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# Kinetic Mechanism and Structural Requirements of the Amine-Catalyzed Decarboxylation of Oxaloacetic Acid

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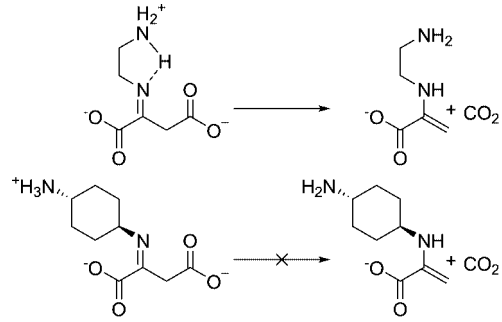

The kinetic and chemical mechanism of amine-catalyzed decarboxylation of oxaloacetic acid at pH 8.0 has been reevaluated using a new and versatile assay. Amine-catalyzed decarboxylation of oxaloacetic acid proceeds via the formation of an imine intermediate, followed by decarboxylation of the intermediate and hydrolysis to yield pyruvate. The decrease in oxaloacetic acid was coupled to NADH formation by malate dehydrogenase, which allowed the rates of both initial carbinolamine formation (as part of the imination step) and decarboxylation to be determined. By comparing the rates observed for a variety of amines and, in particular, diamines, the structural and electronic requirements for diamine-catalyzed decarboxylation at pH 8.0 were identified. At pH 8.0, monoamines were found to be very poor catalysts, whereas some diamines, most notably ethylenediamine, were excellent catalysts. The results indicate that the second amino group of diamines enhances the rate of imine formation by acting as a proton shuttle during the carbinolamine formation step, which enables diamines to overcome high levels of solvation that would otherwise inhibit carbinolamine, and thus imine, formation. The presence of the second amino group may also enhance the rate of the carbinolamine dehydration step. In contrast to the findings of previous reports, the second amino group participates in the reaction by enhancing the rate of decarboxylation via hydrogen-bonding to the imine nitrogen to either stabilize the negative charge that develops on the imine during decarboxylation or preferentially stabilize the reactive imine over the unreactive enamine tautomer. These results provide insight into the precise catalytic mechanism of several enzymes whose reactions are known to proceed via an imine intermediate.

## Introduction

Oxaloacetic acid (OAA) plays a central role as an intermediate in several metabolic pathways such as the tricarboxylic acid cycle, gluconeogenesis, fatty acid biosynthesis, amino acid degradation, and amino acid biosynthesis. As such, the chemistry of OAA has been studied extensively, and because OAA is a $\beta$-ketocarboxylic acid, the $\beta$-decarboxylation reaction has received the most attention. $\beta$-Decarboxylation can be catalyzed by a number of agents including divalent cations,[1] protons,[2] and enzymes (OAA decarboxylase,[3] phosphoenolpyruvate carboxykinase,[4] malic enzyme,[5] and pyruvate kinase[6]), each of which serves as an electron sink that stabilizes the incipient carbanion formed with the loss of $CO_2$.

Amines have also been shown to catalyze the decarboxylation of OAA.[7] It has been widely postulated that such decarboxylations proceed through an imine intermediate formed from

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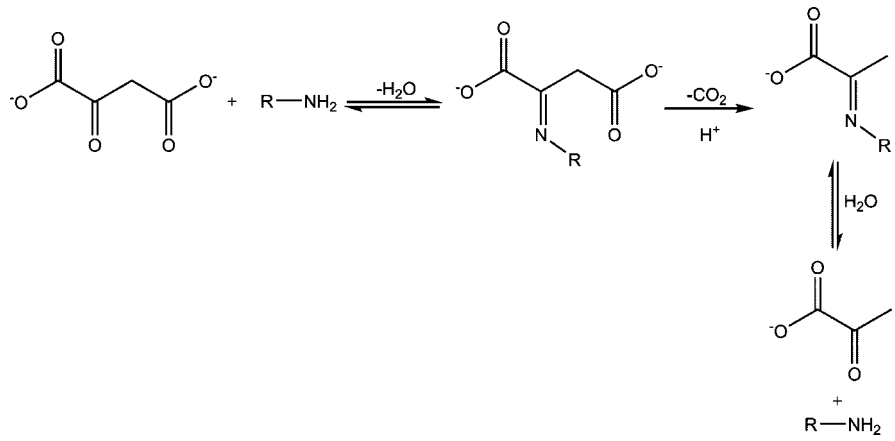
**SCHEME 1. General Mechanism of Amine-Catalyzed Decarboxylation of OAA**




condensation of the ketocarbonyl of OAA and the amine.[8] Following imination of OAA, the intermediate decarboxylates to yield the corresponding pyruvate imine, which subsequently undergoes hydrolysis to generate pyruvate and the amine catalyst as shown in Scheme 1.[9] The imine formation step can be further divided into nucleophilic attack by the amine on OAA to form a carbinolamine and dehydration of the carbinolamine to form the imine intermediate.

While amine-catalyzed OAA decarboxylation has been known for a number of years,[11] surprisingly, there has never been any attempt to correlate the structure of different amines with the rates of the different steps in Scheme 1. Several enzymes, such as aldolase in glycolysis, transaldolase in the pentose phosphate pathway, and amino acid transaminases involved in amino acid catabolism all utilize imine formation to catalyze their respective reactions.[12] In addition, studies of amine-catalyzed decarboxylation are particularly relevant to acetoacetate decarboxylase which has two proximal lysine residues in the active site that work in concert to decarboxylate acetoacetate via imine formation.[13] Thus, an understanding of the amine-catalyzed decarboxylation of OAA will provide insight into enzymatic transformations that utilize imine formation as part of their catalytic cycle. Herein, we report on the kinetic mechanism and structural requirements for the $\beta$-decarboxylation of OAA. Furthermore, we introduce a novel, enzyme-coupled assay that is more versatile than previous assays because it can be used either for presteady-state kinetics to analyze imine formation or for steady-state kinetics to examine the decarboxylation step.

Of particular interest in this study were diamines, which are effective catalysts at neutral pH. A previous study of amine-catalyzed imine formation and $\beta$-decarboxylation of OAA suggested that increased catalyst basicity would promote tautomerization of the OAA-imine to the unreactive enamine, thus decreasing the rate of decarboxylation.[14] Since aliphatic diamines with greater separation between the two amino groups have been shown to be poorer catalysts than diamines with amino groups in close proximity,[15] the authors concluded that the decreased catalytic ability of long-chain diamines was due to their higher basicity.[14] However, this assumption does not address the possibility that the second amino group is directly involved in the decarboxylation reaction. Through systematic and detailed kinetic analysis of a variety of diamines and diamine analogs, we provide strong evidence that the second amino group of diamines plays a crucial role in diamine-catalyzed $\beta$-decarboxylation.

