Published in final edited form as:

J Biotechnol. 2014 June 20; 180: 17–22. doi:10.1016/j.jbiotec.2014.03.026.

Metal-Dependent Amyloid β -Degrading Catalytic Antibody Construct

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Abstract

Catalytic antibodies (catabodies) that degrade target antigens rapidly are rare. We describe the metal-dependence of catabody construct 2E6, an engineered heterodimer of immunoglobulin light chain variable domains that hydrolyzes amyloid β peptides ($A\beta$) specifically. In addition to the electrophilic phosphonate inhibitor of serine proteases, the metal chelators ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline completely inhibited the hydrolysis of $A\beta$ by catabody 2E6. Formation of catabody-electrophilic phosphonate inhibitor adducts was unaffected by EDTA, suggesting that the metal exerts a favorable effect on a catalytic step after the initial catabody nucleophilic attack on $A\beta$. The EDTA inactivated catabody failed to disaggregate fibrillar $A\beta$, indicating the functional importance of the $A\beta$ hydrolytic activity. Treating the EDTA-inactivated catabody with Zn^{2+} or Co^{2+} restored the $A\beta$ hydrolytic activity, and Zn^{2+} -induced catabody conformational transitions were evident by fluorescence emission spectroscopy. The studies reveal the absolute catabody dependence on a metal cofactor.

Keywords

Alzheimer disease; amyloid β clearance; catalytic antibody light chain; metal activation

1. Introduction

Accumulation of amyloid β 1-42 ($A\beta_{42}$) and 1-40 ($A\beta_{40}$) peptides is thought to play a central role in the pathogenesis of Alzheimer's disease (AD), and developing a safe and efficient means to reduce brain $A\beta$ burden is an important objective in AD research (Liu *et al.* 2012). Small quantities of intravenously infused $A\beta$ -binding immunoglobulin (Ig) G class antibodies that cross the blood-brain barrier (BBB) decrease parenchymal $A\beta_{1-42}$ ($A\beta_{42}$) due to microglial uptake of IgG- $A\beta$ immune complexes, but this process also activates neurotoxic inflammatory pathways (Bard *et al.* 2000; Liu *et al.* 2012). Most AD

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patients present with cerebral amyloid angiopathy (CAA) caused by A β 40 deposits in the microvasculature (Jellinger 2002). A β -binding IgGs increase vascular A β 40 deposition, exacerbating the pre-existing CAA and causing increased microhemorrhages (Liu *et al.* 2012; Zago *et al.* 2013). Reminiscent of the infused IgGs, appearance of A β -binding autoantibodies in the cerebrospinal fluid correlates with exacerbated CAA (Piazza *et al.* 2013).

Catalytic antibodies (catabodies) to several autoantigens and microbial antigens have been identified (Shuster *et al.* 1992; Paul *et al.* 2012). Catabodies frequently utilize a hydrolytic mechanism similar to the serine/threonine protease enzyme family, *i.e.*, acylation of the active site nucleophile followed by water attack and hydrolysis of the acylated catabody. Enzyme-like nucleophilic sites composed of a Ser-His-Asp triad and its variants were found in catabody V-domains by mutagenesis, crystallography and molecular modeling (Gao *et al.* 1995; Ramsland *et al.* 2006; Taguchi *et al.* 2008a). Catabodies offer the advantage of digesting A β into harmless soluble fragments with no dependence on accessory inflammatory cells. As age-associated A β accumulation has no useful function, A β -degrading IgM class catabodies found in healthy humans may fulfill a homeostatic anti-amyloid function (Taguchi *et al.* 2008b). No catabody with medical utility has been developed, however, because of insufficient catalytic rates and limited information about factors that stabilize the Ig variable domains (IgVs) into their high-activity, catalytic conformations (Zhou *et al.* 2002). We and others reported recombinant IgVs with high-level A β -degrading activity (Taguchi *et al.* 2008a; Kasturirangan *et al.* 2010). IgV 2E6, a single chain heterodimer of light chain variable (V_L) domains (Fig. 1a) isolated from a human IgV library, degraded A β with no evidence of nonspecific proteolysis or formation of immune complexes that activate inflammatory cells (Taguchi *et al.* 2008a).

