Mutants of 4-Oxalocrotonate Tautomerase Catalyze the Decarboxylation of Oxaloacetate through an Imine Mechanism

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A designed single amino acid substitution can alter the catalytic activity and mechanism of 4-oxalocrotonate tautomerase (4-OT). While the wild-type enzyme catalyzes only the tautomerization of oxalocrotonate, the Pro1Ala mutant (P1A) catalyzes two reactions—the original tautomerization reaction and the decarboxylation of oxaloacetate. Although the N-terminal amine group of P1A is involved in both reactions, our results support a nucleophilic mechanism for the decarboxylase activity, in contrast to the general acid/base mechanism that has been previously established for the tautomerase activity. These findings demonstrate that a single catalytic group in a 4-OT mutant can catalyze two reactions by two different mechanisms.

KEYWORDS:

decarboxylation · imines · oxaloacetate · protein engineering · tautomerases

Introduction

It has been observed that certain enzymes have an inherent functional promiscuity in their catalytic mechanisms or substrate specificities.[1–5] In principle, this promiscuity could be utilized to generate new enzymatic activities from existing protein scaffolds that could be developed into biotechnologically useful catalysts. Additionally, this conversion could give insight into the natural process of evolution. Although different chemical reactions have been observed in these enzymes, most examples of catalytic promiscuity have involved a generally conserved mechanism.

4-oxalocrotonate tautomerase (4-OT, E.C. 5.3.2.–), discovered in the soil bacterium *Pseudomonas putida* mt-2, is a homohexameric enzyme with 62 amino acids per monomer.[6] It catalyzes the isomerization of the unconjugated α-ketoacid, 2-oxo-4*E*-hexenedioate (4-oxalocrotonate; **1**), to its conjugated isomer, 2-oxo-3*E*-hexenedioate (**3**; Scheme 1). Extensive structural and kinetic analyses of this enzyme have enabled the determination of the catalytic mechanism.[7] The secondary amine of the N-terminal Pro residue, which is essential to the catalytic mechanism, acts as a general base ($pK_a \approx 6$) that transfers a proton from C3 in **1** to C5, to afford **3** through the dienolate intermediate **2**. It is likely that this step is facilitated by an arginine residue ($ArgH^+$ in Scheme 1) that polarizes the ketone function and stabilizes the initial enolate product. This anion stabilizing group is probably Arg39, as has already been proposed on the basis of both mutagenesis[7] and calculations.[8]

Our goal was to convert 4-OT from an acid/base tautomerase into an enzyme that utilizes an imine (Schiff base) mechanism, since such enzymes have been engineered to catalyze synthetically useful transformations such as the aldol and retroaldol reaction.[9, 10] Understanding the catalytic role played by the secondary amine group of Pro1 in the wild-type enzyme (wt4-OT) was crucial for this rational design. An interesting characteristic of amines is their functional duality, which is well documented for small amine molecules in solution.[11] In particular, amines can act either as general bases or as nucleophilic catalysts. As nucleophiles, they can form imine intermediates or enamines that facilitate many carbonyl transformation reactions, including decarboxylation of β-ketoacids. The balance between these two catalytic modes depends in part on the level of substitution at the nitrogen atom: secondary amines exhibit stronger basicity than that of primary amines.[12] In addition, secondary amines react with carbonyl compounds to favor formation of enamine intermediates while primary amines favor the imine tautomers (Scheme 1).[11]

