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Metalloantibodies: Mercury(II)-Dependent Acyl Transferases

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Abstract—A new approach for the elicitation of metal-dependent catalytic antibodies for ester hydrolysis is described. A coordinatively unsaturated mercury complex **1-(Hg)**, has been utilized as a hapten to elicit antibodies that incorporate mercury(II) as a Lewis acid cofactor. From a panel of monoclonal antibodies generated to **1-(Hg)**, antibody 38G2 was found to hydrolyze the ester **3** in the presence of $HgCl_2$ [$K_m$app(**3**) = 345 μM; $K_m$app(Hg^{2+}) = 87 μM; k_{cat}app/$k_{uncat} = 3\times10^2$]. This is the first example of a biocatalyst that enlists mercuric ion as a cofactor and it is anticipated that this approach will open new avenues for exploitation of metals thought previously beyond the scope of protein catalysts.

Introduction

The preliminary reports that antibodies could be programmed to perform chemical catalysis[1,2] stimulated intensive efforts to explore their utility and scope as probes to answer fundamental questions regarding the evolution of biocatalysis.[3,4] Arguably, one of the most remarkable aspects of catalytic antibodies is the facility with which they can, by appropriate design, catalyze reactions thought both beyond the realm of biocatalysis[5–7] and even chemical catalysis.[8–11]

The metallo-peptidase and esterase enzymes, such as carboxypeptidase A[12] and the matrix metalloproteinase family,[13] are one of the most mechanistically well-characterized class of enzymes.[14,15] The metal ion cofactor, Zn(II), acts as a Lewis acid and polarizes the carbonyl oxygen bond of the amide or ester substrate, thus facilitating attack of the nucleophile, Glu-270 and stabilizes the tetrahedral intermediate thus formed (Fig. 1).[12,15]

One of the intriguing aspects of these enzymes is the conserved nature of the metal center. In most cases it is Zn(II) [or occasionally Co(II)].[16] This is in contrast to chemical studies, where the complete family of transition metals with a range of ligands is routinely explored as mimics of biochemically relevant hydrolyses.[17]

As part of an on-going exploration into the elicitation of antibodies that catalyze acyl transfer processes we anticipated merging both the biocatalytic and chemical approaches to elicit new metal-dependent catalysts. Thus, we hoped to evolve catalytic proteins within the time scale of the immune response, that could utilize a transition metal cofactor not normally exploited in vivo.

Results and Discussion

A number of methods have been developed to elicit metal-dependent catalytic antibodies.[18–22] The standard approach involves immunization with either the free ligand, or a metal–ligand complex.[23] The crux however, is that the antibody does not contribute to the ligand-sphere of the metal, but rather binds the ligand itself and the metal is inserted after the ligand binding. To design a system more closely mimicking carboxypeptidase A, where the enzyme supplies the active ligand sphere of the Zn(II) center, an extension of the standard methodology is required. Secondly, existing

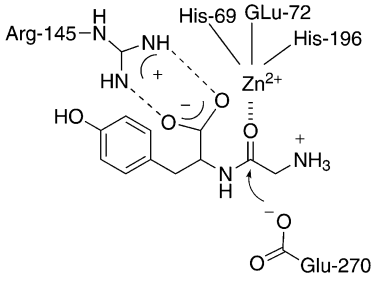

Figure 1. Carboxypeptidase A mechanism. Proposed Zn(II)–substrate complex which facilitates nucleophilic attack by Glu-270.[12]

