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Pyridoxal-mediated abzyme system for aldol and retro-aldol reactions

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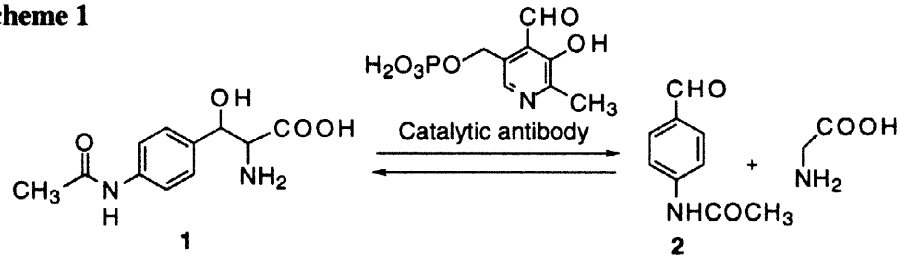
Abstract

A pyridoxal-mediated abzyme system for aldol and retro-aldol reactions is demonstrated. Antibody 10H2 catalyzes the aldol and retro-aldol reactions of 4-acetamidobenzaldehyde and glycine to β-hydroxy α-amino acid, using pyridoxal 5'-phosphate as a cofactor.

Key words: aldol ractions; antibodies; catalysis; amino acids and delivatives

Since the aldol reaction and its reverse reaction, the retro-aldol reaction, are the most basic reactions for C-C bond formation/cleavage in chemistry as well as biology [1], much attention has been directed toward the creation of antibody catalysts tailored for these reactions [2-5]. Recently, an antibody-catalyzed aldol reaction has been examined, based on the strategies mimicking natural class I aldolases [2]. In this work, we focused on threonine aldolases [6] as our model. The natural enzymes catalyze the aldol reactions from glycine and aldehydes to β-hydroxy α-amino acids, and its reverse reactions, by using pyridoxal 5'-phosphate (PLP) as a cofactor [7]. Here, we demonstrate the first example of a pyridoxal-mediated abzyme system for aldol and retro-aldol reactions (Scheme 1).

Scheme 1



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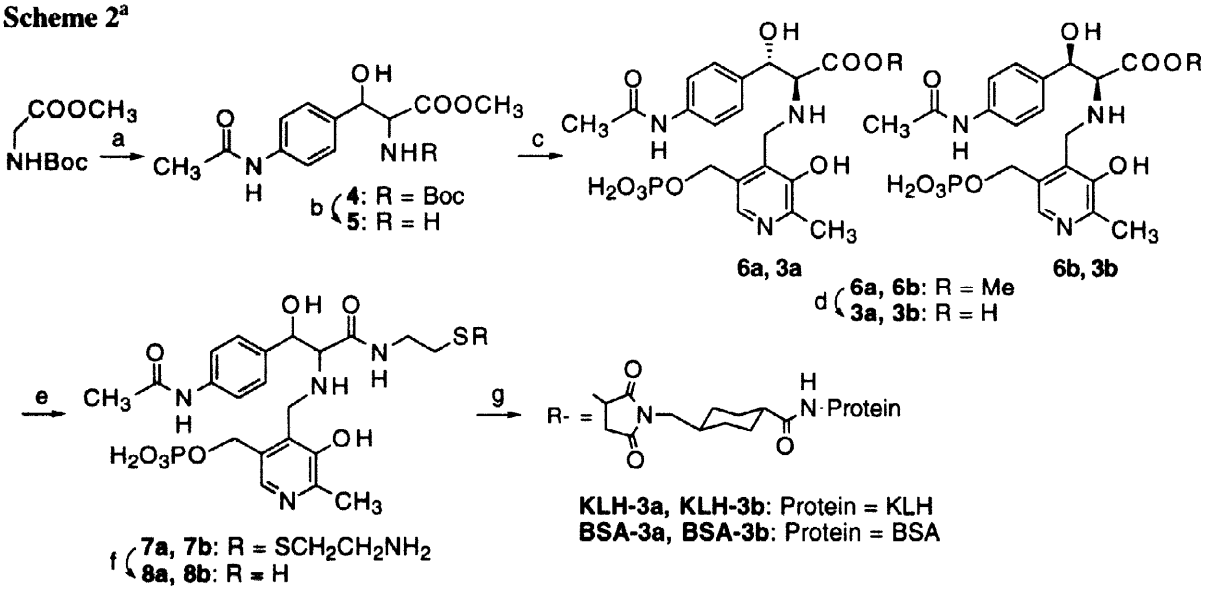
To generate antibodies that catalyze the aldol and retro-aldol reactions of 4-acetamidobenzaldehyde (**2**) and glycine to the β-hydroxy α-amino acid **1**, an immunized hapten **3** was designed to incorporate the three components, to facilitate the proximity effect for the multi-molecular reaction. Haptens **3a** and **3b** were synthesized and were conjugated to carrier proteins, keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) (Scheme 2). Balb/c mice were immunized with a mixture (1:1) of KLH-**3a**/KLH-**3b**, and monoclonal antibodies were generated by standard protocols [8]. Hybridoma supernatants were screened for antibodies with binding affinities to a mixture (1:1) of BSA-**3a**/BSA-**3b** by ELISA. Finally, in competitive ELISA with the haptens, the highest binder, 10H2, was chosen among twelve purified monoclonal antibodies.