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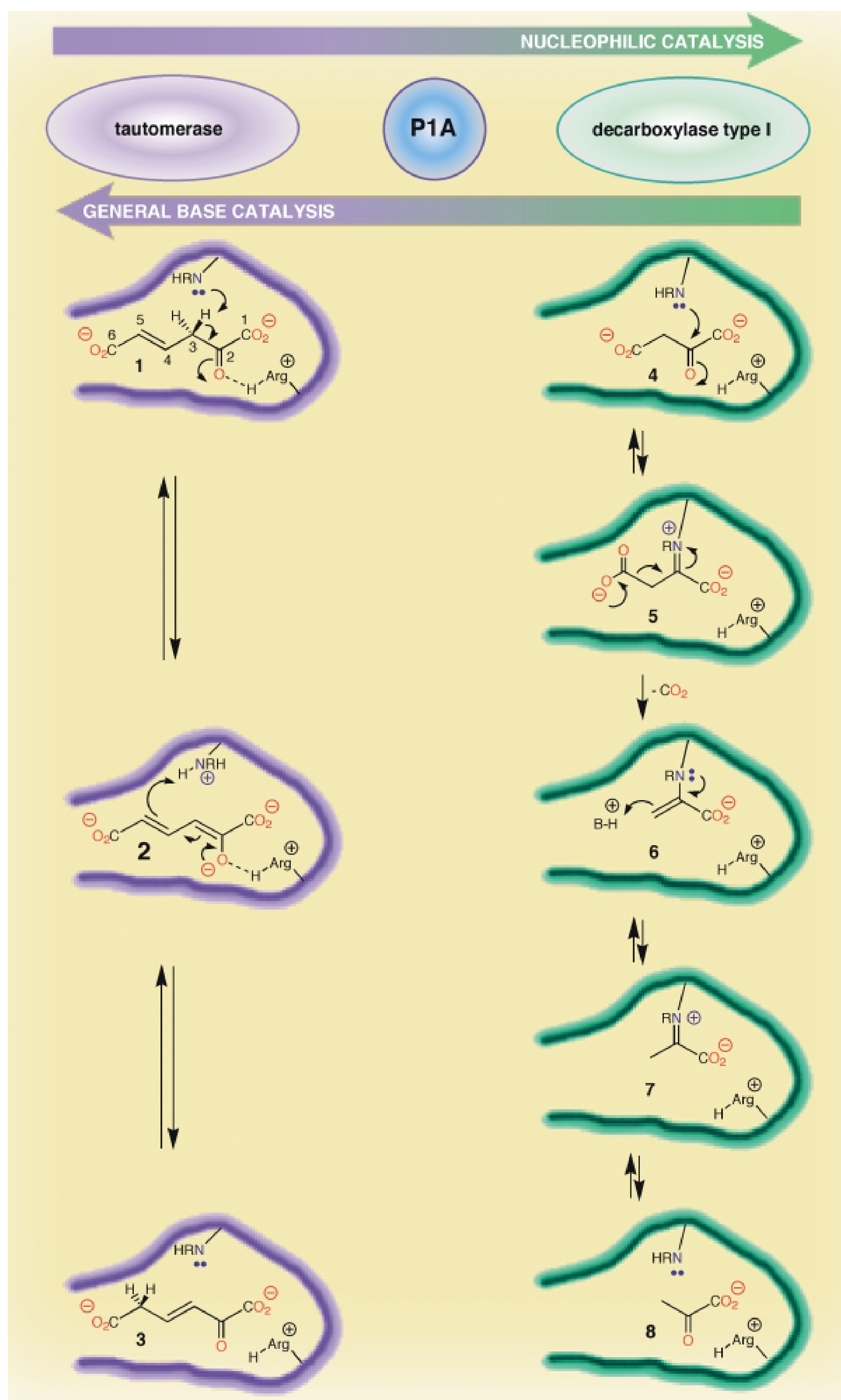

Scheme 1. A plausible scenario for evolution of a new mechanism in 4-OT enzymes. Either of the two specialized enzyme families, tautomerase (such as wt4-OT, left) or "decarboxylase type I" (right) could evolve from one another, with a bifunctional enzyme, such as P1A, being an intermediate in the evolution process. The catalytic amine group can be either primary (R = H) or secondary (R = alkyl). It was proposed that in the isomerization reaction Arg39, which interacts electrostatically with the C1 carboxylate, also polarizes the ketone by hydrogen bonding. It has not been determined whether Arg39 is directly involved in the decarboxylation mechanism as well.

In principle, the mechanistic duality of amines could change the active site mechanism of 4-OT with natural or similar substrates. Replacing the secondary amine functional group with a primary amine could introduce a nucleophilic mechanism into the active site chemistry at the expense of the original general acid/base mechanism. Theoretically the natural transformation, the tautomerization of the α-keto acid, 4-oxalocrotonate, could proceed through an imine mechanism.[13] Alternatively, it has been shown that small-molecule amines,[14–16] designed peptides, and catalytic antibodies can use an imine mechanism to catalyze the decarboxylation of biologically interesting α-keto acids such as oxaloacetate.

In the case of 4-OT, the terminal secondary amine of Pro1 can be converted into a primary amine through the previously studied mutations Pro1Ala and Pro1Gly.[17] Here we report that these single mutations convert 4-OT from a monofunctional enzyme into a bifunctional enzyme that can catalyze two mechanistically distinct reactions in which each reaction utilizes a different substrate.

Results and Discussion

Synthesis of wt4-OT was carried out by using the in situ neutralization protocol for *tert*-butoxycarbonyl (Boc) chemistry.[18] The synthetic protein was purified and folded following previously reported procedures.[19] For all proteins, Met45 was replaced with norleucine to eliminate the possibility of oxidizing the enzyme during sample handling.[20] Two mutants in which the N-terminal proline was replaced by either alanine (P1A) or glycine (P1G) were also synthesized. (The enzymatic activities of the biologically expressed mutants P1A and P1G in the isomerization reaction of **1** into **3** were previously described in detail by Whitman and co-workers.[17]) The synthetic proteins were characterized by ESI-MS, HPLC and, CD spectroscopy. The CD

spectra of the synthetic wt4-OT, P1A, and P1G proteins were identical over a pH range between 5.8 and 8.0. This indicates that the mutations did not cause any gross conformational changes in the synthetic proteins and agrees with results shown previously with the biologically expressed proteins. The synthetic mutants P1A and P1G were also examined for the isomerization reaction of **1** into **3**. The tautomerase kinetics were similar to those previously reported for the recombinant enzymes:[17] (Enzyme: rate of catalysis $k_{cat.}$ (s^{-1}), Michaelis constant K_M (μM)) wt4-OT: 2047 ± 140, 100 ± 20; P1A: 12 ± 2, 35 ± 10; P1G: 12 ± 3, 60 ± 10. This confirms that the synthetic proteins with the additional mutation (Met45Nle) behave similarly to those obtained by biological means.

