Copying Nature's Mechanism for the Decarboxylation of β-Keto Acids into Catalytic Antibodies by Reactive Immunization

Robert Björnestedt, Guofu Zhong, Richard A. Lerner,* and Carlos F. Barbas III*

Contribution from The Skaggs Institute for Chemical Biology and the Department of Molecular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037

Received June 20, 1996[⊗]

Abstract: Reactive immunization was used to generate catalytic antibodies that use the enamine mechanism common to the natural class I aldolase enzymes. In order to investigate the possibility of exploiting the imine and enamine intermediates programmed into antibody catalysts by reactive immunization and the features which antibody aldolases share with naturally evolved catalysts, we have studied their ability to catalyze the decarboxylation of structurally related β-keto acids. Both aldolase antibodies were shown to efficiently catalyze the decarboxylation of two hapten-related β-keto acids with rate enhancements (k_{cat}/k_{uncat}) between 4959 and 14 774. Inhibition studies support the role of an essential lysine residue in the active site of the antibodies and the formation of a cyanide accessible imine intermediate in the mechanism. Investigation of the decarboxylation reaction of 2-{3′-(4″-acetamidophenyl)propyl}-acetoacetic acid, **4**, to 6-(4′-acetamidophenyl)-2-hexanone, **12**, in the presence of ^{18}O-labeled water by electrospray mass spectrometry revealed obligatory incorporation of ^{18}O in the antibody-catalyzed reaction consistent with decarboxylation proceeding via an imine intermediate. These studies demonstrate that reactive immunization may be utilized to program in fine detail the mechanism of catalytic antibodies and our ability to exploit the programmed reaction coordinate for different catalytic tasks.

Introduction

The study of amine-catalyzed decarboxylations of β-keto acids occupies a special place in the history of bioorganic chemistry and enzymology. In a series of elegant studies Westheimer and colleagues elucidated in detail the mechanism by which the enzyme acetoacetate decarboxylase catalyzes the decarboxylation of acetoacetic acid.[1] These studies demonstrated that the reaction proceeds by the formation of a Schiff base between the ϵ-amino group of a lysine residue in the enzyme and acetoacetate, followed by decarboxylation to form an enamine which is then tautomerized to a Schiff base and finally hydrolyzed to release acetone and the free enzyme. The enzymatic mechanism is analogous to that originally proposed by Pederson in 1934 for simple amine-catalyzed decarboxylations.[2] Herein we report the programming of the chemical mechanism used by the natural enzyme acetoacetate decarboxylase into catalytic antibodies by the process of reactive immunization.

Previously, antibodies capable of catalyzing intermolecular aldol reactions with control of stereochemistry in both Cram and anti-Cram directions were produced.[3a] These catalytic antibodies were shown to use the enamine mechanism common to the natural class I aldolase enzymes.[4] The reaction mechanism of these aldolase antibodies was programmed using reactive immunization[3] with the β-diketone hapten **1** as shown in Scheme 1. The β-diketone **1** is a chemical trap. Reaction of an ϵ-amino group of lysine within the active site of the antibody with a keto group of **1** results in the formation of a tetrahedral carbinolamine, which is dehydrated to an imine and subsequently tautomerized to the stable vinylogous amide **3** shown in Scheme 1. The imine and enamine intermediates developed along the reaction coordinate of the reaction between the antibody and the diketone and the aldol reaction are also found along the reaction coordinates of other reactions, for example, decarboxylations, racemizations, and alkylation reactions to name a few. Indeed, it is known that some naturally occurring class I aldolases are bifunctional. In addition to catalyzing the aldol reaction, these bifunctional catalysts also catalyze the decarboxylation of β-keto acids.[5] In order to investigate the possibility of exploiting common intermediates programmed into antibody catalysts by reactive immunization and the features which antibody aldolases share with naturally evolved catalysts, we have studied their ability to catalyze the decarboxylation of structurally related β-keto acids.

Results and Discussion

Two antibody aldolases, 38C2 and 33F12, were available for study.[3a,6] These antibodies catalyze the retroaldol reaction of

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1996.

(1) The following constitutes a partial listing of the most relevant references: (a) Hamilton, G. A.; Westheimer, F. H. *J. Am. Chem. Soc.* **1959**, *81*, 6332. (b) Fridovich, I.; Westheimer, F. H. *J. Am. Chem. Soc.* **1962**, *84*, 3208. (c) Laursen, R. A.; Westheimer, F. H. *J. Am. Chem. Soc.* **1966**, *88*, 3426. (d) Tagaki, W.; Guthrie, J. P.; Westheimer, F. H. *Biochemistry* **1968**, *7*, 905. (e) Frey, P. A.; Kokesh, F. C.; Westheimer, F. H. *Biochemistry* **1971**, *14*, 7266. (f) Kokesh, F. C.; Westheimer, F. H. *J. Am. Chem. Soc*. **1971**, *93*, 7270. (g) Schmidt, Jr., D. E.; Westheimer, F. H. *Biochemistry* **1971**, *10*, 1249. (h) Autor, A. P.; Fridovich, I. *J. Biol. Chem*. **1970**, *245*, 5214. (i) Westheimer, F. H. *Proceedings of the Robert A. Welch Foundation Conferences on Chemical Research;* Robert A. Welch Foundation, Houston, 1971; Vol. 15, pp 7−50. (j) Westheimer, F. H. *Tetrahedron* **1995**, *51*, 3.

