Uroporphyrinogen decarboxylation as a benchmark for the catalytic proficiency of enzymes

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The magnitude of an enzyme's affinity for the altered substrate in the transition state exceeds its affinity for the substrate in the ground state by a factor matching the rate enhancement that the enzyme produces. Particularly remarkable are those enzymes that act as simple protein catalysts, without the assistance of metals or other cofactors. To determine the extent to which one such enzyme, human uroporphyrinogen decarboxylase, enhances the rate of substrate decarboxylation, we examined the rate of spontaneous decarboxylation of pyrrolyl-3-acetate. Extrapolation of first-order rate constants measured at elevated temperatures indicates that this reaction proceeds with a half-life of 2.3×10^9 years at 25 °C in the absence of enzyme. This enzyme shows no significant homology with orotidine 5′-monophosphate decarboxylase (ODCase), another cofactorless enzyme that catalyzes a very slow reaction. It is proposed that, in both cases, a protonated basic residue (Arg-37 in the case of human UroD; Lys-93 in the case of yeast ODCase) furnishes a counterion that helps the scissile carboxylate group of the substrate leave water and enter a relatively nonpolar environment, stabilizes the incipient carbanion generated by the departure of CO_2, and supplies the proton that takes its place.

decarboxylase | catalysis | porphyrin | coproporphyrinogen | evolution

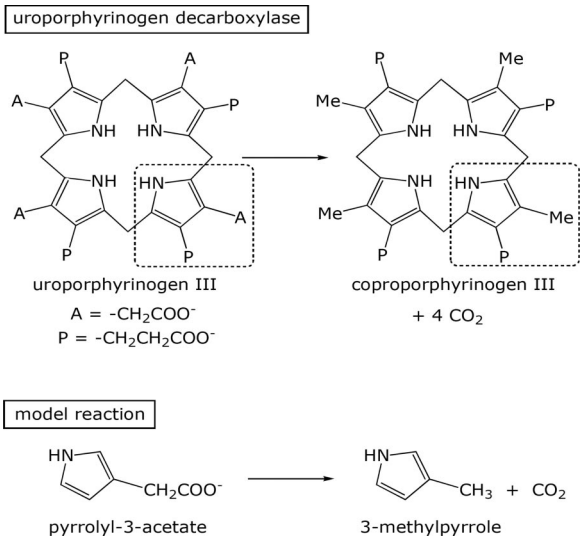

Fig. 1. Decarboxylation Reactions. (*Upper*) The reaction catalyzed by UroD. (*Lower*) The model reaction examined in this work.

The catalytic power of an enzyme can be judged from the rate enhancement that it produces. In general, enzymes act on their substrates at somewhat similar rates, with k_{cat} values ranging between 50 and 5,000 s^{-1}. However, the rate constants of the same reactions in the absence of a catalyst vary over a range of at least 15 orders of magnitude. And the rate enhancements produced by enzymes vary over a similarly broad range ($\approx 10^{15}$-fold), indicating the magnitude of the increase in the enzyme's affinity for the substrate as it passes from the ground state to the transition state (1). Particularly remarkable are those cases in which the enzyme acts as a simple protein catalyst, without the assistance of metals or other cofactors. Such a reaction, the decarboxylation of orotidine 5′-phosphate, was found earlier to proceed in the absence of enzyme with a half-time of 7.8×10^7 years in the absence of enzyme (2).

Here, we describe another reaction, involving a very different substrate (uroporphyrinogen, Uro'gen), that proceeds with a half-life of 2.3×10^9 years at 25 °C in the absence of enzyme. The enzymes catalyzing these reactions act as pure protein catalysts. Their amino acid sequences show no significant homology.

In plants and animals, uroporphyrinogen decarboxylase (UroD, EC 4.1.1.37) catalyzes the first committed step in the biosynthesis of heme, chlorophyll, and the cytochromes. The pioneering experiments of Mauzerall and Granick established that during the action of this single enzyme, the acetate group at the 3-position of each of the 4 pyrrole rings of uroporphyrinogen III (Uro'gen III) is converted to a methyl group, yielding coproporphyrinogen III (Fig. 1) (3–6). The decarboxylations of these acetate groups proceed in a preferred order (7), to generate the tetramethyl product (8). Subnormal levels of UroD activity are responsible for porphyria cutanea tarda, in which Uro'gen III accumulates and severe clinical consequences ensue. The human enzyme has been shown to reach half-maximal activity at low concentrations of substrate (Uro'gen III $K_m = 7 \times 10^{-8}$ M), decarboxylating all 4 of the tetrapyrrole rings with $k_{cat} = 0.16\ s^{-1}$ and $k_{cat}/K_m \approx 3 \times 10^6\ s^{-1}\ M^{-1}$ (9).

