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Electrostatic Interactions Dominate the Catalytic Contribution of Arg39 in 4-Oxalocrotonate Tautomerase

Norman Metanis,[†,‡,§] Ashraf Brik,[†,‡,§] Philip E. Dawson,*[,§,∥] and Ehud Keinan*[,†,‡,§]

Department of Chemistry and Institute of Catalysis Science and Technology, Technion—Israel Institute of Technology, Technion City, Haifa 32000 Israel, and Departments of Molecular Biology and Cell Biology and Chemistry and The Skaggs Institute of Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received June 18, 2004; E-mail: dawson@scripps.edu, keinan@scripps.edu

The hexameric enzyme 4-oxalocrotonate tautomerase (EC 5.3.2, 4-OT) catalyzes the isomerization 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 intermediate **2** (Figure 1).[1] Kinetic studies have shown that this efficient enzyme transfers a proton from C-3 to C-5 using an N-terminal Pro residue as a general base ($pK_a \sim 6.4$).[2−5]

Structural studies have shown that, in addition to Pro1, the active site contains residues on neighboring subunits of the hexamer, including Arg11′, Arg39″, and Arg61′, that might be involved in catalysis and substrate binding.[6] Kinetic analysis of Ala mutations, Arg11Ala, Arg39Ala, and Arg61Ala,[4] confirmed the importance of Arg11 and Arg39 in binding and catalysis.[7,8] These Ala mutations eliminate the entire guanidinium side chain and replace it with active site water. These studies could not distinguish the relative contributions of hydrogen bonding and electrostatic interactions.

The noncoded amino acid citrulline (Cit) has an uncharged side chain that is isosteric with arginine but replaces the guanidinium functionality with a neutral urea moiety (Figure 1). Arginine to citrulline substitutions have been used in experimental and computational studies in order to dissect the hydrogen bonding and electrostatic interaction of arginine in enzymes.[9,10] This unnatural amino acid substitution can be achieved by chemical synthesis, as previously established for various 4-OT analogues.[11]

Here, we report on the synthesis and catalytic properties of a full set of 4-OT analogues with arginine to citrulline substitutions at positions 11, 39, and 61. These data suggest that the main contribution of Arg39″ to catalysis is by electrostatic stabilization of the anionic transition state leading to intermediate **2**, and not by hydrogen bonding.

All 4-OT analogues were chemically synthesized using a resin splitting strategy (Scheme 1), purified and folded as described previously.[12] The CD spectra of the folded proteins were nearly identical to that recorded for the wild-type enzyme, indicating that the mutations did not cause significant conformational changes in the synthetic proteins.

The kinetic parameters (k_{cat} and K_M) were determined by monitoring the enzyme-catalyzed conversion of **1** (which exists in solution mainly in the dienol form, 2-hydroxymuconate) to **3**.[13] Table 1 summarizes the kinetic parameters of all synthetic enzymes.

As shown in Figure 1, the Cit residue at positions 39 and 61 can interact with the substrate to contribute two hydrogen bonds to the proposed substrate and intermediate structures. The results show that the (Arg61Cit) analogue, **RRÇ**, isomerizes **1** with kinetic parameters that are of the same order of magnitude as those of the wt4-OT (2-fold decrease of k_{cat}, 4-fold increase of K_M). Taken together with previous observations with the (Arg61Ala)-4-OT mutant, **RRA**.[4] These small differences suggest that Arg61′ has a minor role in catalysis and substrate binding, despite the proximity of this residue to the carboxylate group in the crystal structure of the modified enzyme.[6]

In contrast, the activity of the Arg39Cit analogue, **RÇR**, was reduced significantly. In this context, k_{cat} decreased 1600-fold while K_M increased only by 2.5-fold, leading to 4000-fold decrease in k_{cat}/K_M. This observation suggests that the main role of the positively charged Arg39″ residue is to stabilize the development of a negatively charged transition state leading to intermediate **2**, as shown in Figure 1. Interestingly, this $>10^3$ decrease in k_{cat} for the **RÇR** analogue is a factor of 10 larger than the corresponding Ala

