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Rapid analysis of solvent effects on enamine formation by fluorescence: how might enzymes facilitate enamine chemistry with primary amines?

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Abstract—In order to determine factors that facilitate the use of primary amines in biological chemistry, solvent effects on enamine formation with glycine were studied. Solvent effects were rapidly analyzed by monitoring the increase in fluorescence resulting from the reaction between a fluorogenic maleimide and in situ-generated enamine of acetone. These studies suggest that in addition to a simple hydrophobic microenvironment, a microenvironment that also affords for hydrogen bond formation facilitates enamine-based reactions involving primary amines.

The enamine is one of the most important intermediates for carbon–carbon bond formation in both organic chemistry and the biological world. In organic synthesis, pyrrolidine derivatives are used to efficiently form enamines with carbonyl compounds in many reactions. In nature, on the other hand, enzymes such as aldolases and decarboxylases utilize the ε-amino groups of lysine residues to form an enamine during their catalytic cycle.[1,2] Antibodies and peptides have also been demonstrated to catalyze reactions via an enamine mechanism in aqueous solution.[3] While biological catalysts can contain the pyrrolidine-based amino acid proline, the amine functionality is masked as an amide except when proline is the amino terminal residue of the polypeptide. How does nature use the ε-amino group of lysine as effectively as organic chemists use pyrrolidine-based catalysts?

The ε-amino group in the free amino acid lysine has a pK_a of 10.7 in aqueous solution[4] and is protonated at neutral pH. Therefore the amino groups of lysine residues of proteins typically do not function as nucleophiles to form an enamine or imine in aqueous solution at neutral pH. Enzymes must provide suitable environments and interactions to change the reactivity of their amino groups.[1,2,3b,5] Two strategies for reactivity change have been reported: One involves an electrostatic mechanism and the other is the creation of hydrophobic microenvironments. In the former mechanism, a positive charged residue within an interactive distance of the active site lysine ε-amino group acts to modulate the pK_a of a proximal amino group facilitating its reactivity. Natural decarboxylase and aldolase enzymes that use an enamine intermediate typically use this mechanism,[1,2,5] and this electrostatic mechanism has been analyzed in model systems.[5a] On the other hand, man-made aldolase antibody catalysts and only a few proteins[6] have been suggested to use the later mechanism. Investigation of reactions involving an enamine intermediate related to hydrophobic microenvironments has lagged behind.[7] In order to understand the requirements for the reactivity changes of primary amino groups of proteins and for the formation of enamine intermediates in hydrophobic microenvironments, simplified model systems, as well as whole proteins, must be analyzed.[8] We are interested in exploring factors, in addition to simple hydrophobic microenvironments, that may be necessary for proteins to facilitate enamine catalysis with primary amines. Therefore, we have investigated solvent effects on enamine-based reactions. The development of simple and rapid systems to analyze reactivity changes of amino groups should facilitate these investigations. We have

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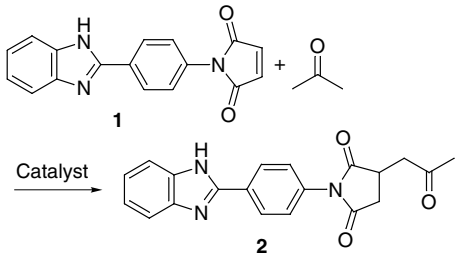
applied our fluorescence assay[9] (see below) to address the reactivity of a primary amine in a model system of an enzymatic reaction and evaluated solvent contributions and characteristics that facilitate this reaction.

Here we study glycine as a model system for protein catalysts that utilize the ε-amino group of lysine residues to form an enamine during catalysis. Glycine is a good model system for the following reasons: (1) Glycine has a single amino group and this feature simplifies analysis. If lysine were used in these experiments, discrimination between reactions at the two amino groups of lysine would be necessary. In enzymes, the lysine residue is incorporated in the context of the protein and the reactivity of its α-amino group is effectively blocked as an amide. (2) The pK_a of the amino group of glycine is 9.6,[4] and like the lysine ε-amino group is protonated at neutral pH. (3) A solution of glycine in H_2O is neutral. If alkylamines were to be used for these experiments, the alkaline pH in aqueous medium would be another factor to be considered.[10]

We recently developed a fluorescent detection system for monitoring C–C bond formation by using fluorogenic maleimide **1** (Scheme 1).[9] This substrate reacts with an in situ-generated enamine of acetone and yields fluorescent product **2**. This system is useful for rapid screening of reaction conditions on a small scale by monitoring fluorescence. We analyzed a series of solvents for their efficiency in the glycine-catalyzed reaction of acetone with **1**. The relative reaction velocities observed in the various solvents are shown in Table 1. Although the rate-limiting step of amine-catalyzed aldol reactions is typically C–C bond forming step rather than enamine formation,[8c,11] the in situ-generated enamine intermediate is efficiently trapped by the reactive maleimide functionality. Thus, comparison of the velocities of the increase in fluorescence (i.e., comparison of the velocities of the formation of fluorescent product **2**) will indicate an approximate efficiency ranking of solvents for enamine formation.