To determine the extent to which UroD enhances the rate of substrate decarboxylation, we sought a model reaction for comparison. Because Uro'gen III itself is rapidly degraded by exposure to air and light, and its autoxidation is sensitized by the product uroporphyrin (4), the rate of spontaneous decarboxylation of Uro'gen III could not be measured directly. However, the individual pyrrole rings of Uro'gen III are not conjugated with each other, so the rate of uncatalyzed decarboxylation of a simpler analogue, containing a single pyrrole ring, would be expected to be similar to the rate of decarboxylation of Uro'gen III. At first glance, the monopyrrole porphobilinogen might appear to be an attractive choice, but because of the presence of an exocyclic aminomethyl group, that molecule undergoes relatively rapid lactamization and polymerization to porphyrins (10). To circumvent those difficulties, we chose to examine the decarboxylation of pyrrolyl-3-acetate (PAA) (Fig. 1), and found that decarboxylation of this molecule proceeds smoothly to completion in water at elevated temperatures.

Results

By heating PAA [prepared by the method of Kakushima *et al.* (11)] (0.02 M) under argon at 200 °C in sealed quartz tubes, we observed quantitative conversion to 3-methylpyrrole (3MP; Fig. 1) in anionic buffers (0.1 M acetate, phosphate, borate, and

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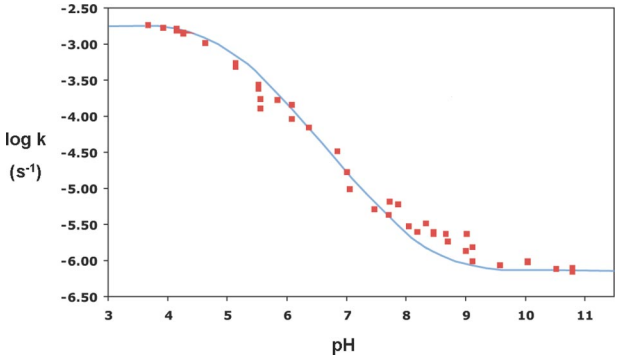

Fig. 2. First-order rate constants for the decarboxylation of PAA at 200 °C, in acetate, borate, phosphate, and carbonate buffers (0.1 M).

carbonate) at pH values ranging from 3 to 12 (measured at 25 °C). At all pH values, decarboxylation followed simple first-order kinetics to completion, and the rate of decarboxylation was not significantly affected by changing ionic strength, in the presence of added KCl at concentrations ranging from 0.1 M to 1.0 M. When the logarithms of the observed first-order rate constants for PAA decarboxylation (based on the disappearance of PAA) were plotted as a function of pH, the results showed a sigmoid dependence on pH, reaching a maximal plateau below pH 4 and a minimal plateau above pH 9 (Fig. 2). The solid line in Fig. 2 is calculated for the equation:

$$k = 1.8 \times 10^{-3} \times [\mathrm{PAAH}] + 7 \times 10^{-7} \times [\mathrm{PAA}^-] \text{ at } 200\ ^\circ\mathrm{C}$$

in which [PAAH] represents the concentration of the conjugate acid of PAA, with a pK_a value of 4.95, and PAA^- represents the concentration of its conjugate base. After establishing the pH dependence of the reaction at elevated temperatures, it became desirable to determine the corresponding rate constants for each form of PAA at 25 °C.

