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Research Paper

'Morphs' (MRFs): metal-reversible folding domains for differential IgG binding

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Abstract

Background: Selective recognition and binding of IgG molecules is the basis for a host of immunological and affinity purification techniques. Capture of an IgG in these procedures relies chiefly on its interaction with one of a variety of reengineered bacterial receptors which bind to the Fc region of IgG molecules with very high affinity. While this interaction is extremely efficient in trapping IgG molecules, the tight interaction between the binding partners often requires denaturing conditions for disruption of the complex, which can adversely affect the yield of purified IgG and also limit the lifetime of the receptor matrix. An effective receptor/IgG binding system which could be modulated by less extreme conditions is of considerable general interest.

Results: We describe the properties of a series of modified Fc receptor domains which are competent to bind IgG with high affinity but which can be reversibly unfolded upon addition of modest amounts of transition metal ions. Data are presented demonstrating loss of the secondary structural content of the domains as a function of increasing metal concentration, with a concomitant decrease in IgG binding affinity. Variants of the Fc receptor differing at a single amino acid position display increased sensitivity to metal-induced unfolding, while retaining comparable IgG binding ability in the absence of metal.

Conclusions: The interaction of this series of Fc receptors with metal ions abolishes IgG binding, but removal of metal ions allows refolding of the domains with restoration of the IgG binding function. Examples of the utility and potential applications of these metal-modulated IgG binding domains are discussed.

Keywords: IgG; B1 domain; Affinity purification; Metal binding; Protein design

1. Introduction

A variety of commonly employed biochemical and molecular biological procedures rely on the recognition of binding targets by specific antibody molecules, particularly class G immunoglobulins (IgG) [1]. Purification of IgG molecules is typically achieved via affinity separation by binding to one of a number of bacterially derived IgG receptor proteins [2]. The most common of these receptors are protein A from *Staphylococcus aureas*, protein G from group G *Streptococcus* and protein L from *Peptostreptococcus magnus*. Each of these cell wall proteins comprises several small extracellular domains that bind the Fc regions of IgG molecules with high affinity [3–6]. Engineered derivatives of these proteins retaining one or more of the IgG binding domains have been used to construct agarose- or sepharose-based affinity matrices for purification purposes [7–10]. Although these reagents are specific for IgG from numerous species (with varying species selectivity depending on the receptor used) [11] and therefore effect very clean separations, the necessary dissociation of the IgG–receptor complex is difficult to achieve because of the high affinity of the interaction. As a result, denaturation of the complexes – typically by extreme pH changes – is the most commonly used method for releasing the matrix-bound IgG targets. While these harsh treatments are effective for disrupting the complexes, the recovery of correctly folded, and therefore active, IgG may vary [12–14]. Any procedure employing a more gentle method of IgG receptor release is therefore of considerable general interest.

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Here we describe a series of modified Fc receptors based on the B1 IgG binding domain from protein G that can be reversibly unfolded by addition of transition metal ion salts. In the absence of metal ions, these domains are fully folded and retain the ability to bind IgG molecules with high affinity. However, addition of metal ions results in disruption of the folded structure of the domains sufficient to eliminate IgG binding. This effect is fully reversible: upon removal of metal ions by chelators such as EDTA, the domains refold and are once again competent to bind IgG. We present data illustrating the effect of metal ions on the structure and binding functions of a series of these domains – responsive over a range of metal ion concentrations – and suggest potential applications of these domains in IgG binding procedures, affinity separations of IgG molecules and receptor fusion constructs.

2. Results and discussion

2.1. Domain construction and disruption of secondary structure by metal

The Fc receptors reported here are all derivatives of two designed metal binding variants of the B1 domain of protein G (Fig. 1). These proteins, which bind metal with high affinity, each incorporate a designed metal binding site positioned at the interface of the domain's single α-helix and second β-strand. By contrast, the high affinity Fc binding site is located on the opposite side of the domain, and incorporates residues from the helix and third β-strand with no overlap with the metal site region. The site is comprised of three histidine residues at positions 16, 18 and 30 and a cysteine residue at position 33 [15]. The two metal binding B1 variants are differentiated by an additional mutation in one of them – a residue 5 substitution of methionine for the wild-type leucine – and are referred to as Zβ1M and Zβ1L reflecting this residue 5 difference. Both of these domains are folded and stabilized toward thermal denaturation in the presence of metal. Several variant domains have been constructed, in both the Zβ1M and Zβ1L backgrounds, in which the metal site Cys33 residue is substituted with a series of non-chelating amino acids. The mutants are named based on the individual Cys33 substitution and the background in which it was made, i.e. 'LMet' denotes Zβ1L with a methionine substituted for cysteine, 'MVal' denotes Zβ1M with valine substituted for cysteine. In all cases the substitution results in folded IgG binding domains in the absence of metal which, upon metal addition, display dramatic changes in secondary structure content.