(2) Pederson, K. J. *J. Phys. Chem.* **1934**, *38*, 559.

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(5) Kobes, R. D.; Dekker, E. E. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 607. Nishihara, H.; Dekker, E. E. *J. Biol. Chem.* **1972**, *247*, 5079. Vlahos, C. J.; Dekker, E. E. *J. Biol. Chem*. **1986**, *261*, 11049.

(6) Nucleic acid sequencing of the genes coding for these antibodies has revealed that they are somatic mutants of one another and differ by 18 amino acids in their variable regions.

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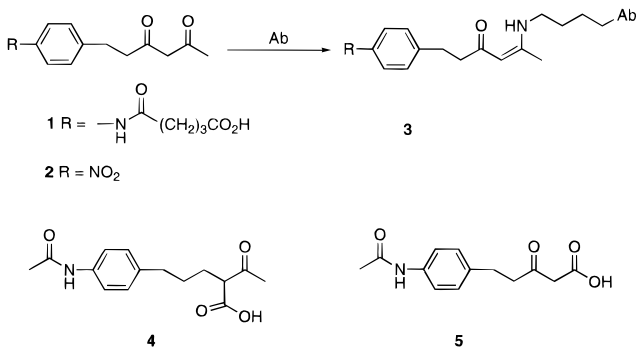
Scheme 1



4-hydroxy-6-phenyl-2-hexanone but not 2-hydroxy-6-phenyl-4-hexanone, suggesting preferential attack of the lysine at the 2 position. This reactivity and the hapten structure suggested **4** would be a suitable β-keto acid substrate if 38C2 and 33F12 could tolerate substitution at the 3 position of the hapten. In order to map the geometry of the active sites of these antibodies, we studied their reactions with a series of structurally related β-diketones. Both antibodies 38C2 and 33F12, shown to be capable of reacting with the linear diketones **2** and 2,4-pentanedione,[3a] also reacted with the branched diketones 3-methyl-2,4-pentanedione, 2-acetylcyclopentanone, and 2-acetylcyclohexanone to form stable vinylogous amides which exhibited intense ultraviolet absorptions, λ_{max}, at 328, 335, and 335 nm, respectively, in a spectral region clear of absorption from protein. These experiments suggested an active site geometry which tolerates considerable substitution at the 3 position of the hapten molecule. On the basis of these experiments, the β-keto acids **4** and **5** were synthesized.

The lithium salt **11** of compound **4** was prepared in six steps from 4-iodoaniline as shown in Scheme 2. Acetylation of 4-iodoaniline (85%) followed by palladium-catalyzed arylation of allyl alcohol under Heck conditions[7] generated compound **7** (69%). Reduction of compound **7** followed by reaction with triphenylphosphine and bromine provided compound **9** (84%). α-Alkylation of ethyl acetoacetate with **9** under basic conditions[8] afforded the ester **10** (61%) which upon saponification with LiOH provided compound **11** (94%). The ketone **12** was prepared by saponification of compound **10** followed by acid-catalyzed decarboxylation of the resulting β-keto acid. The lithium salt **14** of compound **5** was prepared from compound **6** as shown in Scheme 3. In this synthesis, palladium-catalyzed arylation[7] of 1-methyl-2-propen-1-ol with compound **6** produced the ketone **13** (81%). Direct carboxylation of ketone **13** with magnesium methyl carbonate[9] in DMF afforded **14** (51%) upon workup with LiOH and purification by RP-HPLC.

The β-keto acids **4** and **5** prepared by acidification of their respective lithium salts **11** and **14** were tested as substrates for decarboxylation by antibodies 38C2 and 33F12. The initial rates of decarboxylations catalyzed by 38C2 and 33F12 were studied as a function of the concentration of substrates **4** and **5**. The kinetic parameters are summarized in Table 1. As shown in Figure 1, the decarboxylation of **4** by antibody 38C2 followed Michaelis−Menten saturation kinetics. For both antibodies, catalysis of decarboxylation of **4** or **5** was efficient and exhibited multiple turnovers and no product inhibition. Catalysis of the decarboxylation of **5** by 38C2 at a rate lower than that observed for substrate **4** is indicative of the preferential reactivity of the antibody with the keto functionality at the 2 position as observed in the aldol reaction. Other antibodies which bound hapten **1** with high affinity albeit in a noncovalent fashion were also studied and were found not to catalyze the decarboxylation reactions.