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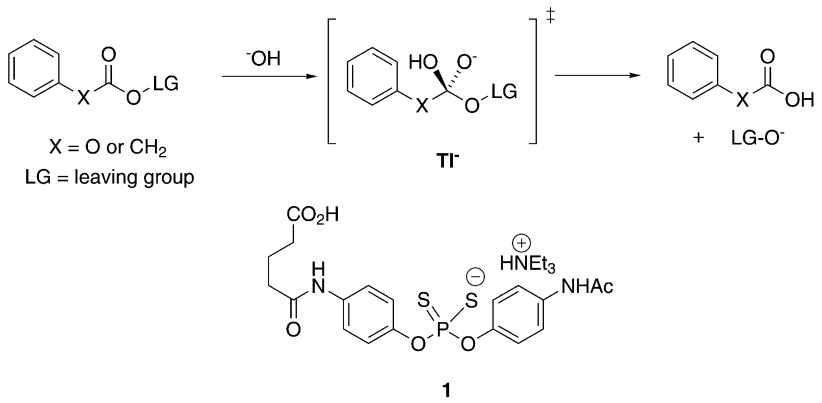

Figure 2. Phosphorodithioate **1** is a stable mimic of the anionic tetrahedral intermediate formed during acyl transfer reactions.

approaches do not allow for the simultaneous incorporation of transition state stabilization and metal-recognition by antibodies, a factor that ultimately limits the potential efficiency of any catalysts elicited. This is imposed, in general, by the inherent kinetic instability of such a complex of the transition-state-metal ion.

Phosphorodithioate **1**, a mimic of the anionic tetrahedral intermediate generated during acyl transfer reactions, was recently used as a hapten and elicited catalytic antibodies that were amongst the most efficient yet at catalyzing bisaryl carbonate hydrolysis (Fig. 2).[24]

It was anticipated that **1** could be exploited further, by utilizing the known metal coordinating ability of the non-bridging sulfur atoms, to overcome the limitations of existing approaches for the elicitation of metal-dependent catalytic antibodies (vide supra). The phosphorodithioate moiety classically binds soft metals and at the same time leaves the complexed metal atom coordinatively unsaturated.[25] Therefore by utilizing a metal complex of **1** as a hapten, antibody combining-sites may be generated that simultaneously coordinate directly to the displayed metal and bind, hence stabilizing the tetrahedral intermediate for acyl transfer reactions.

The success of this approach is linked to the in vivo integrity of the metal–**1** complex during the evolution of the preliminary immune response. Therefore, tight binding was a primary requisite during metal selection. Determination of the dissociation constant, K_d, of various metal ions complexed to the phosphorodithioate moiety was accomplished routinely using the phosphorodithioate charge transfer dye **2** (Fig. 3). It should be noted that the K_d determined in this fashion will be an upper estimate value for the metal–**1** complex which binds in a bidentate manner compared to the tridentate complex formed with dye **2**.

Upon metal binding a significant reduction and hypsochromic shift of the dye's absorption spectra between 415 and 450 nm is observed.[26] Among the metal ions tested (Co^{2+}, Zn^{2+}, K^+, Cd^{2+}, Eu^{3+}, Gd^{3+}, Hg^{2+}, In^{3+}, La^{3+}, Pb^{2+}, Tb^{3+}) soft metals were found to bind most tightly to the phosphorodithioate moiety. Equilibrium dissociation constant, K_d, measurements were performed for both Pb^{2+} and Hg^{2+} and found to be 80 and 1.6 nM, respectively. On this basis, Hg^{2+} was the metal of choice for the immunization protocols.

Mercury is non-essential in biosystems and is, in fact, toxic in either its inorganic [Hg(II) or (I)] or elemental (vapor) forms and extremely toxic in its organic (alkyl mercury) form.[27] A considerable reason for its toxicity is its affinity for cellular sulfur residues coupled with its action as an enzyme inhibitor. All of these factors explain, perhaps, why mercury has not evolved as an acceptable cofactor for naturally occurring biocatalysts. Chemically however, mercury is ideal for this role. It possesses partially filled *d*-orbitals, a feature it shares with its transition metal congeners, and Hg^{2+} has a strong tendency to form complexes.[28] Therefore, antibodies that can bind mercury(II) may catalyze acyl transfer reactions via a productive ternary complex in which the carbonyl of the ester group is coordinated to the mercury atom.