Although the acid/base mechanism has been well characterized for wt4-OT and it is likely to be maintained in the P1A and P1G mutants,[17] in princlple an imine mechanism could also catalyze the same tautomerization through a 1,2 nucleophilic addition.[13] In order to rule out the alternative mechanism, we carried out the tautomerization reaction in $H_2{}^{18}O$ with both wt4-OT and P1A. If the reaction proceeds by the acid/base mechanism, no ^{18}O will be incorporated in the product. Conversely, since the imine mechanism involves hydrolysis of the immonium intermediate by water, ^{18}O labeling should be observed in the C2 carbonyl group. The reaction was carried out in buffered $H_2{}^{16}O$ and $H_2{}^{18}O$, monitored by UV to completion, and analyzed by ESI-MS. Only unlabeled product (158 Da) was observed with both enzymes with no indication of the ^{18}O-labeled compound (160 Da) within the detection limits ($< 2\%$). These results further support the conclusion that the acid/base mechanism is maintained in wild-type enzymes as well as in the P1A mutant.[17]

Another known reaction that can be catalyzed by nucleophilic amines is the decarboxylation of β-ketoacids. For example, oxaloacetate (OAA) is an α,β-keto acid that is a key metabolic intermediate, acting as an acceptor for C_2 units that enter the citric acid cycle. The naturally occurring decarboxylation of oxaloacetate involves a decarboxylase enzyme that requires a metal cofactor.[21] Although there is no oxaloacetate decarboxylase that employs an imine mechanism, the well-studied enzyme acetoacetate decarboxylase (AAD, E.C. 4.1.1.4) has been shown to proceed through an imine mechanism following a 1,2 nucleophilic addition.[22–24] Small amine molecules[14–16] and designed peptides[25, 26] that catalyze the decarboxylation of oxaloacetate by the imine mechanism have aroused increasing interest.

We assayed wt4-OT for its ability to catalyze the decarboxylation of oxaloacetate and could not detect any catalytic activity above the background reaction (rate of background reaction $k_{un} = 2 \times 10^{-5}\,s^{-1}$ at pH 6.2, 22 °C) under various reaction conditions, including different concentrations of enzyme and substrate and a broad pH range (5.8 – 8.0). By contrast, both P1A and P1G were found to be efficient catalysts of oxaloacetate decarboxylation. Catalysis with both mutants exhibited Michaelis – Menten saturation kinetics (Figure 1) and the catalytic parameters were found to be $k_{cat.} = 0.02\,s^{-1}$, $K_M = 2$ mM for P1G and $k_{cat.} = 0.08\,s^{-1}$, $K_M = 0.7$ mM for P1A. This new catalytic activity is consistent with the expectation that an active site primary

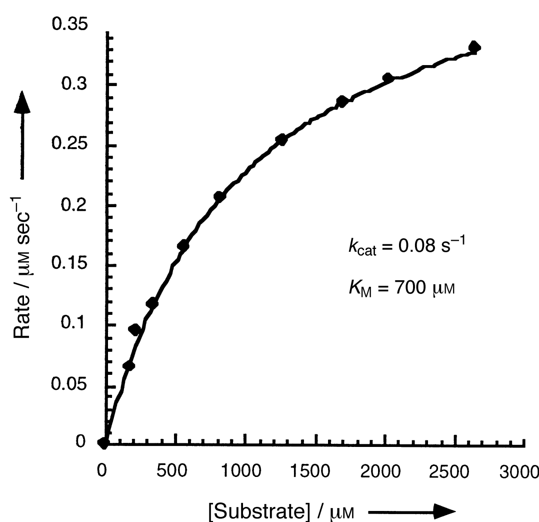

Figure 1. *P1A-catalyzed decarboxylation of oxaloacetate. The reaction was carried out at 22 °C in NaH_2PO_4 buffer (50 mM, pH 6.2).*

amine would be less basic and more conformationally flexible than the secondary amine of the wt4-OT.[11] Irreversible inhibition experiments were carried out either by alkylation of the active site amine with bromopyruvate[28] or by reductive alkylation of the amine with acetone and $NaBH_3CN$. In both cases complete, irreversible inhibition of P1A was achieved. The lack of decarboxylase activity with the otherwise identical wt4-OT provides additional support for the role of the N-terminal primary amine as a key catalytic group in P1A and P1G.