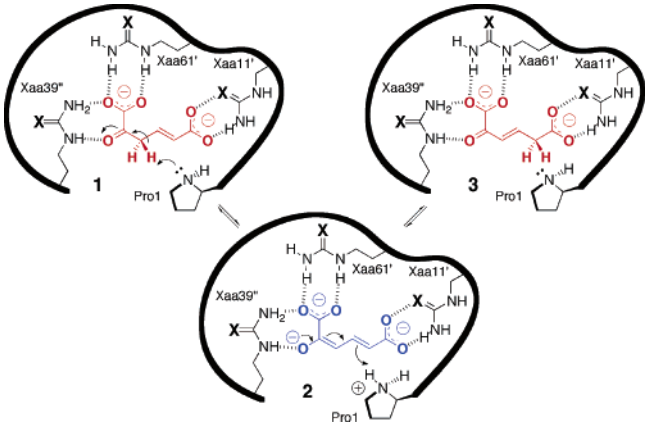
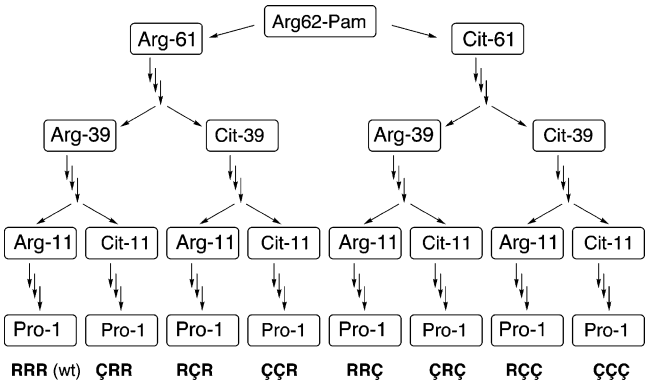


Figure 1. Proposed general-base mechanism for the 4-OT-catalyzed tautomerization of **1**. The three arginine residues ($\mathbf{X} = NH_2^+$) of the wild-type were replaced by citrulline ($\mathbf{X} = O$).

Scheme 1. Split Resin Assembly of the 4-OT Analogues[a]



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

[†] Technion—Israel Institute of Technology.
[‡] Department of Molecular Biology, The Scripps Research Institute.
[§] The Skaggs Institute of Chemical Biology, The Scripps Research Institute.
[∥] Department of Cell Biology and Chemistry, The Scripps Research Institute.

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Table 1. Kinetic Parameters for the Isomerization of **1** Catalyzed by the Synthetic wt4-OT (**RRR**) and the Citrulline Analogues

analogue[a]	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M (M^{-1} sec^{-1})	k_{cat}/k_{uncat} [b]
RRR	62 ± 10	2500 ± 150	4.0×10^7	2.8×10^6
ÇRR	780 ± 120	24 ± 3	3.0×10^4	2.8×10^4
RÇR	160 ± 40	1.5 ± 0.2	9.8×10^3	1.8×10^3
RRÇ	260 ± 25	1200 ± 70	4.5×10^6	1.4×10^6
ÇÇR	nd	< 0.5	nd	nd
ÇRÇ	780 ± 270	5.4 ± 1.5	7.0×10^3	6.2×10^3
RÇÇ	nd	< 0.5	nd	nd
ÇÇÇ	nd	< 0.5	nd	nd
RRR[c]	*180 ± 30*	*3500 ± 500*	*1.9×10^7*	
ARR[c]	*1600 ± 300*	*40 ± 6*	*2.5×10^4*	
RAR[c]	*290 ± 40*	*28 ± 2*	*9.7×10^4*	
RRA[c]	*290 ± 40*	*3500 ± 240*	1.2×10^7	

