1G is preserved while the active-site primary amine residue is more mobile than the secondary amine of the wild-type.[6]

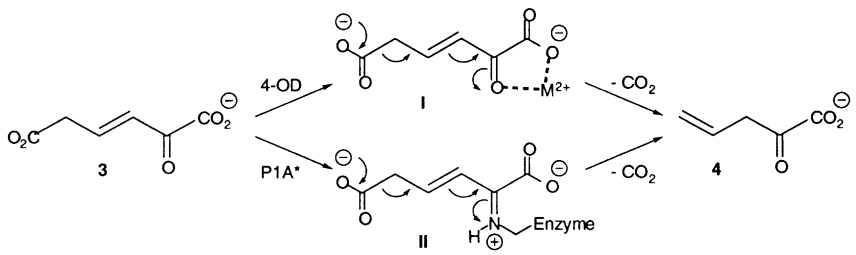
Interestingly, a decarboxylation step follows the tautomerization of **1** to **3** in the degradative pathway in the soil bacterium *P. putida* mt-2, which converts various aromatic hydrocarbons into intermediates in the Krebs cycle.[11] It has been proposed that 4-oxalocrotonate decarboxylase, 4-OD[11,12] catalyzes the decarboxylation of **3** to **4** using a divalent metal ion cofactor in analogy to the natural oxaloacetate decarboxylase.[13] These enzymes form a metal complex with the α-ketoacid portion of the substrate and this complex acts as an electron sink that facilitates the subsequent release of CO_2 (Scheme 3).

Considering the apparent nucleophilicity of the active-site amine residue in either P1A or P1G and their conformational flexibility, we reasoned that further mutations in the active site could lead to an enzyme that uses an imine mechanism to catalyze the decarboxylation reaction of **3** to produce **4** (P1A* Scheme 3). However, although the P1A and P1G mutants catalyze both the isomerization of **1** to **3** and the decarboxylation of oxaloacetate, they do not catalyze the decarboxylation of **3** to **4**. Instead, these mutants undergo a specific 1,4-addition to enone **3** (Michael addition) to form a stable covalent adduct.

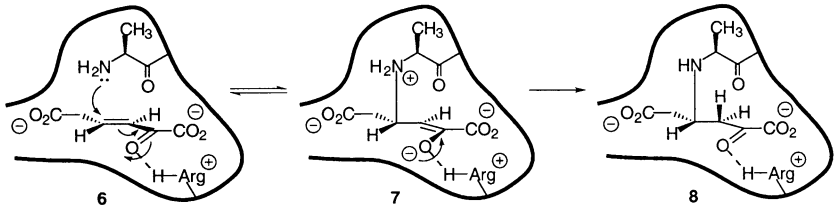
Results and Discussion

The proposed decarboxylation of 2-oxo-3E-hexenedioate, **3**, by a 4-OT mutant would proceed through an imine intermediate. To check whether P1A can form an imine intermediate with **1** or **3**, the enzyme was incubated with **1** (1 mM) and $NaBH_3CN$ for 45 min (the reversible isomerization reaction of **1** to **3** reaches equilibrium at 90% product within 10 s). The protein was then purified and analyzed by ESI-MS. Surprisingly, the mass increase corresponded to the entire mass of **3**, suggesting that 1,4 addition of the protein to the enone system had occurred (Scheme 4) instead of 1,2 addition to the carbonyl followed by reduction.

The alkylation of P1A was monitored by ESI-MS over time without reducing agent. As shown in Figure 1A the mass corresponding to the modified enzyme (6925 Da) increased as a function of time. Although ESI-MS is not



Scheme 3. Two possible mechanisms for the decarboxylation of **3** to **4**. The upper route, used by the natural enzyme 4-OD involves activation of the substrate with a metal ion. The alternative, lower route utilizes imine intermediate as an electron sink.



Scheme 4. Proposed mechanism for the alkylation of 4-oxalocrotonate with P1A.

strictly quantitative, it is reasonable to assume that the ionization efficiency for both proteins is nearly identical and the relative intensities of the MS signals reflect relative molar ratios. The mass difference between these species (158 Da), corresponds to the mass of the substrate and reflects the addition of the protein N–H group across the C–C double bond of **3**. As can be seen from Figure 1B, the first 50% of the active sites were alkylated within 12 min and essentially no further alkylation of the other 50% was observed even after 6 h. Identical results were observed with the mutant P1G. The wild type 4-OT, however, failed to show any covalent addition products even after 24 h with high concentration (2 mM) of substrate.