## Results and Discussion

**Assay for Amine-Catalyzed Oxaloacetic Acid Decarboxylation.** Early assays of amine-catalyzed OAA decarboxylation involved measuring the products of the reaction, $CO_2$, or pyruvate. The amount of $CO_2$ released was measured manometrically[17] while formation of pyruvate was coupled to the NADH-requiring enzyme lactate dehydrogenase.[18] A more recent study utilized absorbance at 280 nm corresponding to the formation of the OAA−enamine.[14] All three of these experimental approaches have significant shortcomings. The manometric assay is a fixed-time assay that relies on the

(1) (a) Pedersen, K. J. *Acta Chem. Scand.* **1952**, *6*, 285. (b) Gelles, E.; Clayton, J. P. *Trans. Faraday Soc.* **1956**, *52*, 353. (c) Grissom, C. B.; Cleland, W. W. *J. Am. Chem. Soc.* **1986**, *108*, 5582.

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(6) Kiick, D. M.; Cleland, W. W. *Arch. Biochem. Biophys.* **1989**, *270*, 647.

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(8) (a) Hay, R. W. *Aust. J. Chem.* **1965**, *18*, 337. (b) Pedersen, K. J. *Acta Chem. Scand.* **1954**, *8*, 710. (c) Leussing, D. L.; Raghavan, N. V. *J. Am. Chem. Soc.* **1980**, *102*, 5635.

(9) An analogous mechanism has also been proposed for amine catalyzed decarboxylation of acetoacetate, another $\beta$-ketocarboxylic acid.[10]

(10) (a) Guthrie, J. P.; Jordan, F. *J. Am. Chem. Soc.* **1972**, *94*, 9136. (b) Pedersen, K. J. *J. Am. Chem. Soc.* **1938**, *60*, 595.

(11) Wohl, A.; Oesterlin, C. *Ber. Dtsch. chem. Ges.* **1901**, *34*, 1139.

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(14) Leussing, D. L.; Raghavan, N. V. *J. Am. Chem. Soc.* **1980**, *102*, 5635.

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(17) (a) Hay, R. W. *Aust. J. Chem.* **1965**, *18*, 337. (b) Kaneko, S. *J. Biochem. (Japan)* **1938**, *28*, 1. (c) Gelles, E.; Clayton, J. P. *Trans. Faraday Soc.* **1956**, *52*, 353.

(18) Spetnagel, W. J.; Klotz, I. M. *J. Am. Chem. Soc.* **1976**, *98*, 8199.

**SCHEME 2. Reaction Catalyzed by Malate Dehydrogenase**

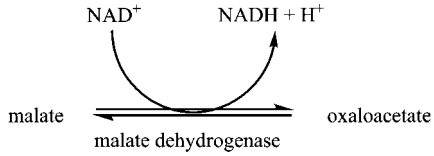



**TABLE 1. Reagent Combinations of the OAA Decarboxylation Assay[a]**

| reagents in Cuvette | initial velocity × $10^{-1}$ ($dA_{340}$/min) |
|---|---|
| malate, $NAD^+$, ethylenediamine | 0 |
| malate, malate dehydrogenase, ethylenediamine | 0 |
| malate dehydrogenase, $NAD^+$, ethylenediamine | 0 |
| malate, malate dehydrogenase, $NAD^+$ | 0.02 ± 0.01 |
| malate, malate dehydrogenase, $NAD^+$, ethylenediamine | 2.97 ± 0.09 |

[a] Reagent concentrations: 20 mM malate, 50 mM $NAD^+$, 76 U/mL malate dehydrogenase, 70 mM ethylenediamine. All assays performed in 2.0 M Tris−HCl, pH 8.0. The velocities are the average ± S.D. of three measurements.

assumption that all of the released $CO_2$ is in the gas phase and not in solution. Moreover, measuring the production of $CO_2$ or pyruvate only provides information on the overall rate of decarboxylation. Measuring the absorbance at 280 nm is complicated by the fact that OAA (enol tautomer) also absorbs at 280 nm. Thus, the previous assays only allow for effective measurement of the rate of decarboxylation and provide little information on the rate of imine formation. Therefore, we developed a novel approach for measuring the rates involved in amine-catalyzed decarboxylation.

Instead of using OAA directly as the substrate for the reaction, it was generated in situ via the malate dehydrogenase-catalyzed oxidation of malate by $NAD^+$ (Scheme 2). Upon incubating the enzyme, $NAD^+$, and malate, the reaction rapidly comes to equilibrium in favor of malate (equilibrium constant is $2.86 \times 10^{-5} = [NADH][OAA]/[NAD^+][malate]$).[19] Reaction of an amine with OAA to form a carbinolamine intermediate results in oxidation of malate to form OAA to restore the equilibrium. Thus, the rate of malate oxidation corresponds to the rate of carbinolamine formation and is monitored by following the increase in NADH which absorbs at 340 nm. Since ethylenediamine has been previously reported to catalyze the decarboxylation of OAA at near-neutral pH conditions,[14] it was used to characterize the assay. Different combinations of the reagents of the assay were used to exclude the possibility of alternative reactions and to show that the assay does, in fact, measure the rate of OAA decarboxylation. The results of this study are summarized in Table 1.

A representative time course for the reaction measured using this assay at pH 8.0[20] is shown in Figure 1. The time course is biphasic with an initial rapid increase in absorbance followed by a slower linear phase. The fast phase cannot be explained as a rapid initial decarboxylation of the equilibrium concentration of OAA because the OAA concentration is maintained by the coupling enzyme, which is not rate-limiting at the concentrations

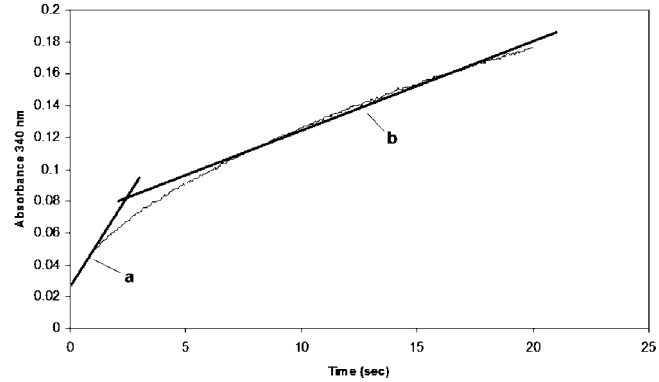



**FIGURE 1.** Representative time course of the malate dehydrogenase assay for amine-catalyzed decarboxylation of OAA showing (a) the burst phase corresponding to rapid combination of OAA and the amine and (b) the slow phase representing the steady-state decarboxylation of the imine followed by hydrolysis of the product and generation of another molecule of the OAA−imine. Reagent concentrations: 2.0 M Tris−HCl, pH 8.0, 76 U/mL malate dehydrogenase, 20 mM malic acid, 50 mM $NAD^+$, and 545 mM ethylenediamine.