As shown in Table 1, reactions in DMSO with 7% and 2% H_2O (entries 4 and 5) were dramatically faster than the reactions in H_2O (entry 1) or in DMSO with 52% and 22% H_2O (entries 2 and 3). The reactions in DMF demonstrated a similar trend with faster velocities observed in lower concentrations of H_2O: The reactions in DMF with 22%, 7%, and 2% H_2O (entries 7–9) were faster than the reaction in H_2O (entry 1) or in DMF with



Scheme 1. Reaction of fluorogenic maleimide **1** and in situ-generated enamine of acetone to yield the fluorescent product **2**.

Table 1. Relative velocity of glycine-catalyzed reaction of acetone and **1**, determined by increase in fluorescence[a]

Entry	Solvent	Relative velocity
1	79% H_2O	<0.5
2	52% H_2O–27% DMSO	1
3	22% H_2O–57% DMSO	2
4	7% H_2O–72% DMSO	40
5	2% H_2O–77% DMSO	100
6	52% H_2O–27% DMF	5
7	22% H_2O–57% DMF	30
8	7% H_2O–72% DMF	90
9	2% H_2O–77% DMF	70
10	2% H_2O–77% 2-PrOH	50
11	2% H_2O–77% MeOH	<0.5
12	2% H_2O–77% CH_3CN	<0.5

[a] Reactions were initiated by adding 1 μL of a stock solution of substrate **1** (5 mM) in CH_3CN to a mixture of acetone (20 μL), 2 μL of glycine solution (100 mM) in H_2O, and H_2O (77 μL) at 25 °C for entry 1. For entry 2, a mixture of H_2O (50 μL) and DMSO (27 μL) was used instead of H_2O (77 μL) in entry 1 experiment. The final conditions: [**1**] 50 μM, [acetone] 20% (v/v) (2.7 M), [glycine] 2 mM in 1% CH_3CN–79% solvent mixture indicated. The reactions were monitored by the increase in fluorescence (λ_{ex} 315 nm, λ_{ex} 365 nm) for 20 min. The relative velocity was determined by comparison with the velocity of the reaction at entry 5. Error limits for these measurements were within ±15%. The reaction was heterogeneous in the absence of added H_2O, so this condition was not studied.

52% H_2O (entry 6). The reaction in 2-PrOH with 2% H_2O (entry 10) also proceeded. Neither MeOH (entry 11) nor CH_3CN (entry 12) were good solvents for this reaction.

Glycine's amine is located at the α-position relative to the acid functionality. To analyze whether the distance between the amine and acid functionalities affects the reactivity of the amine, reactions with β-alanine were also analyzed. When β-alanine was used in this reaction and studied in mixtures of H_2O–DMSO, the relative velocities were the same as those of the reactions involving glycine in the corresponding mixtures of H_2O–DMSO. Thus, the distance between the amine and acid functionalities did not affect the reactivity of the amine, suggesting that the solvent effects observed for glycine should be general for a series of amino acids.

To examine the solvents effect on enamine formation with glycine more directly, glycines ability to form an enaminone with 2,4-pentanedione (**3**) was analyzed in a series of solvents. The initial velocities for enaminone formation were determined by the increase in UV absorption at 318 nm.[3a,b] The results are shown in Figure 1. Enaminone formation was faster in DMSO, DMF, and 2-PrOH with a low concentration of H_2O than in the same solvents with a high concentration of H_2O.[12] Enaminone formation was not observed in H_2O–CH_3CN or in H_2O–MeOH.[12] When β-alanine was studied as a substitute for glycine in the enaminone formation reaction in mixtures of H_2O–DMSO, the reaction velocities were the same as those observed with glycine.

