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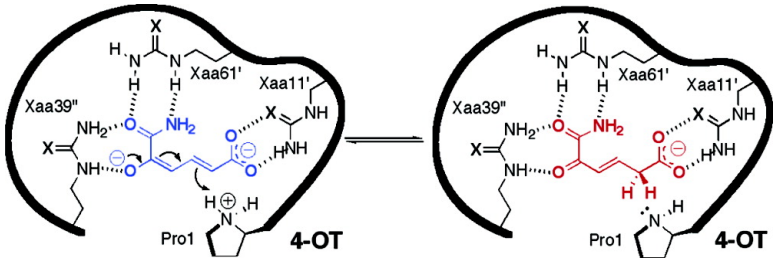

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A Designed Synthetic Analogue of 4-OT Is Specific for a Non-Natural Substrate

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Abstract: The substrate specificity of 4-oxalocrotonate tautomerase (4-OT) is characterized by electrostatic interactions between positively charged arginine (Arg) side chains on the enzyme and the dianionic substrate, 4-oxalocrotonate. To generate specific hydrogen-bonding interactions with a monoanionic substrate analogue, we have introduced a urea functional group into the active site by replacing arginine side chains with isosteric citrulline (Cit) residues. This design was based on the complementarity between the urea functionality of citrulline and the uncharged amide function of the substrate, as opposed to the guanidinium−carboxylate electrostatic interaction between the wild-type enzyme and the natural substrate. Indeed, the synthetic (Arg39Cit)4-OT analogue catalyzed the tautomerization of the non-natural monoamide−monoacid substrate while it was a poor catalyst for the natural diacid substrate. The specificity of (Arg39Cit)4-OT for the monoamide−monoacid substrate relative to that of the diacid substrate was found to be 740-fold greater than that of the wild-type enzyme for tautomerization of the non-natural substrate as compared with the natural one. The role of electrostatic interactions in the tautomerization of the monoamide−monoacid substrate was probed in detail with several other Arg to Cit analogues of this enzyme. This study has demonstrated that chemical manipulation of the functional groups within the active site of an enzyme can modify its catalytic activity and substrate specificity in a predictable way, suggesting that the incorporation of noncoded amino acids into proteins has great promise for the development of new enzymatic mechanisms and new binding interactions.

Introduction

The generation of new catalytic activities through structural perturbation of existing enzymes is a powerful approach for enzyme design.[1] The resulting enzymes have potential as biotechnologically useful catalysts, and these studies have led to a better understanding of the molecular basis of enzyme catalysis. One approach for the generation of new enzyme activities is through screening of enzyme libraries generated by methods of directed evolution.[2−4] Alternatively, the rational design of site-directed mutations[5] within the enzyme active site has been used successfully for the perturbation and mechanistic analysis of enzymes that are well-characterized and has led in some cases to altered enzymes with properties different from those of the original wild-type enzymes.[6] In addition, site-specific chemical modification methods have enabled the introduction of a broad range of functional groups into enzymes, typically through modification of uniquely reactive amino acids or cysteine side chains.[7]

In principle, the ability to introduce noncoded amino acids into the active site of enzymes would facilitate the rational design of new catalytic activities. The strategy of using noncoded amino acids enables a precise placement of functional groups that are typically not found in proteins into the binding and catalytic machinery. This maneuver can be achieved by either chemical synthesis of the enzyme or by a biosynthetic approach.[8,9] Using these methods, several noncoded amino acids have been used to probe enzyme mechanisms.[10−15]

4-Oxalocrotonate tautomerase (4-OT) is a 62 amino acid homohexameric enzyme that is amenable to total chemical synthesis.[16] This enzyme is a member of a growing superfamily

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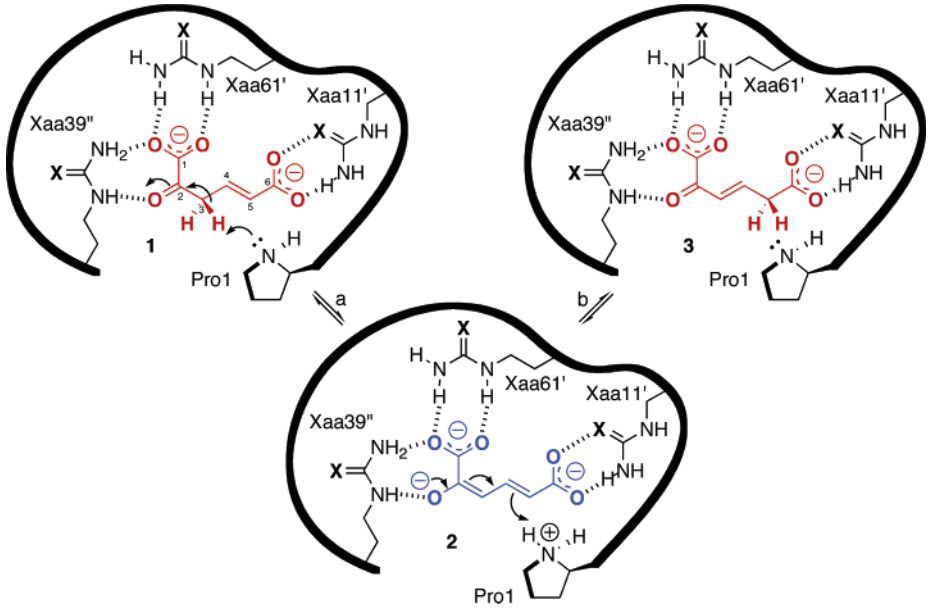
10.1021/ja050110b CCC: $30.25