At and above pH 5, the carboxylate anion is the dominant species of PAA in solution. That species of PAA corresponds to the species of Uro'gen III that undergoes enzymatic decarboxylation at pH values near 7 where this enzyme is optimally active. To estimate the rate of spontaneous decarboxylation of PAA^- at the temperatures where UroD is active, we measured the rate of its uncatalyzed decarboxylation as a function of changing temperature in the range between 180 and 250 °C in sodium carbonate buffer (0.1 M, pH 10.0). The resulting rate constants yielded a satisfactory Arrhenius plot (Fig. 3) that was extrapolated to yield a rate constant of $9.5 \times 10^{-18}\ s^{-1}$ at 25 °C ($\Delta H^{\ddagger} = 41.2$ kcal/mol and $T\Delta S^{\ddagger} = 0.7$ kcal/mol). That rate constant corresponds to a half-life of 2.3×10^9 years, ≈30-fold longer than the half-life for decarboxylation of 1-methylorotate at 25 °C recorded earlier (2). Comparing the rate constants for decarboxylation of Uro'gen III by human UroD (see the introduction) with the extrapolated rate of spontaneous decarboxylation of PAA^-, we estimate that UroD enhances the rate of substrate decarboxylation by a factor of 3×10^{16} (or a factor of 1.2×10^{17} if one takes into account that the enzyme actually performs 4 decarboxylations in turning over each molecule of Uro'gen), and that the nominal dissociation constant of the altered substrate in the transition state $[k_{non}/(k_{cat}/K_m)]$ is 1.7×10^{-24} M (or 4×10^{-25} M if each decarboxylation event is considered individually). Its reciprocal, $2.5 \times 10^{24}\ M^{-1}$, is the largest catalytic proficiency (2) that has been recorded for an enzyme that acts without cofactors.

At low pH values, the species of PAA undergoing decarboxylation is mainly the conjugate acid PAAH. Extrapolation of an Arrhenius plot of rate constants observed at pH 3, at temperatures ranging from 110 to 180 °C, yielded a rate constant of $2.1 \times 10^{-12}\ s^{-1}$ ($\Delta H = 33.9$ kcal/mol; $T\Delta S = 0.6$ kcal/mol; $t_{1/2} =$

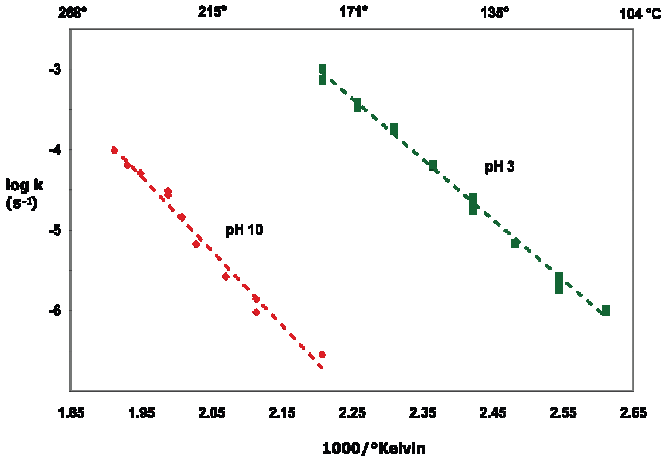

Fig. 3. Arrhenius plot of the decarboxylation of PAA to 3MP conducted at pH 10 (◆) and pH 3 (■). The linear fits were used to obtain the equation for the lines to calculate the rates and thermodynamic parameters.

1.1×10^4 years) at 25 °C. Because carboxylic acids must ordinarily be ionized for decarboxylation to occur (12), the species that actually undergoes decarboxylation at low pH is likely to be the rare zwitterionic tautomer in which the carboxylate group is ionized and the pyrrole ring is protonated at C-2. Based on an acidity function in concentrated sulfuric acid solutions developed by Chiang and Whipple (13, 14), we used UV spectrophotometry to estimate the pK_a value of protonated $PAAH^+$ as −3.8 (Fig. 4), very similar to that reported for unsubstituted pyrrole by those authors. At constant pH, no catalysis of decarboxylation was observed in the presence of potassium acetate buffers (pH 4.0–5.5) ranging in concentration from 0.1 M to 1.0 M.

Discussion

Fig. 5 compares the half-time for decarboxylation of the pyrrolyl-3-acetate anion with those of other biological reactions proceeding at room temperature in the absence of enzymes. Reactions that are subject to catalysis by enzymes without cofactors are shown in red. Based on the enzyme kinetic constants mentioned in the introduction, UroD enhances the rate of substrate decarboxylation by a factor of 1.2×10^{17}, one of the largest rate enhancements that appears to have been reported for an enzyme that acts without cofactors. The only enzymes that are presently known to surpass these values are phosphomonoesterases such as those that act on inositol, fructose 1,6-bisphosphate, and phosphorylated serine and threonine residues in proteins (15).