The current study differs from decarboxylations of activated model compounds catalyzed previously[10] with antibodies in studies designed to elucidate medium effects in enzyme-catalyzed reactions. Our goal is to create an enzyme that uses a mechanism common to nature's catalyst, thereby testing the ability of the experimenter to program a detailed catalytic mechanism into antibodies. To test whether this was achieved, the mechanism by which antibodies 38C2 and 33F12 operate was studied further. Inhibition of decarboxylation activity, as well as aldolase activity,[3a] was complete in the presence of the hapten **2** or 2,4-pentanedione when spectrophotometric titration indicated the formation of a single vinylogous amide species per active site. This is analogous to the inhibition of the enzyme acetoacetate decarboxylase by acetopyruvate.[1d] Additionally, incubation of antibody 38C2 and **4** in the presence of 1% v/v acetone and 50 μM cyanide resulted in 88% inhibition of decarboxylase activity. Activity decreases of 23% and 15% were observed when the study was performed with the addition of either acetone or cyanide alone. The synergistic effect of cyanide and acetone suggests that inhibition is due to attack of the acetone−antibody imine by cyanide to form a covalent aminonitrile adduct. These data are similar to those reported for acetoacetate decarboxylase and the bifunctional enzyme 2-keto-4-hydroxyglutarate aldolase.[1h,5] Cyanide alone inhibits the antibody-catalyzed decarboxylation reaction in a concentration dependent manner, indicating that imine **15** is accessible to attack by cyanide. These experiments confirm that decarboxylation and aldol reactions occur at the same site and are dependent on an essential ϵ-amino group of a lysine residue in the active site.

To confirm not only that decarboxylation is dependent on a lysine residue but that imine formation is obligatory for decarboxylation, we studied the 38C2-catalyzed decarboxylation of **4** in buffered ^{18}O-labeled water. If the reaction proceeds as described in Scheme 4, the product ketone **12** must incorporate ^{18}O in the keto functionality which is obtained following decarboxylation and hydrolysis of the imine **15**. While **4** proved unstable to GC−MS analysis, electrospray MS allowed for the study of this reaction. Initially we determined that purified substrate **4** and product **12** containing ^{16}O produced M^+ ions at the expected *m/z* of 278 for the protonated acid of **4** and *m/z* 256 for the product **12**, corresponding to a (**12** + Na^+) ion. A reaction mixture containing **4,** 2% of the ^{16}O-containing product **12** as an internal standard, and antibody 38C2 in ^{18}O water was followed by direct analysis of the reaction using electrospray MS. The antibody-catalyzed conversion of **4** to **12** results in an obligatory increase of two mass units to yield a new peak at *m/z* of 258 corresponding to the incorporation of ^{18}O into **12**, Figure 2. The appearance of a peak at *m/z* 280 at 160 min corresponds to the exchange of ^{18}O into the substrate **4**. Further study of the simple exchange reaction in the absence of protein or in the presence of a noncatalytic antibody showed that the incorporation of ^{18}O into the *m/z* 280 species is not significantly catalyzed by antibody 38C2 and constitutes the background exchange rate. From these experiments we conclude that the reaction proceeds as described in Scheme 4 where the decarboxylation step is rapid compared to formation and hydrolysis of **15**.

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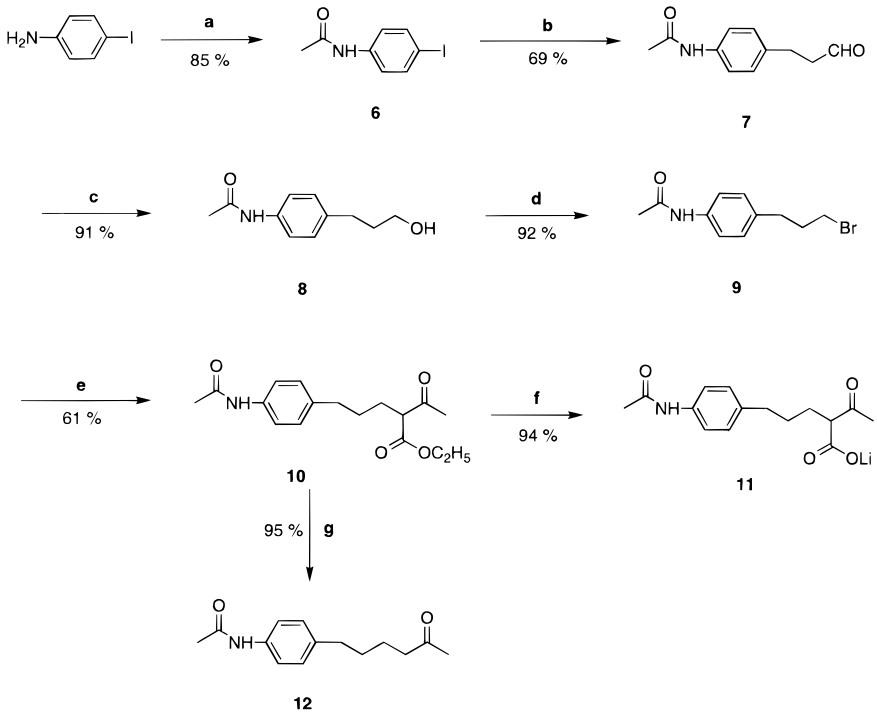
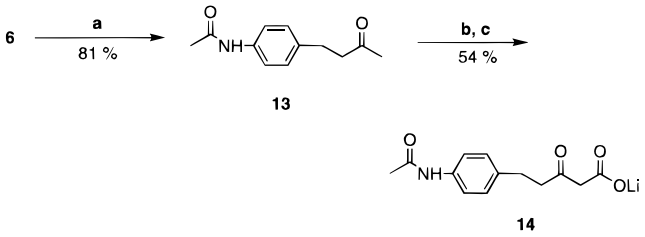
Scheme 2[a]



