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Creation of a Novel Biosensor for Zn(II)

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The use of metal ions to augment the catalytic potential of the 20 natural amino acids is a recurring theme in enzymology. In a similar vein, we[1,2] and others[3–5] have sought to incorporate metal ions into antibodies in order to expand their scope of ligand binding and their catalytic repertoire. Our initial efforts have focused on introducing a Zn(II) binding site into the antigen combining site of antibody 43C9, which accelerates the hydrolysis of amide **1a** by a factor of 10^6 over the background rate (Scheme 1).[6] This antibody was chosen since it is one of the few catalytic antibodies for which both the mechanistic[7–9] and the structural[10] information necessary to guide our design effort is available. We selected Zn(II) as our target metal since its participation in facilitating hydroxide attack on a bound carbonyl center has been well characterized in enzymes such as carboxypeptidase A[11] and carbonic anhydrase.[12] We report here the construction and characterization of a derivative of 43C9 that is highly selective for binding Zn(II) and whose optical properties make it attractive as the basis of a biosensor for this metal ion.

Computer modeling techniques were used to construct a Zn(II) site in the antigen combining site of 43C9 using the metal binding site of carbonic anhydrase as a prototype.[1] The Zn(II) binding site was designed to include three histidine residues: an existing histidine at position H35[13] and two site-directed mutations, N-H33-H and Y-H95-H (Figure 1). The two mutations were sequentially introduced by the overlap extension method,[14] and the mutant protein was expressed and purified in single-chain antibody (SCA) form as described in detail elsewhere.[15]

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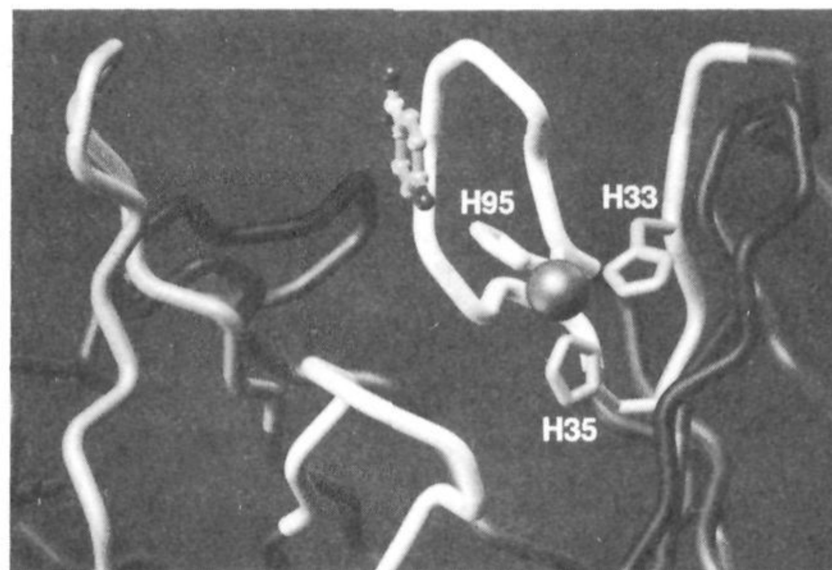
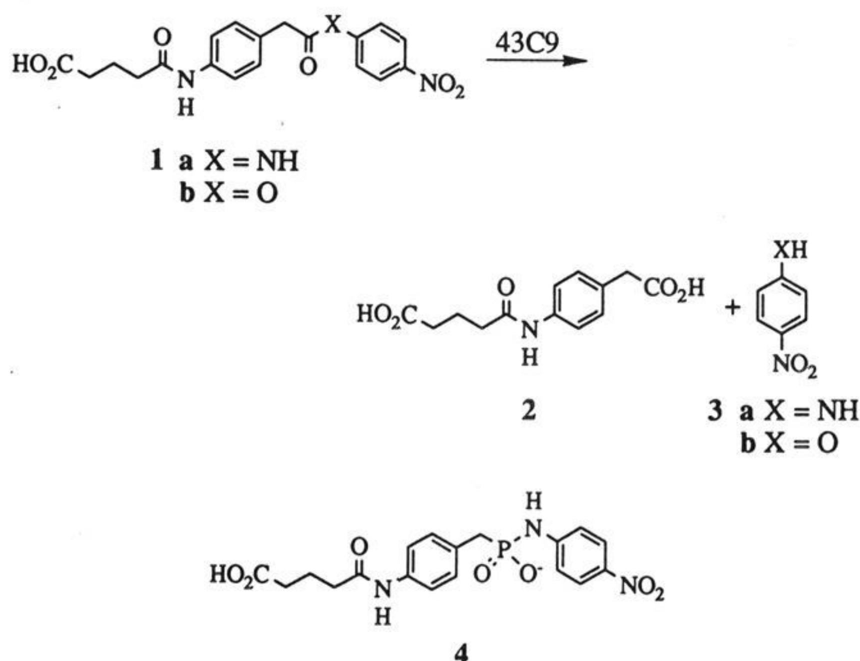


Figure 1. Model of the combining site of the N-H33-H, Y-H95-H 43C9 SCA with simultaneously bound *p*-nitrophenol and Zn(II). The variable light chain (left) and variable heavy chain (right) are shown as tubes of their Cα backbones, and each contributes three complementary-determining regions (CDRs) to the binding site: CDR1 (grey), CDR2 (black) and CDR3 (white). The designed site, consisting of three His side chains with bound Zn(II) (dark grey sphere), allows concurrent binding of *p*-nitrophenol (ball-and-stick model with dark grey spheres for oxygen atoms, top center).

