Thiazolium-dependent catalytic antibodies produced using a covalent modification strategy†

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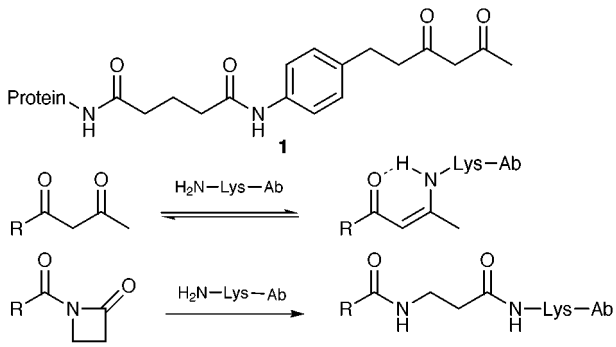
A thiazolium cofactor was introduced at a unique active site residue within antibodies that were generated by reactive immunization; the modified antibodies catalyzed a new cofactor-dependent reaction.

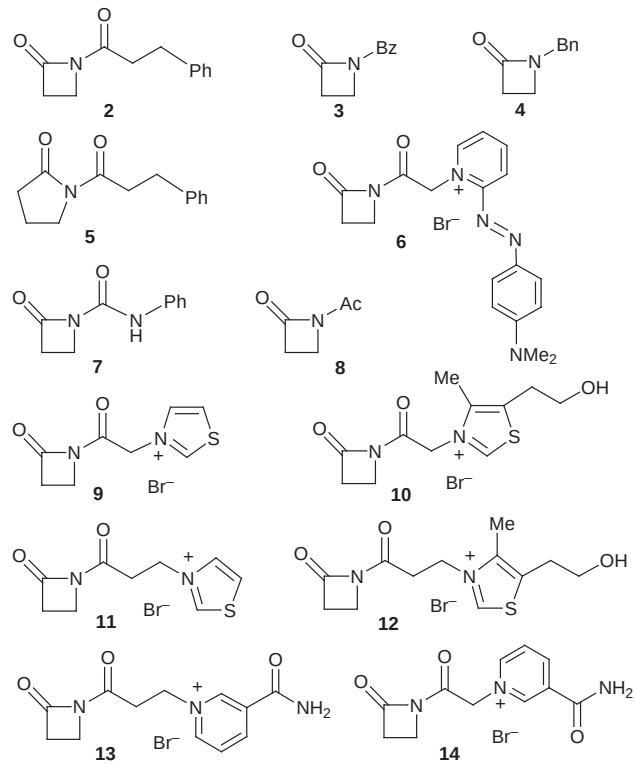
Enzymes effectively extend their catalytic scope by employing cofactors.[1] Consequently, in efforts to create novel cofactor-dependent protein catalysts several strategies have been developed.[2] Herein we report a novel approach which combines the unique features of catalytic antibodies derived from reactive immunization with a strategy for covalent modification that allows for the stable incorporation of a variety of cofactors. We further demonstrate the applicability of this strategy by preparing the first thiazolium-dependent catalytic antibodies.

Antibodies 38C2 and 33F12 were previously generated by reactive immunization with 1,3-diketone **1**.[3] These antibodies possess two key features which make them attractive as model systems for the development of cofactor-dependent catalysts: (i) they have unusually promiscuous active sites capable of binding a large variety of molecules and further transforming them chemically, and (ii) they contain a single highly reactive low pK_a lysine residue in their active site that is essential to their catalytic mechanism. The pK_as of the ε-amino groups of the LysH93 of 38C2 and 33F12 were determined to be 6.0 and 5.5, respectively.[3] Since this reactive lysine is the active nucleophile in Schiff base formation during the aldol reaction, we sought to take advantage of this reactivity and use it as a unique chemical handle for modification of these antibody catalysts. In earlier studies we have demonstrated that many β-dicarbonyl compounds are covalently yet reversibly bound as enaminones by these antibodies.[3] While β-dicarbonyl modified cofactors might be used to decorate the active sites of these antibodies with novel functionalities, we sought to develop a labeling strategy that would be irreversible and compatible with a wide range of cofactor chemistries. We reasoned that β-lactams[4] would form of a stable amide linkage to LysH93 (Scheme 1).



Scheme 1

To test this concept we synthesized a variety of β-lactam derivatives and studied their reactivity towards 38C2. The antibody 38C2 (20–30 μM active site) was first incubated with β-lactams (263 μM) in 5% MeCN– or DMSO–PBS (pH 7.4) at 25 °C. After 15–30 min and 14.5–40 h, the antibody active site was titrated by the addition of pentane-2,4-dione, followed by measurement of the characteristic enaminone absorption at 318 nm.[3] This assay effectively allows us to quantitate modification of the active site. Experiments with compounds **2**, **3** and **4**



suggest that the enhanced reactivity of the β-lactam ring provided by the 1-acyl group present on **2** and **3** is required for reaction with the antibody. The 1-acyl group might also facilitate the reaction by positioning the molecule in the active site of the antibody. The β-lactam ring is in fact required for reactivity since γ-lactam **5**, which contains a stable amide linkage, does not react with the antibody. Although formation of an enaminone upon reaction of the antibody with pentane-2,4-dione is typically complete within 5 min, modification by the β-lactam compounds proceeds in a slow, time dependent fashion.[5] In the case of β-lactam **2**, 95% of the active sites remain unmodified after a 15 min incubation period. After 14.5 h of incubation, only 45% of the active sites remain unmodified. Reaction with these β-lactams was quantitatively inhibited by prior addition of pentane-2,4-dione to the antibody.

