

Direct Observation of an Enamine Intermediate in Amine Catalysis

Xueyong Zhu, Fujie Tanaka, Richard A. Lerner,* Carlos F. Barbas III,* and Ian A. Wilson*

Departments of Chemistry and Molecular Biology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received August 27, 2009; E-mail: wilson@scripps.edu; foleyral@scripps.edu; carlos@scripps.edu

The advent of aldolase catalytic antibodies¹ and the translation of this chemistry to small organic molecules has led to the burgeoning field of organocatalysis.² The centerpiece of amine-catalyzed reactions is presumed to be an enamine, the nascent nucleophile in enantioselective C–C and C–X bond-forming reactions.^{1,2} To more fully understand amine catalysis via an enamine mechanism, we have sought to observe enamine intermediates of aldolase antibodies directly. Enamines, however, are typically unstable in water; equilibrium between formation and hydrolysis of an enamine makes direct observation of an enamine within protein crystals difficult.³ Aldolase antibodies were initially raised against a 1,3-diketone hapten under the assumption that its reaction with a nucleophilic lysine in the binding pocket would yield an enaminone or vinylogous amide, a stabilized enamine (Figure 1A). Spectroscopic analysis of reactions of these aldolase antibodies with 1,3-diketones suggested that the enaminone was, indeed, formed.^{1a–c} We now report direct observation of the enamine by crystallographic analysis of the adduct formed when aldolase antibody 33F12 reacts with a 1,3-diketone derivative **1** (Figure 1A).

To obtain crystals of enzymes containing unstable enamine and related intermediates (i.e., carbinolamine and iminium), special conditions and procedures, such as soaking of crystals in a substrate solution and flash freezing, are typically required.³ To isolate unstable intermediates within crystals, additional interactions between substrate functional groups and amino acid residues of the catalyst (such as charge interactions between substrate phosphate groups and acidic residues) may be needed.³ However, such interactions involving substrates often complicate analysis of the essential features required for catalysis. In the analysis of enamine complexes with natural enzymes, the assignment of functional roles to amino acid residues involved in catalysis or in substrate binding has been difficult.³ Amino acid residues that interact with particular substrate functional groups may play less of a role in the catalytic machinery than in substrate binding. To understand the essentials of enamine catalysis, it may be necessary to investigate catalysts that have more limited interactions with their substrates. Due to its mechanism of elicitation, aldolase antibody 33F12 (and its sequence-related variant 38C2) acquired a promiscuous active site and, as a consequence, is able to catalyze aldol, retro-aldol, and enamine/iminium-based transformations of a very wide range of substrates.¹ It was originally proposed that substrate interactions with the binding site of the catalyst would be based on hydrophobic interactions and covalent imine and subsequent enamine formation.¹ Therefore, aldolase antibodies such as 33F12 should serve as a simplified model system to observe the requirements for an effective aminocatalyst.

We cocrystallized 33F12 Fab' with 1,3-diketone **1** and determined its structure by molecular replacement to a 1.9 Å resolution (Table S1). The Fab' complex resembles that of the native Fab'^{1c} with an overall rmsd of 1.0 Å (C $_{\alpha}$ atoms), and only 0.5 Å for the Fv domain