Scheme 1. A Proposed Mechanism of the Tautomerization of 4-Oxalocrotonate, **1**[a]



[a] The three arginine residues Arg11′, Arg39″, and Arg61′ (**X** = NH_2^+) of the wild-type were replaced by citrulline (**X** = O). The unprimed and singly primed residues belong to different monomers of the same dimer, and the doubly primed residues belong to a third monomer from a neighbor dimer.

of enzymes that have been shown to catalyze isomerization, decarboxylation, and dehalogenation reactions.[17] These enzymes utilize the secondary amine of the N-terminal proline as a general acid or base as a common element in their catalytic mechanisms. Mutation of this residue to glycine in 4-OT results in a primary amine that can participate in both general acid/base[18,19] and nucleophilic catalysis.[19,20] It has been shown that 4-OT catalyzes the tautomerization of a β,γ-unsaturated ketone, 2-oxo-4*E*-hexenedioate (4-oxalocrotonate, **1**), to the α,β-unsaturated isomer, 2-oxo-3*E*-hexenedioate (**3**), through a proposed enolate intermediate, **2** (Scheme 1).[21] Kinetic studies have shown that this efficient enzyme transfers a proton from C-3 to C-5 using the N-terminal Pro residue as a general-base ($pK_a \sim 6.4$).[17,18,21−26] Interestingly, although all six active sites appear to be structurally identical, suicide inhibitors have been shown to initially alkylate only three subunits of the enzyme. This suggests that, when interacting with substrates, all six active sites may not be functionally equivalent.[23]

It has been proposed that the tautomerization reaction with **1** involves two steps, as illustrated in Scheme 1. Deprotonation (step a) is followed by protonation (step b) with the two corresponding transition states, TS1 and TS2, having approximately equal energy barriers.[27] Since the keto and enol forms of **1** coexist in aqueous solution in rapid equilibrium, the first step (a) involves abstraction of a proton from either carbon C-3 or the oxygen at the C-2 position of the substrate. This step is catalyzed by the N-terminal secondary amine (Pro1). In the second step (b), Pro1 donates a proton to the substrate at carbon C-5. Accordingly, the two main features of the catalytic machinery of this enzyme are Pro1, which acts as a general acid/base, and Arg39″, which provides electrostatic stabilization for the negatively charged oxygen at the C-2 position in both transition states and the enolate intermediate.[28] The active site 4-OT has been previously studied by site-directed mutagenesis and has proven to be robust toward modification. For example, the mutants Arg11Ala and Arg39Gln were characterized by NMR and were found to display little perturbation to the active site.[24]

The importance of electrostatics in the 4-OT catalytic mechanism was recently probed using noncoded amino acid side chains.[28] Arginine was substituted at three positions in the active site by citrulline, an uncharged isostere of arginine (Scheme 1). Interestingly, substitution at positions 11 and 39 of 4-OT

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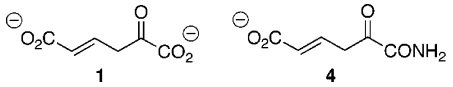
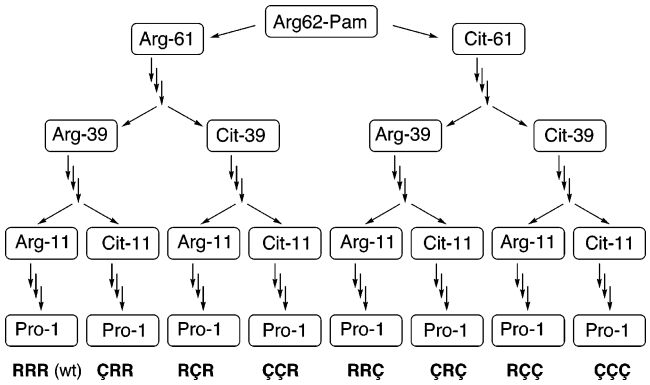
Scheme 2. 4-Oxalocrotonate (**1**) and the Analogous Monoamide−Monoacid Substrate, 4-Oxalamidocrotonate (**4**)

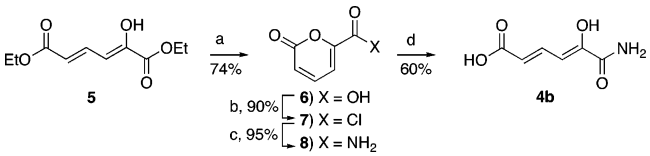


Scheme 3. Split Resin Assembly of the 4-OT Analogues[a]



[a] Citrulline (Cit) was incorporated at positions 11, 39, and 61, **R** = Arginine, **Ç** = Citrulline.