Overall, the results of the fluorescent studies and the enaminone studies with respect to the solvent conditions

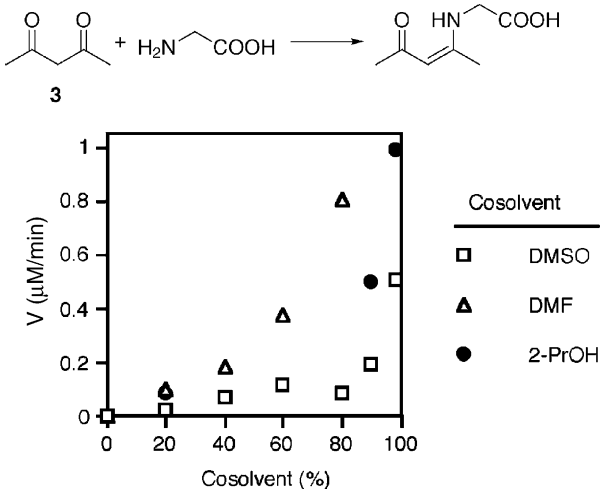
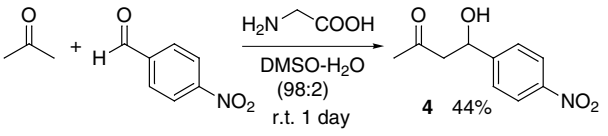


Figure 1. Enaminone formation of 2,4-pentanedione (**3**) with glycine. Conditions: [**3**] 1 mM, [glycine] 1 mM in 1% CH_3CN–99% a mixture of H_2O and cosolvent. The velocities were measured by the increase in UV absorption at 318 nm. Concentration of the enaminone of **3** with glycine was corrected by the absorption of the enaminone of **3** with methylamine. Error limits for these measurements were within ±15%.



Scheme 2. Glycine-catalyzed aldol reaction.[†]

were in close agreement. These results support the use of the rapid fluorescent analysis with **1** to investigate conditions for the enamine formation reaction with glycine. The conditions studied for the enamine formation reaction were also confirmed by studies of the aldol reaction. Since enamine formation with glycine was faster in the conditions determined above, reactions with electrophiles other than maleimide **1** should also be faster under similar solvent conditions. When the aldol reaction of acetone and *p*-nitrobenzaldehyde was performed in the presence of glycine, the reaction in DMSO–H_2O (98:2) approximating the condition in Table 1 at entry 5 provided aldol product **4**[13] (Scheme 2). The same reaction in CH_3CN–H_2O (98:2) approximating the conditions in Table 1 at entry 12 did not generate detectable amounts of **4** after 1 day. These results indicate that facilitation of enamine formation can be key to accelerating reactions involving enamine intermediates.

We observed significant solvent effects on the reactivity of the amino group of glycine in reactions designed to study enamine formation. A rapid fluorescence assay with fluorogenic maleimide **1** was used to evaluate solvent effects of the reaction of glycine with **1**. The data was confirmed by the UV analysis of the enaminone formation reaction with 2,4-pentanedione (**3**) and subsequently with the aldol reaction.

DMSO, DMF, and 2-PrOH with a low concentration of H_2O—that is hydrophobic conditions—facilitated enamine formation with glycine significantly better than the same solvents with a high concentration of H_2O. MeOH, a less hydrophobic solvent than 2-PrOH, was a poor solvent for the enamine-based reactions. These results are consistent with nature's strategy for the activation of primary amines which involves their placement in hydrophobic microenvironments within proteins.[3b,6] Our results, however, suggest that other factors beyond simple hydrophobicity are key. Note that DMSO, DMF, and 2-PrOH are all protophilic solvents and have higher Kamlet–Taft β values reflecting their solvent hydrogen-bond acceptor basicity.[14] It may be that the oxygens of these solvents form hydrogen bonds with protons that act as acid catalysts for enamine formation and/or stabilize protonated transition states along the reaction coordinate from imine to enamine formation via hydrogen bond formation. CH_3CN, which does not have the potential to form hydrogen bonds, was a poor solvent for the enamine-based reaction.[14] These results indicate that enamine formation with glycine is not accelerated simply by reasons that can be attributed to the solvent's polarity. Consistent with this is the fact that the rates of enamine formation with glycine do not correlate with solvent dielectric constants[15] or their Dimroth–Reichart E(T)N solvent polarity values.[16] Thus, hydrophobic conditions together with the capacity to engage in hydrogen bonds are important factors that facilitate the reactivity of primary amino groups to form enamines. Therefore we suggest that in addition to a simple hydrophobic microenvironment, a microenvironment that also affords for hydrogen bond formation may be necessary for the lysine ε-amino group of enzymes to efficiently form enamines.

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[†] A mixture of acetone (1.0 mL), *p*-nitrobenzaldehyde (0.5 mmol), and glycine (0.15 mmol) in DMSO (3.92 mL)–H_2O (80 μL), (net 20% acetone–1.6% H_2O–78.4% DMSO) was stirred at room temperature for 1day. Workup and purification afforded **4**.

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