In principle, two different mechanisms could explain the P1A- and P1G-catalyzed decarboxylation of OAA. Both mechanisms involve withdrawal of electron density from the carbonyl carbon atom, which facilitates the subsequent release of CO_2. In the first mechanism, the nucleophilic N-terminal amine of P1A or P1G attacks the ketone of OAA to form an imine (or iminium). The decarboxylated product (pyruvate) would then be liberated by hydrolysis. In the alternative mechanism, activation of the substrate is achieved by noncovalent polarization of the ketone through a Lewis acid or hydrogen bonding. The distinction between these two mechanisms is not trivial since carbonyl polarization by hydrogen bonding could facilitate nucleophilic attack by nitrogen. This polarization could be relevant to the proposed imine mechanism in P1A, since Arg39 is appropriately positioned to polarize the ketone, as has been proposed in the tautomerization mechanism.[7] Alternatively, Arg39 could polarize the carbonyl group sufficiently to directly catalyze the decarboxylation. However, since the wt4-OT did not show any detectable decarboxylase activity under various reaction conditions, this mechanism is unlikely.

Further support in favor of the involvement of an imine mechanism in the decarboxylation of oxaloacetate by P1A is provided by the following experiments. Incubation of P1A with 1 mM oxaloacetate in the presence of $NaBH_3CN$ for 15 min at room temperature followed by HPLC and ESI-MS analyses indicated that more than 95 % of the protein was monoalkylated (Figure 2). The mass increase of the protein monomer ($M+72$)

corresponded to reductive alkylation by one pyruvate molecule (the product of the decarboxylation reaction). Nevertheless, incubation of P1A with pyruvate (1 mM) under the same conditions for 1 h did not produce any reductive alkylation product. Furthermore, pyruvate was found to be a poor inhibitor of the decarboxylation activity of P1A (inhibition constant (K_i) > 8 mM). These results suggest that the initial decarboxylation product, the imine of pyruvate (intermediate **7**, Scheme 1), is reduced before its dissociation from P1A. Finally, when the reductive alkylation experiment with oxaloacetate was carried out with wt4-OT instead of P1A no reductive amination product could be detected, even after incubation for more than 1 h. These results are consistent with the decarboxylase activity of P1A and P1G, which is not observed with wt4-OT.

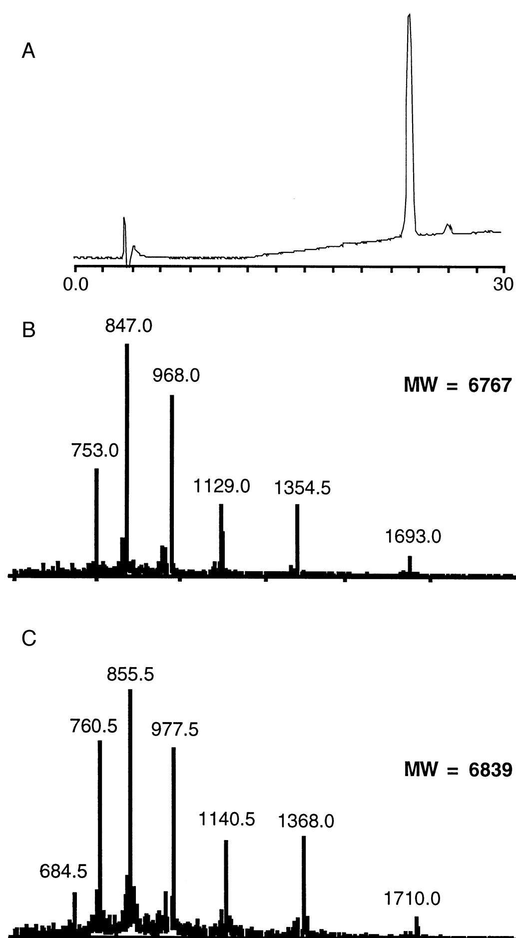

Figure 2. A) Analytical HPLC chromatogram (C-18 reversed-phase column, 0–60% acetonitrile over 30 min, monitored at 214 nm) of the crude peptide $^{\alpha}N$-$C_3H_5O_2$-P1A. The enzyme P1A (1 mg, 100 μM) was incubated with oxaloacetate (1 mM) in NaH_2PO_4 buffer (100 mM, pH 7.4) at 22 °C for 5 min and then the mixture was treated with $NaBH_3CN$ (4 mM) at 27 °C for 10 min. B) ESI-MS analysis of HPLC-purified P1A. C) ESI-MS of the modified enzyme. The crude protein was not purified prior to the ESI-MS analysis except for removal of salts by HPLC.