used. Thus, if rapid initial decarboxylation were occurring, only one phase would be observed. The likely alternative is that the rapid phase in Figure 1 must correspond to the formation of a covalent intermediate that cannot be reduced by malate dehydrogenase, thus driving the equilibrium toward increased production of OAA and, in turn, toward increased production of NADH. Since the rapid phase is the first phase observed, the most likely possibility is that it corresponds to the nucleophilic attack of OAA by the amine to yield a carbinolamine. It is plausible that the rapid phase may correspond to the rate of carbinolamine dehydration, but only if carbinolamine formation is faster than dehydration and the equilibrium between OAA and the carbinolamine lies far toward OAA such that the first step cannot be seen. The slow phase, on the other hand, represents the rate of decarboxylation (which appears to be rate-limiting), hydrolysis of the pyruvate imine, and formation of a new OAA−imine, which drives the coupling enzyme to produce more NADH.[22] While it is possible that the rate-limiting step of the reaction is, in fact, the hydrolysis of the pyruvate imine, this is unlikely because it is chemically analogous to the rapid reaction observed in the burst phase, and thus decarboxylation of the OAA imine is likely to be the rate-limiting step. The biphasic time course is consistent with the conclusions of Leussing and Raghavan,[14] who observed that above pH 7.5 decarboxylation is much slower than imine formation when

(19) Guynn, R. W.; Gelberg, H. J.; Veech, R. L. *J. Biol. Chem.* **1973**, *248*, 6957.

(20) The choice of pH was motivated by several factors. The studies of Leussing and Raghavan[14] found that decarboxylation was slower than imine formation above pH 7.5. Therefore, pH 8.0 afforded the ability to observe the fast consumption of oxaloacetate as well as the slower steps involving decarboxylation. Moreover, measuring malate dehydrogenase activity at pH 8.0 using Tris buffer was well established, and from a practical standpoint malate dehydrogenase is a homodimer which dissociates into monomers as the pH decreases.[21] Lastly, control experiments in HEPES buffer showed that while Tris is an amine it did not react with oxaloacetate, whereas ethylenediamine did.

(21) Wood, D. C.; Jurgensen, S. R.; Geesin, J. C.; Harrison, J. H. *J. Biol. Chem.* **1981**, *256*, 2377.

(22) Two other assays were used to confirm that pyruvate was indeed being produced by the decarboxylation reaction. NMR analysis confirmed the product was pyruvate, but the rates of decarboxylation could not be compared to the assay used here because the analysis was performed in DMSO. In addition, lactate dehydrogenase also detected the production of pyruvate. However, the very low concentrations of oxaloacetate used in the malate dehydrogenase assay were not feasible for the lactate dehydrogenase assay and thus the rates from the two assays cannot be compared directly, which underscores the sensitivity as well as the versatility of the malate dehydrogenase assay used in this study.

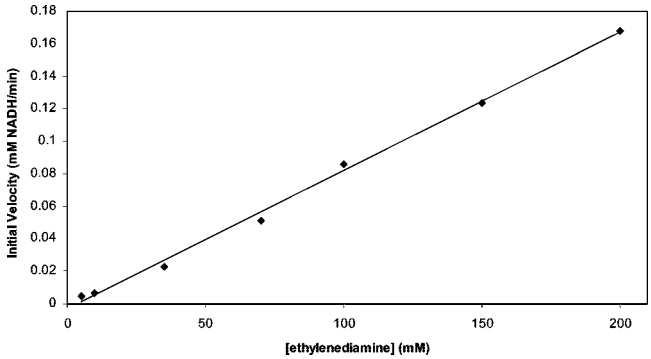



**FIGURE 2.** Plot of ethylenediamine concentration versus initial velocity of the decarboxylation step showing linear dependence of the rate of decarboxylation on catalyst concentration. Reagent concentrations: 2.0 M Tris−HCl, pH 8.0, 76 U/mL malate dehydrogenase, 20 mM malic acid, and 50 mM $NAD^+$ with varying ethylenediamine concentration.

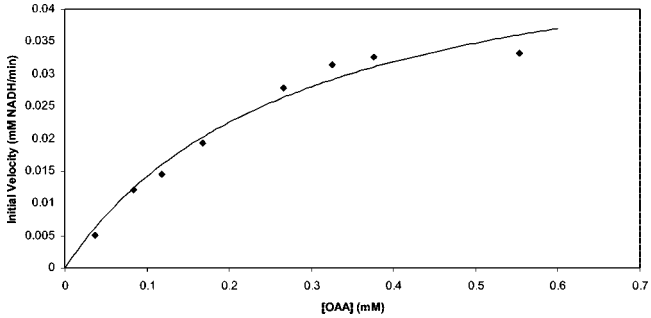



**FIGURE 3.** Plot of OAA concentration versus initial velocity of the decarboxylation step. The line represents the best fit of the data to the Michaelis−Menten equation. Malic acid concentration was converted to OAA concentration prior to regression using the equilibrium expression for malate dehydrogenase: $2.86 \times 10^{-5} = [NADH][OAA]/[NAD^+][malate]$.

ethylenediamine is used as the catalytic amine.[23] In addition, Leussing and Raghavan's pH profile of the rates of imine formation suggested that, with ethylenediamine, the rate of carbinolamine formation was faster than its subsequent dehydration at pH 8.0. Thus, the measured rates of carbinolamine formation do not also correspond to the rate of imine formation as long as carbinolamine formation is the fastest step.

The rate of the slow phase varied in a linear fashion with increasing amounts of the amine (Figure 2) but exhibited saturation kinetics when the concentration of malate was varied (Figure 3). The Michaelis−Menten model for enzyme-catalyzed reactions was used to derive the parameters $K_M$ and $V_{max}$ to describe the saturation observed. It is important to note that the $K_M$ values determined are only analogous to the corresponding enzymatic parameter in that they represent the substrate concentrations needed to achieve half-maximal velocity. The Michaelis−Menten equation does not apply to this reaction because, unlike enzyme-catalyzed reactions, where enzyme concentration is much lower than substrate concentration, in our assay, the concentration of the amine catalyst is much greater than that of OAA. However, the reason amine-catalyzed decarboxylation exhibits saturation kinetics at high catalyst concentrations is due to the relatively low turnover rate of the decarboxylation step with respect to the rate of formation of the imine intermediate, which allows for the development and maintenance of a steady-state concentration of the OAA−imine. This results in saturation of the amine since the decarboxylation of the OAA−imine must occur before the catalyst is released to form the imine with a new molecule of OAA. Several factors confirm that the saturation observed is not a consequence of simply saturating malate dehydrogenase: First, the reaction rate increases linearly with increasing amounts of the catalyst. Second, when a variety of amines were tested as catalysts for OAA decarboxylation, saturation curves were obtained despite the fact that the lowest concentration of malate used was 1.0 mM. Since the $K_M$ value of malate for malate dehydrogenase is 1.0 mM,[25] only the upper portion of the saturation curve would have been observed at the malate concentrations used. Third, when the reaction was run with three different concentrations of malate dehydrogenase with malate held at a saturating concentration of 200 mM the rate did not change, indicating that the saturation phenomena were involved with the amine catalysis of OAA.