The integrity of enzyme (Radisky *et al.* 2006) and catabody (Taguchi *et al.* 2008a) active sites depends on precise topographical arrangements of the catalytic amino acids. In addition to structural stability within the active site, remote polypeptide regions can influence catalysis by regulating the catabody conformation (Sapparapu *et al.* 2012). Metal ions participate directly in the mechanism of catalysis at the active site (Rawlings and Barrett, 2013), and metal binding to remote sites can influence the catalytic site conformation (Betzl *et al.* 1988; Vadivel and Bajaj 2012). We describe the metal-activated hydrolysis of A β by catabody 2E6.

2. Methods

2.1. A β peptides

Synthetic A β 40 and A β 42 (EZBiolab, Carmel, IN) were treated with 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich, Saint-Louis, MI, USA) before use (1 mg/mL, 1 h). Preparation of ¹²⁵I-A β 40 was described previously (Taguchi *et al.* 2008a). Fibrillar A β 42 was prepared by incubating A β 42 (50 μ M) in 10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS) containing 1% dimethylsulfoxide for 24 h (37°C).

2.2. Antibodies

IgV 2E6 and the control IgV MMF6 with identical scaffold structure were isolated from a single chain immunoglobulin V domain library cloned in the pHEN2 bacterial expression vector prepared from human peripheral blood lymphocytes (Taguchi *et al.* 2008a). The two IgVs contain the same N-terminal V_{L1} domain and different C-terminal V_{L2} domains with a his6 tag (respectively, GenBank accession numbers FJ231715 and KF018653). The mutant IgV 2E6 gene was synthesized by Mutagenex Inc (Hillsborough, NJ, USA) by replacing the V_{L2} domain framework regions 1-4 (FRs; Kabat residues 1–23, 35–49, 57–88 and 98–107) with the corresponding FRs of IgV MMF6 V_{L2} domain and cloned into pHEN2 vector (NcoI/XhoI sites). Except as specified, the studies were done with the IgVs purified from bacterial periplasmic extracts by His6 tag binding to Ni-nitrilotriacetic acid (NTA) immobilized metal affinity chromatography (IMAC) columns, followed by acid elution as described (Taguchi *et al.* 2008a). His6 protonation at this pH elutes the IgVs without dissociating the strong Ni-NTA chelates on the column. Unfractionated culture supernatants were obtained by centrifugation (5,000xg, 30 min) from the IgV-secreting and control bacteria grown to equivalent density (~0.8 A₆₀₀ units; 24 h, 2YT medium containing 1 mM isopropyl-β-D-thiogalactopyranoside and 0.1 mg/ml ampicillin: IgV expression 1.5–5.4 mg/L). The partially-fractionated IgV 2E6 (paIgV) was obtained by two FPLC-anion exchange chromatography cycle (Hitrap Q FF column, GE Healthcare; 3 mL/min; from 1 Liter culture supernatant concentrated 25-fold on a 10 kDa Pellicon 2 membrane followed by dialysis against chromatography buffer). The first and second chromatography cycles were conducted in buffers adjusted to pH 7.4 and 8.0, respectively (20 min 0–1 M NaCl gradient in 50 mM Tris-HCl, 0.1 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)). IgV 2E6 eluted in the non-bound fraction from the first chromatography cycle and the paIgV was recovered in the bound fraction in the second chromatography cycle (retention volume 26–34 mL; protein content 1.8 mg, IgV content 0.11 mg; determined, respectively, by the microBCA method and dot-blotting with antibody to the c-myc-peptide tag (Taguchi *et al.* 2008a). Control paIgV MMF6 eluted in the bound fraction from the first chromatography cycle conducted as described for paIgV 2E6 (retention volume 44.1–56.2 mL, protein content 3.8 mg, paIgV MMF6 content 0.11 mg). EDTA-inactivated IgV 2E6 was prepared by 1 hour incubation of the IgV in PBS containing 0.1 mM CHAPS and 1 mM EDTA, followed by dialysis against the same buffer without EDTA.