Metal titration experiments of all of the domains show a progressive loss of secondary structure with increasing Zn(II) concentration as demonstrated by the loss of intensity in the respective circular dichroism (CD) spectra (Fig. 2A and B). The amount of metal required to achieve the maximum change is higher for the Zβ1L-based variants, but varies within that set for the different substitutions of Cys33, likely reflecting differing affinities for metal for each of the variants. Similar titrations followed by [^{1}H]NMR show well dispersed spectra for the apo proteins, similar to those of the wild-type B1 domain, which progressively broaden upon addition of increasing amounts of Zn(II) (Fig. 2C). Both of these experiments are consistent with a major alteration in the folded structure of the variants upon their interaction with metal.

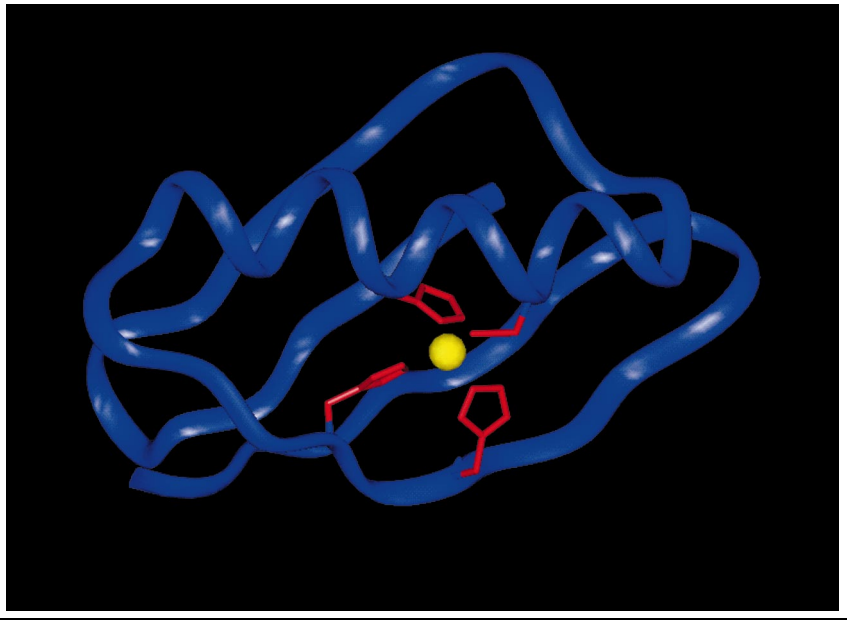

Fig. 1. Model of Zβ1. Ribbon drawing of the Zβ1 scaffold showing the metal site residues His16 and His18 on β-strand 2, and His30 and Cys33 on the helix. Based on the NMR structure of the B1 domain [31].

These metal-induced structural changes were also indicated in experiments with the fluorescent dye 8-anilino-1-naphthalene sulfonic acid (ANS). ANS is commonly used to follow changes in exposed hydrophobic surface area upon disruption of a protein's native structure [16,17]. Addition of ANS to the Fc receptor variants showed both intensity increases and λ_{max} shifts in the fluorescence emission spectrum of the dye upon addition of Zn(II) (Fig. 2D). As Zn(II) alone had no effect on ANS fluorescence, these results are consistent with the metal-induced perturbations indicated by the CD and NMR titration spectra. Together these experiments indicate that association with metal results in at least a partial unfolding of all of the Cys33 substitution variants.

2.2. Metal binding disrupts IgG binding

The effects of the metal-induced unfolding of the Fc receptor variants on their IgG binding properties were assessed in two different IgG binding assays. The first employed a competition assay for binding to IgG immobilized on microtiter plates. Each of the variant proteins was titrated against a fixed amount of wild-type protein G conjugated with alkaline phosphatase (G/AP) (Fig. 3A). Conjugate cleavage of the chromogenic alkaline phosphatase substrate *p*-nitrophenyl phosphate (PNPP) resulted in an increase in absorbance at 405 nm proportional to the amount of G/AP remaining after equilibration and washing of the IgG-coated wells. Thus, variants which were competent to bind IgG and, therefore, capable of competing away the G/AP conjugate, caused a decrease in A_{405} with increasing concentration. As shown in Fig. 3B, in the absence of metal all of the variants displayed an ability to effectively compete with the G/AP conjugate that was nearly identical to that of the unmodified B1 domain. Addition of Zn(II) to the equilibration mixtures when the concentrations of both G/AP and Fc receptor variants were held constant resulted in a corresponding increase in A_{405} with increasing amounts of added metal, indicating that the presence of Zn(II) disrupts the ability of the variants to effectively bind the target IgG (Fig. 3C). No effect of metal was seen on the ability of the unmodified B1 domain to compete for IgG binding. These data demonstrate that, while the domains retain nearly wild-type IgG binding activity, the presence of metal disrupts their folded structure and renders them unable to recognize and bind their targets.