Hapten **1** was synthesized as described previously[24] and conjugated to carrier proteins bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) via their sulfo *N*-hydroxysuccinimide esters.[29] Mercuric ion insertion was then performed by addition of excess $HgCl_2$ (100 mM in water) to the KLH–**1** and BSA–**1** conjugates in *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid (EPPS, 50 mM, pH 7.5) for 2 h. The carrier protein solutions were then dialyzed exhaustively in EPPS buffer (50 mM, pH 7.5) to remove the unbound Hg^{2+}, thus nullifying any potential in vivo toxicity of the excess Hg(II).

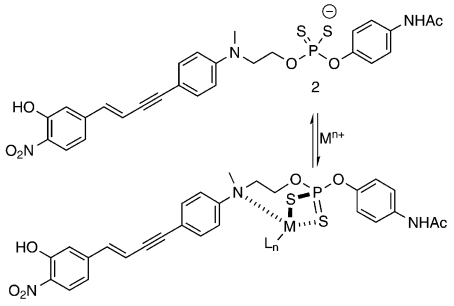

Figure 3. Phosphorodithioate phase transfer dye sensor **2** exhibits a unique reduction and hypsochromic shift in its absorption spectrum, between 415 and 450 nm, on binding of metal ions.

Balb/c mice were immunized with the dialyzed KLH–**1(Hg)** conjugate solutions and monoclonal antibodies were generated and purified by standard hybridoma protocols.[30,31] A panel of 29 monoclonal antibodies were generated that bound to the BSA–**1–(Hg)** complex as determined by enzyme-linked immunosorbent assay (ELISA). Each member of this panel was then screened for the ability to catalyze the cleavage of ester **3** and amide **4** in the presence of different metals (Hg^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, In^{3+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Al^{3+}, Y^{3+}, La^{3+}, Cd^{2+}, Pb^{2+}, Ag^{+}, Lu^{3+}, Ce^{3+}, Eu^{3+}) (Fig. 4).

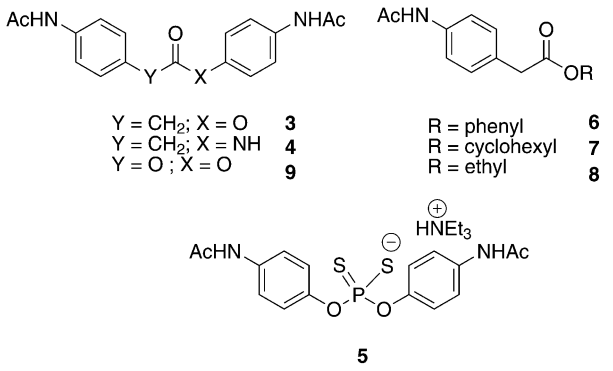

Figure 4. Esters **3** and **6–8**, amide **4** and carbonate **9** substrates investigated during this study. Phosphorodithoate **5** was used as a hapten analogue inhibitor of 38G2 activity.