The half-site stoichiometry, which has previously been reported for the inactivation of wt4-OT by either **4** or **5b**, probably reflects the X-ray hexameric structure of wt4-OT, which is a trimer of dimers. Since residues of at least two subunits participate in each active site,[5] the phenomenon of half-site stoichiometry was proposed to result from an intradimer conformational changes where the modification of Pro1 in one monomer may slow or preclude the modification of Pro1 in the the neighboring subunit.[8] Indeed, the crystal structure of the protein that was inactivated by **5b** showed that the modified adduct interacts with residues from the two neighboring subunits.[9] Examination of the catalytic activity of the alkylated P1A under various concentrations of substrate (40–400 μM) and alkylated P1A enzyme (5–50 nM) revealed that the 50% alkylated P1A was essentially inactive as a catalyst in the isomerization reaction of **1** to **3** (see Experminental). This observation points at specific modification of an active site residue and is consistent with the proposal that the enzyme uses only half of its active sites to catalyze the isomerization reaction.

To identify the specific amino acid residue in P1A that was alkylated with **3**, the modified P1A was reduced with $NaBH_4$ to give alcohol **9** (Scheme 5) prior to the trypsin digestion in order to prevent elimination back to **3** and P1A under the trypsin cleavage conditions. The reduced adduct was purified by HPLC and subjected to proteolytic digestion by trypsin. The resultant peptide mixture was separated by HPLC to give six well-defined components (Fig. 2). A mass of 1381 Da, which corresponds to the modified amino-terminal fragment, $^{\alpha}N$-$C_6H_9O_5$-AIAQIHILEGR was found at retention time of 14.7 min (fraction 4). This fragment was not found in the peptide mixture that resulted from the proteolytic digestion of the unmodified P1A and had a different retention time from the unmodified peptide AIAQIHILEGR (fraction 3). These results indicate that a single site on the enzyme has been modified and that site is located within the 11-residue amino-terminal portion. The only chemically plausible sites for alkylation within this portion are Ala1 and His6. Peptide mapping of the $^{\alpha}N$-$C_6H_9O_5$-AIAQIHILEGR (see Experimental) fragment using MS/MS strongly confirmed that monoalkylation had occurred on the Ala1 residue.

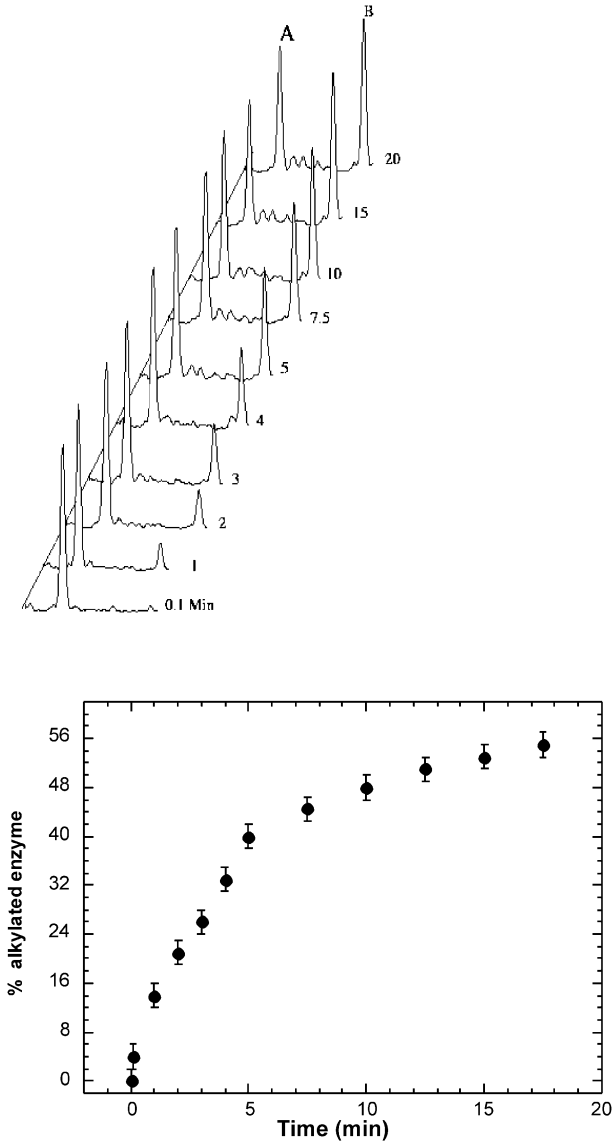
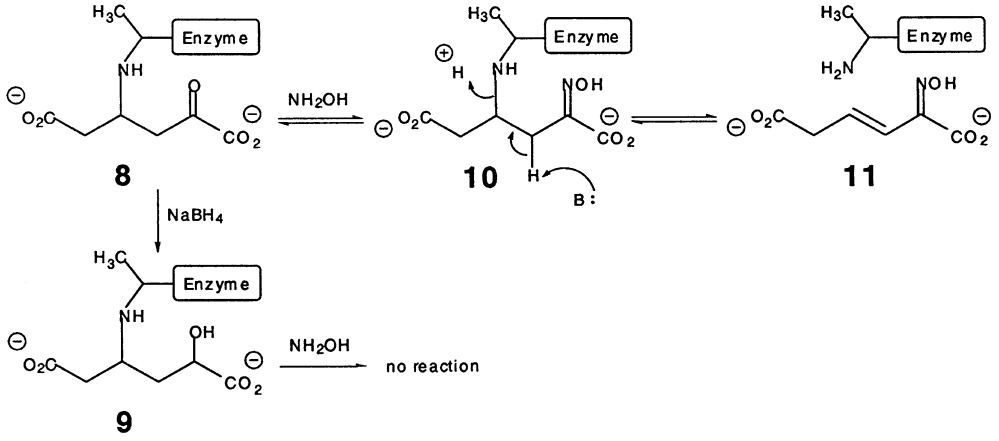