To identify the alkylation site of P1A, the alkylated enzyme was subjected to proteolytic digestion by trypsin. HPLC separation of the resultant peptide mixture afforded nine well-defined components (Figure 3). Analysis of these components by ESI-MS revealed that each peak corresponded to an individual peptide. A mass of 1292.5 Da, which corresponds to the modified N-terminal fragment, *N*-alkyl-A1–R11 ($C_3H_5O_2$-AIAQIHILEGR) was found in the HPLC chromatogram with a retention time of 16.2 min (fraction 6). This fragment was not found in the peptide mixture that resulted from the proteolytic digestion of the unmodified P1A, in which the unmodified peptide AIAQIHILEGR exhibited a different retention time (fraction 5). These results indicate that a single site on the enzyme has been modified and that site is located within the first 11 amino acids of the protein. Peptide mapping for *N*-alkyl-A1–R11 with an MS/MS experiment strongly supports the assumption that monoalkylation occurred on the Ala1 residue. The presence of the b ions 456.1, 569.3, 706.30, 819.3, 932.4, and 1061.3 corresponded to N-terminal fragments beginning with N-alkyl-AIAQ and ending with *N*-alkyl-AIAQIHILE. These results determined the modification site within the first four N-terminal amino acids. Since the possibility that Gln4 acts as a nucleophile to form an imine intermediate is highly unlikely,[27] it can be concluded that the alkylation site was Ala1.

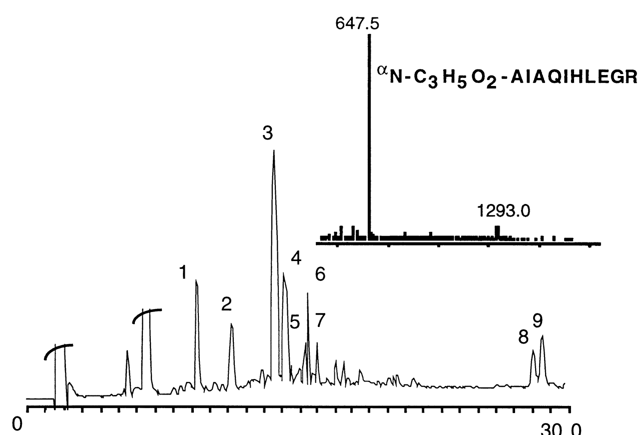

Figure 3. HPLC analysis (C-18 reversed-phase column, 0–60% acetonitrile over30 min, monitored at 214 nm) of the trypsin digestion mixture of the modified P1A. The modified P1A enzyme (100 μM) was incubated with trypsin (0.4 μM) in tris(hydroxymethyl)aminomethane buffer (pH 8.0) at 37 °C, for 3 h. The numbered fractions were collected and analyzed by ESI-MS in comparison with the corresponding digestion mixture of the P1A. Fraction (retention time, M_W, peptide): 1 (9.4 min, 890 Da, E22–R29), 2 (11.3 min, 631 Da, E17–R21) 3 (13.6 min, mixture of two peptides, 1058 Da, S30–R39, and 1172 Da, G48–K59), 4 (14.3 min, 886 Da, V41–K48), 5 (15.3 min, 1221 Da, A1–R11), 6 (15.6 min, 1292.5 Da, $^{\alpha}N$-$C_3H_5O_2$-A1–R11), 7 (16.2 min, 2492 Da, $^{\alpha}N$-$C_3H_5O_2$-A1–R21), 8 (28.1 min, 6839 Da, $^{\alpha}N$-$C_3H_5O_2$-P1A), 9 (28.7 min, 6427 Da, $^{\alpha}N$-$C_3H_5O_2$-A1–K59).

The progress of the reductive alkylation of P1G with oxaloacetate and $NaBH_3CN$ was followed by HPLC and ESI-MS in order to demonstrate that imine formation and turnover of the substrate occur at comparable rates. As can be seen from Figure 4, 50% of the active sites of P1G were reductively alkylated in 40 s, while the rest of the active sites were alkylated in the next 150 s. A previous study of the affinity labeling of wt4-OT with 3-bromopyruvate also exhibited half-site stoichiometry.[28] It has been proposed that only half of the active sites are