[a] Conditions: (a) Ac_2O, Et_3N, CH_2Cl_2, rt, 2 h. (b) Allyl alcohol, $PdCl_2$, Bu_4NCl, $NaHCO_3$, DMF, rt. (c) $NaBH_4$, CH_3OH. (d) Br_2, Ph_3P, DMF. (e) Ethyl acetoacetate, CH_3ONa, CH_3OH, reflux. (f) LiOH, H_2O, rt. (g) (i) NaOH, H_2O. (ii) 0.05 M H_2SO_4, 50 °C.

Scheme 3[a]



[a] Conditions: (a) 1-Methyl-2-propen-1-ol, $PdCl_2$, Bu_4NCl, $NaHCO_3$, DMF, rt. (b) (i) MMC, DMF, 120 °C, 5 h. (ii) HCl, 0 °C. (c) LiOH, H_2O.

Table 1. Kinetic Parameters for Antibody-Catalyzed Decarboxylation Reactions[a]

substrate	k_{cat} (min^{-1})	K_m (mM)	k_{un} (min−1)	k_{cat}/k_{un}
4	0.164	0.95	1.11×10^{-5}	14774
4[b]	0.125	1.82		11261
5	0.082	0.85	1.23×10^{-5}	6666
5[b]	0.061	0.20		4959

[a] Assay conditions: 10 mM phosphate, 150 mM NaCl, pH 7.4, in the presence of 1.25 μM antibody. [b]Reaction using antibody 33F12.

The catalytic efficiency of antibody 38C2 as a decarboxylase can be brought into perspective by comparison with decarboxylation reactions catalyzed by simple amines,[11] designed[12a] and combinatorially optimized peptides,[12b] and the enzyme acetoacetate decarboxylase.[1,13] The most effective simple amine catalyst of acetoacetate decarboxylation is aminoacetonitrile, AAN.[11b] This amine has served as a model for acetoacetate decarboxylase catalyzed decarboxylations since its p*K*a approximates that of the active-site lysine of the enzyme. Studies

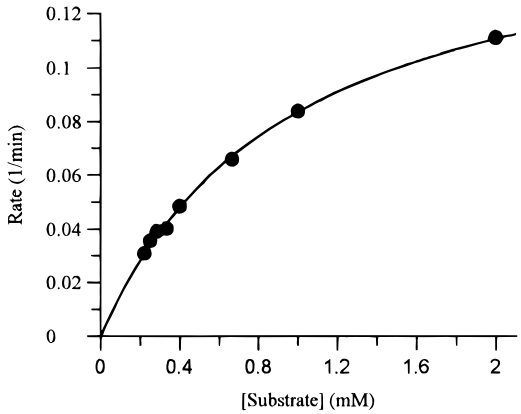


Figure 1. Michaelis−Menten plot for the conversion of **4** to **12** catalyzed by antibody 38C2. Product formation was monitored by high-performance liquid chromatography. The curve was fit to the experimental data by nonlinear regression analysis using GraFit software.[17]

of AAN-catalyzed decarboxylation of acetoacetate have demonstrated that the imine formed in this reaction decarboxylates at a rate 300 000 times faster than acetoacetic acid itself.[11b,d] The rate of acetoacetate decarboxylase-catalyzed decarboxylation is only 156 times that of the rate of decarboxylation of the *N*-cyanomethylimine of acetoacetate allowing most of the catalytic power of the enzyme to be assigned to the enzyme's ability to facilitate imine formation with the amine group of the active site lysine.[11b,13] Since imine formation is optimal at a given pH[11c] with an amine whose p*K*a approximates the given pH, phenylalanine ethyl ester (PheOEt) whose amine has a p*K*a of 7.2[12a] is an appropriate model compound for the study of amine catalyzed decarboxylations at neutral pH. We determined the second-order rate constant for PheOEt-catalyzed decarboxylation of **4** to be 6.9×10^{-6} min^{-1} mM^{-1}. PheOEt has been studied as a model compound for peptide-catalyzed decarboxylation of oxaloacetate, and the second-order rate constant for