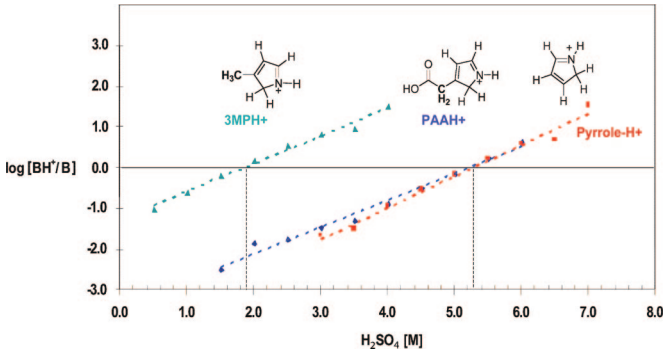

Fig. 4. UV titration of 3-methylpyrrole-H^+, pyrrole-3-acetic acid-H^+ ($PAAH^+$), and pyrrole-H^+ in sulfuric acid, showing that the conjugate acid of PAA has a pK_a for protonation of C2 nearly identical with that of pyrrole (H_o −3.8, corresponding to 5.2 M H_2SO_4) (14).

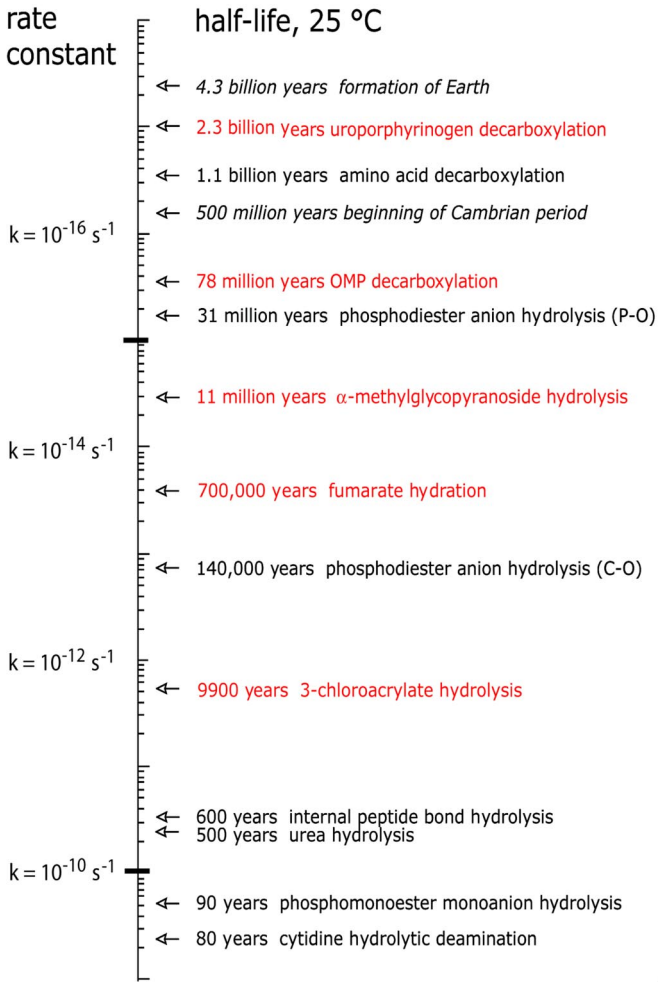

Fig. 5. Half-lives of reactions proceeding spontaneously in water. Reactions that are subject to catalysis by enzymes that act without cofactors are shown in red.

Without exception, those phosphatases are metal-dependent, and metal ions are effective catalysts of phosphomonoester hydrolysis by themselves.