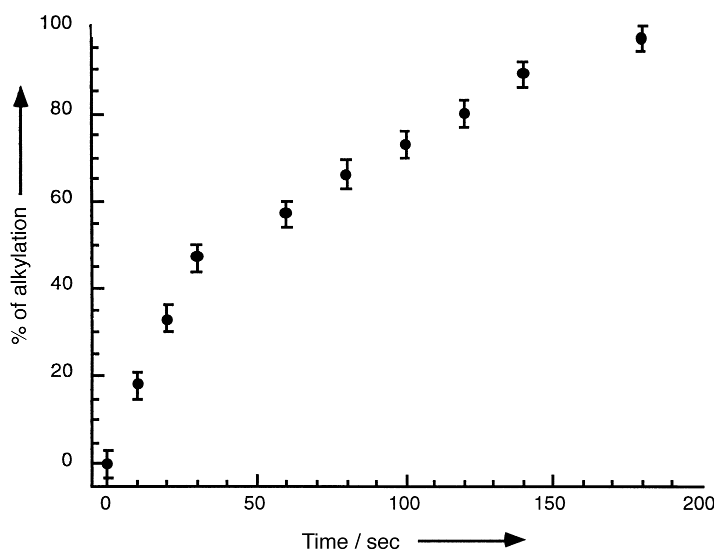

Figure 4. Reductive alkylation of P1A in 1 mM oxaloacetate, 4 mM $NaBH_3CN$, and 100 mM NaH_2PO_4 at pH 7.4 and 22 °C. The percent alkylation was determined from ESI-MS spectra following quenching and desalting by HPLC.

catalytically active in the 4-OT hexamer.[27, 28] Considering our observation that the decarboxylation of oxaloacetate takes 50 s ($k_{cat.} = 0.02\ s^{-1}$) for a single turnover, the 40 s reductive alkylation suggests that formation of the imine species is kinetically competent with the overall reaction. When this experiment was carried out with P1A, 50% of the active site was alkylated within 10 s and these results are in agreement with the 13 s turnover rate ($k_{cat.} = 0.08\ s^{-1}$) with P1A.

An important experiment in the characterization of the imine mechanism of AAD was the decarboxylation of acetoacetate in the presence of $NaBH_3CN$ to trap the imine intermediate by reduction.[22] This irreversible inhibition experiment not only confirmed the imine mechanism but also identified the active site amine (Lys115) by hydrolysis of the modified protein and isolation of an ε-isopropyl lysine. Two important features of this reductive alkylation experiment with AAD should be highlighted: 1) the intercepted imine intermediate was that of the decarboxylation product (acetone) rather than the substrate (acetoacetate) and 2) an independent attempt to reductively alkylate AAD with acetone and $NaBH_3CN$ indicated that acetone is an inefficient substrate for the alkylation of AAD. Additional support for the mechanism was provided by incorporation of ^{18}O into the product, acetone.[22]

These characteristics of ADD are analogous to our 4-OT-based decarboxylases. Treatment of P1A with oxaloacetate and $NaBH_3CN$ resulted in reductive alkylation of an active site amine. This amine was shown to be the N-terminal Ala by proteolysis and MS/MS analysis. As was the case with AAD, the intercepted imine intermediate was that of the decarboxylation product (pyruvate) rather than the substrate (oxaloacetate) and pyruvate does not alkylate P1A in the presence of $NaBH_3CN$. Efforts to observe pyruvate by mass spectrometry have been unsuccessful, which has prevented the analysis of ^{18}O labeling. However, in the case of P1A, we have further demonstrated that reduction of the imine intermediate and turnover of the substrate occur at comparable rates. The direct analogy between the characterization of AAD and the results obtained for our decarboxylase catalysts agrees with our proposed imine mechanism.

The $k_{cat.}/K_M$ value (114 $s^{-1}M^{-1}$) of our designed decarboxylase P1A is noteworthy considering the fact that wt4-OT exhibited no detectable catalytic activity. When compared to catalysis by a small-molecule amine (ethylamine: $k_2/K_M = 8 \times 10^{-4}\ s^{-1}M^{-1}$),[29] P1A has a catalytic proficiency[2] of 1.4×10^5. In principle, such catalytic activity might afford sufficient selective advantage to initiate the optimization process of adaptive evolution.[2] In order to reach the proficiency of the naturally occurring oxaloacetate decarboxylase, this primordial enzyme would have to improve $\approx$4500 fold (Table 1) and would require a much more sophisticated sequence of evolutionary events than the single amino acid change described in this work.

Table 1. Catalytic decarboxylation of oxaloacetate ($k_{un} = 2 \times 10^{-5}\ s^{-1}$ in 50 mM NaH_2PO_4 at pH 6.2 and 22 °C). In all cases the kinetic parameters were determined under the optimal pH value for the individual catalyst.

Catalyst	$k_{cat.}$ [s^{-1}]	K_M [µM]	$k_{cat.}/K_M$ [$s^{-1}M^{-1}$]
oxaloacetate decarboxylase	360	670	540000
4-OT (P1A)	0.08	700	114
4-OT (P1G)	0.02	2000	10
YLK-18	0.015	5660	2.7
oxaldie 1	0.0067	14000	0.5