Figure 1. Progress of the alkylation reaction of P1A by **3**. Top: Hypermass reconstruction of the raw MS data to single charge states. The measured mass for A is 6767 Da and for B is 6925 Da. Bottom: Plot of conversion as a function of time.



Scheme 5. Proposed mechanism for the hydrolysis of the alkylated species by hydroxylamine.

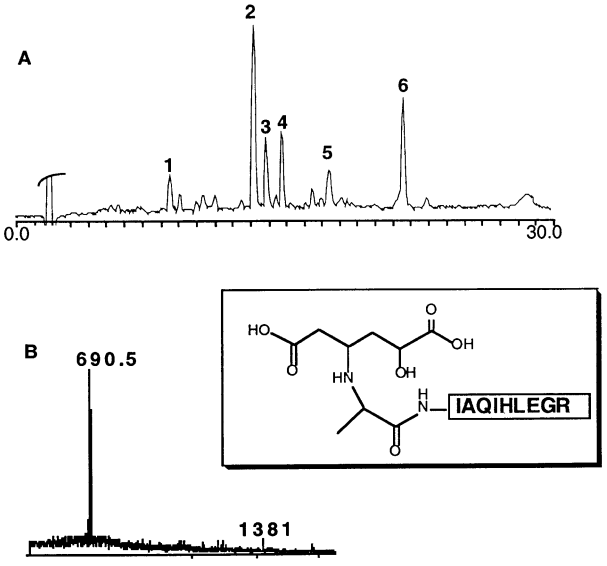


Figure 2. HPLC analysis of the trypsin digestion mixture of the modified P1A. The enzyme (100 μM), which was modified with **1** and $NaBH_4$, was incubated with trypsin (0.4 μM) in Tris buffer (pH 8.0) at 37 °C for 3 h and analyzed by HPLC (C-18 reversed-phase column, 0–60% acetonitrile/30 min, monitored at 214 nm). The numbered fractions were collected and analyzed by ESI-MS: fraction (retention time, M_r, peptide). 1 (8.6 min, 890, E22-R29), 2 (13.2 min, mixture of two peptides, 1058, S30-R39, and 1172, G48-K59), 3 (13.8 min, 1221, A1-R11) 4 (14.7 min, 1381, $^{\alpha}N$-$C_6H_7O_5$-A1-R11), 5 (17.5 min, 1584, G48-R62), 6 (21.6 min, 5356, $^{\alpha}N$-$C_6H_7O_5$- A1-K47).