The second assay was performed to further investigate the effect of metal binding on IgG binding, specifically, whether metal would have the same effect on the structure and binding of the modified Fc receptor domains if added to a pre-formed IgG/Fc receptor complex. For this experiment, a new variant was constructed incorporating a Gly-Ser-Gly-Cys linker at the C-terminus of one of the Fc receptor variants, LIle. The resulting protein, LIleC, was then quantitatively immobilized via disulfide linkage to a thiol-agarose resin (Fig. 4A). After addition and binding of whole human IgG to the column, the resin was washed and IgG eluted by addition of 100 mM $CdCl_2$ (Fig. 4B). Subsequent addition of EDTA to strip metal from the column resulted in refolding of the domains, allowing the resin to be used again for productive IgG binding (not shown). Repeated cycles of metal binding and EDTA stripping could be visualized in the absence of IgG by adding Co(II) to the column and monitoring the color change of only the portion of the resin that had been saturated with Fc receptor (not shown). Control experiments using underivatized thiol-agarose resin showed no retention/metal elution of IgG (Fig. 4B). These results indicate that the effect of metal on the Fc variants is a disruption of the native structure that results both in the prevention of IgG binding and in the disruption of pre-formed IgG/Fc receptor complexes.

2.3. Applications

We envision utilizing these domains, alone or in combination, as alternatives to the Fc receptors currently in use. Our domains, while retaining their high affinity IgG binding functions in the absence of metal ions, can be gently and reversibly unfolded without the need to denature their target IgG molecules required for the currently used systems. If necessary, removal of residual metal ions and/or chelator–metal complexes from the final sample is easily achieved via a gel filtration step subsequent to the unfolding of the domains and elution of the desired product. The reversibility of the unfolding reaction will likely result in affinity reagents with longer useful lifetimes than those which must be completely denatured for IgG release. This reversibility also makes possible the in situ activation/deactivation of the binding functions of these domains simply by adding transition metal ions or chelators to the buffer. These domains can be used in any application where immobilization and/or capture of intact, active IgG is desired.

The behavior of the domains in the ANS experiments also illustrates their potential utility as metal ion biosensors. The coupling of the binding event to an easily monitored signal, combined with the differing metal affinities exhibited by this series of variants, makes possible the construction of sensor arrays for detecting the presence of metal ions across a range of concentrations [18–21]. It may also be possible to design constructs with intrinsic reporter groups, to eliminate the need for external additions.

We also anticipate the utility of these domains as general affinity reagents to be used as protein expression tools. The successful overexpression of recalcitrant proteins by fusion to efficiently expressed partners has been repeatedly demonstrated [22–26]. The domains reported here all can be expressed in *Escherichia coli* at up to 20 mg/l of cell