2.3. Aβ40 hydrolysis

¹²⁵I-Aβ40 hydrolysis by the IgVs was measured in duplicate as in (Taguchi *et al.* 2008b). ¹²⁵I-Aβ40 (~0.1 nM, 30,000 cpm/reaction) was treated with the IgVs in PBS containing 0.1 mM CHAPS and 0.1% (w/v) bovine serum albumin, and radioactive product fragments were as measured as the amount of radioactivity rendered soluble in 5% trichloroacetic acid. Data are corrected for background hydrolysis in diluent (<5%). Errors are SD values. Aβ hydrolysis determined by this method correlates with depletion of the intact ¹²⁵I-Aβ40 peak and product appearance quantified by FPLC gel filtration (Pearson's test, two tailed, P<0.01). IgV 2E6 also hydrolyzed non-radioactive Aβ40 (Taguchi *et al.* 2008a). ¹²⁵I-Aβ40 hydrolysis was measured in the presence of the following protease

inhibitors: EDTA (1 mM), 1,10-phenanthroline (0.5 mM), iodoacetamide (1 mM), pepstatin A (10 μ M), and diphenyl *N*-(benzyloxycarbonyl)amino(4-amidinophenyl)methanephosphonate (compound **1a**; 1 mM). The residual hydrolytic activity (%) was determined as: $100 \times (\% \text{ hydrolysis in the presence of inhibitor})/(\% \text{ hydrolysis in the absence of inhibitor})$. Metal activation of EDTA-treated IgV 2E6 (10 μ g/mL) was tested by measuring ^{125}I -A β 40 hydrolysis in the presence of 0.075 mM MgCl₂, CaCl₂, MnCl₂, FeCl₂, CoCl₂, NiCl₂, CuCl₂, ZnCl₂ or HgCl₂. Restoration of IgV hydrolytic activity was studied in the same manner at 0.75, 0.225, 0.075, and 0.0225 mM CoCl₂ and ZnCl₂.

2.4. Fibrillar A β dissolution

Fibrillar A β 42 (20 μ M) was treated for 24 h with the IgV 2E6 (4 μ g/mL) in PBS, 0.1 mM CHAPS and 1% dimethylsulfoxide (37°C). The IgV was tested without or with EDTA (10 mM) pre-treatment for 1 h. EDTA was removed by dialysis prior to the dissolution assay. Dissolution of fibrillar A β was measured by from binding of Thioflavin T (ThT, 5 μ M) and fluorimetry (λ_{ex} 450 nm, λ_{em} 482 nm; PMT 600 V).

2.5. Fluorescence spectroscopy

Emission spectra (290–400 nm) of EDTA-inactivated IgV 2E6 (52 μ g/mL) in PBS containing 0.1 mM CHAPS and increasing ZnCl₂ concentrations (0.0225, 0.075, 0.225, and 0.75 mM) were collected using a Cary Eclipse spectrofluorimeter (Agilent Technologies; λ_{ex} 278 nm; excitation slit 10 nm, emission slit 10 nm, data interval 0.5 nm, PMT voltage 500 V).

2.6. Phosphonate binding

Formation of irreversible IgV adducts with diphenyl *N*-[6-(biotinamido)hexanoyl]amino(4-amidinophenyl)methanephosphonate (compound **1b**, prepared as in (Nishiyama *et al.* 2002)) was determined by SDS-gel electrophoresis as in (Planque *et al.* 2004). Briefly, the IgV (22 μ g/mL) pretreated for 1 h with EDTA (1 mM) or diluent was incubated with **1b** (0.1 mM) for 18 hours, boiled (5 min) in 2% SDS and subjected to SDS-PAGE (4–20% gels). After transfer to nitrocellulose membranes, biotin-containing bands were detected using streptavidin conjugated with horseradish peroxidase and a chemiluminescent substrate, and images were acquired with a ChemiDoc XRS system (Bio-Rad Lab).