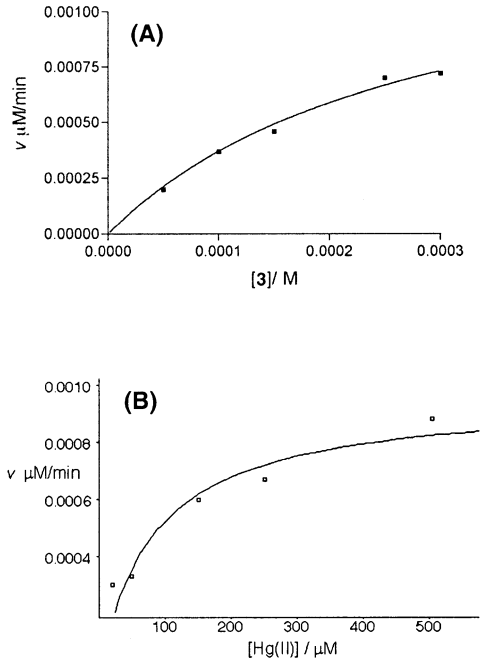

Figure 5. (A) Hanes–Woolf plot of the 38G2 kinetic data for hydrolysis of ester **3**. Kinetic analysis of ester **3** cleavage in the presence of Hg^{2+} was measured at pH 6.1 (50 mM PIPES, 50 mM NaCl with 5% DMSO as co-solvent) at rt. Reactions were performed with ester (**3**) (50–350 μM) in the presence or absence of antibody 38G2 (10 μM) and $HgCl_2$ (500 μM) concentration. Each point is the mean value of at least two measurements ($y = 635.2x + 216229.9$). (B) Nonlinear regression plot (versus S) of the mercury (II) (20–500 μM) dependence of the 38G2 catalyzed hydroysis of ester **3** (250 μM). (C) Inhibition of 38G2 (10 μM) in the presence of $HgCl_2$ (500 μM). Data is plotted as the percentage of the maximal rate (with no inhibitor). The $IC_{50} = 60$ μM.

Two antibodies were found to catalyze ester **3** hydrolysis and both exhibited mercuric-ion dependence. No antibodies catalyzed the hydrolysis of anilide **4**. The most efficient esterase antibody, 38G2, was extensively purified by a process of ammonium sulfate precipitation, DEAE ion exchange, protein G affinity, and Mono Q column chromatography. In addition it was regrown from the original cell lines to firmly establish that the catalysis was not due to a contaminating esterase. Perhaps the most convincing evidence is its unique dependence on mercuric ion for activity.

Kinetic analysis of 38G2 was performed at pH 6.1 [50 mM 1,4-piperazinediethanesulfonic acid (PIPES), 50 mM NaCl], with 5% dimethylsulfoxide as co-solvent. Antibody 38G2 exhibits Michaelis–Menten kinetics (k_{cat}app $= 2\times10^{-4}$ min^{-1}; $K_m$app(**3**) $= 345$ μM; $K_m$app(Hg^{2+}) $= 87$ μM; k_{cat}/k_{uncat}, of 3×10^2) and performs multiple turnovers without any reduction in activity (Figs 5A and B). The pseudo-first order non-catalyzed rate constant, k_{uncat}, of ester **3** is 6.75×10^{-7} min^{-1} and was determined by the method of initial rates using the buffer system vide supra. Interestingly this catalyst has no esterase activity in the absence of Hg(II). Inhibition experiments confirmed that the hapten analogue, phosphorodithioate **5**, is a tight-binding inhibitor of 38G2 activity and therefore the inhibition constant value, $K_i = 28$ μM, was measured by the method of Copeland[32] in the presence of excess Hg^{2+} (500 μM).

Preliminary substrate specificity studies with antibody 38G2 in the presence of Hg^{2+} have revealed limited tolerance for any change to the core structure of aryl ester **3**. Removal of the *para*-acetamido group of **3** gives ester **6** that is a much poorer substrate.[33] The corresponding cyclohexyl **7** and ethyl **8** esters are not substrates (Fig. 4). In addition the bisaryl carbonate **9** is not a substrate.

Conclusion

Immunization with the haptenic phosphorodithioate–mercury complex **1–(Hg)** resulted in the elicitation of antibodies that hydrolyze ester bonds via mercury-mediated Lewis acid catalysis. While the rate acceleration observed in this case was modest, the results presented here demonstrate that with the appropriate design, new biocatalysts can be evolved that utilize cofactors thought beyond the realm of enzymes.

Experimental

Materials and methods

Unless otherwise stated, reactions were performed under an inert atmosphere with dry reagents and solvents and flame-dried glassware. Analytical thin layer chromatography (TLC) was performed using 0.25 mm

silica gel coated Kieselgel 60 F_{254} plates. Visualization of the chromatogram was by UV absorbance, methanolic sulfuric acid, aqueous potassium permanganate, iodine, and *p*-anisaldehyde. Liquid chromatography was performed using compressed air (flash chromatography) with the indicated solvent system and silica gel 60 (230–400 mesh). Preparative TLC was performed using Merck 1 mm coated silica gel Kieselgel 60 F_{254} plates. ^{1}H NMR spectra were recorded on either a Bruker AM-250 or a Bruker AMX-400 spectrometer. Chemical shifts are reported in parts per million (ppm) on the δ scale from an internal standard. ^{13}C NMR (proton decoupled) were recorded on a Bruker AMX-500 spectrometer at 125 MHz. ^{31}P NMR (proton decoupled) spectra were recorded on a Bruker AMX-400 spectrometer and 101 MHz. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-VSE mass spectrometer.