Scheme 4. Synthesis of 4-Oxalamidocrotonate (**4**)[a]



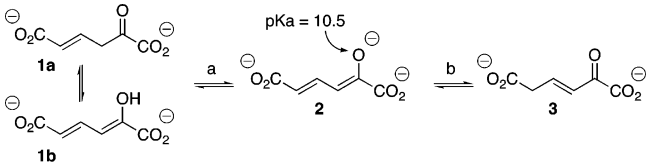
[a] Key: (a) concd HCl, reflux, 8 h; (b) $SOCl_2$ reflux, 14 h; (c) (i) HMDS, CH_2Cl_2, rt, 10 h, (ii) 5% H_2SO_4; (d) (i) aq NaOH (2 M) 16 h, (ii) HCl (6 M).

resulted in a significant decrease of catalytic proficiency, suggesting that the positively charged guanidinium group of arginine binds the negatively charged acid groups of 4-oxalocrotonate **1** through predominantly electrostatic interactions. These mechanistic insights suggested that the arginine−citrulline substitutions could be harnessed for the design of a modified enzyme specific for a new substrate with different electrostatic properties than **1**. In particular, Arg39Cit could form a productive binding interaction with a monoamide−monoacid substrate, **4**, by hydrogen-bond complementarity rather than through electrostatic interactions (Scheme 2). Here we demonstrate that citrulline analogues of 4-OT are up to 740-fold more specific than the wild-type enzyme for tautomerization of the ketoamide substrate **4** compared to the natural substrate **1**.

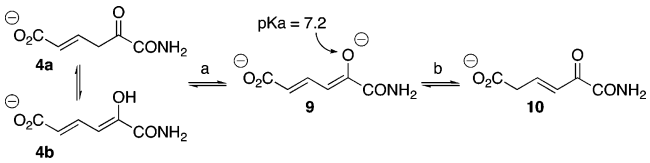
Results and Discussion

Synthesis and Characterization of Enzymes and Substrates. All 4-OT analogues as well as the wild-type enzyme (wt4-OT, **RRR**) were synthesized as described previously (Scheme 3).[28] 4-Oxalamidocrotonate (**4**), was synthesized from diethyl 2-hydroxy-2,4-hexadien-1,6-dioate (**5**) (Scheme 4),[29] by converting it to 6-hydroxycarbonyl-2-pyron (**6**) and then to the corresponding acyl chloride (**7**).[30] Reaction of the latter with hexamethyldisilizane yielded amide **8**,[31] which was converted to **4** by treatment with base and then with acid. Titration of substrates **1** and **4** with base established the pK_a values of

Scheme 5. Tautomerization of 4-Oxalocrotonate (**1**)



Scheme 6. Tautomerization of 4-Oxalamidocrotonate (**4**)



compound **1**—2.5, 4.5, and 10.5 (Scheme 5)—and of compound **4**—4.2 and 7.2 (Scheme 6). The corresponding UV absorption maxima of **1** at various pH values were 302 nm (pH 2.3), 294 nm (pH 7.4), and 350 nm (pH 12.4). The absorption maxima of **4** were 300 nm (pH 2.3), 298 and 349 nm (pH 7.4), and 349 nm (pH 12.4).

Mechanistic Considerations. The titration analysis indicates that the enol function of **4b** has a much lower pK_a (7.2) than that of **1b** (pK_a = 10.5). As a result, under the experimental conditions (pH 7.38) used for the 4-OT catalysis, **4** is expected to exist predominantly in its enolate form, **9** (Scheme 6). Experimental and computational studies suggest that both transition states of the reaction with the natural diacid substrate are similar in energy.[21,27] Since the pK_a of the 4-oxalamidocrotonate substrate is significantly reduced, the first step is expected to be rapid for the enol (**4b**) and nonexistent for the enolate (**9**); as a result, it is likely that the second step is rate-determining with these substrates. Since the enolate corresponds to intermediate **2** in the proposed tautomerization mechanism of **1**, the second step (b) should become rate-limiting in the case of **4**. We propose that in step b, the general acid Pro1H$^+$ transfers a proton to C-5, thus producing the ketoamide product **10**, in analogy to the mechanism proposed for **1**. Consequently, neither the general base property of Pro1 nor the anionic stabilization effect of Arg39″ would be required for efficient catalysis in the case of **4** (Scheme 7).

A change of the rate-determining step in enzymatic catalysis as a function of the substrate is not unprecedented. The case of α-chymotrypsin-catalyzed hydrolysis of amides and esters represents a classic example: with an amide substrate the rate-determining step is acylation of the catalytic serine residue to form the covalent acyl−enzyme intermediate, while with an ester substrate, the deacylation step of the intermediate becomes rate-determining.[32] In addition, mutant enzymes have been shown to have a rate-determining step different from that observed for the corresponding wild-type enzymes.[12,33,34]

Kinetic Analysis. Table 1 summarizes the kinetic parameters of the catalytic tautomerization of **4** using eight analogues of 4-OT. For clarity, Table 2 highlights the comparison of **RRR**, **RÇR**, and **RÇÇ** with substrates **1** and **4**. In the case of **4**, the kinetic parameters were measured using standard assay condi-

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Scheme 7. Proposed General-Acid Mechanism for the 4-OT-Catalyzed Tautomerization of **4** (arginine, **X** = NH_2^+; citrulline, **X** = O)