3. Results

3.1. Metal chelator inhibition of A β degradation

IgV 2E6 prepared by Ni-NTA IMAC was reported to hydrolyze A β rapidly (Taguchi *et al.* 2008a). We screened the inhibition of IgV 2E6-catalyzed ^{125}I -A β 40 hydrolysis by inhibitors of the major enzyme classes (Fig. 1a, selected inhibitor structures shown in Fig. 1b): metalloprotease inhibitors, ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline; the cysteine protease inhibitor iodoacetamide; the Asp/Glu protease inhibitor pepstatin A; and the serine peptidase inhibitor diphenyl (benzyloxycarbonyl)amino(4-amidinophenyl)methanephosphonate **1a**. The tested inhibitor concentrations yield readily detectable enzyme inhibition (Beynon and Salvesen, 2001). Cysteine protease and Asp/Glu protease inhibitors were without effect. Phosphonate **1a**

inhibited the hydrolytic activity, consistent with the serine protease character of this catabody (Taguchi *et al.* 2008a). EDTA and 1,10-phenanthroline also inhibited the IgV hydrolytic activity completely, suggesting that a metal ion is required for the hydrolytic reaction. A β is known to bind certain metal ions (Warmlander *et al.* 2013). To exclude metal binding to A β as a factor, we repeated the ^{125}I -A β 40 hydrolysis test after removing EDTA from the chelator-pretreated IgV 2E6. The EDTA-free IgV did not hydrolyze A β (Fig. 1c). The metal chelator exerted a consistent inhibitory effect on IgV 2E6 catalyzed A β hydrolysis regardless of the IgV fractionation method. EDTA inhibited the A β hydrolytic activity of the anion exchange-fractionated paIgV 2E6 that had not been subjected to the IMAC procedure, and there was no detectable hydrolysis of A β upon removal of the EDTA from paIgV (Fig. 1c). It may be concluded that removal of an A β -bound metal ion is not the reason for the EDTA inhibitory effect.

Like the purified IgV, unfractionated culture supernatants of bacteria secreting IgV 2E6 hydrolyzed radiolabeled ^{125}I -A β 40. At the 1:30 fold dilution of the IgV 2E6 supernatant shown in Fig. 1d, A β 40 was hydrolyzed negligibly by equivalently prepared control supernatants from bacteria expressing empty vector, non-catalytic IgV MMF6 with the same scaffold structure as IgV 2E6, and mutated IgV 2E6-containing the IgV MMF6 framework regions. Background hydrolysis by bacterial proteases was observed only when undiluted control supernatants were used and reaction time was prolonged from 3h to 18h (*e.g.*, ^{125}I -A β 40 expressed per unit time for undiluted supernatant from bacteria expressing empty vector *versus* IgV 2E6, respectively, 383 ± 27 and $63,870\pm 8,040$ cpm/h/100 μL). As the unfractionated IgV 2E6 hydrolyzed A β , the hydrolytic activity was not acquired by IgV-metal interactions occurring during the IMAC procedure.

The IgV 2E6 catalytic activity was largely retained despite experimental manipulations over prolonged durations that can dissociate bound ligands. For example, the A β hydrolytic activity of paIgV 2E6 obtained by two rounds of anion-exchange chromatography was 86% of the starting IgV-containing bacterial culture supernatant. Similarly, 67% of the A β hydrolytic activity observed in IMAC-purified IgV was maintained following dialysis for 40 h against the catalysis assay buffer. It may be concluded that the IgV can be maintained in a sufficiently metal-bound stable catalytic state.

Nucleophilic attack by the catalyst is a rate limiting step in peptide bond hydrolysis (Fig. 1e). To determine whether removal of the IgV-bound metal decreases IgV nucleophilicity, we compared the reactivity of the hydrolytic IgV and EDTA-inactivated IgV with electrophilic phosphonate **1b** (Fig. 1a), a validated probe for protein nucleophilicity (Planque *et al.* 2004; Nishiyama *et al.* 2005). Formation of covalent phosphonate **1b** adducts with the catalytically active IgV and EDTA-inactivated IgV was comparable (bands with mass 30 kDa, Fig. 1f). Removal of the IgV-bound metal ion, therefore, does not attenuate the IgV nucleophilic reactivity, and the bound metal must facilitate a step in the catalytic cycle after completion of initial nucleophilic attack on the substrate (Fig. 1e).