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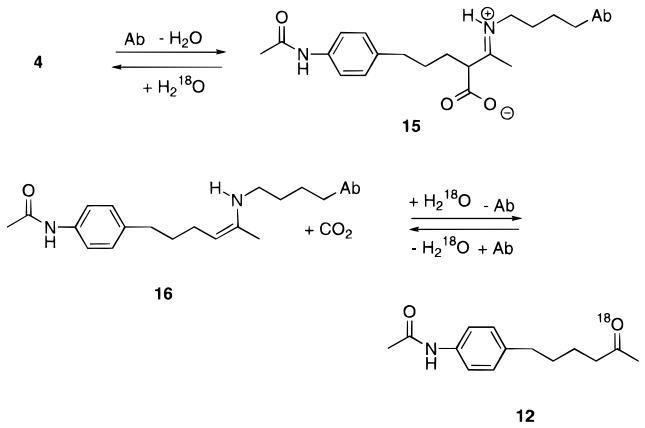
Scheme 4



PheOEt-catalyzed decarboxylation of oxaloacetate has been reported. This together with the new data provided here and the data on acetoacetate decarboxylase-catalyzed decarboxylations allows the relative efficiencies over amine catalysis (k_{cat}/K_m)/k_{NH2}, the rate enhancement over background (k_{cat}/k_{uncat}), and the effective molarity (k_{cat}/k_{NH_2}) of the active site amines of peptide and protein catalysts to be compared (Table 2). The antibody 38C2 provides a 25 000-fold enhancement of the efficiency of the decarboxylation of **4** as compared to the nonenzymic PheOEt amine-catalyzed process. This is a significantly greater enhancement than observed for the peptide catalysts oxaldie-1[12a] and Ox-Opt[12b] and much lower than that observed for acetoacetate decarboxylase.[1,13] The effectiveness of the active site amine of 38C2 is also indicated by an effective molarity of 24 M which exceeds that observed in most model systems[14] and other catalytic antibodies.[15] With respect to catalysis of the aldol reaction by 38C2, steps following imine formation are rate limiting in contrast to the decarboxylase activity described here where imine formation is rate limiting.

Conclusions

One of the goals of bioorganic chemistry is to create new catalysts that utilize defined chemical mechanisms. These studies highlight the ability of reactive immunization to accomplish this.[3] The programming of the identity of amino acid side chains involved in covalent catalysis constitutes a unique feature of this approach and is a significant advance over the use of transition state analogs alone to induce catalytic antibodies.[3a,14] Ultimately, one would wish to combine both methods wherein the experimenter controls both the global aspects and mechanistic details of the reaction coordinate. This approach offers significant advantages over peptide model systems which use simple electrostatic interactions to bind their substrates and are thus very limited with respect to their ability to achieve the concert of effects that characterize an efficient and specific catalyst.[12,1j] The rate enhancement (k_{cat}/k_{uncat} = 14 774) for antibody 38C2-catalyzed decarboxylation of **4**, while significant, is 10^5-fold lower than that reported for acetoacetate decarboxylase.[1i,13] Thus, antibody 38C2 represents a catalyst which having solved the problem of chemical mechanism awaits fine tuning of interactions along the reaction coordinate of decarboxylation. The evolution of catalysis of this antibody to even greater levels may shed light on the factors responsible for the enormous rate enhancements observed for natural decarboxylase enzymes.[16]

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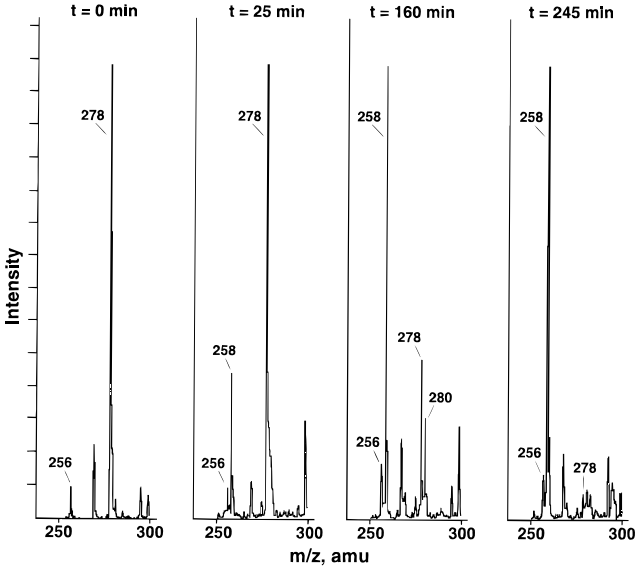

Figure 2. Mass spectra from electrospray MS showing the antibody-catalyzed conversion of β-keto acid **4** to ketone **12** in buffered 98%-^{18}O-labeled water. The *m/z* of 256 corresponds to ketone **12** already present at $t = 0$ whereas the antibody catalyzed conversion of **4** (*m/z* 278) to **12** incorporated ^{18}O to produce an *m/z* of 258. The reaction contained 1.5 mM β-keto acid **4**, 11 mg/mL antibody 38C2, and phosphate-buffered saline in ^{18}O-labeled water. Immediately before analysis the samples were diluted 10-fold in methanol.