Scheme 2[a]



[a] Reagents: (a) (1) LDA/THF (2) 4-acetamidobenzaldehyde, 57% (1:1 mixture of the diastereomers); (b) CF_3COOH/CH_3CN-CH_2Cl_2, 91% (1:1 mixture of the diastereomers); (c) (1) pyridoxal 5'-phosphate, $NaBH_3CN/MeOH$-H_2O-NaOH (pH 7), 68% (1:1 mixture of **6a** and **6b**) (2) HPLC separation of **6a** and **6b**; (d) $NaOH/H_2O$, **3a** 77% from **6a**, **3b** 97% from **6b**; (e) *N*-hydroxysuccinimide, $S_2(CH_2CH_2NH_2)_2$, EDC/phosphate buffer (pH 7.2)-CH_3CN, **7a** 13% and **7b** 24% from 1:1 mixture of **3a** and **3b**; (f) dithiothreitol/phosphate buffer (pH 7.2)-CH_3CN, **8a** 80% from **7a**, **8b** 52% from **7b**; (g) maleimide activated KLH or maleimide activated BSA/phosphate buffer (pH 7.1).

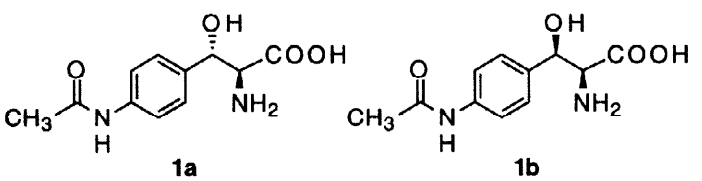
Prior to the catalytic assay, the ε-amino groups of lysine residues within the antibody were masked by acetylation, to eliminate the side reaction of PLP with the lysine residues, which could interfere with the reaction of PLP with the substrate:[1] In fact, when antibody 10H2 and PLP were mixed in 50 mM HEPES (pH 8.0), an undesired imine formation was detected by monitoring the increase of absorbance at 430 nm [7]. Antibody 10H2 was treated with acetic acid *N*-hydroxysuccinimide ester in the presence of hapten, and the excess hapten and reagents were removed by both gel filtration and dialysis.[2] The acetylated 10H2 was found

[1] PLP is known as a chemical modifying reagent for amino groups located on enzymes [9].

[2] Examination of the MALDI-TOF mass spectra for 10H2 and the acetylated 10H2 (10H2: 148886 [M^++H], acetylated 10H2:

to maintain the same binding affinity to the hapten as the unmodified 10H2, and was examined for the catalytic activities.

Initially, the antibody-catalyzed aldol reaction was examined. When the reaction was carried out using glycine (5.0 mM), aldehyde **2** (1.0 mM), PLP (20 μM), and antibody 10H2 (10 μM), in 5% DMSO/50 mM HEPES (pH 8.0) at 25 °C, the antibody was found to catalyze the reaction to provide the aldol products. The initial rates of formation of **1a** and **1b**[3] were approximately 2-fold higher than those of the background reaction.[4] The reaction was completely inhibited by the addition of a mixture of **3a** (200 μM)/**3b** (200 μM). Next we examined the retro-aldol reaction of the β-hydroxy α-amino acid **1a** (100 μM) or its diastereoisomer **1b** (100 μM) with PLP (20 μM) in the presence of antibody 10H2 (10 μM) in the same buffer conditions. The antibody-catalyzed retro-aldol reaction displayed a moderate *threo*-stereoselectivity for the substrates; antibody 10H2 catalyzed the reaction of the *threo*-isomer **1b** with a 4-fold increased rate over that of the background reaction,[5] but catalyzed the reaction of the *erythro*-isomer **1a** with a 2.5-fold increased rate. In the contrary, the background reaction of the *erythro*-isomer **1a** proceeded with a rate 2.5-fold faster than that of the *threo*-isomer **1b**. Thus, the antibody 10H2 predominately catalyzed the disfavored reaction in the background. There is no doubt that the PLP cofactor is essential for the catalyzed reactions, because the antibody showed no catalytic activity under the reaction conditions in the absence of PLP.