In order to determine the stoichiometry of the β-lactam modification, we first used mass spectrometry. The Fab

† Experimental details for the preparation and characterization of **2**–**14**, antibody labeling, characterization, and kinetics are provided as electronic supplementary information, see http//www.rsc.org/suppdata/cc/1999/1383/.

fragment of 38C2 (22 μM) was treated with compounds **2** and **4** (500 μM) for 65 h and the proteins were subsequently purified by gel filtration chromatography. MALDI-TOF mass spectra of the unmodified Fab 38C2, Fab 38C2 treated with **2**, and Fab 38C2 treated with **4** show centroid masses of *m*/*z* 48331, 48534 and 48326, respectively. Given the molecular weight of **2** (*m*/*z* 203), these data indicate that one molecule of **2** is incorporated per molecule of Fab 38C2. The Fab 38C2 was not modified with **4**, as expected based on our active site titration data.

To further support our conclusions, a spectrophotometric assay was also investigated. Violet colored compound **6** was reacted with 38C2 as described above. Absorbance measurements at 566 nm suggest that 65–80%[6] of the antibody was modified with **6.** Titration of this modified antibody by the addition of pentane-2,4-dione verifies that 69% of the active sites were modified. Further, an equivalent loss of aldolase activity from 38C2 was observed. To examine the chemistry by which compound **6** reacts with the active site, **6**-modified antibody 38C2 was treated with 500 mM hydroxylamine at room temperature for 27 h and subsequently purified. Spectrophotometric measurements of the resulting antibody at 566 nm revealed no loss of the chromophore, consistent with the formation of a stable amide linkage. While other active site residues such as tyrosine L41[3*d*] might be candidates for modification, hydroxylamine treatment would be expected to readily deacylate such an ester modification, thereby liberating the chromophore.[5] Additional studies indicated that antibody 38C2 was also covalently modified by compounds **7**–**14**.[7]

We also extended these studies to antibody 33F12, which differs from 38C2 by 18 amino acids. Examination of the rate of modification of 33F12 with 1-acylazetidin-2-ones **2**, **3** and **9** under the conditions described above revealed that antibody 33F12 reacted with these compounds at a rate 70–80% of that observed for 38C2. The studies described above are all consistent with a model wherein 1-acylazetidin-2-ones react with a single amine functionality within the active site of antibodies 38C2 and 33F12. Because of its unique chemical reactivity and structural accessibility, the ε-amino group of LysH93, common to both of these antibodies, is the most likely site for modification.

Given the diverse array of chemistries accessible to the B_1-dependent enzymes,[1,8,9] we chose to study the catalytic activity of antibodies modified with compounds **9** and **10**. We chose α-decarboxylation as a model reaction for the well studied thiamin-dependent enzyme pyruvate decarboxylase.[9] Antibody 38C2 (166 μM) was treated with **9** and **10** (10 mM) in 5% MeCN–PBS at room temperature for 51 and 66 h, respectively, followed by purification by gel filtration chromatography. More than 95% of the active sites were modified as determined by the enaminone assay previously described. Thiazolium-mediated decarboxylation of $PhCOCO_2H$ **15** was studied by HPLC using 40 μM of **9**-modified antibody 38C2 and 500 μM of **15** in 10% DMSO–aqueous buffer [(PBS, pH 7.4)/(200 mM bicine, pH 8.5) = 1:1]. Antibody 38C2 modified with **9** catalyzed the formation of BzOH[10] **16**, whereas unmodified antibody 38C2 and the antibody treated with **10** did not catalyze the reaction. The bulky substituents (Me and $HOCH_2CH_2$ groups) of **10** may present steric barriers to binding of the substrate **15**. When treated with **9,** antibody 33F12 also catalyzed this decarboxylation reaction. The modified antibodies 38C2 and 33F12 displayed typical Michaelis–Menten saturation kinetics; kinetic parameters were as follows: **9**-modified antibody 38C2, k_{cat} 2.1 × 10^{-3} min^{-1} and K_m 5.8 × 10^2 μM; **9**-modified antibody 33F12, k_{cat} 7.8 × 10^{-4} min^{-1} and K_m 2.7 × 10^2 μM. The rates of the antibody catalyzed reactions were compared with the rate of the thiazolium-catalyzed reaction, k_{thz} 1.0 × 10^{-8} μM^{-1} min^{-1}. Antibodies 38C2 and 33F12 modified with **9** therefore yield rate enhancements over thiazolium catalysis alone [$(k_{cat}/K_m)/k_{thz}$] of 370 and 290, respectively. The effective molarity of the cofactor (k_{cat}/k_{thz}) is therefore 0.21 M for 38C2 and 0.078 M for 33F12. These studies suggest that catalysis arises from sequestration of the substrate in proximity to the thiazolium cofactor. The relative hydrophobicity of the active site may also contribute to solvent mediated catalysis of the reaction.[11]

In summary, we have demonstrated that aldolase antibodies 38C2 and 33F12, prepared using reactive immunization, are specifically and covalently modified with a diverse range of 1-acylazetidin-2-ones. We have found that this methodology can be used for the production of novel thiazolium-dependent catalytic antibodies. The attachment system described above should also be useful for the diverse post-translational modification of biological libraries, such as phage displayed antibody libraries[12] which retain LysH93. This would allow for the *in vitro* evolution of cofactor-dependent catalysis. Because reactive immunization selects for catalysts that operate through covalent mechanisms, it presents us with a simple strategy for the generation of diverse antibody combining sites that retain a reactive chemical group in their active site.[3] As we have demonstrated here with aldolase antibodies, the active site remains amenable to labeling with cofactors, thereby providing a handle for extending the scope of catalytic antibodies. Since the chemist is not constrained to select cofactors from nature's pallet, unnatural yet catalytically interesting cofactors can be studied.

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