We also tested the functional outcome of IgV inactivation caused by metal removal. Treatment with the hydrolytically active paIgV 2E6 solubilized pre-aggregated fibrillar A β 42 compared to the diluent and non-catalytic MMF6 controls, evident from reduced

Thioflavin-T binding to the IgV-treated fibrillar A β 42 (Fig. 1g). The EDTA-inactivated pIgV did not solubilize fibrillar A β . It may be concluded that the metal-assisted IgV hydrolytic activity was the cause of A β solubilization.

3.2. Zn²⁺ and Co²⁺ activation

The following metals were tested for the ability to restore the ¹²⁵I-A β 40 hydrolytic activity of EDTA-inactivated IgV 2E6: Mg²⁺, Ca²⁺, Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Cd²⁺, Hg²⁺ (0.075 mM metal dichloride salts). The hydrolytic activity was restored partially in the presence of Co²⁺ and Zn²⁺ (Fig. 2a). Other metals were without appreciable effect. Dose-response studies identified the optimal Co²⁺ and Zn²⁺ concentrations supporting IgV catalysis. At 0.225 mM Co²⁺ or Zn²⁺, the EDTA-inactivated IgV hydrolysed ¹²⁵I-A β 40 at levels comparable to the control IgV without EDTA treatment, indicating complete restoration of the activity by these metals (Fig. 2b,c). The IgV hydrolytic activity was markedly reduced at the highest Co²⁺ concentration tested (0.75 mM) and a smaller reduction of activity was also evident at this Zn²⁺ concentration. The inhibitory effect of 0.75 mM divalent metal was consistent in 3 repeat experiments. The findings suggest that low Zn²⁺ and Co²⁺ concentrations restore the IgV to its catalytic state. The differing functional effect at the high Zn²⁺ and Co²⁺ concentration may be explained by the distinct chemical properties of the two metals (different ionic radii, potentially different coordination numbers depending on the protein microenvironment)(McCall *et al.* 2000; Hashida and Inouye 2007).

3.3. Zn²⁺-stabilized IgV conformation

The fluorescence emission spectrum of the EDTA-treated IgV in the absence of Zn²⁺ contained an emission maximum (λ_{max}) at 336 nm (Fig. 3a; λ_{exc} 278 nm), a characteristics of tryptophan fluorescence. No change of emission intensity or λ_{max} was evident at the lowest Zn²⁺ concentration (0.0225 mM; no restoration of A β -hydrolytic activity was evident at this concentration). With increasing Zn²⁺ up to 0.225 mM, a concentration-dependent decrease of emission intensity at 336 nm evident (Fig. 3b), suggesting decreased IgV fluorophore exposure. There was no noticeable λ_{max} shift. The metal concentration yielding least emission intensity (336 nm) matches the concentration needed for full restoration of IgV hydrolytic activity. A further increase of Zn²⁺ to 0.75 mM induced a slight λ_{max} red-shift to 341 nm and a marked increase of emission intensity, suggesting increased fluorophore exposure. At this Zn²⁺ concentration, a break in the IgV hydrolytic activity curve was evident. Several potential Zn²⁺ binding residues were identified using METSITE (Sodhi *et al.* 2004) (17 sites with false positive rate set at 1%; top scoring sites with predicted accuracy of 99 and 80%, respectively, V_{L1} FR3 Glu81 and V_{L1} CDR2 Glu50; Kabat numbering, see Fig. 1a for IgV structure). The results suggest that Zn²⁺ binding induces IgV conformational transitions that influence the magnitude of the IgV catalytic activity.

4. Discussion

The studies reveal Zn²⁺ and Co²⁺ as alternate cofactors for IgV 2E6 catalyzed hydrolysis of A β . Removal of IgV-bound metal by chelating compounds inhibited the hydrolytic activity,

provision of $\text{Zn}^{2+}/\text{Co}^{2+}$ restored the activity, and loss of A β hydrolytic activity due to metal removal rendered the IgV incapable of dissolving fibrillar A β . Spectroscopic studies provided supporting evidence for Zn^{2+} induced conformational changes that stabilize the IgV in its catalytic conformation. Of 9 divalent metals tested, only the period 4 transition metals Zn^{2+} and Co^{2+} activated the IgV, indicating a comparatively strict metal requirement. Zn^{2+} binds and stabilizes numerous proteins, including enzymes, receptors and transporter proteins. $\text{Zn}^{2+}/\text{Co}^{2+}$ ions are coordinated by the side chain O, N and S atoms of Asp, Glu, His, Lys or Cys residues. As Co^{2+} can exert toxic effects (Llobet *et al.* 1986), Zn^{2+} is the preferred cofactor for the IgV.