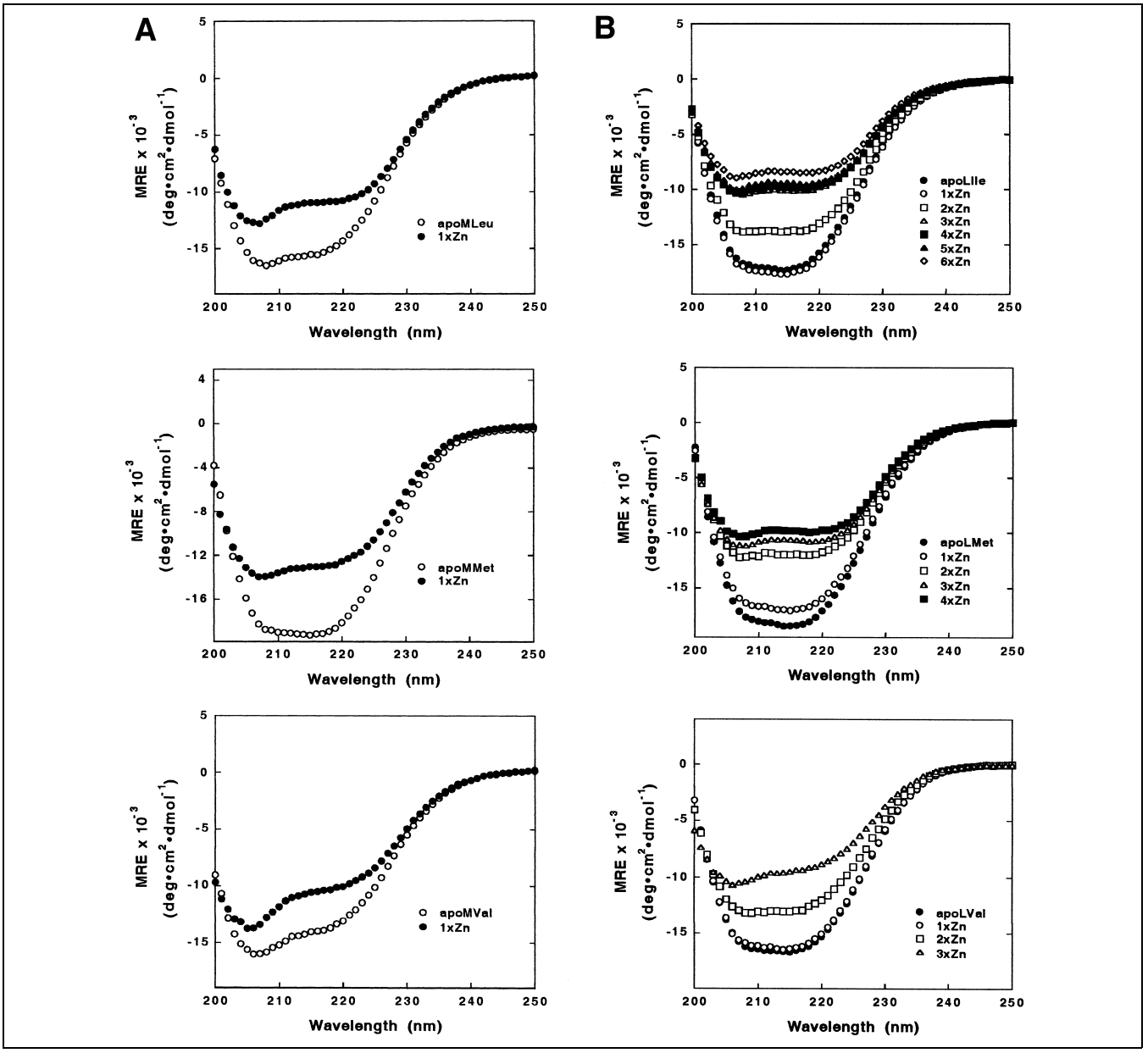

Fig. 2. Zn(II) titration spectra of ZB1 variants: A: CD spectra of MLeu (upper panel), MMet (middle panel) and MVal (lower panel) in the presence (filled symbols) and absence (open symbols) of one molar equivalent of Zn(II). The protein concentration in each case was 20 μM. B: CD spectra of LIle (upper panel), LMet (middle panel) and LVal (lower panel) with between three and six molar equivalents of Zn(II). The protein concentration in each case was 20 μM. C: ^{1}H NMR spectra of LIle without (i) and in the presence of 1 (ii), 2 (iii) and 3 (iv) molar equivalents of Zn(II). Protein concentration was approximately 2.5 mM. D: Zn(II)-induced changes in the fluorescence intensity and λ_{max} of emission in solutions of ANS (200 μM) and MVal (upper right), LMet (lower left) and LIle (lower right), containing one, three and five molar equivalents of $ZnCl_2$ respectively. All protein concentrations were 20 μM. No effect was observed with Zn(II) additions to ANS/buffer alone (upper left).

culture. Fusion proteins prepared with these domains could be overexpressed and then easily purified by passage over an IgG affinity matrix [27], after which the complex may be released by adding metal ions to the buffer, or the fusion cleaved by means of a protease cleavage signal engineered into the linker connecting the two proteins (Fig. 5). We believe that the combination of inexpensive, gentle, reversible unfolding conditions and ease of production will make these domains useful tools in many different applications.

3. Significance

Receptor-mediated recognition and capture of IgG molecules are important steps in many immunological protocols, both for immobilization/purification of the antibodies themselves and for affinity purification of desired antigens. Because the harsh conditions generally required for disruption of the receptor/IgG complex can adversely affect product yield, systems employing milder elution methods have broad application. We describe a series of modified