We compared the catalytic parameters of P1A with those reported for several oxaloacetate decarboxylases that use the imine mechanism (Table 1). These enzymes include the naturally occurring enzyme,[30] and representative helical-bundle-forming, synthetic, catalytic peptides, YLK-18 (YKLLKELLAKLKWLLRKL-$CONH_2$)[26] and oxaldie 1 (LAKLLKALAKLLKK-$CONH_2$)[25] (Table 1). As can be seen, both 4-OT mutants are significantly better catalysts for this reaction than the de novo designed peptides that employ the imine mechanism. Another relevant catalyst that utilizes the imine mechanism is the catalytic antibody 38C2.[9] In addition to efficiently catalyzing the aldol, retro-aldol,[10, 31] and deuterium-exchange reactions,[32] this antibody was also found to catalyze the decarboxylation of 2-(3′-(4″-acetamidophenyl)propyl)acetoacetic acid ($k_{cat.} = 0.0027\ s^{-1}$, $k_{cat.}/K_M = 2.9\ s^{-1}M^{-1}$).[33]

It has been proposed that enzyme evolution can be a continuum in which a new catalytic mechanism is gained while the parent activity declines.[2, 34] This is supported by several recent studies in which elements of a given mechanism could be altered by small changes in the amino acid sequence.[35–43] In addition, the functional and structural duality of biopolymers has been demonstrated with RNA.[44, 45] Since a different catalytic mechanism has been designed in 4-OT, an interesting consequence of the continuum hypothesis would be that the imine and acid/base catalytic mechanisms (Scheme 1) are not mutually exclusive. The previous results reported by Whitman and co-workers, and further supported by our ^{18}O-labeling experiments, suggest that the P1A and P1G mutants promote the isomerization reaction of **1** into **3** through an acid/base mechanism with $k_{cat.}/K_M$ values approximately 100-fold lower than the wild-

type enzyme.[17] However, the decarboxyase activity of P1A and P1G, with the substrate oxaloacetate, is consistent with the catalytic amine acting initially as a nucleophile, and proceeds through an imine mechanism. The presence of significant levels of both decarboxylation and tautomerization activity in P1A and P1G demonstrates that different catalytic mechanisms can coexist in the 4-OT active site.

Conclusion

We have shown that a rational change of the catalytic activity and mechanism of 4-OT can be accomplished by a single mutation. The wild-type enzyme catalyzes a tautomerization reaction through a general acid/base mechanism while the P1A and P1G mutants also catalyze the decarboxylation of oxaloacetate by a nucleophilic mechanism. Interestingly, these mutants are bifunctional—they catalyze two different reactions by unique mechanisms that use the same catalytic group. Taking a cue from nature, a new synthetic family of nucleophilic catalysts could be generated on the basis of the 4-OT scaffold through selection methods and rational engineering. In addition, since 4-OT is amenable to chemical synthesis, a variety of unnatural amino acids can be incorporated into this scaffold. These enzymes could catalyze aldol, retroaldol, Michael addition, alkylation, and other carbonyl transformation reactions. Work along these lines is currently underway in our laboratories.

Experimental Section

Kinetic experiments based on UV measurements were carried out with a Varian-CARY 100 Bio spectrometer using microcuvettes (1 cm optical path, 80-μL capacity). CD experiments were carried out with a AVIV 202 SF spectrometer using microcuvettes (1 cm optical path, 500-μL capacity). Buffers for the kinetic measurements were freshly prepared with deionized water. NaH_2PO_4 and Na_2HPO_4 were purchased from Fisher Biotech (IN). All Boc-protected amino acids were obtained from Midwest BioTech (Fisher, IN). 2-(*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and *N*,*N*-diisopropylethylamine (DIEA) were obtained from Quantum Biotechnologies (Montreal, CA). Oxaloacetate was purchased from Acros Organics. *N*,*N*-Dimethylformamide (DMF) and HPLC-grade acetonitrile were purchased from Fischer. Trifluoroacetic acid was obtained from Halocarbon (Hackensack, NJ). HF was purchased from Matheson (Cucamonga, CA).

Synthesis of 4-OT and mutants: The polypeptide chain of the 62 amino acid monomeric unit of the wt4-OT was synthesized manually on a 0.4-mmol scale by using 11-fold excess of Boc-protected amino acids following the in situ neutralization protocols for Boc chemistry, as described earlier.[19] After polypeptide assembly was completed, histidine(dinitrophenyl) groups were removed by treatment of the Boc–peptide–resin with a solution of 20% 2-mercaptoethanol and 5% DIEA in DMF. The cleavage of the side-chain-protected group, simultaneously with the cleavage of the peptide from the resin, were achieved by treatment of the dry peptide–resin with HF containing 4% *p*-cresol for 1 h at 0 °C. The crude peptide product was precipitated and washed with cold anhydrous diethyl ether, dissolved in 6 M guanidine hydrochloride (pH 2–3) and directly purified by preparative C-18 reversed-phase column. Similarly to previous reports, the synthetic yields were ≈10%. For the synthesis of P1A and P1G, Boc-Ala and Boc-Gly were substituted for Boc-Pro in the final coupling. For all proteins, Met45 was replaced with norleucine to eliminate the possibility of oxidizing the enzyme during sample handling.[20]