IgV 2E6 uses the same serine protease-like nucleophilic mechanism for A β hydrolysis as catabodies directed to other polypeptide targets (Planque *et al.* 2004; Reshetnyak *et al.* 2007; Hifumi *et al.* 2012). As the nucleophilic reactivity is a germline antibody property developed by Darwinian evolution, the consistent serine protease-like mechanism of catabodies is not surprising. The possibility of applying co-dependence on a metal for identifying high-activity catabodies suitable for medical application, however, has not been examined adequately. Two groups reported metal-activated proteolysis by catabodies directed to HIV proteins and an autoantigen (Hifumi *et al.* 2000; Polosukhina *et al.* 2006; Baranova *et al.* 2010). Like IgV 2E6, these catabodies utilize the serine protease catalytic mechanism, but their metal-dependency appears to be less strict.

The his6 tag is unlikely to contribute in metal-activated IgV catalysis, as the his6-binding Ni^{2+} and Cu^{2+} ions (Terpe, 2003) did not substitute effectively for $\text{Zn}^{2+}/\text{Co}^{2+}$. Moreover, if the his6 tag is occupied by a metal ion, the hydrolytic IgV present in the bacterial culture supernatants cannot be fractionated by binding to immobilized Ni-NTA as observed. Unlike physiological antibodies, IgV 2E6 is composed only of the V-domains, eliminating metal binding to the constant domains as a factor regulating catalysis. The antibody V-domain repertoire is diverse, and individual V-domains express ligand-interacting sites with differing specificity, including sites located outside the complementarity determining regions responsible for antigen binding (Kang and Kohler 1986; Kohler and Paul 1998; Neshat *et al.* 2000). Allosteric stabilization of the IgV catalytic site in a high-activity conformation upon coordination of $\text{Zn}^{2+}/\text{Co}^{2+}$ at a remote site can be conceived. Precedents for allosteric metal activation of serine proteases are available, *e.g.*, Proteinase K activation by remote Ca^{2+} coordination (Betzl *et al.* 1988). Excess Co^{2+} binding appeared to down-regulate the IgV hydrolytic activity, and a break in the activity *versus* Zn^{2+} concentration dependence curve was also evident. Biphasic metal effects on enzymes due to binding at distinct metal-interacting site have been documented, *e.g.*, Zn^{2+} accelerates proteolytic auto-activation of Factor XII, but the rate is reduced by excess Zn^{2+} (Bernardo *et al.* 1993). Co^{2+} can serve as the replacement metal ion at protein Zn^{2+} -binding sites (Auld 1995; Hu *et al.* 2008), but differing concentrations of the two metals are required for activation and inhibition of metal-dependent enzymes displaying multiple metal-binding sites (Hashida and Inouye 2007; Sabel *et al.* 2011). Removal of bound metal did not decrease the IgV nucleophilic reactivity, suggesting that the metal is not involved in initiating IgV nucleophilic attack on A β . However, this leaves open the possibility that $\text{Zn}^{2+}/\text{Co}^{2+}$ activates the water molecule needed to hydrolyze the covalent A β -antibody intermediate,

analogous to the role of Zn^{2+} in hydrolysis of the covalent Ser-phosphate intermediate formed by an alkaline phosphatase (Stec *et al.* 2000). Our studies do not rule out, therefore, the alternative of a direct metal ion participation in the mechanism of catalysis.