How does UroD generate such a large rate enhancement? At saturating substrate concentrations, the activity of this enzyme reaches a maximum near pH 6.85, for the enzymes from human erythrocytes (7), *Saccharomyces cereviseae* (16), and *Rhodobacter sphaeroides* (17). According to the simplest scenario, that behavior would be consistent with a requirement, for substrate turnover, of at least 2 functional groups—the conjugate acid of 1 group and the conjugate base of another—with pK_a values that straddle this maximum. Either (or both) of those groups might in principle be part of the enzyme or part of the substrate. Because carboxylic acids must ordinarily be ionized for decarboxylation to occur in water (12), it seems reasonable to conjecture that the conjugate base that is required by the pH dependence of the enzyme reaction is the scissile carboxylate group of the substrate itself (Fig. 2).

In considering the identity of the conjugate acid whose presence is implicated by the pH dependence of the enzyme reaction, it is of interest to consider crystal structures of human UroD that have been determined in the presence (18) and absence (19) of the coproporphyrinogen reaction product (Figs. 6 and 7). Apart from several basic residues that appear to anchor the nonreacting propionate side chains of the reactant and

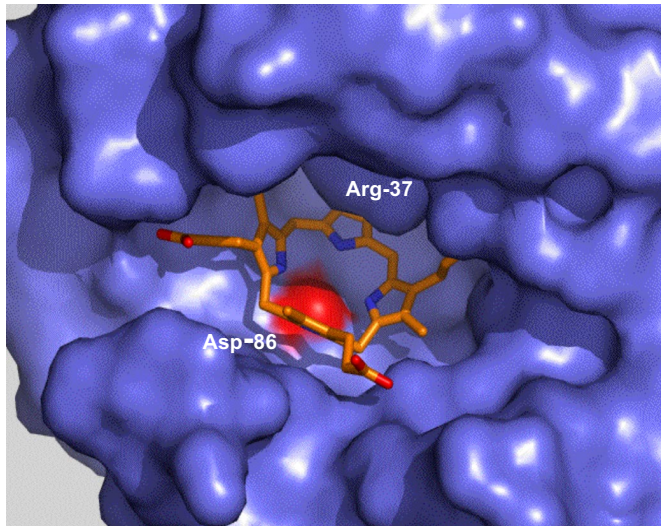

Fig. 6. Coproporphyrinogen I bound in the active site of Uro D Protein Data Bank ID code 1R3Q (18). The carboxylate group of Asp-86 is in red.

product, the only active-site residues that seem to be in a position to play a direct role in catalysis are Tyr-164, Asp-86, and Arg-37. Because mutation of Tyr-164 to phenylalanine results in retention of 29% of the activity of the wild-type enzyme (18), it seems reasonable to infer that Tyr-164 does not make an essential contribution to substrate decarboxylation.

Before the structure of UroD was determined, it was proposed that an acidic group might transfer a proton to C-2, yielding an sp_3-hybridized species that subsequently undergoes rapid decarboxylation (20). Consistent with that possibility, the present findings indicate that the decarboxylation of PAA is accelerated 2,500-fold by simple protonation. General acid catalysis is known to be extremely sensitive to orientation effects, so that the actual factor by which an appropriately situated general acid could enhance the rate of reaction might be much greater, especially

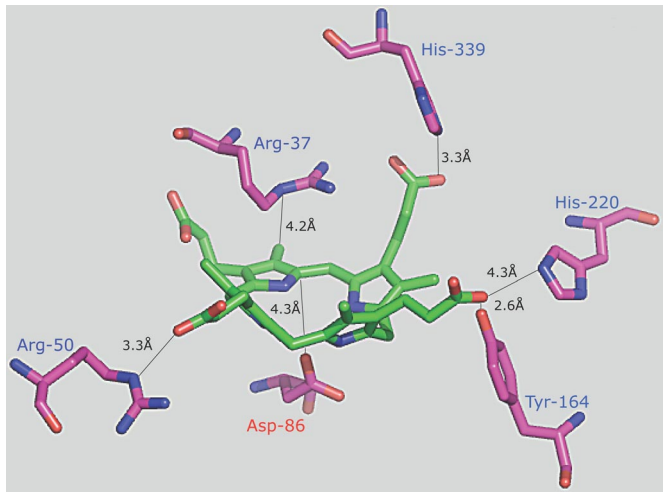

Fig. 7. Polar amino acids in the active site of Uro D within 5.0 Å of bound coproporphyrinogen I (Protein Data Bank ID code 1R3Q; ref. 18). Asp-86 is below the tetrapyrrole ring and can interact with the 4 pyrrole nitrogen atoms. Arg-37, Arg-50, His-220, His-339, and Tyr-164 appear to interact with the propionate side chains. The carboxylate oxygen atom of Asp-86 lies ≈4.3 Å from C2 of the pyrrole ring and the γ-nitrogen atom of Arg-37 lies ≈4.2 Å from the exocyclic methyl carbon atom of one of the pyrrole rings of coproporphyrinogen.