Table 2. Kinetic Parameters for Antibody-Catalyzed Decarboxylation Reactions[a]

catalyst	k_{cat}/k_{uncat}	$(k_{cat}/K_m)/k_{amine}$	k_{cat}/k_{amine} (M)
AAD	5.2×10^9 [a]	9.5×10^6 [b]	7.8×10^7
38C2	14774	2.5×10^4 [c]	23.8[c]
oxaldie I	423[d]	48.5[d]	0.42[d]
Ox-Opt[e]	1159[e]	205[e]	1.16[e]

[a] k_{cat} and K_m of the acetoacetate decarboxylase (AAD)-catalyzed decarboxylation of acetoacetate from ref 13, k_{uncat} from ref 1i. [b] k_{amine}, aminoacetonitrile-catalyzed decarboxylation of acetoacetate from ref 1i. [c] Amine = phenylalanine ethyl ester. [d] 14-mer polypeptide catalyzed decarboxylation of oxaloacetate from ref 12a, amine = phenylalanine ethylester. [e] 18-mer polypeptide (YKLLKELLAKLKWLLRKL-NH_2)-catalyzed decarboxylation of oxaloacetate from ref 12b, amine = phenylalanine ethyl ester.

Experimental Section

General Procedures. Solvents were ACS grade from Fischer. Methanol and methylene chloride were dried over powdered magnesium and CaH_2, respectively. Reagents were purchased from Aldrich or Fluka and used without further purification, unless otherwise stated. NMR was recorded on a Brucker AM-300 spectrometer for samples in $CDCl_3$ solution. Chemical shifts δ are given in parts per million, and coupling constant *J* values are given in hertz. Mass spectra were provided by the Scripps Research Institute facility (G. Siuzdak). Flash chromatography was performed with Merck silica gel 60 (230−400 mesh).

***N*-(4-Iodophenyl)acetamide (6).** A solution of 4-iodoaniline (10.0 g, 45 mmol) and triethylamine (19.2 mL, 137 mmol) in 100 mL of methylene chloride was cooled to 0 °C. Acetic anhydride (13.0 mL, 135 mmol) was added dropwise. The reaction mixture was left at 0 °C for 10 min, and then it was allowed to reach room temperature. The mixture was kept at ambient temperature for 2 h. The volatile materials were removed under vacuum, and the solid residue was dissolved in 30 mL of hot methylene chloride. The white solid product **6** (9.9 g) was obtained by filtration, yield 85%.

3-(4′-Acetamidophenyl)propanal (7). *N*-(4-Iodophenyl)acetamide (**6**) (3.6 g, 14 mmol) was added to 16 mL of dried DMF, and then tetrabutylammonium chloride (3.9 g, 14 mmol), sodium bicarbonate

(16) Preliminary studies suggest that the catalytically active lysine possesses an ε-amino group with a highly perturbed p*K*a analogous to that observed in acetoacetate decarboxylase.[1e−g]

(2.9 g, 35 mmol), and allyl alcohol (1.4 mL, 21 mmol) were added. The mixture was stirred for 10 min, and then palladium chloride (0.57 g, 3.2 mmol) was added. The reaction mixture was kept stirring at room temperature for 36 h under an atmosphere of nitrogen. It was then diluted with 100 mL of ethyl acetate, washed with 25 mL of 5% hydrochloric acid and 2 × 25 mL of brine, and dried over magnesium sulfate. Evaporation of solvent gave a crude product, which was purified by column chromatography on silica gel (ethyl acetate/hexane = 70/30), 1.85 g of pure product **7** was obtained, yield 69%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.32 (t, J = 1.3, 1 H), 7.43 (d, J = 8.4, 2 H), 7.27 (s, br, 1 H), 7.13 (d, J = 8.4, 2 H), 2.92 (t, J = 7.5, 2 H), 2.77 (t, J = 7.5, 2 H), 2.17 (s, 3 H). MS: *m/z* (rel intens) 192 ($M + H^+$, 2), 171 (100), 149 (62), 121 (12), 107 (34). $C_{11}H_{13}O_2N$ (191.23).