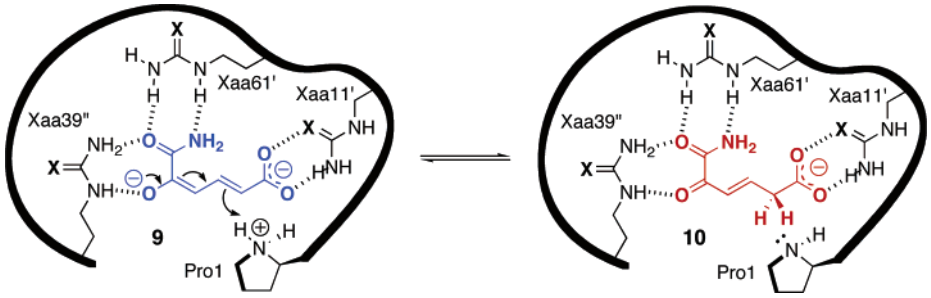

Table 1. Kinetic Parameters for Synthetic Wt4-OT, **RRR**, and Citrulline Analogues with 4-Oxalamidocrotonate **4**

analogue	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\cdot s^{-1}$)	k_{cat}/k_{uncat}[a]
RRR[b]	90 ± 25	290 ± 35	3.1×10^6	2.7×10^4
ÇRR	19 ± 5	4.6 ± 0.4	2.4×10^5	431.0
RÇR	130 ± 26	72 ± 8	5.6×10^5	6.8×10^3
RRÇ	36 ± 10	27 ± 2	7.4×10^5	2.5×10^3
ÇÇR	nd	>0.5	nd	nd
ÇRÇ	49 ± 14	4.1 ± 0.4	8.3×10^4	384.0
RÇÇ	125 ± 18	21 ± 2	1.7×10^5	2.0×10^3
ÇÇÇ	nd	>0.5	nd	nd

[a] Uncatalyzed rate for **4**: 1.064×10^{-2} s^{-1}. [b] Coding refers to positions 11, 39, and 61 of the protein: **R** = Arg, **Ç** = citrulline.

Table 2. Kinetic Parameters for Synthetic Analogues **RRR**, **RÇR**, and **RÇÇ** with the Substrates 4-Oxalocrotaonate (**1**) and 4-Oxalamidocrotonate (**4**)

reaction	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\cdot s^{-1}$)	k_{cat}/k_{uncat}[a]
RRR[b] + **1**	62 ± 10	2500 ± 150	4.0×10^7	2.8×10^6
RRR + **4**	90 ± 25	290 ± 35	3.1×10^6	2.7×10^4
RÇR + **1**	160 ± 40	1.5 ± 0.2	9.8×10^3	1.8×10^3
RÇR + **4**	130 ± 26	72 ± 8	5.6×10^5	6.8×10^3
RÇÇ + **1**	nd	> 0.5	nd	nd
RÇÇ + **4**	125 ± 18	21 ± 2	1.7×10^5	2.0×10^3

[a] Uncatalyzed rate for **1**, 8.7×10^{-4} s^{-1}; for **4**, 1.064×10^{-2} s^{-1}. [b] Coding refers to positions 11, 39, and 61 of the protein: **R** = Arg, **Ç** = citrulline. Data for reactions with substrate **1** are taken from ref 28.

tions for 4-OT (20 mM NaH_2PO_4, pH 7.38) following the disappearance rate of enolate **9** (λ_{max} = 350 nm). The kinetic parameters for substrate **1** were taken from our previous work.[28]

Wt4-OT Catalyzes the Tautomerization of Both Substrates. As can be seen in Table 2, the wt4-OT, **RRR**, catalyzes the tautomerization of **4** 8.5-fold slower than that of **1**, with little change in substrate binding. The overall effect is a 13-fold decrease in k_{cat}/K_M. This minor change in K_M indicates that charge complementarity does not play a significant role in the binding of either the carboxylate-1 or carboxamide-1 of either the ketoacid substrate or the ketoamide substrate, respectively, and either Arg39″ or Arg61′. This hypothesis is strongly supported by our previous studies of the **RÇR** and **RRÇ** analogues with **1**, where removal of the positive charge from either Arg39″ or Arg61′ resulted in a small increase in the K_M values, 2.5- or 4.2-fold, respectively.[28] The modification of the substrate from a negatively charged ketoacid to a neutral ketoamide is analogous to arginine to citrulline substitutions in the enzyme.