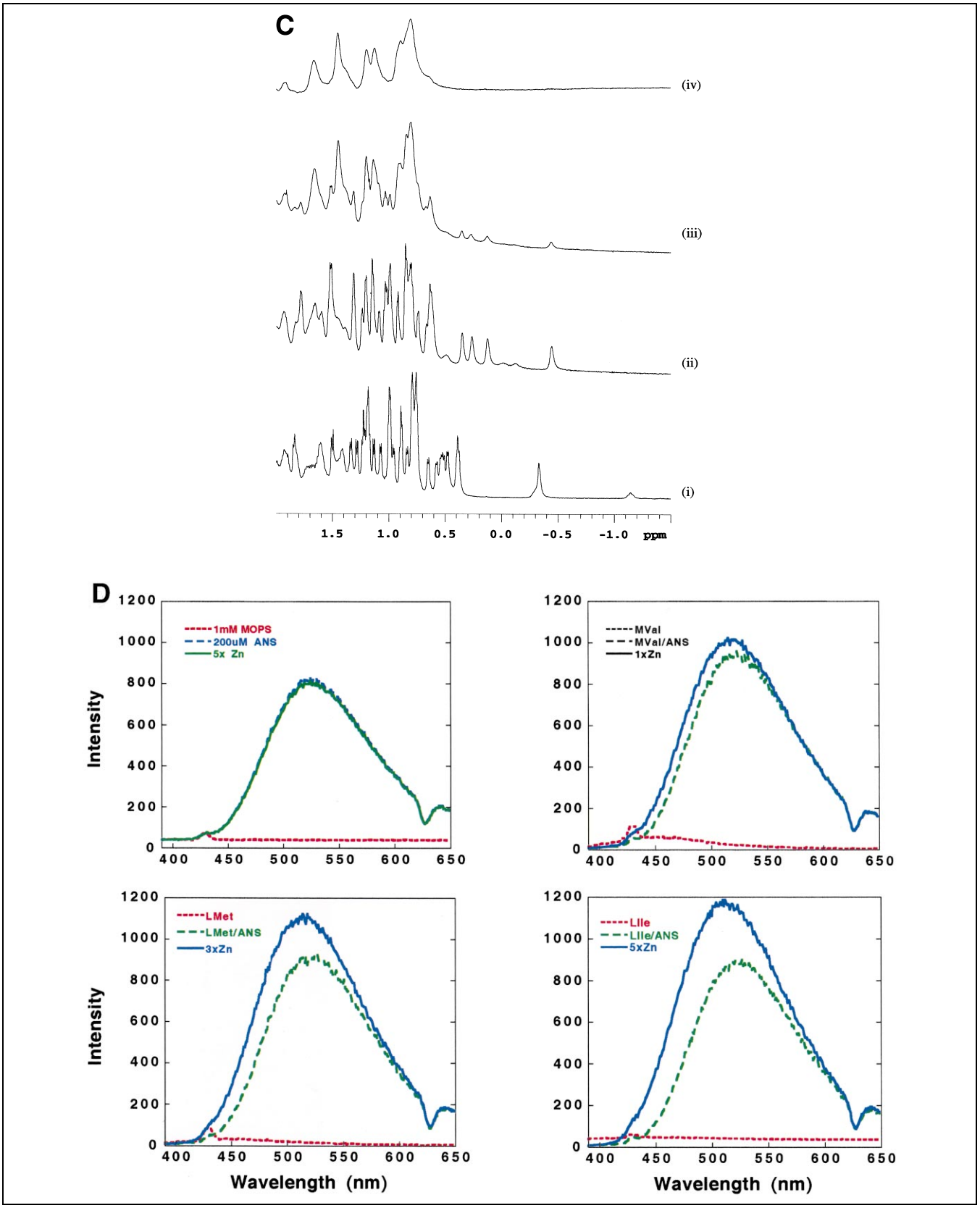

Fig. 2 (*Continued*).