3-(4′-Acetamidophenyl)-1-propanol (8). At 0 °C, sodium borohydride (0.12 g, 3 mmol) was portionly added to 3-(4′-acetamidophenyl)propanal (**7**) in 25 mL of dried methanol. The reaction mixture was kept at 0 °C for 1 h, and then it was poured into 200 mL of ammonium chloride-saturated ice−water. The reaction mixture was extracted with 3 × 70 mL of ethyl acetate. The combined organic phases were dried over sodium sulfate. Evaporation of solvent gave 0.47 g of alcohol product **8**, yield 91%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.43 (s, br, 1 H), 7.40 (d, J = 8.5, 2 H), 7.13 (d, J = 8.5, 2 H), 3.66 (t, J = 6.4, 2 H), 2.68 (t, J = 7.7, 2 H), 2.17 (s, 3 H), 1.90 (m, 2 H). MS: *m/z* (rel intens) 216 ($M + Na^+$, 52), 194 ($M + H^+$, 27), 176 (100), 154 (46), 136 (39), 107 (16); $C_{11}H_{15}O_2N$ (193.25).

N-[4-(3′-Bromopropyl)phenyl]acetamide (9). 3-(4′-Acetamidophenyl)-1-propanol (**8**) (0.40 g, 2 mmol) and triphenylphosphine (0.54 g, 2.1 mmol) were added to 10 mL of dried DMF. At room temperature, a solution of bromine (0.37 g, 2.3 mmol) in 6 mL of DMF was added dropwise to the reaction mixture until it became a red solution (a small excess of bromine). Then the reaction mixture was kept at room temperature for 1 h. Evaporation of the solvent gave a residue which was separated by column chromatography (ethyl acetate/hexane = 60/40) on silica gel to afford 0.47 g of product **9**, yield 92%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.58 (s, br, 1 H), 7.43 (d, J = 8.3, 2 H), 7.14 (d, J = 8.3, 2 H), 3.39 (t, J = 6.4, 2 H), 2.74 (t, J = 7.3, 2 H), 2.17 (s, 3 H), 2.14 (pent, J = 7.0, 2 H). MS: *m/z* (rel intens) 257 ($M + H^+$, 19). $C_{11}H_{14}OBrN$ (256.14).

Ethyl 2-{[3′-(4″-Acetamido)phenyl)propyl}acetoacetate (10). Sodium (15 mg, 0.65 mmol) was added to 1 mL of dried methanol. After 5 min of stirring, ethyl acetoacetate (0.075 mL, 0.63 mmol) was added to the sodium methoxide solution. The reaction mixture was warmed to slowly reflux. The solution of *N*-[4-(3′-bromopropyl)phenyl]-acetamide (**9**) (130 mg, 0.5 mmol) in 2 mL of dried methanol was then added dropwise to the reaction mixture in 10 min. The reflux was kept for an additional 4 h. Evaporation of solvent under vacuum gave a residue which was separated by column chromatography (ethyl acetate/hexane = 60/40) on silica gel to afford 93 mg of product **10**, yield 61%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.53 (s, br, 1 H), 7.40 (d, J = 8.3, 2 H), 7.09 (d, J = 8.3, 2 H), 4.18 (q, J = 7.0, 2 H), 3.42 (t, J = 7.5, 1 H), 2.59 (t, J = 7.5, 2 H), 2.21 (s, 3 H), 2.15 (s, 3 H), 1.85 (m, 2 H), 1.59 (m, 2 H), 1.27 (t, J = 7.0, 3 H). MS: *m/z* (rel intens) 289 (M^+, 42); $C_{17}H_{23}O_4N$ (305.37).

Lithium Salt 11 of 2-{3′-(4″-Acetamidophenyl)propyl}acetoacetic Acid (4). Ethyl 2-{3′-(4″-acetamidophenyl]propyl}acetoacetate (**10**) (70 mg, 0.23 mmol) and lithium hydroxide (5.4 mg, 0.22 mmol) were added to 7.5 mL of water. The mixture was stirred at room temperature, and after 24 h, the milk-like suspension turned into a clear solution. The mixture was extracted with 3 × 5 mL of ethyl acetate. The water phase was evaporated using a freezer−vacuum evaporator, resulting in a white solid salt product, **11**, yield 61 mg, 94%. ^{1}H NMR (300 MHz, D_2O/DSS): δ 7.47 (s, br, 1 H), 7.35 (d, J = 8.3, 2 H), 7.05 (d, J = 8.3, 2 H), 3.36 (t, J = 7.4, 1 H), 2.54 (t, J = 7.4, 2 H), 2.16 (s, 3 H), 2.11 (s, 3 H), 1.81 (m, 2 H), 1.53 (m, 2 H). MS: *m/z* (rel intens) 283 (M^+, 22); $C_{15}H_{18}O_4LiN$ (283.25). After a solution of **11** was acidified by TFA, **4** was obtained. HR-MS of **4**: 278.1391; $C_{15}H_{20}O_4N^+$ (calcd 278.1392).