An interesting consequence of the change in the rate-determining step is that the reaction with **4** requires the availability of the protonated state of Pro1 ($Pro1H^+$), whereas the reaction with **1** requires the neutral state of Pro1 (Scheme 8). In the wild-type enzyme the pK_a of Pro1 is ~6.4,[35] and presumably, Pro1 has been evolutionary optimized to be 90% neutral at physiological pH 7.4. In contrast, for the catalysis with substrate **4** at pH 7.4, only 10% of this enzyme is available in the required protonated state. If the protein concentration is corrected for protonation state, the k_{cat} becomes 10-fold higher, or equal to that of the diacid reaction. The expected rate profile as a function of pH would show low activity below pH 6, where the enzyme would be protonated but the substrate would be predominantly in the enol form, and also above pH 8, where the enzyme would be deprotonated and the substrate would be an enolate. These extremes suggest that the optimized pH for this enzyme-catalyzed reaction would be between 6 and 8. These considerations suggest that an appropriate redesign of the active site to increase the pK_a of the catalytic Pro1 residue would result in increased concentration of $Pro1H^+$ at neutral pH. For example, the pK_a of Pro1 in the mutant Phe50Ala has already been shown to be ~7.3 as compared with 6.4 in the wild-type enzyme, probably due to the increased dielectric constant of the active site.[36]

Arg39Cit Is Specific for the Tautomerization of the Ketoamide Substrate 4. In the reaction of **4** under the catalysis of the Arg39Cit analogue, **RÇR**, k_{cat} decreased only 4-fold with insignificant change in K_M (1.4-fold increase) in comparison to catalysis with the wild-type enzyme, **RRR** (Table 2). Consistent with our previous results, Arg39″ has little effect on the K_M of either **1** or **4**. In addition, **RÇR** showed similar k_{cat} (only 4-fold decrease) to that of **RRR**, suggesting that electrostatic effects play a minor role in the transition state stabilization with **4**, as compared to the reaction with **1**. Although K_M is not necessarily equal to a K_D value, treatment of K_M as an apparent dissociation constant has been used previously in the context of 4-OT.[24] This result supports our proposed mechanism for the tautomerization of **4**. As the positive charge of Arg39″ does not play a critical role in the catalysis reaction with **4**, deletion of this positive charge causes only minor changes in k_{cat}. The **RÇR** analogue shows 740-fold greater specificity for the catalysis with **4** versus **1** compared to the wild-type enzyme, **RRR**. This result is also supported by the double mutant, **RÇÇ**, which exhibits a similar k_{cat} to that of **RÇR**. Mutant **RÇÇ** represents a specific catalyst for the nonnatural substrate, **4**, as it shows no detectable catalysis with the natural substrate, **1**.

Arg11 Controls Substrate Orientation. The (Arg11Cit)-4-OT analogue, **ÇRR**, is a poor catalyst for **4**, with k_{cat} being

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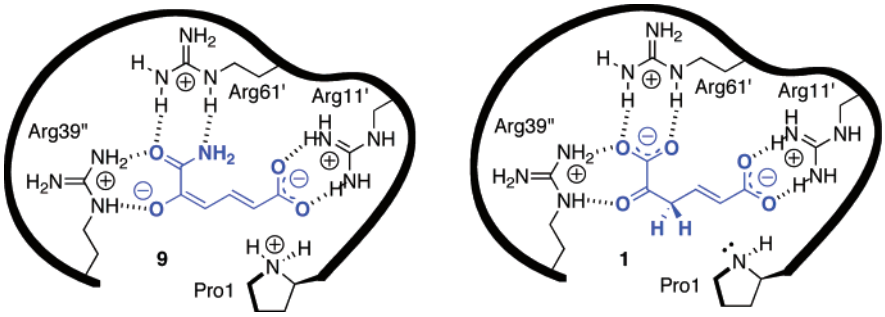
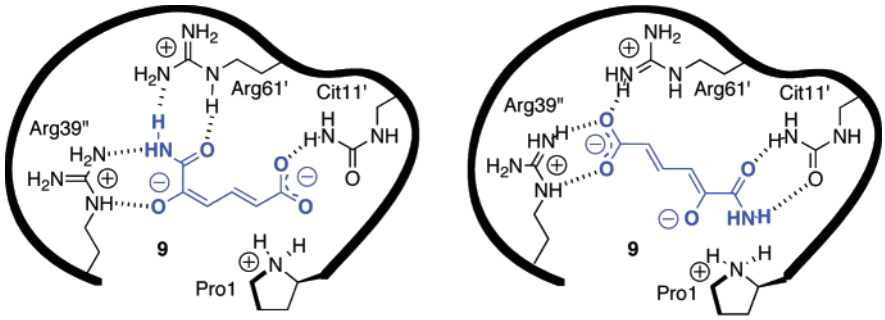
Scheme 8. Similar Binding between the wt-4OT and Either the Natural Diacid **1** or Non-Natural Monoamide−Monoacid Substrate **9**



Scheme 9. Two Alternative Binding Modes of **9** within the Active Site of Analogue **ÇRR**



64-fold smaller in comparison with wt4-OT (Table 1). Interestingly, however, as reflected by the small K_M value, **ÇRR** binds **4** more tightly than wt4-OT binds either **1** or **4**. This is a rather unexpected observation, considering the major role played by Arg11′ in binding of the natural substrate through charge complementarity with carboxylate-6, as manifested by the observation that substitution to citrulline increased K_M by 13-fold.[28] In contrast, if the orientation of **4** is similar to the natural orientation proposed for **1** (Scheme 9), then the K_M of **ÇRR** would be expected to be similar to that of **1**. We propose that a 180° switch in the binding orientation of **4** within the active site could explain the decrease in both k_{cat} and K_M observed with **ÇRR**. The inverted orientation (Scheme 9) would place the negatively charged carboxylate-6 of **9**, the enolate of **4**, in close proximity to the positive charges of Arg39″ and Arg61′ and would form hydrogen-bond complementarities between carboxamide-1 of **9** and the amide functionality of Cit11′. Although this binding mode would result in better substrate binding, it adversely affects catalysis. The catalytic general acid in Pro1 is no longer positioned at the optimal distance and in the optimal orientation to react with the γ-carbon of the enolate.