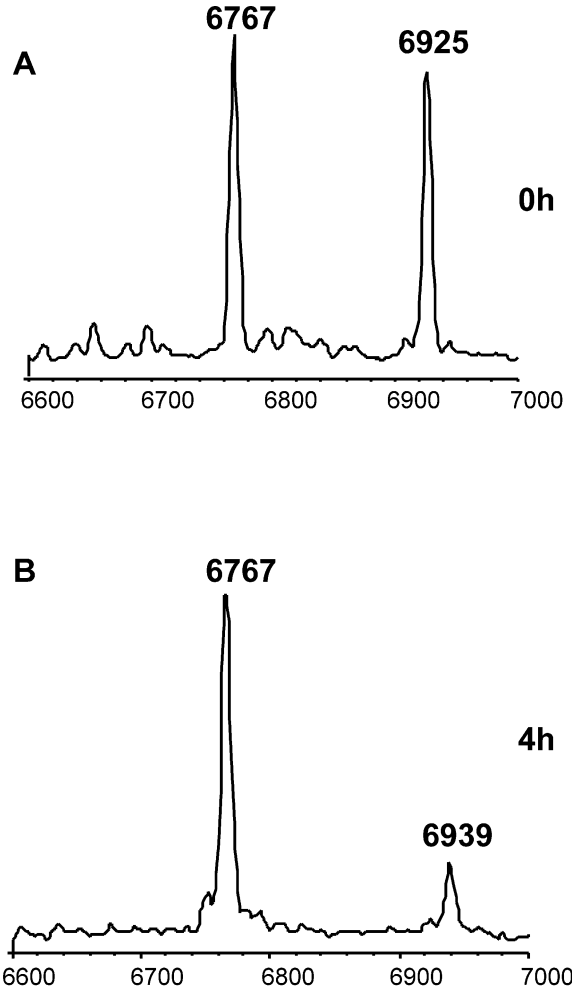


Figure 3. (A) ESI-MS analysis of HPLC-purified P1A with 4 oxalocrotonate, the mass of 6767 Da corresponds to free P1A, and 6925 Da corresponds to modified P1A; (B) ESI-MS analysis of HPLC-purified $^{\alpha}N$-$C_6H_7O_5$-P1A and free P1A after 4 h incubation with NH_2OH, the mass of 6939 Da corresponds to the aminoxy intermediate that eventually hydrolyze to the free P1A (6767 Da).

The structure of the covalent adduct **8** was further confirmed by incubation of the modified P1A with 1 mM hydroxylamine. As illustrated in Scheme 5, this treatment led to 90% recovery of the free enzyme within 4 h. Monitoring the progress of this reaction by HPLC and ESI-MS (Fig. 3) showed complete disappearance of the original adduct **8** (6925 Da) and formation of the free enzyme (6767 Da, 90%) and the oxime derivative **10** (6939 Da). Additional support for this mechanism was obtained from the fact that alcohol **9**, which was obtained from $NaBH_4$ reduction of **8**, was found to be stable upon treatment with hydroxylamine for more than 10 h.

These results show that the product of the isomerization reaction, **3**, becomes an affinity label of the P1A mutant. Three lines of evidence support the N-terminus being the modified active-site residue: (1) the wt4-OT does not covalently react with **3**, (2) the MS data showed that only one residue per mutant subunit was modified, and (3) trypsin digestion combined with MS/MS experiment

confirmed that this monoalkylation occurred in the first residue (Ala1 residue).

Although the irreversible alkylation reaction of **6** to **8** (Scheme 4) is essentially complete (for half-site enzyme stoicheometry) within 12 min, it is still slower than the reversible isomerization reaction of **1** to **3**, which is complete within 10 s under the same conditions (the equilibrium ratio of **1** and **3** is 1:10). The kinetic parameters for the isomerization reaction, which have already been determined for these proteins,[6] were confirmed by us for the Met45Nle mutans:[10] P1A, $k_{cat} = 12$ s^{-1}, $K_M = 35$ μM; P1G, $k_{cat} = 12$ s^{-1}, $K_M = 60$ μM; wt4-OT, $k_{cat} = 2050$ s^{-1}, $K_M = 100$ μM. Thus, proton abstraction by the N-terminal amine is preferred over the nucleophilic 1,4 addition with this substrate, allowing the enzyme to turn over many times before being alkylated. Nucleophilic 1,4 addition of the N-terminal amine group to **3** would form an enolate intermediate, **7**, and then, after two proton transfer steps, the saturated ketone **8**. This transformation could be facilitated by the carbonyl polarization effect of Arg39, in analogy to the proposed mode of interaction between wt4-OT and its substrate, **1**,[2] and with **5**.[9]

Suicide inhibitors are characterized by high efficiency of covalent modification of the enzyme. The alkylation of P1A by **3** is consistent with a very poor suicide inhibition since only 1 in ~1000 turnovers results in a productive covalent modification. Thus, while substrate **1** could be defined, at least partially, as a suicide inhibitor of P1A, the product, **3**, is better defined as an affinity label.