Thus, the malate dehydrogenase assay is unique for measuring amine-catalyzed OAA decarboxylation because it measures starting material consumption which gives more information because the point of measurement is prior to the rate-determining step. The assay provides evidence to support the proposed mechanism: the burst phase indicates initial OAA combination with the amine to form the corresponding carbinolamine, and the slow phase shows both the irreversible decarboxylation step and the hydrolysis of the pyruvate imine to release an amine that can then form a new OAA imine for another catalytic cycle. Formation of this new OAA imine results in the production of more NADH, which would not have been observed without both the irreversible step and the regeneration of the amine. The fast rate of carbinolamine formation can be monitored by presteady-state methods while the rectangular hyperbola derived from the rates of the slow phase can be fitted to the Michaelis−Menten equation to yield a maximal velocity corresponding to the rate of decarboxylation. Because the decarboxylation rates observed in the steady-state analysis were obtained at saturating concentrations, they allow for direct comparison of the abilities of the imine complexes to undergo decarboxylation. The presteady-state rates of decarboxylation were not used in analysis of decarboxylation, and can be found in Supporting Table 1 (Supporting Information). An experimental system is now in place for determining the structural requirements for how amines affect the rates of carbinolamine formation, carbinolamine dehydration, and the decarboxylation step.

**Structural Requirements for Amine-Catalyzed Decarboxylation of Oxaloacetic Acid.** Table 2 shows the amines tested along with their fast phase (carbinolamine formation) rates measured from the pre-steady-state analysis. Table 3 contains the same amines and the measured maximal slow-phase (decarboxylation) velocities at steady state. For the pre-steady-state analysis, the level of amine and malate was the same for each amine, which allows for a direct comparison of the rates of carbinolamine formation for each amine. Similarly, the results of the steady-state study were useful in the comparison of the ability of the imine complexes to decarboxylate because all rates were measured at saturating concentrations.

**Requirements for Carbinolamine Formation.** The monoamines aniline[26] and aminoacetonitrile[14] have been reported to

(23) Even at acidic pH values, the decarboxylation step for amine catalysis of acetoacetate is still partially rate-limiting.[24]

(24) O'Leary, M. H.; Baughn, R. L. *J. Am. Chem. Soc.* **1972**, *94*, 626–630.
(25) Zimmerle, C. T.; Alter, G. M. *Biochemistry* **1993**, *32*, 12743.

**TABLE 2. Rates of Burst Phase**

| catalyst | rate of burst phase × $10^{-3}$ (dA/min) |
|---|---|
| 1,2-diaminopropane | 23.7 ± 0.4 |
| ethylenediamine | 28.7 ± 0.2 |
| 1,3-diaminopropane | 41.2 ± 0.9 |
| 1,4-diaminobutane | 48.4 ± 0.3 |
| 1,5-diaminopentane | 52.7 ± 0.5 |
| 1,6-diaminohexane | 49.5 ± 0.4 |
| 1,7-diaminoheptane | 49.4 ± 0.3 |
| *trans*-1,4-diaminocyclohexane | 44.7 ± 1.1 |
| *cis*-1,2-diaminocyclohexane | 38.1 ± 1.2 |
| *trans*-1,2-diaminocyclohexane | 23.8 ± 0.5 |
| 2-dimethylaminoethylamine | 30.9 ± 0.8 |
| *N*,*N*′-dimethylethylenediamine | 29.0 ± 0.8 |
| ethanolamine | 19.7 ± 0.5 |
| cysteamine | 16.8 ± 0.1 |
| diethylenetriamine | 44.8 ± 0.9 |
| 2-aminoethyltrimethylammonium chloride | 0 |
| phenylenediamine | 0 |
| aminoacetonitrile | 0 |
| aniline | 0 |

Reaction conditions: 2.0 M Tris−HCl, pH 8.0, 76 U/mL malate dehydrogenase, 20 mM malic acid, 50 mM $NAD^+$, and 545 mM amine. The velocities are the average ± S.D. of three measurements.

**TABLE 3. Maximal Steady-State Decarboxylation Rates**[a]

| catalyst | decarboxylation rate × $10^{-5}$ ($min^{-1}$) |
|---|---|
| 1,2-diaminopropane | 336.2 ± 48.5 |
| ethylenediamine | 156.1 ± 17.7 |
| 1,3-diaminopropane | 16.7 ± 1.4 |
| 1,4-diaminobutane | 7.8 ± 0.7 |
| 1,5-diaminopentane | 5.6 ± 1.0 |
| 1,6-diaminohexane | 7.9 ± 1.5 |
| 1,7-diaminoheptane | 2.4 ± 0.1 |
| *trans*-1,4-diaminocyclohexane | 0 |
| *cis*-1,2-diaminocyclohexane | 100.5 ± 15.3 |
| *trans*-1,2-diaminocyclohexane | 127.0 ± 6.3 |
| 2-dimethylaminoethylamine | 18.4 ± 2.6 |
| *N*,*N*′-dimethylethylenediamine | 0 |
| ethanolamine | 6.9 ± 0.9 |
| cysteamine | 8.8 ± 0.8 |
| diethylenetriamine | 154.9 ± 34.0 |
| 2-aminoethyltrimethylammonium chloride | 0 |
| phenylenediamine | 0 |
| aminoacetonitrile | 0 |
| aniline | 0 |

[a] Reaction conditions: 2.0 M Tris−HCl, pH 8.0, 76 U/mL malate dehydrogenase, and 50 mM $NAD^+$, with varying malate concentration from 1 to 200 mM. Amines were generally used at 35 mM, except for 1,4-diaminobutane, 1,5-diaminopentane, and 1,7-diaminoheptane, which were held at 200 mM, ethanolamine, which was held at 140 mM, cysteamine, which was held at 50 mM, 1,6-diaminohexane, which was held at 100 mM, and 2-dimethylaminoethylamine, which was held at 70 mM. The maximal velocities were determined by fitting a plot of OAA concentration (as determined by the equilibrium constant for the malate dehydrogenase reaction) versus initial velocities to the Michaelis−Menten equation. Velocities were converted from $dA_{340}$/min to $min^{-1}$ by dividing by the product of the extinction coefficient of NADH and the amine concentration, which enabled direct comparison of rates despite differences in amine concentration.

catalyze the decarboxylation of OAA under certain conditions. Aminoacetonitrile has also been reported to be a very effective catalyst for acetoacetate decarboxylation.[27] Both of these decarboxylations have been proposed to proceed through an imine intermediate. Therefore, we investigated the ability of these monoamines to form an imine with OAA at pH 8.0. Using a stopped-flow spectrophotometer to measure the rate of the

**TABLE 4. Rates of Carbinolamine Formation with Monoamines**[a]

| amine | rate of carbinolamine formation × $10^{-2}$ (dA/min) |
|---|---|
| ethylamine | 0 |
| propylamine | 1.59 ±0.05 |
| butylamine | 2.26 ±0.04 |
| pentylamine | 2.66 ±0.09 |
| hexylamine | 2.57 ±0.05 |
| isopropylamine | 0.96 ±0.14 |
| *tert*-butylamine | 1.26 ±0.10 |
| cyclohexylamine | 3.54 ±0.05 |

[a] Reaction conditions: 2.0 M Tris−HCl, pH 8.0, 76 U/mL malate dehydrogenase, 20 mM malic acid, 50 mM $NAD^+$, and 545 mM amine. The velocities are the average ± S.D. of three measurements.