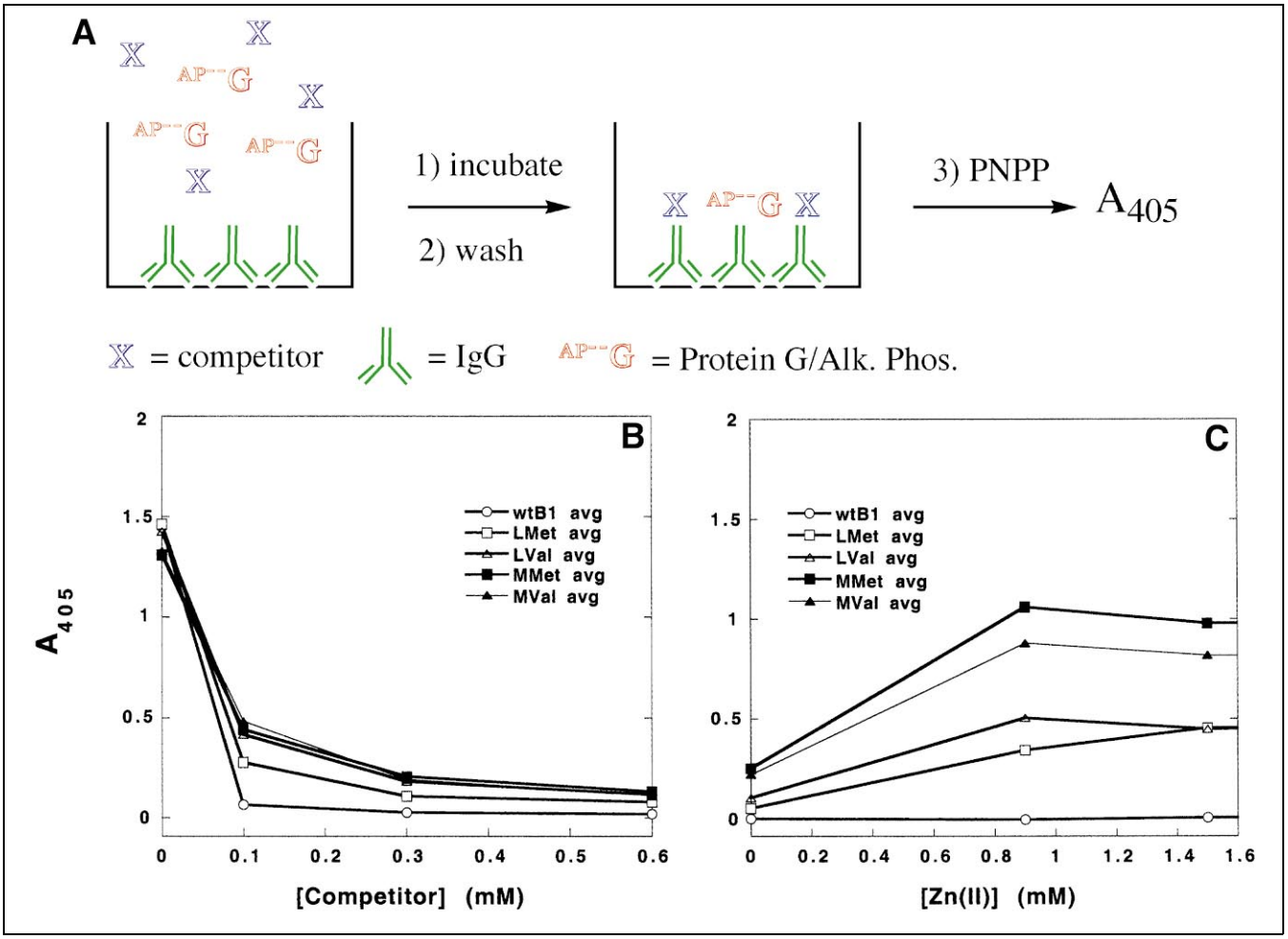

Fig. 3. Competition IgG binding assay for assessment of the folded structure of the Zβ1Cys33 variants. A: Diagram of assay for binding of immobilized IgG in the presence of wild-type protein G. B: Decrease in A_{405} as a function of increasing Zβ1 variant concentration, demonstrating that all of the apo proteins retain the ability to bind IgG as compared with the wild-type B1 domain and can efficiently compete away protein G with increasing concentration. C: In the presence of Zn(II), all of the variants lose their IgG binding function, consistent with a metal-dependent unfolding of the domains.

Fc receptors that are competent to form high affinity complexes with IgG molecules that are reversibly disrupted upon addition of transition metal ions. The binding of metal ions results in a dramatic change in the folded structure of the domains which is sufficient to prevent IgG binding and disrupt pre-formed receptor/IgG complexes. Removal of the metal ions results in the complete refolding of the domains, thus allowing the same domains to be used for repeated IgG binding steps. The range of metal sensitivities demonstrated for the receptor variants and their potential for use also in non-immunological procedures, define a flexible and versatile system amenable to a variety of applications.

4. Materials and methods

4.1. Construction of B1 variants

All Zβ1 variants were cloned according to standard protocols [28] into the vector pET11aΔ. pET11aΔ is a T7 expression vector and is identical to the pET11a vector except that it lacks a *Hin*dIII restriction enzyme site [29]. The genes for the Cys33 substitution mutants were constructed by PCR using the pZβ1M vector as template. The oligonucleotide SM6 (GCTA**CCGCGG**AAAAAGTTCACAAACAG[(AGT)T(CG)]GCTAA CGACAACG) was used as the forward primer in a PCR reaction with primer Asp4 (GCTCAGCGGTGGCAGCAGCCAAC) to introduce the Cys33 mutations. SM6 was synthesized with the degenerate sequence (AGT)T(CG) for codon 33 in order to produce a pool of primers containing six possible codons at this position, coding for the amino acids Ile, Leu, Met, Phe and Val [30]. The PCR products were then digested with *Sal*I and *Bam*HI and ligated into both pZβ1M and pZβ1L. All variants were expressed and purified as described previously [15].

4.2. Circular dichroism

Circular dichroism spectra were recorded on an Aviv 62DS instrument in 2 mm pathlength quartz cuvettes and were all acquired at 4°C. All protein samples were prepared by passage over