The above-described observations raise an intriguing question: what is the origin of reactivity of the P1A and P1G mutants towards the tautomerization product, **3**, as compared with the absolute inertness of wt4-OT? Whitman et al. have suggested that replacement of the conformationally rigid, secondary cyclic amine function of Pro1 by the more flexible primary amine Gly1 or Ala1 affects the catalytic efficiency in the tautomerization reaction.[6] Their NMR experiments suggested that essentially no structural changes other than increased flexibility had occurred in these mutants.

Accordingly, it is likely that the increased mobility and decreased steric demand of the primary amine in P1A and P1G as compared with wt4-OT allow for the amine function of the mutants to reach over to the C-4 position in **3** in a productive orientation to form a covalent bond. Interestingly, the enhanced nucleophilic character of the amine group in P1A and P1G in comparison with that of the wt4-OT is reflected not only in their ability to react with **3** but also in their ability to catalyze the decarboxylation of oxaloacetate.[10]

If P1A or P1G is reactive towards the C-2 carbonyl, required for the decarboxylation of **3** by an imine mechanism, then it occurs at a significantly slower rate than the Michael addition. It is possible that further mutations in the active site of 4-OT could shift the regioselectivity of the nucleophilic attack on the enone system of **3** from the currently observed 1,4 addition mode to the 1,2 addition mode. A mutant capable of a direct nucleophilic attack on the carbonyl carbon with subsequent formation of an imine intermediate could become a new 2-oxo-3-hexenedioate decarboxylase. However, this reactivity would have to compete with both the isomerization reaction and the Michael addition. Since an X-ray structure of the complex of wt4-OT with its natural substrate is not yet available[7] further structural analysis of the product-alkylated P1A and P1G mutants could shed light on the catalytic mechanisms of these enzymes. Work towards this goal is currently underway.

There are a few examples in the literature where the product of a catalyzed reaction by natural enzyme or its mutants reacts covalently with the enzyme. For example the observation that a single mutation, E197D, in histidine decarboxylase led to inactivation of the enzyme by the natural substrate.[14] Another example along this line is the inactivation of morphine dehydrogenase by the product of the catalyzed reaction, in which the free Cys-80 undergoes Michael addition to morphinone, a reactive α, β-unsaturated ketone.[15] In this study and in the contrary to our example, it was found that the mutant enzyme (Cys80Ser), which has similar kinetic properties to the wild type, is less prone to alkyation.

The enzyme formamidopyrimidine-DNA glycosylase (Fpg), which plays a prominent role in the repair of oxidatively damaged DNA, contains a catalytic N-terminal proline residue, which is conserved in all Fpg proteins.[16] It was proposed that the enzyme uses its proline to form an iminium intermediate that acts as an electron sink to facilitate formation of an α,β-unsaturated aldehyde via β-elimination of an oxygen function. Interestingly, the P1G mutant of this enzyme was found to be a poor catalyst of the natural reaction although it retained its ability not only to bind the substrate but also to form an imine intermediate with the substrate.[16] Although the origin of low activity of the P1G mutant of Fpg has not yet been verified, it is tempting to draw an analogy between Fpg and wt4-OT and suggest that one reason for the conservation of an N-terminal proline in these enzymes could be the need to protect the enzyme from Michael-type alkylation by the product of the natural reaction.

One driving force in enzyme evolution is the optimization of the catalytic efficiency of the desired reaction.[17] Yet, the avoidance of either undesired reactions or those that release toxic products is probably another important factor in adaptive evolution.[17,18] Such undesired activities may be unraveled in the mutants of any natural enzyme. For example, it is not surprising that wt4-OT does not catalyze the decarboxylation of oxaloacetate,[10] because this catalytic activity could be detrimental to aerobic organisms, which use oxaloacetate as an essential component of the citric acid cycle. Nevertheless, the P1A and P1G mutants of this enzyme do catalyze this decarboxylation reaction. Similarly, compound **3**, which was found here to alkylate P1A and P1G, is non-toxic for wt4-OT.