6-(4′-Acetamidophenyl)-2-hexanone (12). Ethyl 2-{3′-(4″-acetamido)phenyl)propyl}acetoacetate (**10**) (30 mg, 0.10 mmol) was added to 1 mL of sodium hydroxide (0.25 M, 0.25 mmol). The reaction mixture was stirred until all starting material **10** was dissolved in water. Then sulfuric acid (0.05 M, 3.0 mL) was added, and the reaction mixture was warmed to 50 °C for 30 min. After cooling to room temperature, the reaction mixture was extracted with 3 × 15 mL of ethyl acetate and dried over magnesium sulfate. Evaporation of solvent gave 20 mg of solid product **12**, yield 95%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.52 (s, br, 1 H), 7.41 (d, J = 8.2, 2 H), 7.09 (d, J = 8.2, 2 H), 2.55 (t, J = 7.2, 2 H), 2.45 (t, J = 7.0, 2 H), 2.21 (s, 3 H), 2.14 (s, 3 H), 1.64 (m, 4 H). MS: *m/z* (rel intens) 233 (M^+, 46). $C_{14}H_{19}O_2N$ (233.31).

4-(4′-Acetamidophenyl)-2-butanone (13). Synthesis was performed as described for compound **7** with the exception that allyl alcohol was substituted with 1-methyl-2-propen-1-ol. Yield 81%.

5-(4′-Acetamidophenyl)-3-oxopentanoic Acid (5) and Its Salt 14. 4-(4′-Acetamidophenyl)-2-butanone (**13**) (100 mg, 0.48 mmol) was added to magnesium methyl carbonate (MMC; 2 M in DMF, 2.4 mL). The mixture was heated to 120 °C for 5 h. The mixture was cooled to 0 °C, and then 30 mL of hydrochloric acid was added dropwise. The lithium salt **14** was obtained by adding an excess of lithium hydroxide to the reaction mixture. After filtration, a part of the filtrate was purified by RP-HPLC to obtain a pure salt, **14**. MS: *m/z* (rel intens) 255 (M^+, 11). $C_{13}H_{14}O_4LiN$ (255.20). The yield of **14** from **13** was 54%. A sample of **5** was obtained by acidification of its salt **14**. 5-(4′-Acetamidophenyl)-3-oxopentanoic acid (**5**): ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.51 (s, br, 1 H), 7.44 (d, J = 8.2, 2 H), 7.19 (d, J = 8.2, 2 H), 3.42 (s, 2 H), 2.60 (t, J = 7.1, 2 H), 2.44 (t, J = 7.1, 1 H), 2.20 (s, 3 H). HR-MS: 250.1081; $C_{13}H_{16}O_4N^+$ (calcd 250.1079).

Assays. (a) Antibody Assays. The kinetic measurements were performed in phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4) in the presence of 2.5 μM antibody active sites (based on two active sites per 150 kDa). The product formation was followed by RP-HPLC (Microsorb MV, C-18, 300 Å pore size, 0.45 × 22 cm, flowrate 1.5 mL/min) monitored at 243 nm. The following isocratic HPLC conditions were used to separate the substrates from the products in the antibody assays: 17.5% acetonitrile/82.5% water (0.1% trifluoroacetic acid), t_R(**4**) = 8.3 min and t_R(**12**) = 16.1 min; 15% acetonitrile/85% water (0.1% trifluoroacetic acid), t_R(**5**) = 6.8 min and t_R(**13**) = 9.1 min. The kinetic parameters k_{cat} and K_m were determined by nonlinear regression analysis of experimental data using the GraFit program package.[17] The remaining decarboxylation activity of antibody 38C2 in the presence of 2,4-pentanedione or cyanide was determined as above. The antibody was preincubated with the diketone for 10 min before addition of substrate.

(b) Amine-Catalyzed Decarboxylation. The primary amine-catalyzed decarboxylations were performed under the same experimental conditions as the antibody assay. The second-order rate constants were determined from plots of experimental data where the concentration of the amine was varied.

(c) Enamine Formation. The enamine formation between the catalysts and β-diketones (**2**, 2,4-pentanedione, 3-methyl-2,4 pentanedione, 2-acetylcyclopentanone, and 2-acetylcyclohexanone) was followed spectrophotometrically at 318−335 nm in thermostated (20 °C) 100 μL cuvetts using a Cary 3 spectrophotometer.

(d) ^{18}O Incorporation. The electron spray ionization (ESI) mass spectrometry used to monitor ^{18}O incorporation into **12** was performed on an API III Perkin Elmer SCIEX triple quadropole mass spectrometer. In preparation of the sample, lyophilized antibody 38C2 was resuspended in ^{18}O-labeled water (95−98% ^{18}O, Cambridge Isotope Laboratories, Andover, MA) to give a final concentration of 11 mg/mL. The reaction was started by addition of β-keto acid **4** (1.5 mM), and aliquots were taken out for analysis of ^{18}O incorporation over time. Immediately before analysis the samples were diluted 10-fold in methanol.

Acknowledgment. We are grateful to Brian Bothner and Gary Suizdak for assistance with MS and Jürgen Wagner for early contributions to this work. This study was supported by the NIH (Grant CA27489) and the Skaggs Institute for Chemical Biology. C.F.B. acknowledges an Investigator Award from the Cancer Research Institute.

JA9620797

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