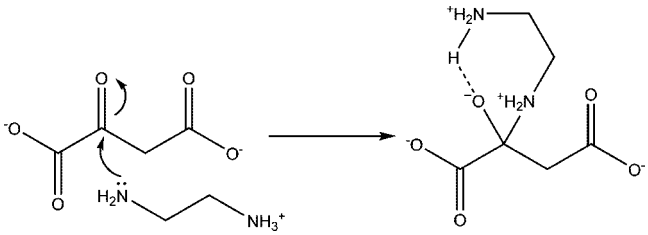
distinct burst phase associated with carbinolamine formation, we demonstrated that, at pH 8.0, the burst phase was noticeably absent when aminoacetonitrile and aniline were used as catalysts. The most likely explanation for this observation is that the electron-withdrawing nitrile group of aminoacetonitrile renders it an extremely poor nucleophile, while aniline is a poor nucleophile because of the electron-withdrawing nature of the aromatic ring, and thus, it is likely that an acid catalyst is needed to promote carbinolamine formation. In fact, previous reports of aniline-[26] and aminoacetonitrile-catalyzed[14] OAA decarboxylation observed catalysis only under acidic conditions, which is consistent with our findings that carbinolamine, and subsequent imine, formation (which is necessary for decarboxylation) does not occur at pH 8.0, and which supports the conclusion that acid catalysis is needed for imine formation with these weakly nucleophilic amines.[28]

Ethylamine also failed to react with OAA to form a carbinolamine, despite the fact that the ethylamine amino group should be much more nucleophilic than that of aminoacetonitrile or aniline due to the lack of the electron-withdrawing nitrile or aromatic group. It appears that the absence of imine formation with ethylamine can be attributed to solvation effects resulting in a significant decrease in nucleophilicity that prevents carbinolamine formation. This was demonstrated by measuring the rate of the burst phase with the monoamines propylamine, butylamine, pentylamine, hexylamine, isopropylamine, cyclohexylamine, and *tert*-butylamine. As the length of the alkyl chain increases from ethylamine to hexylamine, solvation of the amino group should decrease due to the presence of a longer hydrophobic chain. As shown in Table 4, the rate of carbinolamine formation increases as solvation of the amino group decreases from ethylamine to hexylamine, suggesting that the nucleophilicity of the amino group is heavily dependent on the degree of solvation of the nucleophilic amine.

A similar solvation trend can be seen among diamines. The rate of carbinolamine formation was found to increase significantly as the size of the diamine increased, with ethylenediamine exhibiting the slowest rate and with 1,5-diaminopentane, 1,6-diaminohexane, and 1,7-diaminoheptane exhibiting much faster rates. Furthermore, for diethylenetriamine, which was expected to be more poorly solvated than ethylenediamine because of the increased ratio of nonpolar methylene groups to polar amino groups, the rate of the burst phase was faster than that of ethylenediamine.

(26) Hay, R. W. *Aust. J. Chem.* **1965**, *18*, 337.
(27) Guthrie, J. P.; Jordan, F. *J. Am. Chem. Soc.* **1972**, *94*, 9136.
(28) A helpful reviewer has pointed out that the lack of reactivity of aniline and aminoacetonitrile could also be the consequence of unfavorably low equilibrium constants for imine formation at pH 8.0.

**SCHEME 3. Intramolecular Assistance during Carbinolamine Formation**



A burst phase was also observed with the cyclic diamines *trans*-1,4-diaminocyclohexane, *trans*-1,2-diaminocyclohexane, and *cis*-1,2-diaminocyclohexane. The rate of this burst was fastest for *trans*-1,4-diaminocyclohexane and was comparable to that of 1,4-diaminobutane. However, both of these rates were slightly slower than that of the monoamine cyclohexylamine. This can be rationalized by noting that both of these diamines, in their monoprotonated forms, have an electron-withdrawing ammonium group that decreases the nucleophilicity of the other nitrogen. On the other hand, cyclohexylamine, which can only react from its neutral form (even though the protonated form predominates at equilibrium), is more nucleophilic and thus forms the carbinolamine more rapidly. This is also consistent with the lower rates observed for *trans*-1,2-diaminocyclohexane and *cis*-1,2-diaminocyclohexane, which, when monoprotonated, would be less nucleophilic than *trans*-1,4-diaminocyclohexane because the electron-withdrawing ammonium group is closer to the nucleophilic amino group.

It was rather surprising that ethylenediamine formed the carbinolamine at all since it would be expected to be solvated at least as well as ethylamine, if not better because of the presence of the second polar amino group. The fact that it does form the carbinolamine suggests the possibility that the second amino group of ethylenediamine plays a role in carbinolamine formation, either by hydrogen bonding to stabilize the oxyanion generated by nucleophilic attack of the carbonyl as shown in Scheme 3 or else by donating a proton to the oxyanion to stabilize it. Further evidence for this effect is seen with ethanolamine and cysteamine, both of which exhibit the burst kinetics, albeit slowly. With ethanolamine, it is probable that the hydroxyl group acts as a hydrogen bond donor to stabilize the incipient oxyanion, whereas with cysteamine the reasonably acidic thiol group can donate a proton to the oxyanion.