Conclusions

It is well known that highly evolved enzymes are optimized not only to catalyze a desired reaction but also to avoid undesired processes. Mutation of active site residues designed to decrease the optimized catalytic activity may also enhance alternative reaction pathways. Thus, even a minor change in the active site residues could result in a dramatic change in the delicately optimized balance of their chemical reactivities. 4-oxalocrotonate tautomerase (4-OT) catalyzes the isomerization of **1** to **3** using a general acid/base mechanism that involves a conserved N-terminal proline residue. The P1A mutants has been shown to catalyze this isomerization but at a reduced rate. Here, we have demonstrated that the P1A mutant of wt4-OT also undergoes specific 1,4-addition to the tautomerization product, enone **3**, to form a stable covalent adduct. This nucleophilic reactivity of P1A is remarkable considering the complete lack of reactivity of the wild-type enzyme in this reaction. Conservation of the N-terminal Pro could be a strategy used by Nature to restrict the nucleophilic reactivity of an amine in the active site of this enzyme.

Materials and Methods

Materials

Buffers for the kinetic measurements were freshly prepared using de-ionized water. Mono- and dibasic sodium NaH_2PO_4 was purchased from Fisher Biotech. All Boc-protected amino acids were obtained from Midwest BioTech (Fisher, IN, USA). 2-(H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIEA) were obtained from Quantum Biotechnologies (Montreal, CA, USA). *N*,*N*-Dimethylformamide (DMF) and HPLC-grade acetonitrile were purchased from Fischer. Trifluoroacetic acid (TFA) was obtained from Halocarbon (Hackensack, NJ, USA). HF was purchased from Matheson (Cucamonga, CA, USA).

Synthesis of 4-OT and mutants

The polypeptide chain of the 62 amino acid monomeric units of the wt4-OT was synthesized manually on a 0.4-mmol scale using 11-fold excess of Boc-protected amino acids following the in situ neutralization protocols for Boc chemistry, as described earlier.[19] Following chain assembly, His(DNP) groups were removed by treatment of the Boc-peptide-resin with a solution of 20% 2-mercaptoethanol and 5% DIEA in DMF. The polypeptide was deprotected and cleaved from the resin by treatment of the dry peptide-resin with HF, 4% v/v *p*-cresol for 1 h at 0 C. The crude peptide product was precipitated and washed with cold anhydrous ether, dissolved in 6 M Gn·HCl, pH 3 and immediately purified by preparative reversed-phase HPLC. For the synthesis of P1A and P1G, Boc-Ala and Boc-Gly were used instead of Boc-Pro. For all proteins Met45 was replaced with norleucine to prevent oxidation of the enzyme during sample handling.[20]

ESI-MS monitoring of the reaction between P1A and 3

The enzyme P1A (95 μL, 13.3 μM, in NaH_2PO_4 buffer, 100 mM, pH 7.4) was incubated with 4-oxalocrotonate (5 μL, final concentration 1.25 mM) at 24 C. Eleven experiments were carried out at various incubation times between 10 s and 6 h. In each experiment, the reaction was quenched with TFA (pH <2) and immediately analyzed by HPLC (C-18 reverse-phase column, acetonitrile gradient, 0–60%/10 min, monitored at 214 nm). The entire peak (retention time $\sim$7 min) that corresponded to the mixture of both alkylated and unmodified proteins was collected and analyzed by ESI-MS. The wt-4OT was incubated for 24 h and the products analyzed under identical conditions.

Trypsin cleavage

A 1:1 mixture of the modified and unmodified P1A (100 μM) was treated with $NaBH_4$ for 15 h, purified by HPLC as described above and incubated with trypsin (0.4 μM, Tris buffer, pH 8.0) for 3 h at 37 C. Analytical HPLC (acetonitrile gradient 0–60%/30 min) showed that 90% of the proteins were cleaved, resulting in six well-defined HPLC peaks (Fig. 2). The fractions were collected and analyzed by ESI-MS in comparison with the corresponding digestion mixture of an unmodified P1A. For details (fraction, retention time, MW, peptide), see legend to Figure 2. Fraction 4 (14.7 min), which corresponded to $^{\alpha}N$-$C_6H_9O_5$-AIAQIHILEGR was concentrated to dryness under reduced pressure and was dissolved in methanol/water for MS/MS analysis.