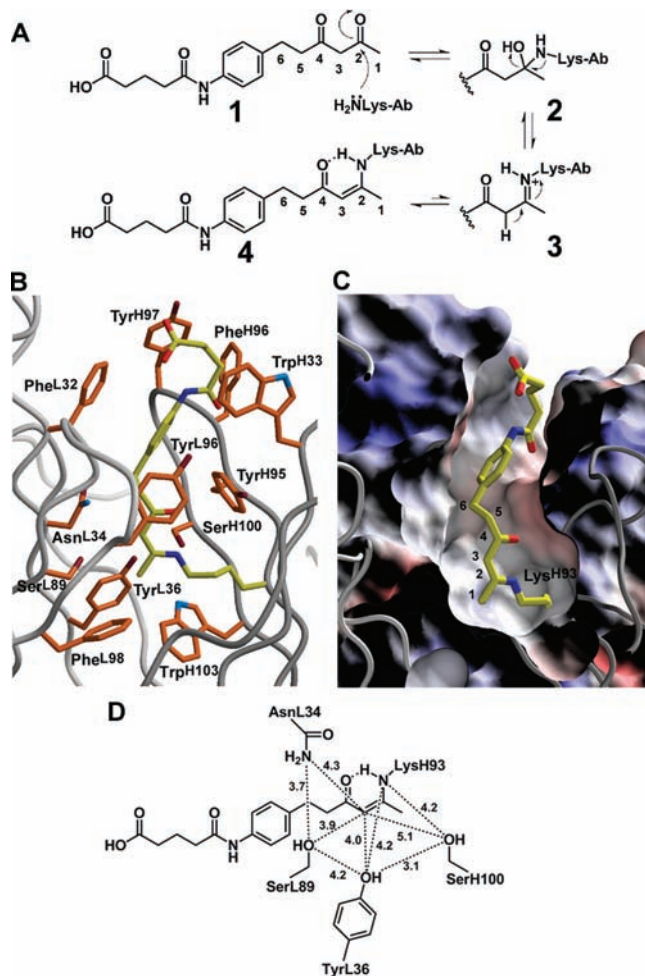


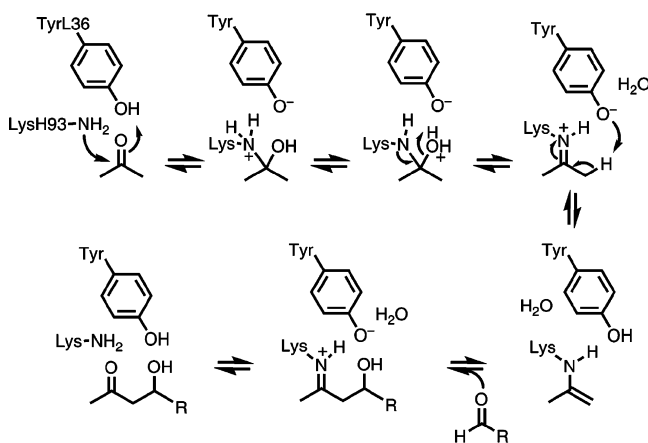
Figure 1. Enamine-forming reaction and the aldolase antibody binding site. (A) Reaction of antibody 33F12 with 1,3-diketone hapten **1**. A reactive lysine in 33F12 attacks one of the carbonyl groups of **1** to form carbinolamine **2** that subsequently collapses to iminium ion **3**. A stable covalent adduct, enaminone **4** is formed when the imine tautomerizes. (B) Binding site of the 33F12-hapten complex. The light chain and heavy chain backbone is represented as a tube in light and dark gray, respectively, with side chains of residues discussed in the text shown in stick form and labeled. (C) Shape complementarity of the enamine lysine-diketone adduct. A slice is shown through the antibody molecular surface and colored by electrostatic potential with 1.4 Å sphere radius. (D) 33F12-enamine interactions with some important binding site residues. Atom contact distances are given in angstroms.

(Figure S1). The binding site pocket is a narrow, elongated cleft more than 11 Å deep with the reactive catalytic lysine, LysH93, at its base (Figure 1B and 1C). LysH93 is surrounded by mostly hydrophobic residues that substantially lower its pK_a, a mechanism that appears now to be shared with the natural enzyme acetoacetate decarboxylase.^{1c,4} The LysH93-diketone covalent adduct is clearly

present in the complex structure (Figures 1B, 1C and S1) and conclusively demonstrates enamine formation. The enaminone portion is well ordered in the electron density maps, but the rest of the 1,3-diketone is less well-defined, consistent with the experimental observations that the antibody accepts a wide range of substrate through nonspecific hydrophobic interactions.^{1c}

The ϵ -amine of LysH93 is covalently linked to the C2 atom of the 1,3-diketone, and the C1, C2=N ϵ , C3, C4=O, and C5 atoms of the enaminone moiety are all in the same plane (Figure 1). Upon covalent adduct formation, no significant conformational changes occur within the antibody-binding site, except for a few side-chain rotamers, as in the case for AsnL34 (Figure 1B). The enamine moiety makes van der Waals interactions with TyrL36, SerL89, PheL98, TyrH95, and TrpH103, while the phenylamide substituent interacts with AsnL34, GlyL91, TrpH33, TyrH95, PheH96, and TyrH97 (Figure 1B). Importantly, hydrophilic residues (AsnL34, SerL89, SerH100, and TyrL36) are in close proximity to the enamine moiety (Figure 1B, 1D). The distances between the phenolic oxygen of TyrL36 and the enamine N ϵ (from LysH93) and C3 (from the diketone) are 4.2 and 4.0 Å, respectively, while the SerH100 hydroxyl is located 4.2 and 5.1 Å from the enamine N ϵ and C3, respectively. Additionally, the SerL89 hydroxyl is within 3.9 Å of the enamine C3.

Scheme 1. Plausible Role of TyrL36 in General Acid/Base Catalysis



Based on these structural observations, we hypothesize that TyrL36 acts as the general acid and base directly (Scheme 1), or indirectly through a water molecule, with the assistance of SerH100, SerL89, and/or AsnL34 for the formation and hydrolysis of enamine and imine intermediates. Water-assisted catalysis has been proposed for aldolases and designed retro-aldol catalysts.⁵ For the aldol reaction of acetone and an aldehyde, as in Scheme 1, LysH93 first makes a nucleophilic attack on the carbonyl carbon of acetone to form a carbinolamine; the phenolic OH of TyrL36 may act as an acid to activate the carbonyl. Next, water elimination from the carbinolamine results in formation of an iminium ion. TyrL36 may assist in the elimination of water by providing a charged environment. The iminium intermediate is then converted to an enamine, in which Tyr L36 could act as the base to abstract the α -proton of the iminium. For the C–C bond formation step, TyrL36 may then activate the carbonyl group of the aldehyde substrate through a hydrogen bond or proton transfer. Hydrolysis of the iminium affords the aldol product, where TyrL36 could again act as a base to form OH[−] by abstraction of a proton from a water molecule in the

hydrolysis step. Water molecules may also be involved in the catalysis depending on the actual distances between TyrL36 and the lysine ϵ -amino group during catalysis.^{5,6} The role of TyrL36 was further supported by the TyrL36Phe mutant of aldolase antibody 38C2 which did not bind to diketone **1** derivatives (unpublished).

Once the enaminone is formed, no additional interactions are required to retain the enaminone. TyrL36 does not need to provide direct interactions with the enaminone formed in the crystal structure. The distances between the TyrL36 and the enaminone moiety suggest its role in general acid/base catalysis either directly or through water. No amino acid residues other than TyrL36, SerL89, SerH100, and AsnL34 are observed in close enough proximity that could potentially provide charged interactions or hydrogen bonds during catalysis. Thus, this enamine complex structure provides strong evidence that fewer residues are essential for amine catalysis within the hydrophobic environments of this catalytic antibody than speculated for natural aldolase enzymes,³ and this structure should serve to guide future studies aimed at the rational design of these types of catalysts⁵ as well as organocatalysts.² Indeed, enamine catalysis in proteins might be more simplistic than previously imagined.

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Supporting Information Available: Experimental procedures, crystal structure determination, and structure of the Fab complex (Table S1; Figure S1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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