Zn^{+2} is found at micromolar concentrations in peripheral blood (Chia *et al.* 2000), the preferred route for delivering proteinaceous candidate drugs. Initial tests indicated an A β -clearing effect of IgV 2E6 injected into the brain or intravenously in transgenic animal models of AD (Planque *et al.* 2012). The enzymes neprilysin and insulinase are candidates for AD immunotherapy (Fernandez-Gamba *et al.* 2009; Park *et al.* 2013). IgV 2E6 hydrolyzes A β at the His14-Gln15 bond, and when the IgV is maintained in its metal-activated state, A β is hydrolyzed at a rate comparable to the enzymatic rate (Taguchi *et al.* 2008a). Enzymatic hydrolysis at the His14-Gln15 bond generates non-amyloidogenic, non-toxic fragments A β fragments (Mukherjee *et al.* 2000). However, unlike neprilysin and insulinase (Duckworth *et al.* 1998; Sumitomo *et al.* 2005), the IgV does not hydrolyze irrelevant polypeptides (Taguchi *et al.* 2008a). Moreover, unlike A β -binding monoclonal antibodies, IgV 2E6 dissolves A β without forming immune complexes that activate inflammatory cells, thereby reducing the likelihood of counteracting neurotoxic effects (Paul *et al.* 2010). The metal-stabilized catalytic state of the IgV may be useful for accomplishing specific A β clearance.

Acknowledgments

This work was supported in part by National Institutes of Health grants R01AG025304 and U01AG033183. YN, SAP and SP have a financial interest in patents on catalytic antibodies and Covalent Bioscience Inc.

Abbreviations

AD	Alzheimer's disease
Aβ	amyloid β peptide
Aβ42	A β 1-42
Aβ40	A β 1-40
APP	A β precursor protein
BBB	blood-brain barrier
catabody	catalytic antibody
CDR	complementarity determining region
CAA	cerebral amyloid angiopathy
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
EDTA	ethylenediaminetetraacetic acid
FPLC	fast-protein liquid chromatography
FR	framework region
Ig	immunoglobulin

IgV	immunoglobulin variable domains
paIgV	partially purified IgV
IMAC	immobilized metal affinity chromatography
NTA	nitrilotriacetic acid
PBS	phosphate buffered solution
V domain	variable domain
V_H domain	heavy chain V domain
V_L	light chain V domain
ThT	thioflavin

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Highlights

- Chelation of bound metal inhibited A β hydrolysis by a catalytic antibody fragment.
- The metal accelerated a reaction step after initial antibody nucleophilic attack.
- The hydrolytic but not chelator-inactivated antibody dissolved A β aggregates.
- Zn-induced conformational transitions were associated with restored catalysis.
- The antibody requires bound metal for efficient A β hydrolysis and dissolution.

Fig. 1. EDTA inactivation of IgV 2E6 hydrolytic activity

(a) Enzyme inhibitor effects. ^{125}I -A β 40 was treated (18 h) with IMAC-purified IgV 2E6 (10 $\mu\text{g}/\text{mL}$) that had been preincubated (1 h) with phosphonate **1a** (1 mM), Pepstatin A (0.01 mM), 1,10-phenanthroline (0.5 mM), EDTA (1 mM) or iodoacetamide (1 mM). Data show % inhibition of hydrolytic activity compared to ^{125}I -A β 40 hydrolysis by the IgV in diluent ($7,165 \pm 193$ CPM). *Inset*, heterodimeric IgV composed of $\text{V}_{\text{L}1}$ and $\text{V}_{\text{L}2}$ domains. Filled and unfilled boxes correspond to framework regions (FRs) and complementarity determining regions (CDRs), respectively. **(b)** Phosphonate inhibitors and EDTA. **1a**, **1b** contain the electrophilic phosphonate diester. The biotin group of **1b** allows detection of irreversible protein adducts. EDTA chelates divalent metals. **(c)** Irreversible IgV inhibition by EDTA. IMAC-purified IgV 2E6 was pretreated with EDTA (10 mM, 1 h). ^{125}I -A β 40 hydrolysis was measured without or with removal of EDTA (- removal and + removal, respectively). Also shown are hydrolytic activities of EDTA-pretreated paIgV 2E6 (10 mM, 1 h) with and without EDTA removal (1:2,000 dilution of paIgV prepared by anion exchange chromatography). **(d)** ^{125}I -A β 40 hydrolysis by IgV 2E6-containing bacterial culture supernatants (diluted 1:30). Control culture supernatants displayed negligible hydrolysis (bacteria without vector or empty vector, bacteria expressing IgV MMF6 or IgV 2E6 with mutated FR1-FR4 segments). The activity of reference IMAC purified IgVs is also shown (10 $\mu\text{g}/\text{mL}$). ^{125}I -A β 40 treated for 18 h with culture supernatants (mean $\pm$ SD of 3 replicates). *Inset*, Lane 1, anti-myc antibody stained SDS-electrophoresis gel of IgV 2E6-containing bacterial culture supernatant. Lanes 2 and 3, respectively, anti-c-myc antibody-stained and silver-stained SDS-gels of IMAC-purified IgV 2E6. The 30 kDa and 18 kDa bands correspond to the hydrolytically active heterodimeric IgV and its inactive C-terminal V_{L} fragment produced by IgV autolysis respectively (Taguchi *et al.* 2008a). **(e)** Peptide bond hydrolysis pathway. $\text{R}_\text{N}-\text{CONH}-\text{R}_\text{C}$, substrate peptide; $\text{HNu}-\text{CAB}$, catabody containing a nucleophile ($\text{HNu}-$); $\text{NH}_2-\text{R}_\text{C}$, C-terminal substrate fragment; $\text{Wact}-\text{CAB}$, catabody containing a water activating group; $\text{R}_\text{N}-\text{CO}_2\text{H}$, N-terminal substrate fragment. The initial catabody reaction consists of nucleophilic attack on a peptide bond. Hydrolysis of the acyl-catabody intermediate requires a water activating group *e.g.*, a metal ion that facilitates water nucleophilic attack or stabilizes the oxyanionic ester bond transition state (not shown).