Tandem MS/MS analysis

Fraction 4 ($^{\alpha}N$-$C_6H_9O_5$-AIAQIHILEGR) was analyzed by tandem mass spectrometry mapping on a Thermoquest/Finnigan LCQ Deca Ion-Trap mass spectrometer using a standard electrospray ionization source. The parent ion ($m/z=1381$) was isolated and fragmented at a normalized collision energy of 35%. Activation Q and activation time were set at standard values (0.250 and 30 ms, respectively). The presence of the b-ions, 776.2, 889.4, 1002.5, 1036.5 Da, corresponded to N-terminal fragments $^{\alpha}N$-$C_6H_7O_5$-AIAQIH, $^{\alpha}N$-$C_6H_7O_5$-AIAQIHI, $^{\alpha}N$-$C_6H_7O_5$-AIAQIHIL and $^{\alpha}N$-$C_6H_7O_5$-AIAQIHILE. The presence of y-ions, 724.4, 837.5, 965.6, 1037.7, corresponded to the unmodified C-terminal fragments HILEGR, IHILEGR, QIHILEGR and AQIHILEGR. Fragments observed at $m/z=776.2$, 889.4, 1002.5, 1131.5 corresponded to the b-ions of the parent peptide, and the fragments at $m/z=724.4$, 837.5, 965.6, 1037.5 corresponded to the y-ions of the parent peptide.

Hydrolysis of the addition product using hydroxylamine

P1A (1 mg) was folded in 1 mL NaH_2PO_4 buffer (50 mM, pH 7.4) for 3 h, followed by the addition of 4-oxalocrotonate (50 μL of a stock solution of 25 mM in ethanol). The mixture was kept at room temperature for 10 min. The product was purified on HPLC (C-18 reverse-phase acetonitrile gradient 0–60%/10min, monitored at 214 nm) and the desired peak was collected. ES–MS analysis

showed a 1:1 mixture of modified and unmodified P1A. The solution was concentrated to dryness under reduced pressure and the residue was re-dissolved in NaH_2PO_4 buffer (500 μL, 50 mM, pH 7.4). Hydroxylamine (50 μL, final concentration 1 mM) in NaH_2PO_4 buffer (50 mM, pH 7.4) was added and the reaction was kept at 37 C for 6 h. The reaction was purified by HPLC (C-18 reverse-phase column, acetonitrile gradient, 0–60%/10 min, monitored at 214 nm) with the entire peak being collected before analysis by ESI-MS (Fig. 3).

Enzymatic activity of P1A at 50% inactivation

To 95 μL of P1A (18.8 μM, in NaH_2PO_4 buffer, 100 mM, pH 7.4) was added 4-oxalocrotonate (5 μL, final concentration 1.25 mM) and the reaction was kept at room temperature for 12 min. At this point 50% of the active site were alkylated as was indicated by HPLC/ESI-MS analysis on control samples. Part of the solution (22 μL) was was diluted in buffer (378 μL, 100 mM NaH_2PO_4, pH 7.4) to give a final enzyme stock solution of 1 μM. The catalytic activity of the isomerization of **1** to **3** was examined in comparison with the unmodified P1A using established kinetic procedures.[1,6] The enzyme activity was assayed spectrophotometrically at 24 °C by following the appearance of the product at 236 nm. The assay mixture contained 50 mM NaH_2PO_4 buffer, enzyme concentrations ranging from 1 to 50 nM and substrate concentrations between 40 and 400 μM using stock solutions of and a 25 mM substrate in ethanol and 1 μM enzyme (substrate and product concentrations from initial incubation are <1 μM). The initial velocities were determined from the first 10 s of the tracing ($<10\%$ completion of the reaction).

Acknowledgements

This work was supported by the Skaggs Institute for Chemical Biology, (P.D. and E.K.), NIH (GM59380) (P.D.), and the Israel Ministry of Science (A.B.). E.K. is the incumbent of the Benno Gitter & Ilana Ben-Ami Chair of Biotechnology, Technion. We thank Dr. Steven Bark of TSRI open access mass spectrometry facilities for help with the MS/MS experiments.

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