Phosphorodithioate 1 synthesis. Synthesis of **1** was achieved as previously described and gave satisfactory spectroscopic data:[24] ^{1}H NMR (400 MHz, CD_3OD) δ 7.45 (d, $J=9$ Hz, 2H), 7.43 (d, $J=9$ Hz, 2H), 7.26 (d, $J=9$ Hz, 2H), 7.25 (d, $J=9$ Hz, 2H), 2.41 (t, $J=7$ Hz, 2H), 2.32 (t, $J=7$ Hz, 2H), 2.09 (s, 3H), 1.96 (tt, $J=7$/7 Hz, 2H); ^{13}C NMR (62.5 MHz, CD_3OD) δ 177.3, 173.5, 171.4, 150.5, 150.3, 135.6, 123.2, 123.1, 121.9, 121.8, 36.8, 34.2, 23.7, 22.2; ^{31}P NMR (101 MHz, CD_3OD) δ 110.97; ^{31}P NMR (101 MHz, DMSO-d_6) δ 109.27; MS: *m*/*z* (rel intens) 601 (MCs^+, 15), 572 (10), 546 (35), 419 (87); HRMS (FAB) ($C_{19}H_{21}O_6N_2PS_2Cs = MCs^+$) calcd 600.9633, found 600.9655.

Phase-transfer dye 2. Synthesis of **2** was achieved as previously described and gave satisfactory spectroscopic data:[26] ^{1}H NMR (400 MHz, $CDCl_3 + CD_3OD$) δ 8.03 (d, $J=9.0$ Hz, 1H), 7.41 (d, $J=9.0$ Hz, 2H), 7.29 (d, $J=9.0$ Hz, 2H), 7.21 (dd, $J=9.0$, 2.0 Hz, 2H), 7.15 (s, 1H), 7.13 (dd, $J=9.0$, 2.0 Hz, 1H), 6.88 (d, $J=16.0$ Hz, 1H), 6.75 (d, $J=9.0$ Hz, 2H), 6.69 (d, $J=16.0$ Hz, 1H), 4.23 (dt, $J=10$, 6 Hz, 2H), 3.71 (t, $J=6.0$ Hz, 2H, DBU), 3.58–3.55 (m, 2H, DBU), 3.51 (t, $J=6$ Hz, 2H, DBU), 3.31 (t, $J=6.0$ Hz, 2H), 3.05 (s, 3H), 2.66–2.62 (m, 2H, DBU), 2.11 (s, 3H), 2.00 (tt, $J=6.0$ Hz, 2H, DBU), 1.79–1.66 (m, 6H, DBU); ^{13}C NMR (100 MHz, $CDCl_3$ + CD_3OD) δ 171.2, 156.2, 150.7 (DBU), 147.0, 137.1, 135.3, 134.2, 134.0, 126.5, 122.9, 121.7, 118.4, 117.5, 115.9, 112.7, 110.3, 98.4, 87.8, 63.8, 55.3 (DBU), 53.1, 49.5 (DBU), 39.3 (DBU), 39.2, 33.7 (DBU), 29.8 (DBU), 27.4 (DBU), 24.8 (DBU), 23.7 (DBU), 20.3; ^{31}P NMR (100 MHz, $CDCl_3$ + CD_3OD) δ 113.9; ESI-MS for $C_{27}H_{25}O_6N_3S_2P$ [M-DBUH$^+$]: 582.734.