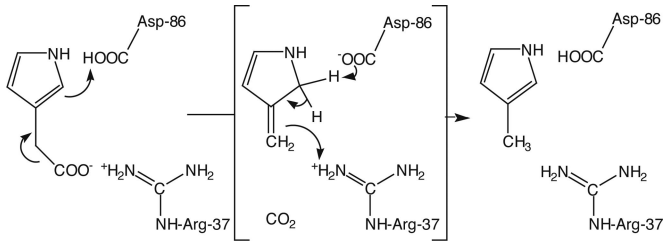

Fig. 8. Possible mechanism of action of UroD, showing assistance by Asp-86 and Arg-37, based on the decarboxylation of PAA^- (present observations).

if it transferred a proton to C-2, which, as the present pK_a value of −3.8 indicates, is much less readily protonated than the carboxylate group of PAA. Asp-86 is situated in a position where it could interact with all 4 of the pyrrole rings of coproporphyrinogen, but Asp-86 may also serve as the conjugate acid whose participation in catalysis is implied by the pH dependence of the enzyme reaction. When Asp-86 is mutated to glycine, UroD loses 98% of its activity toward uroporphyrinogen I (18). Asp-86 may transfer a proton to C-2, to generate an intermediate that undergoes subsequent decarboxylation. In view of the thermodynamic difficulty of protonating C-2 ($pK_a = \approx -3.8$), we consider it more likely that Asp-86 acts in a concerted manner as shown in Fig. 8, generating a doubly protonated C-2 methylene intermediate as CO_2 is eliminated.

Although it seems likely that Asp-86 plays a role in catalysis as suggested above, the fact that a mutant form of UroD, in which Asp-86 has been replaced by glycine (18), retains 2% of the activity of the wild-type enzyme (producing a rate enhancement of 2.4×10^{15}-fold) seems to imply that other factors play an essential role in catalysis.

Of central interest is Arg-37, which appears to be absolutely required for enzyme activity as indicated by the results of mutation experiments by Phillips and his associates (personal communication), and by the inactivation of the bacterial enzyme by phenylglyoxal (16). It seems reasonable to speculate that Arg-37 [earlier assigned a possible role in binding the nonreacting propionyl side-chains of the substrate (21)] may play a more direct role in the catalytic process. After first forming an ion pair with the scissile acetate carboxylate group of the substrate, which allows that part of the substrate to enter the nonpolar pocket where decarboxylation occurs, the guanidinium group of Arg-37 may furnish a proton to the exocyclic $=CH_2$ group attached to C-3, taking the place of the CO_2 that was eliminated. Although the guanidinium groups of arginine residues ($pK_a = 13.5$) have been implicated as proton donors in only a few enzyme reactions (22), the extremely low rate of decarboxylation of PAA^- established in the present experiments implies that the pK_a value of the methyl group of 3-methylpyrrole is in the neighborhood of 36 (23) Thus, the conjugate base of the carbanion generated by CO_2 elimination from Uro'gen III should have no difficulty in abstracting a proton from the guanidinium group of Arg-37. We therefore propose the overall mechanism shown in Fig. 8, in which Asp-86 and Arg-37 act in concert. First, Arg-37 furnishes an anchored counterion that accompanies the scissile carboxylate group of the substrate into a nonpolar cavity within the active site. Asp-86 then protonates C-2 of the substrate as CO_2 is eliminated to yield a carbanionic intermediate, which generates the product by proton abstraction from Arg-37.

Recent density function calculations have been interpreted as supporting the alternative possibility that Arg-37 protonates C-2 of the substrate, performing the function assigned to Asp-86 in Fig. 8 (24). Because the pK_a value of Arg-37 (≈13.5) is greater by ≈17 units than the pK_a value of the C-2-protonated form of the substrate (−3.8; see Fig. 4), we view proton transfer from Arg-37 to C-2 as less likely from a thermodynamic standpoint than proton transfer from Asp-86 (pK_a ≈3.9) to C-2.