The proposed inverted orientation is also consistent with the kinetic parameters of the double mutant **ÇRÇ**, which exhibits both reduced K_M and k_{cat} (Table 1). The importance of electrostatic interactions for substrate binding is highlighted by the measurable loss of catalytic activity in the **ÇÇR** and **ÇÇÇ** analogues in which both Arg11′ and Arg39″ are substituted with the neutral Cit residue. An inverted substrate orientation in the active site has been reported for other enzyme systems.[37]

Arg61 Has a Minor Role in the Tautomerization of Both Substrates. Catalysis of the tautomerization of **4** with the Arg61Cit analogue, **RRÇ**, exhibited a 2.6-fold decrease in K_M in comparison with the wild-type, **RRR**. This small decrease in K_M could result from a productive hydrogen-bonding interaction between the carboxamide-1 of **4** and the urea functionality of Cit61′. In contrast to the case with the diacid substrate, where **RRÇ** had little effect on k_{cat},[28] **RRÇ** exhibited a 11-fold decrease in k_{cat} with **4** (Table 1). This moderate decrease in k_{cat} suggests that substrate binding to the Cit residue may result in nonoptimal orientation of the substrate with respect to the catalytic Pro1 residue.

Conclusions

This work has demonstrated that chemical manipulation of the functional groups within the active site of an enzyme can modify its catalytic activity and substrate specificity in a predictable way. To generate specific hydrogen-bonding interactions with a designed substrate analogue, we have introduced a urea functional group into the active site of 4-oxalocrotonate tautomerase by replacing the natural arginine side chain with an isosteric citrulline residue. This design was based on the complementarity between the urea functionality of citrulline and the uncharged amide function of the substrate, as opposed to the guanidinium−carboxylate electrostatic interaction between the wild-type enzyme and the natural substrate. Indeed, the synthetic (Arg39Cit)4-OT analogue catalyzes the tautomerization of a designed monoamide−monoacid substrate while it is a poor catalyst for the natural diacid substrate. This work suggests that the incorporation of noncoded amino acids into proteins has great promise for the development of new enzymatic mechanisms and new binding interactions.

Experimental Section

General Methods. ^{1}H NMR spectra were recorded on a Bruker AM 200 or Bruker AM 500 spectrometers, using $CDCl_3$ as a solvent (unless otherwise specified). ^{13}C NMR spectra were recorded on a Bruker AV300 UltraShield with robot. Mass spectra were measured on a

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Finnigan MAT TSQ700 (CI with isobutane) and infrared spectra on a Bruker FTIR Vector22. Buffers for kinetic measurements were prepared using deionized water. NaH_2PO_4 and Na_2HPO_4 were purchased from Fisher Biotech. All Boc-amino acids were obtained from Midwest Biotech (Fishers, IN), with the following side chain protecting groups: Arg(Tos), Asp(OcHxl), Glu(OcHxl), Thr(Bzl), Ser(Bzl), Lys(2ClZ), His(Dnp) (OcHxl = cyclohexyl, Bzl = benzyl, 2ClZ = 2-chlorobenzyloxycarbonyl, Dnp = 2,4-dinitophenyl). The solvents *N*,*N*-dimethylformamide (DMF), dichloromethane, acetonitrile (ACN) were of high purity (HPLC-grade) and purchased from Fisher. Trifluoroacetic acid (TFA) was obtained from Halocabon Products (River Edge, NJ). Anhydrous HF was purchased from Matheson Gas (Cucamonga, CA). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*-diisopropylethylamine (DIEA), and Boc-Arg-OCH_2-Pam resin were obtained from Peninsula Laboratories (Belmont, CA).

Analytical reversed-phase HPLC was performed on a Hewlett-Packard HPLC 1050 with 214 nm UV detection using Vydac C-18 columns (5 μm, 0.46 × 15 cm). Preparative reversed-phase HPLC was performed on Waters HPLC system using Vydac C-18 columns (10 μm, 2.5 × 25 cm). Linear gradients of acetonitrile in water with 0.1% TFA were used for all systems to elute bound peptides. The flow rates were 1 mL/min (analytical) and 30 mL/min (preparative). Buffer A is MilliQ water containing 0.1% TFA; buffer B is acetonitrile with 10% water and 0.01% TFA. Electrospray ionization MS was performed on an API-III triple quadruple mass spectrometer (Sciex, Thornhill, ON, Canada). Peptide masses were calculated from the experimental mass to charge (*m*/*z*) ratios from all of the observed protonation states of a peptide by using MacSpec software (Sciex). Theoretical masses of peptides and proteins were calculated by using MacProMass software (Beckman Research Institute, Duarte, CA).