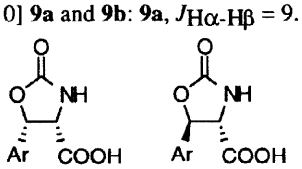


1a **1b**

Based on the principle of microscopic reversibility of these reactions, the catalytic efficiency of the pyridoxal-mediated abzyme system was evaluated in the retro-aldol reactions. The kinetics of the reaction of **1b** was analyzed by measuring the initial velocities of the reaction over 20-200 μM of PLP and 25-250 μM of **1b**. The data were applied to a random bimolecular kinetic model (Scheme 3 and equation 1 [11]); $K_{\mathbf{1b}}$ = 104

151953 [M^++H]) suggests that about 70 lysyl groups per molecule of 10H2 were acetylated.

[3] The relative stereochemistry of **1a** and **1b** was defined to be *erythro* and *threo*, respectively, by the NMR spectra of the corresponding oxazolidone derivatives [10] **9a** and **9b**: **9a**, $J_{H\alpha\text{-}H\beta}$ = 9.1 Hz; **9b**, $J_{H\alpha\text{-}H\beta}$ = 5.0 Hz.



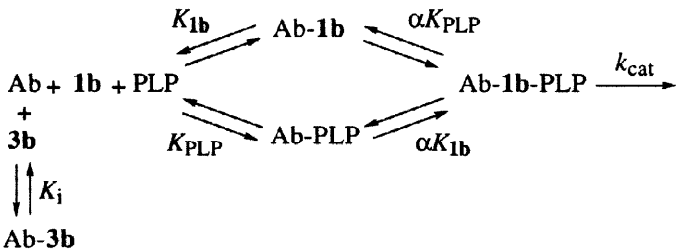
9a **9b** Ar = *p*-$CH_3CONHPh$

[4] The initial rates for the formation of **1a** and **1b** in the antibody-catalyzed reaction were 0.0034 and 0.0013 μM/min, respectively, after background correction. However, it was difficult to obtain meaningful kinetic parameters for the reaction, because the limited solubility of aldehyde **2** precluded to vary the initial concentrations of substrates in the assay.

[5] The enantioselectivity in the catalyzed retro-aldol reaction of **1b** was not observed by HPLC analysis with a chiral column for the substrate **1b** remaining during the reaction.

μM, K_{PLP} = 36 μM, α = 0.2, αK_{1b} = 21 μM, αK_{PLP} = 7 μM, and k_{cat} = 1.3 x 10^{-2} min^{-1}. The effective molarity (k_{cat}/k_{uncat}) of the antibody-catalyzed reaction is 1 x 10^3 μM (the second-order rate constant for the background reaction, k_{uncat} = 1.3 x 10^{-5} $min^{-1}\mu M^{-1}$). The antibody-catalyzed reaction of **1b** was inhibited by the addition of the hapten **3b**, which has the same relative stereochemistry as **1b**, showing that the antibody-catalyzed reactions occurred in the antigen-combining sites: K_i = 0.1 μM (Scheme 4 and equation 2).[6]

Scheme 3



$$V/(k_{cat}[Ab]) = [\mathbf{1b}][PLP]/(\alpha K_{\mathbf{1b}}K_{PLP} + \alpha K_{\mathbf{1b}}[PLP] + \alpha K_{PLP}[\mathbf{1b}] + [\mathbf{1b}][PLP]) \quad (1)$$

$$V/(k_{cat}[Ab]) = ([\mathbf{1b}][PLP]/\alpha K_{\mathbf{1b}}K_{PLP})/(1 + [\mathbf{1b}]/K_{\mathbf{1b}} + [PLP]/K_{PLP} + [\mathbf{3b}]/K_i + [\mathbf{1b}][PLP]/\alpha K_{\mathbf{1b}}K_{PLP}) \quad (2)$$

In this work, we have proposed a pyridoxal-mediated system for antibody-catalyzed reactions, based on natural threonine aldolase as a model. The hapten design allowed the elicited antibody to catalytically assemble PLP and the substrate(s), and to catalyze the aldol and retro-aldol reactions. Since the PLP cofactor participates in a vast number of important enzymatic transformations (including not only aldol and retro-aldol reactions, but also transamination [13], elimination [13], decarboxylation, and racemization) in the biometabolism of amino acids, applications and improvements of the pyridoxal-mediated system will provide useful antibody-catalysts for these transformations.

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[6] Equation 2, in which I is hapten **3b** instead of the product in the original, was adapted from ref.12.