[a] Analogue coding refers to positions 11, 39, and 61 of the protein, respectively. [b] Uncatalyzed rate: 8.7×10^{-4} sec^{-1}. [c] Italicized data were taken from ref 4.

mutation **RAR**.[4] These results suggest that the neutral urea group of citrulline provides less stabilization for the negatively charged intermediate than aqueous solvation. Hilvert has reported a similar destabilizing effect upon substitution of arginine by citrulline in chorismate mutase.[9b,14]

Arginine 11 has been proposed to interact with the substrate in an end-on manner (Figure 1). In this context, the Arg11Cit substitution was expected to introduce a neutral hydrogen bond donor and a neutral hydrogen bond acceptor. The **ÇRR** analogue also exhibited reduced activity, k_{cat} decreased 100-fold, K_M increased 13-fold, and k_{cat}/K_M decreased 1300-fold in comparison with the wt4-OT, **RRR**. These observations indicate that Arg11′ has an important role not only in substrate binding but also in catalysis (Figure 1).[4] Overall, 4-OT analogues **ÇRR** and **RÇR** highlight the significant role of both Arg11′ and Arg39″ in catalysis.

As expected, much more pronounced changes in both catalysis and binding were observed with the doubly substituted enzymes. The (Arg11Cit)(Arg39Cit) analogue, **ÇÇR**, did not exhibit any detectable catalysis above the limits of our assay.[15] However, the greater than 5000-fold decrease in k_{cat} reflects an approximate additivity of the effects of each substitution on k_{cat} and suggests independent functioning of Arg11′ and Arg39″.[16] This observation is consistent with the former studies on Arg to Ala mutations at positions 11 and 39.[4]

Similarly, the k_{cat} value of the double substitution **ÇRÇ** is consistent with the additive contributions to catalysis from the single substitutions at Arg11′ and Arg61′. The K_M of this double mutant is similar to that of the singly mutated enzyme, **ÇRR**, further supporting the minor role of Arg61′ in substrate binding. Finally, **RÇÇ** and the triply modified enzyme, **ÇÇÇ**, showed no detectable catalysis. These observations are not surprising since the activity of the singly modified (Arg39Cit) **RÇR** enzyme was near our detection limit.[15]

These results support the important role of electrostatic interactions in substrate binding and transition state stabilization. Comparison of the kinetic parameters of **RÇR**, **ÇÇR**, and **RÇÇ** supports the critical roles of Arg11′ and Arg39″ in 4-OT catalysis. Arg11Cit affects both binding and catalysis, consistent with binding and orientating the substrate carboxylate group that is present in the ground state and transition state. In contrast, the Arg39Cit substitution significantly perturbs k_{cat}, consistent with Arg39″ interacting with the developing negative charge of the ketoacid group in the transition state. In addition, the Arg39Cit substitution gives further evidence against the role of Arg39″ as a general acid, in agreement with recent computational studies.[17]

This work highlights the utility of unnatural amino acids to probe the role of specific functional groups in enzymes and opens the 4-OT system to further chemical manipulation.[18] For example, the importance of electrostatic stabilization suggests that Arg39″ could be partially replaced by monovalent cationic residues such as ornithine or homolysine. In addition, Arg to Cit substitution used in this study may be a straightforward approach to convert the natural specificity of this enzyme for carboxylate substrates into specificity for amide substrates.

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Supporting Information Available: Experimental details for the synthesis and characterization of wt4-OT and its citrulline mutants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Standard assay conditions were used for all enzymes (20 mM sodium phosphate buffer, pH 7.38) following the appearance rate of **3** at 236 nm and calculating the kinetic parameters by nonlinear regression analysis as shown in ref 2.
(14) Change in k_{cat}/K_M corresponds to a loss of ~5 kcal mol^{-1} of the transition state, compared to 6.5 kcal mol^{-1} observed in ref 9b.
(15) Detection limit of k_{cat} in our kinetic assay was 0.5 s^{-1}.
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