All synthetic proteins were folded as described previously,[16a] by dissolving them (~0.3 mg) in 1 mL of assay buffer (20 mM sodium phosphate, pH 7.38) followed by incubation at room temperature for 2.5 h and ultracentrifugation to remove solid impurities. Circular dicroism (CD) experiments were carried out on AVIV-62DS spectrometer using a microcuvette (1 mm optical path, 300 μL) and the spectra recorded in the range of 200−260 nm. The spectra of all folded mutant proteins were measured at 25 °C in phosphate buffer (20 mM, pH 7.35), with filtered and degassed phosphate buffer as the blank solution. The results as a plot of mean molar ellipticity per residue ([θ], deg·cm^2 dmol^{-1}) versus wavelength in 1-nm increments for the folded proteins were nearly identical to the plot obtained for the wild-type enzyme, indicating that the mutations did not cause any gross conformational changes in the synthetic proteins. The hexameric folded structure of the enzymes was confirmed by gel filtration (data not shown). The protein concentration was determined using the method of Waddell.[38] Kinetic experiments based on UV measurements were carried out on Shimadszu UV-1601 spectrophotometer using a cuvette (1 cm optical path, 1 mL capacity).

Chemical Peptide Synthesis. The polypeptide chain of the 62 amino acid monomeric units of wt4-OT and its mutants were synthesized manually by solid-phase peptide synthesis (SPPS) methods and in situ neutralization protocols for Boc chemistry as described previously,[39] on a 0.4 mmol scale using 11-fold excess of Boc-protected amino acids (except for the noncoded amino acids norleucine and citrulline, which were used in 3-fold excess). Synthesis was initiated with Boc-N^{γ}-tosyl-L-arginine-4-(oxymethylphenylacetoamidomethyl) resin (Boc-L-Arg-(Tos)-Pam resin) with the following side chain protecting groups: Arg(Tos), Asp(OcHxl), Glu(OcHxl), Thr(Bzl), Ser(Bzl), Lys(2ClZ), His(Dnp). After completion of chain assembly, the His(Dnp) groups were deprotected by treatment of the Boc-peptide-resin with a solution of 20% 2-mercaptoethanol and 5% DIEA in DMF. The entire polypeptide was deprotected and cleaved from the resin by treatment of the dry peptide-resin with HF and 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 guanidinium chloride (Gn·HCl) at pH 2.0, and immediately purified by preparative reversed-phase HPLC. In all proteins, Met45 was replaced with norleucine to prevent oxidation of the enzyme during sample handling.[16b]

Diethyl 2-Hydroxy-2,4-hexadien-1,6-dioate, 5. This diester was prepared using a slightly modified procedure of Lapworth.[29,30] Thus, potassium (8.2 g, 0.22 mol) was slowly added to *tert*-butyl alcohol (80 mL) under argon. Diethyl ether (50 mL) was added and the mixture was stirred for 15 min at 0 °C. A solution of diethyl oxalate (27.1 mL, 0.2 mol) in 20 mL ether was added slowly at the same temperature, and the mixture was stirred for an additional 15 min. A solution of ethyl crotonate (24.9 mL, 0.2 mol) in 20 mL ether was added slowly and the mixture was stirred for 30 min at the same temperature. The mixture was kept overnight at 4 °C to allow precipitation of the potassium salt of **5**. The product was collected by filtration and then dissolved in 750 mL of ice water. Aqueous acetic acid (50%, 35 mL) was added and the resultant precipitate was collected by filtration and washed with cold water to give diester **5** in the form of a yellow crystalline powder (23.21 g, 55%). IR (Nujol): 1710 (m), 1635 (m), 1474 (m), 1444 (m), 1371 (m), 1425 (m br), 1024 (w), 877 (m), 774 (m), 725 (m) cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.61 (dd, J = 15.6, 11.6 Hz, 1H), 6.43 (br s, 1H), 6.26 (d, J = 11.6 Hz, 1H), 5.96 (d, J = 15.6 Hz, 1H), 4.32 (q, J = 6.2 Hz, 2H), 4.20 (q, J = 7.1 Hz, 2H), 1.34 (t, J = 5.7 Hz, 3H), 1.24 (t, J = 7.3 Hz, 3H). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 167.0, 165.1, 144.4, 137.2, 123.5, 108.7, 63.3, 60.9, 14.7, 14.5 ppm. MS (CI with isobutane): *m*/*e* 215 $[MH]^+$, 169.

2-Hydroxymuconate, 1b. Diester **5** (1 g, 4.7 mmol) was dissolved in 20 mL of sodium hydroxide (2.0 N), kept overnight at room temperature, and then acidified with hydrochloric acid (6.0 M, in cold water) up to pH ~2.0. The resultant precipitate, which was collected by filtration, was found to be 2-hydroxymuconate, **1b** (0.5 g, 70%). IR (Nujol): 3397 (m), 1674 (m), 1639 (m), 1617 (m), 1259 (m), 1162 (m), 1096 (m), 877 (m), 777 (m), 723 (m) cm^{-1}. ^{1}H NMR (200 MHz, CD_3OD): δ 7.62 (dd, J = 15.6, 11.7 Hz, 1H), 6.14 (d, J = 11.7 Hz, 1H), 5.85 (d, J = 15.5 Hz, 1H). ^{13}C NMR (75.47 MHz, CD_3OD): 170.9, 167.0, 148.6, 140.0, 122.6, 109.4. MS *m*/*e* 161, 159 $[MH]^+$, 141.