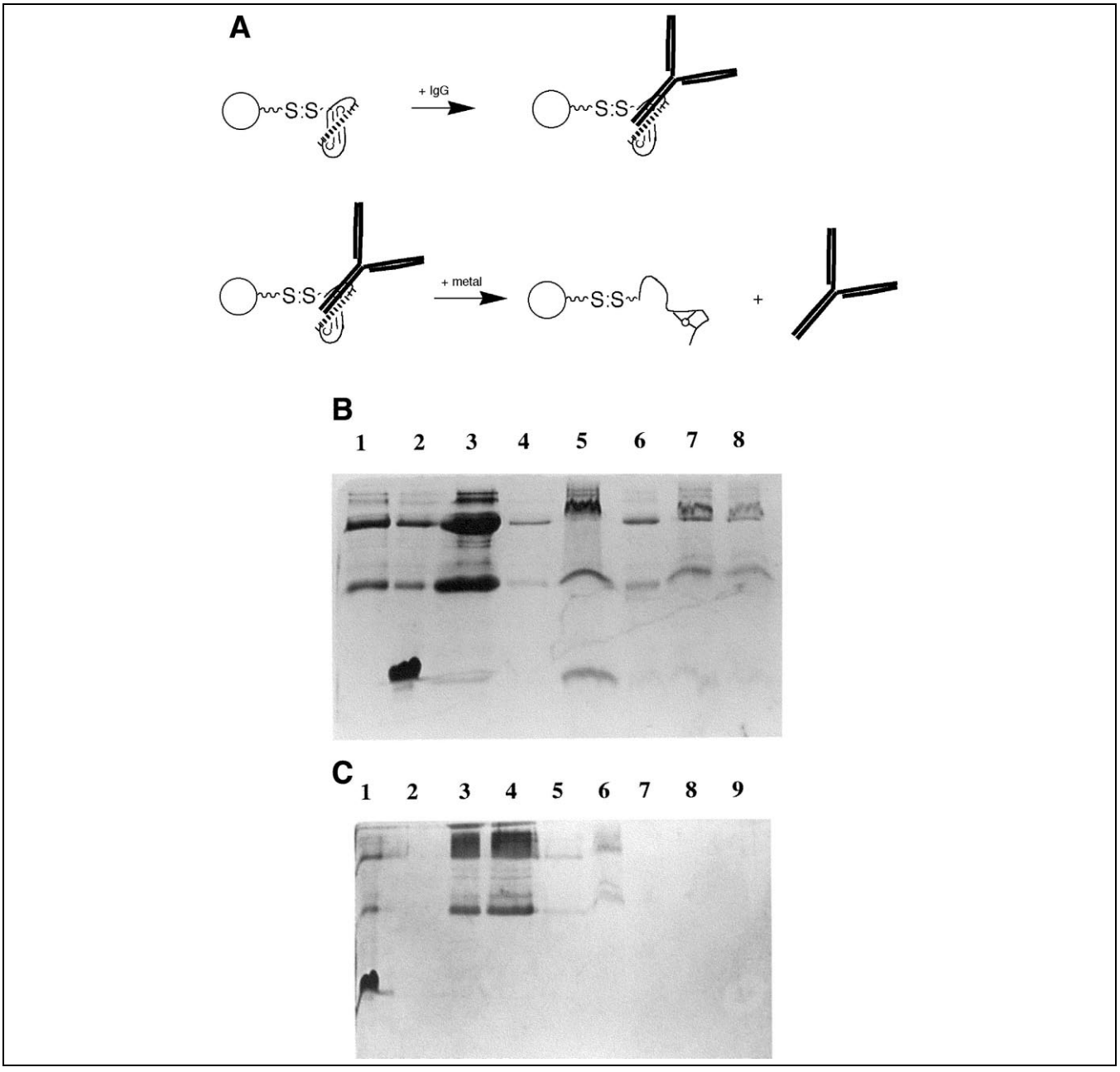

Fig. 4. Metal-dependent IgG binding by immobilized Zβ1LIleC. A: Diagram of affinity purification of IgG by immobilized LIleC. B: Specific IgG binding and elution from thiol-agarose immobilized LIleC: IgG and IgG+LIleC markers (lanes 1 and 2); post-loading column wash (lanes 3 and 4); IgG elution with 100 mM $CdCl_2$ wash (lanes 5–8). C: Control experiment performed with underivatized thiol-agarose resin: IgG and LIleC markers (lane 1); resin wash fraction (lane 2); IgG load and wash (lanes 3–5); resin wash with 100 mM $CdCl_2$ (lanes 6–9).

a PD-10 column (Pharmacia) into 1 mM MOPS, pH 6.8, and diluted to approximately 20 μM. All sample concentrations were verified by A_{280} before and after scans of metal titration samples. Scan data were plotted as MRE (mean residue ellipticity) according to:

$$\text{MRE} = \theta/[P]\times 0.2\times \#\text{ of residues}\times 0.01$$

where θ is the observed ellipticity, $[P]$ is the sample protein concentration (M), 0.2 is the cell pathlength (cm) and 0.01 is a unit conversion factor.

4.3. ANS fluorescence assays

Fluorescence measurements for ANS binding were obtained on an SLM 8100 spectrofluorometer at 4°C. Experiments were performed in a 1 cm quartz cuvette using 20 μM protein in 1 mM MOPS, pH 6.8 in a total volume of 900 μl. Spectra were recorded using an excitation wavelength of 374 nm and were taken with protein alone, after addition of 200 μM ANS and after addition of a specified amount of Zn(II) (as $ZnCl_2$). All spectra were corrected for dilution and baseline offset.

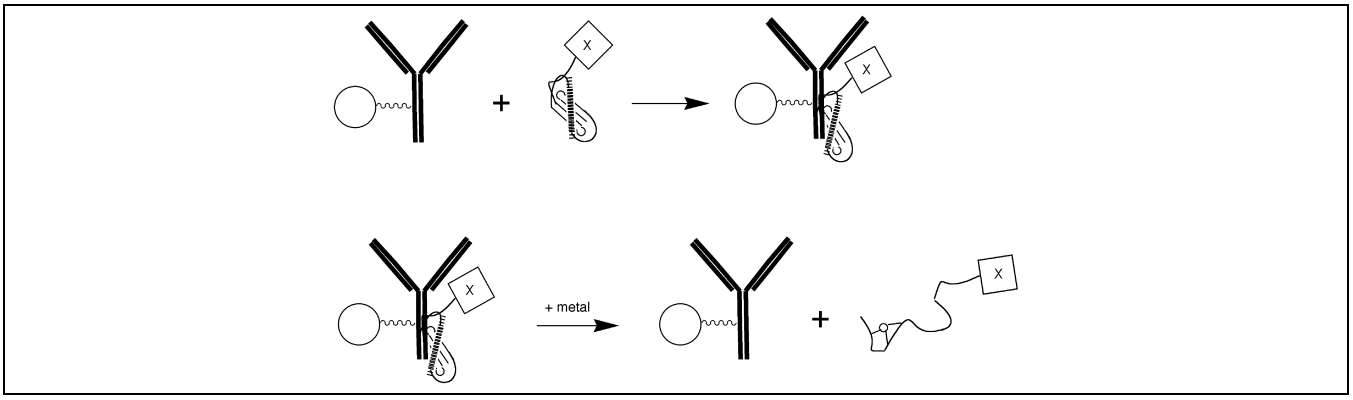

Fig. 5. Affinity purification of Zβ1 fusion constructs. Schematic of an IgG-based affinity purification of a protein of interest fused to a metal-dependent IgG binding Zβ1 variant.