Orotidine 5′-monophosphate decarboxylase (ODCase), is another enzyme that catalyzes another extremely slow reaction without the assistance of cofactors. Of each of the 8 conserved amino acid residues of ODCase, all are present at the active site (25), and 6 have individually been shown by mutation to make major contributions (>5 kcal/mol in free energy) to the stabilization of the altered substrate in the transition state (26). However, only one of those critically important side chains (that of Lys-93 in the yeast amino acid sequence of yeast ODCase)

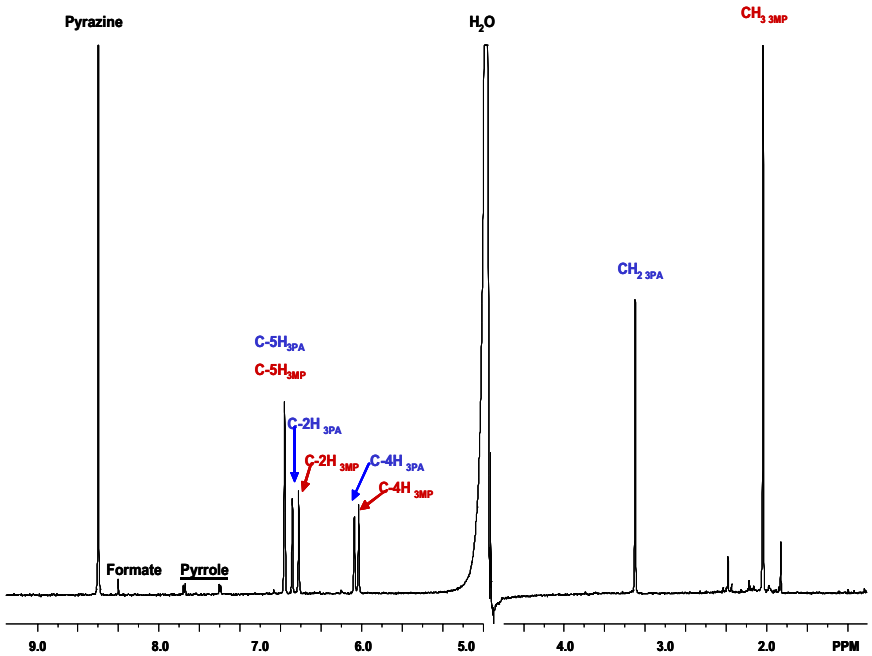

Fig. 9. ^1H NMR spectrum of a ≈50:50 mixture of PAA^- and 3MP obtained after heating at 180 °C for 12 h at pH 10.

makes close contact with the pyrimidine ring in such a way that it might be expected to stabilize a carbanion at the position where decarboxylation occurs. Other active-site residues of ODCase are believed to play a supporting role in orienting the substrate and organizing the closure of loops of the active site around the altered substrate in the transition state (26–28).

Interestingly, comparison of amino acid sequences reveals no significant homology (<8%) between UroD and ODCase. Notwithstanding that lack of homology, we infer that the evolution of these 2 extraordinarily proficient enzymes, UroD and ODCase, may have arrived at a common strategy of catalysis, in which unusually large rate enhancements are largely achieved through the action of a single nitrogenous side chain. In its protonated form, that basic residue (Arg-37 in the case of human UroD; Lys-93 in the case of yeast ODCase) furnishes a counterion that helps the scissile carboxylate group of the substrate leave water and enter a relatively nonpolar environment, stabilizes the incipient carbanion generated by the departure of CO_2, and supplies the proton that takes its place. Preliminary experiments (C.A.L. and R.W., unpublished data) indicate that the decarboxylation of 1-cyclohexylorotate, like that of benzisoxazole-3-carboxylic acid (29), is greatly accelerated by transfer to nonpolar solvents in the presence of amine counterions.