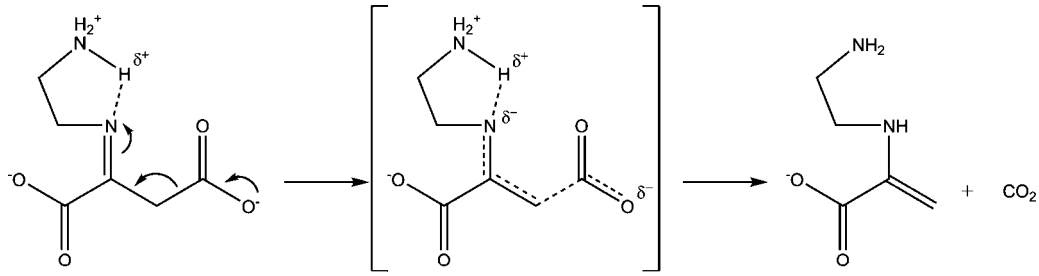
Three ethylenediamine analogues were assayed to help elucidate the factors affecting carbinolamine formation. *N*,*N*′-Dimethylethylenediamine and 2-dimethylaminoethylamine both exhibited a burst phase at nearly the same rate as ethylenediamine. For *N*,*N*′-dimethylethylenediamine, the amino groups are secondary, and thus although the burst phase still corresponds to the formation of a carbinolamine, the dehydration product is an enamine. For 2-dimethylaminoethylamine, which is both a primary and a tertiary amine, the burst can only represent carbinolamine formation via reaction of the OAA with the primary amino group because attack by the tertiary amine cannot form a stable product. In contrast to the first two analogues, 2-aminoethyltrimethylammonium chloride, the trimethylated quaternary ammonium salt of ethylenediamine, did not exhibit burst kinetics. One possible explanation for this is that the quaternary ammonium salt is reasonably well solvated and, thus, like ethylamine, lacks a hydrogen-bonding or acidic group that can enhance imine formation by stabilizing the high energy oxyanionic intermediate. However, another possibility is that ethylenediamine, *N*,*N*′-dimethylethylenediamine, and 2-dimethylaminoethylamine react in their neutral, fully deprotonated form, instead of in their monoprotonated forms. This explanation is attractive because the neutral forms of these amines are expected to be much more nucleophilic than the monoprotonated forms. Since 2-aminoethyltrimethylammonium chloride has a permanent positive charge, it cannot exist in a more nucleophilic form, thus explaining its lack of reactivity.

Thus, it is clear that cooperativity between the amino group and a second polar functional group is not a requirement for carbinolamine formation, but if solvation is significant, as in the case of ethylenediamine, cooperativity seems to be necessary to stabilize the tetrahedral intermediate formed during carbinolamine formation, either by hydrogen bonding or else by proton transfer. As solvation diminishes, the requirement for a second polar group drops. In addition, most diamines probably react in their monoprotonated forms, although it is possible that ethylenediamine, which is a weaker base than the other diamines, reacts in its more nucleophilic neutral form.

**The Decarboxylation Step.** Given a reasonable understanding of the requirements for carbinolamine formation, a thorough structural analysis of the rate of the decarboxylation step was then performed to evaluate whether or not the second amino group of diamines plays a significant role in the catalysis of $CO_2$ loss from OAA. The rate of decarboxylation of OAA by diamines was found to be dependent on the length of the diamine chain, with ethylenediamine exhibiting the fastest decarboxylation rates, and longer diamines such as 1,6-diaminohexane and 1,7-diaminoheptane catalyzing decarboxylation at lower rates. In addition, both *cis*-1,2-diaminocyclohexane and *trans*-1,2-diaminocyclohexane exhibited catalytic activity only slightly lower than that of ethylenediamine. This is reasonable since the proximity of the two amino groups of the cyclic diamines should be comparable to that of ethylenediamine.

The effect of increasing chain length on the rate of the decarboxylation step had been noted previously,[15] and Leussing and Raghavan[14] offered an explanation based on basicity of the imine. They suggested that, with increasing basicity of the imine, the unreactive enamine would begin to predominate, decreasing the overall effectiveness of the diamine catalyst. Since basicity of diamines increases with increasing separation of the two amino groups, this is an enticing explanation for the significant decrease in catalytic rate observed. However, the decarboxylation rates of 1,4-diaminobutane and *trans*-1,4-diaminocyclohexane were found to be significantly different, as *trans*-1,4-diaminocyclohexane failed to catalyze decarboxylation. This is surprising because both compounds possess similar electronic properties, as evidenced by the fact that both are aliphatic diamines with amino groups separated by four carbons. Furthermore, the $pK_a$ values for the two molecules are nearly identical ($pK_1 = 9.24$, $pK_2 = 10.74$ for *trans*-1,4-diaminocyclohexane and $pK_1 = 9.40$, $pK_2 = 10.79$ for 1,4-diaminobutane), and the rates of carbinolamine formation for both catalysts are virtually identical. Thus, according to the mechanism proposed by Leussing and Raghavan, both compounds should catalyze the decarboxylation step at the same rate (since the basicities of the imine nitrogens formed by both catalysts should be similar). Because this was not observed, this suggests that the role of the second amino group in the decarboxylation step is not simply to depress the $pK_a$ of the imine to inhibit tautomerization to the enamine. Considering the lack of electronic differences between the two catalysts, the most reasonable

**SCHEME 4. Catalysis by Intramolecular Stabilization of the Developing Negative Charge**

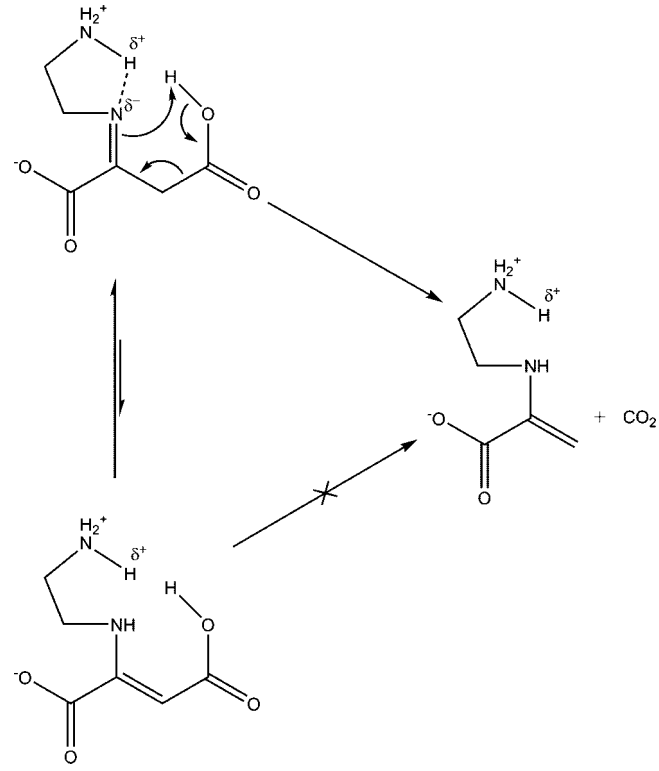


alternative is that the observed discrepancy in catalytic ability arises because the second amino group is directly involved in the catalytic mechanism.[29]