4.4. Structure assessment by one-dimensional (1D) ^1H NMR

Protein samples prepared for NMR experiments were desalted into 20 mM Tris buffer, pH 6.9 and concentrated to approximately 2.5 mM by centrifugation in Centricon concentrators with a 3 kDa molecular weight cutoff (Amicon). Metal titrations were conducted by direct addition of $ZnSO_4$ in Tris buffer to the sample in the NMR tube, and were mixed by inversion. All NMR samples contained 10% D_2O as an internal standard and were collected using a Varian Unity 500 MHz magnet at 15°C and processed with the program Felix (Biosym).

4.5. IgG binding assays

Assessment of the IgG binding ability of each of the Zβ1 variants was performed by using a competition assay with a wild-type protein G/alkaline phosphatase conjugate (G/AP). The bottoms of wells of high protein binding 96-well microtiter plates (Immunosorp, Nunc) were coated with whole human IgG by addition of 50 μl of a 30 μg/ml solution in coating buffer (CB; 50 mM HEPES, 150 mM NaCl, 0.02% NaN_3, pH 7.2) and incubation at room temperature for 2 h. Care was taken to contact only the bottoms of the wells with IgG solution, in order to ensure reproducibility of signal from each set of wells. After incubation, the IgG solution was carefully removed via pipette and the wells were washed two times with 300 μl each of CB; the first wash was allowed to stand for 1 min before CB removal and subsequent wash. Coated, washed wells were then blocked by addition of 300 μl CB with either 10% FCS or 3% BSA added and allowed to stand overnight at 4°C. For IgG binding competition experiments, all components were added in a solution of CB with either 5% FCS or 1.5% BSA and 5 mM TCEP. A constant amount of G/AP (6.0 μg/ml) was added to each mixture, along with increasing amounts of competitor Zβ1 protein (between 0 and 0.6 or 1.2 mM). The 'wild-type' B1 domain used has the substitution T2Q to circumvent incomplete processing of the N-terminal methionine [15], and in all other respects behaves as the original B1 domain from *Streptococcus* (the Zβ1 mutants also contain the T2Q substitution). For experiments with metal present, the same G/AP concentration was used with 0.3 mM competitor protein (determined to be a saturating amount of competitor in all assays) in the same CB/block/TCEP buffer but with increasing amounts of added $ZnSO_4$ (between 0 and 1.3 mM). Final volume in all wells was 50 μl. Solutions were added to coated/blocked plates after washing the wells two times with CB with 5% FCS or 1.5% BSA. Mixtures were incubated at room temperature for 24–48 h before developing. Plates were developed by careful removal of the binding mixtures followed by washing four times with CB. Wells were then washed two times with diethanolamine substrate buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.5) and 50 μl of a 1 mg/ml solution of PNPP was added in the same buffer to initiate the developing reaction. The reactions were allowed to proceed for 15–30 min at room temperature before being quenched by the addition of 50 μl of 0.1 M EDTA followed by reading of A_{405} in a microplate reader. All reactions were performed in duplicate or triplicate and averaged. Deviations between A_{405} of identical wells were generally between 5 and 10% of the total absorbance.

4.6. Construction of Zβ1 variant IgG affinity resin

The Zβ1LileC variant was immobilized on a solid support to create an IgG affinity matrix. The oligonucleotide primer LIleCC was used in a PCR reaction to produce a modified LIle gene which resulted in the addition of a Gly-Ser-Gly-Cys tetrapeptide sequence to the C-terminus of the LIle protein. The resulting mutant was expressed and purified as described previously for the Zβ1 proteins. For column immobilization, pure LIleC protein was incubated with a 50-fold molar excess of DTT at room temperature for 20 min. DTT was removed from the reduced protein by passage over a PD-10 column into buffer containing 100 mM MOPS, 1 mM EDTA, pH 6.8. The resulting sample (typically between 1.2 and 1.6 μmol) was then applied to a column containing 2 ml of TNB-thiol agarose (Pierce), preequilibrated in the MOPS/EDTA buffer. The binding capacity of the TNB-thiol resin was approximately 5 μmol/ml. Binding of the LIleC mutant was quantified by measuring the concentration of TNB eluted during the binding and wash steps and generally exceeded 90% of the total loaded. The column was washed with MOPS/EDTA until the TNB absorbance at A_{410} reached baseline. Between 0.8 and 0.11 μmol of whole human IgG in MOPS/EDTA was then added to the column and the resin was washed with buffer until A_{280} reached baseline. IgG binding to the column was typically 50–60% of the total loaded. Bound IgG was eluted from the column by addition of 100 mM MOPS, 100 mM $CdCl_2$, pH 6.8, with washing until A_{280} reached baseline. Bound and eluted

IgG was assessed by A_{280} and on 18% reducing, sodium dodecyl sulfate (SDS)–polyacrylamide gels, to confirm which species were present in the eluates.

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