Because heme proteins play a central role in the metabolism of modern organisms, it is natural to inquire how evolution overcame the kinetic obstacle raised by the 2.3-billion-year half-life for uncatalyzed decarboxylation of uroporphyrinogen. A primitive decarboxylase that accelerated uroporphyrinogen decarboxylation by a factor of 1 million would have reduced the half-life of the reaction to 2.3 thousand years, offering little or no selective advantage to the host organism. But the potential biological usefulness of decarboxylated tetrapyrroles would have become apparent only with the appearance of an oxidizing atmosphere. Before that stage in evolution, the binding of oxygen by heme proteins was probably unnecessary, and iron-sulfur proteins were presumably capable (at least in principle) of performing many of the redox functions of the cytochromes (30). And the biosynthesis of siroheme, which is present in modern nitrite and sulfite reductases and may have served other functions in ancient organisms, proceeds directly from uroporphyrinogen and requires no decarboxylation (31). Taking these alternatives into consideration, it seems probable that the enzymes involved in uroporphyrinogen metabolism arose by mutation from ancient proteins with other functions, many of which may have existed before oxygen first appeared in the atmosphere and decarboxylation became necessary for the first time.

Materials and Methods

PAA was prepared by the method of Kakushima *et al.* (11). Other chemicals were purchased from Sigma-Aldrich. Typical reaction mixtures contained PAA (2×10^{-3} M) in sodium acetate-d_3, potassium phosphate, sodium borate, and sodium carbonate buffers (0.1 M) ranging in pH from 3 to 12, measured at 25 °C under argon. Samples were sealed in quartz tubes under vacuum and heated in convection ovens (Barnstead/Thermolyne no. 47900) maintained at 110–250 ± 2 °C, as indicated by ASTM thermometers. After cooling, the contents were diluted with D_2O containing added pyrazine (5×10^{-4} M) as a proton integration standard (4H, $\delta = 8.60$ ppm). ^{1}H NMR spectra were acquired using a Varian Inova Unity 500 MHz system equipped with a cold probe and operated by Solaris 9 software. Spectra of PAA and 3MP ($\approx 1 \times 10^{-3}$ M) were readily obtained in 4 or 8 transients, with a 60-s pulse delay (Fig. 9). The methylene protons of PAA and methyl protons of 3MP gave distinct singlet resonances at 3.29–3.54 ppm (depending on pH) and 2.04 ppm, respectively. Integrated intensities using all protons for both species were consistent except below pH 5.5, where exchange of the C-2 and C-4 protons with D_2O became significant during the time required for NMR analysis. For that reason, the integrated intensities of the methylene protons of PAA and the methyl protons of 3MP, which underwent no significant exchange, were used for kinetic analysis.

Effects of pH on the rate of decarboxylation of PAA were examined at 200 °C, showing quantitative conversion of PAA to 3MP at rates that approached a constant high value below pH 4.0 and a constant low value between pH 9 and 10.5 (Fig. 2). The effects of temperature on the rate of decarboxylation were then examined at pH 3.0 for uncharged PAAH, and at pH 10.0 for PAA^-. Times of heating were adjusted so that the reaction had proceeded to between 15% and 85% completion. Samples were examined before and after reaction to establish that no significant change in pH had occurred during the course of reaction. Experiments testing for potential acetate catalysis were performed by using sodium acetate-d_3 buffers at 0.1 and 1.0 M at the temperature range from 110 °C to 180 °C. Effects of salt on the uncatalyzed decarboxylation reaction were studied by comparing rates at pH 3.0 (sodium acetate-d_3) and pH 10.0 (sodium carbonate) in the absence and presence of 0.33 M or 1.0 M KCl at suitable temperatures and incubation times.

Equilibria of C-2 protonation of pyrrole derivatives in strong acid were examined by using the acidity function developed by Chiang and Whipple (13, 14) by recording UV spectra in the presence of varying concentrations of sulfuric acid from 0.5 to 7.5 M. The reversibility of the changes observed showed that no decomposition occurred during the time (≈3 min) required for these experiments. When log $[BH^+]/[B]$ was plotted as a function of the concentration of sulfuric acid, the half-titration point {at which log $([BH^+]/[B]) = 0$} was found to be 5.2 M for PAA, 5.3 M for pyrrole, and 1.8 M for 3MP (Fig. 4). Thus, the pK_a value of the conjugate acid $PAAH^+$ is similar to the value reported for pyrrole-H^+ (≈ -3.8), and lower than the value reported for 3-methylpyrrole-H^+ (-1.0) by Chiang and Whipple (14).

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