We suggest three possible roles for the second amino group in the decarboxylation step. The first possibility (Scheme 4) is that the second amino group, which will exist primarily in its protonated form at pH 8.0,[14] could function to stabilize the anion formed on the imine nitrogen during decarboxylation by either donating a proton to the imine nitrogen as it develops anionic character or else by hydrogen-bonding to stabilize the anionic nitrogen via the protonated second amino group, which is an excellent hydrogen-bond donor. Since *trans*-1,4-diaminocyclohexane is conformationally locked by the cyclohexane ring such that the second amino group cannot come into close proximity with the imine nitrogen to stabilize it, the second amino group cannot participate in the decarboxylation step, which explains its lack of reactivity. On the other hand, the flexible 1,4-diaminobutane can orient the second amino group adjacent to the imine nitrogen, resulting in rapid catalysis. Having the second amino group as a proton donor in the decarboxylation step also allows for a better understanding of why increased amino group separation results in a decreased rate of decarboxylation. By increasing the length of the chain, there is a greater entropic cost associated with bringing the second amino group in close proximity to the imine nitrogen. This reasoning also accounts for 1,2-diaminopropane having a higher maximal velocity of decarboxylation than ethylenediamine because the methyl group which constitutes the difference between ethylenediamine and 1,2-diaminopropane causes the *gauche* orientation of the two amino groups to predominate. Since this orientation in the OAA−imine places the second amino group and the imine nitrogen in the closest proximity, the reaction proceeds at a higher rate. This mechanism is similar to a mechanism proposed by Ogino and associates to explain the low catalytic activity of *trans*-oriented conformationally locked cyclic diamines with respect to their *cis* counterparts.[29]

Leussing and Raghavan[14] postulated that the diprotonated OAA−imine (protonated at the second amino group and on the labile carboxyl group) is the reactive species in the decarboxylation step. If this is the case, no negative charge would develop on the imine nitrogen because the carboxyl group would donate its proton to the nitrogen as $CO_2$ is evolved, and thus, the mechanism suggested above would be invalid. An alternative function of the terminal amino group in the decarboxylation step could be to depress the p$K_a$ of the imine nitrogen by hydrogen bonding as shown in Scheme 5. Lowering the basicity of the imine nitrogen with respect to that of the α-carbon shifts imine/enamine equilibrium toward the reactive imine tautomer, which enhances catalysis.[30]

**SCHEME 5. Catalysis by Preferential Stabilization of the Reactive Imine over the Unreactive Enamine**

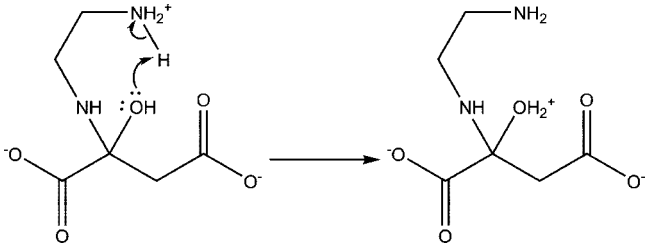


A final possible explanation for the trend in rates as diamine chain length increases is that the rate-limiting step of the reaction changes. Because the burst phase is evident at all times, carbinolamine formation can never be the slowest step. However, there is a distinct possibility that the rate of carbinolamine dehydration becomes the overall rate-limiting step as diamine chain length increases. In this case, we suggest a role for the second amino group that is quite similar to the role it might play if the rate-limiting step were always decarboxylation. Instead of hydrogen-bonding or protonating the imine nitrogen as the negative charge develops, the second amino group may protonate the hydroxyl group of the carbinolamine (Scheme 6), making it a better leaving group and enhancing the rate of

(29) Ogino, K.; Tamiya, H.; Kimura, Y.; Azuma, H.; Tagaki, W. *J. Chem. Soc., Perkin Trans. 2* **1996**, 979.

(30) In addition to enamine tautomerization, other possible unreactive intermediates include imidazolidines (from ring-closing with diamines), thiazolidines (from cysteamine ring-closing), and oxazolidines (from ethanolamine ring-closing). The formation of these intermediates would decrease catalytic rates in much the same way as enamine tautomerization. However, imizadolidine formation does not appear to be as significant as enamine tautomerization in diamine-catalyzed decarboxylation because, otherwise, increasing diamine chain length (which would decrease the stability of the corresponding unreactive cyclic intermediates) would have resulted in increased rates of decarboxylation. On the other hand, thiazolidine and oxazolidine ring-closing may be significant factors in the low decarboxylation rates observed for cysteamine and ethanolamine.

**SCHEME 6. Catalysis of Carbinolamine Dehydration by Intramolecular Protonation of Hydroxyl Group**



dehydration. As the separation between the two amino groups increases, the ability of the second amino group to protonate the hydroxyl group decreases, causing the dehydration step to become rate-limiting.

As expected, *N*,*N*′-dimethylethylenediamine did not exhibit decarboxylation since it only forms an unreactive enamine with OAA. Given the fact that imine formation is necessary for decarboxylation, it becomes clear why phenylenediamine, aniline, aminoacetonitrile, ethylamine, and 2-aminoethyltrimethylammonium chloride do not exhibit significant rates of decarboxylation since none of these molecules form the imine. On the other hand, 2-dimethylaminoethylamine, which can form an imine with OAA, does catalyze decarboxylation. However, it does not catalyze the reaction nearly as rapidly as ethylenediamine. It is likely that two effects contribute to this decrease: First, the dimethyl substitution of one of the amino groups causes it to possess greater steric bulk which hinders the approximation of the amino group and the imine. Second, the protonated tertiary amino group, which is the only form of the amino group that can hydrogen-bond or serve as a proton donor in the reaction, would possess only one N−H bond capable of interacting with the imine nitrogen, as opposed to the three N−H bonds in protonated ethylenediamine. Thus, there is an entropic cost associated with orienting the lone N−H bond in a reactive conformation.

The data for ethanolamine and cysteamine strongly suggest that hydrogen bonding, not proton transfer, is the mechanism by which the second amino group (or other polar group) assists in the decarboxylation step. Both ethanolamine and cysteamine exhibit very low levels of decarboxylation. Cysteamine (thiol $pK_a = 8.6$[31]) is more acidic than monoprotonated ethylenediamine (ammonium $pK_a = 9.57$), but it decarboxylates OAA very slowly. In addition, an ammonium group (as in monoprotonated ethylenediamine) is a much better hydrogen bond donor than a neutral alcohol or thiol.[32] Catalysis by ethanolamine and cysteamine, which are analogs of the amino acids serine and cysteine, respectively, explains why some amino acids catalyze the decarboxylation of OAA.[33]