6-Hydroxycarbonyl-2-pyron, 6. Diester **5** (3.6 g, 16.8 mmol) was dissolved in concentrated hydrochloric acid (100 mL) and the mixture was refluxed for 8 h as described previously.[30] The resultant **6** was collected by filtration (1.74 g, 74%). IR (Nujol): 1732 (m), 1690 (m), 1626 (m), 1237 (m), 1202 (m), 1161 (m), 1125 (m), 886 (m), 737 (m), 723 (m) cm^{-1}. ^{1}H NMR (200 MHz, CD_3OD): δ 7.47 (dd, J = 9.2, 6.6 Hz, 1H), 7.04 (d, J = 6.5 Hz, 1H), 6.42 (d, J = 9.2 Hz, 1H). ^{13}C NMR (75.47 MHz, CD_3OD): 163.0, 162.5, 151.5, 144.9, 121.8, 112.0. MS: *m*/*e* 141 $[MH]^+$.

6-Chlorocarbonyl-2-pyron, 7. Carboxylic acid **6** (1.08 g, 7.71 mmol) was dissolved in thionyl chloride (10 mL) and the mixture was refluxed for 16 h.[30] Excess thionyl chloride was removed under reduced pressure and the resultant crude acyl chloride, **7** (1.1 g, 90%) was taken to the next step without purification. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.47 (dd, J = 9.4, 6.6 Hz, 1H), 7.28 (d, J = 6.6 Hz, 1H), 6.64 (d, J = 9.4 Hz, 1H).

6-Aminocarbonyl-2-pyron, 8. Following the general procedure of amide formation,[31] acyl chloride **7** (1.1 g, 6.94 mmol) was dissolved in dry methylene chloride (15 mL) and the mixture was added to an ice-cold solution of hexamethyldisilizane (5 mL, 20 mmol) in dry methylene chloride (50 mL). Then the reaction mixture was stirred at room temperature for 24 h. Methanol (1.5 mL) was added and the resultant organic phase was washed with 5% sulfuric acid (2 × 20 mL) and then with saturated ammonium sulfate (2 × 20 mL), dried over magnesium sulfate, and filtered. Removal of solvents under reduced pressure afforded amide **8** in the form of white powder (0.92

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g, 95%). IR (Nujol): 3339 (m), 3302 (m), 3187 (m), 1763 (m), 1732 (m), 1703 (m), 1670 (m), 1631 (m), 1614 (m), 1086 (w), 864 (m), 850 (m), 815 (m), 618 (m) cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 8.12 (br s, 1H), 7.88 (br s, 1H), 7.66 (dd, J = 9.3, 6.6 Hz, 1H), 7.01 (d, J = 6.6 Hz, 1H), 6.54 (d, J = 9.3 Hz, 1H). ^{13}C NMR (75.47 MHz, DMSO-d_6): δ 158.3, 158.0, 151.1, 142.2, 116.6, 104.8. MS *m*/*e* 140 $[MH]^+$.

2-Hydroxymucon-1-amide, 4b. Compound **8** (1.0 g, 7.19 mmol) was dissolved in sodium hydroxide (2 N, 50 mL). The mixture was stirred overnight and then acidified with cold hydrochloric acid (6 M). The resultant white solid was filtered, washed with cold methanol, and air-dried to give **4b** (0.67 g, 60%). IR (Nujol): 3459 (m), 3356 (m), 3184 (m), 1732 (m), 1683 (m), 1643 (m), 1622 (m), 1569 (m), 1622 (m), 1540 (m), 1264 (m), 1163 (m), 1107 (w), 873 (m), 722 (m) cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 7.74 (br s, 1H), 7.47 (br s, 1H), 7.63 (dd, J = 15.3, 11.9 Hz, 1H), 6.16 (d, J = 11.9 Hz, 1H), 5.88 (d, J = 15.3 Hz, 1H). ^{13}C NMR (75.47 MHz, DMSO-d_6): δ 168.0 (s), 165.1 (s), 150.1 (s), 138.5 (d), 120.6 (d), 104.0 (d) ppm. MS: *m*/*e* 158 $[MH]^+$, 140.

Enzymatic Activity with 2-Hydroxymuconate, 1b. The kinetic parameters reported in Table 1 were taken from our previous study.[28]

Enzymatic Activity with 2-Hydroxymucon-1-amide, 4b. Each enzyme was assayed spectrophotometrically at 25 °C by monitoring the disappearance rate of **9** ($\epsilon_{max} = 6.97 \times 10^3\ M^{-1}\ cm^{-1}$ at 350 nm) immediately following the addition of **4b** to an assay mixture containing enzyme appropriately diluted in phosphate buffer (20 mM, pH 7.38). Final enzyme concentration varied from 6.6 nM (for wt4-OT, **RRR**) to ~1 μM (for (Arg11Cit,Arg39Cit,Arg61Cit)-4-OT, **ÇÇÇ**). The substrate concentration varied from 20 to 200 μM, and the kinetic parameters K_M and k_{cat} were calculated from nonlinear regression data analysis.

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