Kinetic studies of oxaloacetate decarboxylation: The protein samples were freshly folded before each kinetic experiment by dissolving the protein (0.4 mg) in NaH_2PO_4 buffer (1.2 mL, 50 mM, pH 7.4). After 2–3 h at 22 °C the precipitate was removed by centrifugation and the protein concentration was determined from the difference in absorbance at 215 and 225 nm multiplied by a factor of 144.[46] Each kinetic experiment was carried out in NaH_2PO_4 buffer (50 mM, pH 5.8–8.0) with a constant concentration of the protein (1.5–5 μM) and a series of oxaloacetate concentrations (0–3 mM). The progress of the reaction was monitored by following the decrease of the 265 nm absorption (representing the enol form of oxaloacetate).[47] The kinetic parameters ($k_{cat.}$ and K_M) were calculated from the initial rates by using a nonlinear fit to the Michaelis–Menten model.

Kinetic studies of the isomerization of 1 into 3: As described previously[6] the rate of formation of **3** was monitored at 236 nm for 10 different substrate concentrations ranging from 20 to 160 μM. The kinetic parameters ($k_{cat.}$ and K_M) were calculated from nonlinear regression data analysis.

Inhibition studies: The catalytic activity of P1A was irreversibly inhibited with bromopyruvic acid, which is known to alkylate primary and secondary amines in enzyme active sites, as has already been reported for wt4-OT.[28] In a typical experiment, the enzyme (8 μM) was incubated at 38 °C for 4 h in NaH_2PO_4 buffer (50 mM, pH 6.5) with bromopyruvate (500 μM). The protein was then dialyzed seven times before being examined as a catalyst in the decarboxylation reaction of oxaloacetate (1.75 mM) at 22 °C and pH 6.5. No catalysis above background activity could be detected with the modified enzyme.

Another irreversible inhibition experiment was carried out by reductive alkylation with acetone and $NaBH_3CN$. The enzyme P1A (10 μM) was incubated at 22 °C for 2 h in NaH_2PO_4 buffer (50 mM, pH 7.0) with acetone (5%), and then the mixture was treated with $NaBH_3CN$ (10 mM) at 38 °C for 1 h. The protein was dialyzed seven times before being examined as a catalyst in the decarboxylation reaction of oxaloacetate (1.55 mM) at 22 °C and pH 7.0. No catalysis above background activity could be detected with the modified enzyme.

Reductive amination: To check for imine formation with oxaloacetate, the enzyme P1A (1 mg, 100 μM) was incubated with oxaloacetate (1 mM) in NaH_2PO_4 buffer (100 mM, pH 7.4) at 22 °C for 5 min and then the mixture was treated with $NaBH_3CN$ (4 mM) at 27 °C for 10 min. The reaction was immediately analyzed by HPLC (C18 reversed-phase, 0–60% acetonitrile over 30 min, monitored at 214 nm) and showed a single peak (Figure 2). Analysis of the entire peak by ESI-MS showed a homogenous product corresponding to a single modification of the protein.

The reductive amination rates with P1G were determined by quenching individual reactions at multiple time points with excess trifluoroacetic acid (0.1% solution, pH 2). The samples were desalted by HPLC and analyzed by ESI-MS. The mass spectrum in the range *m/z* 500–2000 was reconstructed to a single charge state (MacSpec, Perkin Elmer) and the relative intensities of the peaks related to the unmodified (6754 Da) and modified (6826 Da) proteins were recorded (Figure 4).

Trypsin cleavage: The purified $^{\alpha}N$-$C_3H_5O_2$-P1A (100 μM) was incubated with trypsin (0.4 μM in tris(hydroxymethyl)aminomethane buffer, pH 8.0) at 37 °C. After 3 h, analytical HPLC (0–60% acetonitrile

over 30 min) showed that 90% of the $^{\alpha}N-C_3H_5O_2$-P1A was cleaved, resulting in 9 well-defined peaks (Figure 3). The fractions were collected and analyzed by ESI-MS in comparison with the corresponding digestion mixture of P1A. For details (fraction, retention time, M_W, peptide), see the legend of Figure 3. Fraction 6 (16.2 min, 1292.5 Da, $^{\alpha}N-C_3H_5O_2$-AIAQIHILEGR) was concentrated to dryness under vacuum and dissolved in methanol/water for MS/MS analysis.

Tandem mass spectrometry analysis: Fraction 6 ($^{\alpha}N-C_3H_5O_2$-AIAQIHILEGR) was further analyzed by tandem mass spectrometry mapping on a Thermoquest/Finnigan LCQ Deca Ion-Trp mass spectrometer with a standard electrospray ionization source. The parent ion (*m/z* 1292.5 Da) was isolated and fragmented at a normalized collision energy of 35%. Activation *Q* and activation time were set at standard values (0.25 and 30 msec, respectively). The fragments that were observed at 456.1, 569.3, 706.30, 819.3, 932.4, and 1061.3 Da correspond to the b-ions of the parent peptide.

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