**Oxyanion Hole Mimics.** Some enzyme-catalyzed decarboxylation and transcarboxylation reactions occur via active-site structures known as oxyanion holes, which consist of two hydrogen bond donors (usually peptidic N−H bonds of small amino acid residues) which stabilize the oxyanion formed in transition states during these reactions.[34] While the burst phase of the kinetics observed with the malate dehydrogenase assay strongly suggests imine formation, it was worth ruling out the possibility that diamines catalyze OAA decarboxyation by simply stabilizing the oxyanion via hydrogen bonding. This was achieved by assaying several compounds which cannot form an imine (or an enamine) but which can act as hydrogen-bond donors. If amino groups are hydrogen bonding to the carbonyl oxygen of OAA, then diols analogous to the diamines might also hydrogen bond and catalyze decarboxylation. Therefore, 1,2-propanediol (analogue of 1,2-diaminopropane), 1,3-propanediol (analogue of 1,3-diaminopropane), *cis*-1,2-cyclohexanediol (analogue of *cis*-1,2-diaminocyclohexane), and *cis*-1,2-pentanediol were examined as possible catalysts. The diols did not exhibit any catalytic activity when compared to ethylenediamine (data not shown). However, the hydrogens in the oxyanion holes of enzymes are usually provided by amides,[34] not hydroxyl groups, and therefore, urea and *N*,*N*′-diacetylethylenediamine (bis-acetylated ethylenediamine) were also tested as catalysts of OAA decarboxylation. It was found that these amides also did not catalyze OAA decarboxylation. It is important to note that Munakata et al.[15] reported that urea did catalyze OAA decarboxylation. However, catalysis was only observed under acidic conditions (pH 5.0) instead of at pH 8.0, and the reported rate constant for urea-catalyzed decarboxylation was more than 10-fold lower than that of ethylenediamine. As a final test to determine if the amino groups are hydrogen bonding to the carbonyl oxygen of OAA, acetone and methyl acetoacetate, both of which contain carbonyls that could compete with OAA for hydrogen bonding to the amine, were tested as inhibitors of the ethylenediamine-catalyzed reaction. As can be seen in Table 5, neither acetone nor methyl acetoacetate inhibited catalysis by ethylenediamine. Thus, diamine-catalyzed decarboxylation of OAA does not occur by stabilization of the oxyanion through hydrogen bonding of the amino groups.

**TABLE 5. Inhibition Studies**[a]

| inhibitor | initial velocity × $10^{-1}$ ($dA_{340}$/min) |
|---|---|
| none | 1.50 ± 0.05 |
| 100 mM methyl acetoacetate | 1.53 ± 0.06 |
| 200 mM methyl acetoacetate | 1.54 ± 0.06 |
| 100 mM acetone | 1.55 ± 0.08 |
| 200 mM acetone | 1.48 ± 0.03 |

[a] Reagent concentrations used: 76 U/mL malate dehydrogenase, 50 mM $NAD^+$, 20 mM malic acid, 35 mM ethylenediamine, and 2.0 M Tris-HCl, pH 8.0. The velocities are the average ± S.D. of three measurements.

## Conclusions

In this report, a new assay for amine-catalyzed decarboxylation of OAA is described. The new assay is a significant improvement over previous assays in that it allows simultaneous examination of the rates of carbinolamine formation and decarboxylation. It also allows kinetic data to be obtained using very low OAA concentrations, providing a more complete description of the kinetic profile. Structural analysis of the rate of carbinolamine formation suggests that imine formation at pH 8.0 is inhibited if the nucleophilic amino group is strongly solvated, but that this inhibition can be overcome by the presence of a second group capable of either intramolecularly hydrogen-bonding with or protonating the oxyanion of the tetrahedral intermediate of carbinolamine formation to stabilize it. Furthermore, analysis of decarboxylation rates shows that diamine-catalyzed decarboxylation requires the approximation of the two amino groups to achieve high rates of decarboxylation. Thus,

(31) Svensson, B. E. *Biochem. J.* **1988**, *253*, 441.
(32) Desiraju, G. R.; Steiner, T. *The Weak Hydrogen Bond in Structural Chemistry and Biology*; Oxford University Press: Oxford, 1999; pp 12−16.
(33) Bessman, S. P.; Layne, E. C. *Arch. Biochem.* **1950**, *26*, 25.
(34) Dodson, G.; Wlodawer, A. *Trends Biochem. Sci.* **1998**, *23*, 347–352.

the role of the second amino group in efficient diamine-catalyzed decarboxylation of OAA is 3-fold: First, it enables well-solvated molecules such as ethylenediamine to form carbinolamines (and thus imines), and second, it assists in decarboxylation by either stabilizing the negative charge that develops on the imine nitrogen during the course of the decarboxylation, or else by preferentially stabilizing the imine tautomer over the enamine, thereby increasing the concentration of the reactive species and enhancing overall decarboxylation rates. Finally, the second amino group may serve to catalyze the carbinolamine dehydration step by protonating the hydroxyl group to enhance its leaving group ability.

## Experimental Section

**Steady-State Kinetic Assay for Decarboxylation of OAA.** Amine-catalyzed decarboxylation of OAA was measured using a malate dehydrogenase-coupled enzyme assay. Each reaction contained 76 U/mL malate dehydrogenase, 50 mM $NAD^+$, 20 mM malic acid, and 2.0 M Tris−HCl pH 8.0. These components were combined and allowed to reach equilibrium (which occurred within seconds). The reaction was started by the addition of the amine and followed by measuring the increase in absorbance at 340 nm associated with production of NADH. Data were collected using a Uvikon 810 (Kontron Instruments) spectrophotometer interfaced to a PC equipped with a data acquisition program. The temperature was maintained at 25 °C by a circulating water bath with the capacity to heat and cool the thermospace of the cell compartment. The initial velocities are reported as $dA_{340}$/min. When malate concentration was varied, saturation kinetics were observed and the parameters $K_M$ and $V_{max}$ were determined by fitting the data to the Michaelis−Menten equation using the nonlinear regression programs of Cleland.[16]

**Rapid Reaction Kinetic Assay for Decarboxylation of OAA.** Rapid reaction kinetics were measured with a SX.18MV-R stopped-flow spectrophotometer. One of the drive syringes contained 0.816 M Tris−HCl pH 8.0 and 1.09 M of the amine, while the other drive syringe contained 3.184 M Tris−HCl, pH 8.0, 152 U/mL, malate dehydrogenase, 40 mM malic acid, and 100 mM $NAD^+$. Equal volumes of these solutions were mixed to give the final concentrations: 2.0 M Tris−HCl, pH 8.0, 76 U/mL malate dehydrogenase, 20 mM malic acid, 50 mM $NAD^+$, and 545 mM of the amine. Data were acquired at 340 nm for up to 20 s. The temperature was maintained at 22 °C with a circulating water bath. Between injections, the flow cell was washed with $dH_2O$. The fast and slow phases were fitted separately by linear least-squares analysis to determine the velocity as $\Delta A$/min. The reported velocity is the average of three separate injections (measurements).

**Determination of p$K_a$ Values.** Approximately 1.0 g of amine was dissolved in approximately 150 mL of $dH_2O$ and titrated against 1.0 M HCl until the final end point was reached. p$K_a$'s were determined as the pH halfway between end points. For 2-aminoethyltrimethylammonium chloride hydrochloride, 0.5 g of amine was dissolved in approximately 150 mL of $dH_2O$ and the mixture titrated against 1.0 M NaOH until the end point was reached. p$K_a$'s were determined as the pH halfway between the beginning of the titration and the end point.

**Acknowledgment.** The authors thank Dr. Reema Thalji and Dr. W.W. Cleland for their expert advice in the preparation of the manuscript.

**Supporting Information Available:** Table of the decarboxylation rates from the pre-steady-state study and $K_M$ values as obtained from the steady-state analysis for most amines. This material is available free of charge via the Internet at http://pubs.acs.org.

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