Carrier protein hapten 1 conjugate preparation. Hapten **1** was activated as its *N*-hydroxysuccinimide ester by treatment with *N*-hydroxysuccinimide (1.2 equiv) and EDC (1.2 equiv) in DMF for 8 h. The DMF solution of hapten **1** was then added to either keyhole limpet hemocyanin (KLH, 5 mg/mL) or bovine serum albumin (BSA, 5 mg/mL) in *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid (EPPS, 50 mM, pH 7.5). The hapten-protein solutions were then allowed to stand at 4 °C overnight. Mercuric ion insertion was then performed by addition of excess $HgCl_2$ (100 mM in water) to the KLH-**1** and BSA-**1** conjugates in EPPS (50 mM, pH 7.5) for 2 h. The carrier protein solutions were dialyzed exhaustively in EPPS buffer (50 mM, pH 7.5) to remove the unbound Hg^{2+}, thus nullifying any potential in vivo toxicity of excess Hg(II).

Monoclonal antibody preparation. Antibodies were produced as previously described.[33]

Kinetic Studies

High-throughput screening for catalysis of ester 3 and amide 4 hydrolysis

The preliminary screen for catalysis was monitored by HPLC on a Vydac C-18 reversed-phase column with a Hitachi D-7000 series machine equipped with an L-2000 autosampler and a D-7500 integrator. Briefly, the assay involved incubation of the ester **3** or amide **4** (500 μM) at pH 6.1 (50 mM PIPES, 50 mM NaCl with 5% DMSO as co-solvent) with each metal(II) chloride (Hg^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, In^{3+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Al^{3+}, Y^{3+}, La^{3+}, Cd^{2+}, Pb^{2+}, Ag^+, Lu^{3+}, Ce^{3+}, Eu^{3+} at 500 μM) in the presence or absence of antibody (20 μM). The reaction was followed by the rate of formation of either 4-acetamidophenyl acetic acid (for hydrolysis of ester **3**) or 4-acetamidoaniline (for hydrolysis of amide **4**) by HPLC [CH_3CN/H_2O (1% TFA) 19:81 mobile phase, with 4′-fluoroacetanilide as an internal standard].

Kinetic studies with 38G2

Kinetic analysis of ester cleavage in the presence of Hg^{2+} was measured at pH 6.1 (50 mM PIPES, 50 mM NaCl with 5% DMSO as co-solvent) at room temperature. Kinetic assays were followed using reversed-phase HPLC [CH_3CN/H_2O (1% TFA) 19:81 mobile phase, with 4′-fluoroacetanilide as an internal standard (IS)] by observing the formation of 4-acetamidophenyl acetic acid at $\lambda = 254$ nm. Reactions were performed with ester (**3**) (50–350 μM) in the presence or absence of antibody 38G2 (10 μM) (purified by protein-G affinity and mono-Q ion-exchange column chromatography) and $HgCl_2$ (500 μM) concentration (total assay volume was 100 μL). At appropriate times during the assay an aliquot (12 μL) of the reaction mixture was removed and added to an equal volume of internal standard solution (100μM in water). This mixture was then analyzed by HPLC vide supra. The assay was followed for no more than 5% of the reaction progress, during which time the progress curves were linear ($r^2 > 0.985$). To measure the mercury(II) dependence of 38G2 activity the assay was repeated at varying concentrations of $HgCl_2$ (20–500 μM) while holding the ester **3** concentration fixed at 250 μM. Kinetic parameters, k_{cat} and K_m were determined by analysis of this raw rate data using the Cricket Graph III v. 1.5.3 (Computer Associates Inc.) and Enzymekinetics v1.1 (Trinity software) computer programs on a Macintosh PC.

Inhibition assay

The inhibition assay was performed in the same fashion as described vide supra. The only changes being that the substrate ester **3** concentration was fixed at 500 μM, and the rate of 38G2 (10 μM) catalyzed hydrolysis of **3** was measured in the presence or absence of inhibitor **5** (0–250 μM). The K_i was then estimated by fitting the IC_{50} value obtained from Fig. 5C to the equation in Copeland et al.[32]

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