(f) **1b**-IgV 2E6 adducts. The **1b** adduct band (30 kDa) formed by IgV 2E6 (22 µg/mL) that had been pretreated with diluent or EDTA (1 mM, 1 h) was comparable. **1b**, 0.1 mM, 18 h incubation. *Inset*, streptavidin-peroxidase stained SDS-gels showing adduct bands formed by EDTA-treated IgV (lane 1) and diluent-treated IgV (lane 2). (g) Reduced fibrillar Aβ disaggregation by EDTA-pretreated paIgV 2E6. Following EDTA pretreatment as in panel C, the EDTA was removed by dialysis, and fibrillar Aβ42 (20 µM) was incubated for 24 h with the EDTA-pretreated paIgV 2E6 (+ bar), control paIgV pretreated with diluent (– bar) or non-catalytic paIgV MMF6 (4 µg/mL). Residual fibrillar Aβ42 was measured by the ThT fluorescence method. Data are expressed as % of ThT fluorescence observed using control fibrillar Aβ42 incubated with diluent (113 ± 6 FU). Mean ± SD of two replicates.

Fig. 2. Reactivation of EDTA-treated IgV 2E6 by Co²⁺ and Zn²⁺

(a) Screening of IgV reactivation by divalent metals. IgV 2E6 was inactivated by EDTA (10 mM, 1 h) and the EDTA was removed by dialysis. The EDTA-inactivated IgV 2E6 treated with Zn²⁺ and Co²⁺ but not the remaining metal ions hydrolyzed ¹²⁵I-Aβ40 detectably. Data are % of ¹²⁵I-Aβ40 hydrolysis by the IgV incubated in diluent (no EDTA/no metal, 8,850±486 CPM). IgV 2E6 10 µg/mL, metal dichlorides 0.075 mM, incubation for 18h. (b) Restoration by Co²⁺. IgV 2E6 was inactivated with EDTA as in panel A. ¹²⁵I-Aβ40 hydrolysis by the EDTA-inactivated treated IgV 2E6 (20 µg/mL, open circles) and the control IgV (closed circles) without EDTA treatment was measured in the presence of CoCl₂ (0.75, 0.225, 0.075, 0.0225 mM). (c) Restoration by Zn²⁺. Experimental details as in panel B.

Fig. 3. Zn^{2+} -induced IgV conformational transitions

(a) Fluorescence emission spectra of EDTA-inactivated IgV 2E6 (46 µg/ml, 1 h) treated with varying Zn^{2+} concentrations. (b) Fluorescence emission intensity at 336 nm plotted versus Zn^{2+} concentration. Data are from panel A.