Scheme 1



Ligand binding affinities for the mutant protein were determined by following the quenching of the intrinsic antibody fluorescence as a function of added ligand (Table 1).[16] Scatchard plots of these data verified the expected 1:1 antibody:ligand stoichiometry, indicating that all of the protein was properly folded. According to the computer model, the side chains of residues H33 and H95 form part of the antigen-binding pocket, and the combination of the N-H33-H and Y-H95-H mutations was found to decrease the binding affinity for hapten **4** by 3.5 kcal/mol. On the other hand, the affinities of the mutant antibody for the products of ester hydrolysis—acid **2** and phenol **3b**—were decreased by *ca.* 1.5 and 0.8 kcal/mol, respectively. Taken together, these results suggest that the binding ability of the protein is not seriously compromised, an important consideration for catalysis.

The binding affinity of the mutant antibody for a number of metal ions was assessed by fluorescence quenching titrations.

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(16) Fluorescence titrations were performed on 0.20 μM N-H33-H, Y-H95-H 43C9 single-chain antibody in a buffer containing 100 mM Na–HEPES, 50 mM NaCl, 10% glycerol, pH 7.5 at 25 °C. The intrinsic protein fluorescence (excitation = 280 nm, emission = 340 nm) was monitored as a function of added ligand, and the data were fit to a quadratic binding equation to determine the K_D value.

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Table 1. Ligand Binding Affinities, K_D, for the Wild-Type and Mutant Protein

	ligand			
protein	hapten **4** (nM)	acid **2** (μM)	phenol **3b** (μM)	Zn(II) (μM)
wild-type[a]	≤1	15 ± 1	0.6 ± 0.1	no binding
N-H33-H, Y-H95-H	370 ± 20	>200	2.5 ± 0.3	1.54 ± 0.09

[a] Reference 15.

Unexpectedly, Zn(II) was found to efficiently quench the antibody fluorescence with a K_D value of 1.54 ± 0.09 μM. The fluorescence of the wild-type 43C9 single-chain antibody was not quenched by the addition of Zn(II) under the same conditions. Quenching was due to reversible Zn(II) binding since the addition of excess EDTA to the antibody–Zn(II) complex restored >60% of the native fluoresence.[17] To assess the pH dependence of Zn(II) binding, the K_D values were determined at pH 6.8, 7.5, and 8.0 (3.78 ± 0.01, 1.54 ± 0.09, and 1.2 ± 0.2 μM, respectively). This slight variation in K_D values indicates that the protein ligands maintained the proper protonation states over this pH range. The binding of Mg(II), Ca(II), Mn(II), Fe(III),[18] Co(II), Ni(II), Cu(II), and Cd(II) (as their chloride salts) was also investigated by fluorescence titrations. None of these metal ions caused appreciable fluorescence quenching at concentrations up to 125 μM, nor did they reverse the fluorescence quenching of Zn(II) in competition experiments using similar metal ion concentrations. Although these metals may bind to the antibody, their K_D values must be at least 2 orders of magnitude higher than that of Zn(II). We believe that the surprisingly high selectivity exhibited by this antibody results from the spacing and orientational constraints placed on the metal site nitrogen ligands by the stereochemistry of the protein fold.

The mechanism of fluorescence quenching by Zn(II) is of interest since this diamagnetic ion normally produces only small changes in protein tryptophan fluorescence (<15%). Of the five Trp residues in the SCA form of 43C9, two (Trp L35 and Trp H36) are adjacent to disulfides and one (Trp H52)[10] is solvent-exposed, suggesting that they are not major contributors to the intrinsic antibody fluorescence.[19] Of the remaining two Trp residues, framework residue Trp H47 lies near the combining site and its Nϵ indole proton forms a hydrogen bond with the Nδ of His H35.[10,14] Perturbation of this hydrogen bond upon Zn(II) binding might have a large effect on the fluorescence of Trp H47 because changes in the environment of a nearby His residue can affect the fluorescence of Trp residues.[20]

Although the bound Zn(II) ion was designed to lie within the antigen-binding pocket, the location was expected to minimally affect the binding of other ligands. However, fluorescence techniques could not be used to demonstrate the simultaneous binding of Zn(II) and other ligands (**2**, **3b**, and **4**) because all of these molecules quench the intrinsic fluorescence of the mutant antibody to a similar extent. However, the absorbance spectrum of *p*-nitrophenol should be sensitive to its environment, and we reasoned that such changes might be useful in quantitating the simultaneous binding of Zn(II). Under conditions where 94% of the *p*-nitrophenol present was bound to the antibody, absorbance spectra were recorded at increasing concentrations of Zn(II) (Figure 2).[21] The binding of Zn(II) red-shifted the λ_{max} of the bound *p*-nitrophenol by *ca.* 2 nm and also altered the appearance of the long-wavelength portion of the spectrum. These spectral changes were completely reversed by the addition of excess EDTA. In addition, in the absence of antibody, a spectrum of 10 μM *p*-nitrophenol and 50 μM Zn(II) was superimposable on that of *p*-nitrophenol alone. The change in A_{440} versus [Zn(II)] yields a saturation curve for Zn(II) binding to the antibody–phenol binary complex (inset to Figure 2). By numerical simulation of the kinetic binding mechanism, the K_D value for Zn(II) binding to the SCA–phenol complex was determined to be 1.8 μM. Therefore, bound *p*-nitrophenol does not inhibit Zn(II) binding, and the two simultaneously bound ligands are close enough to interact electronically.

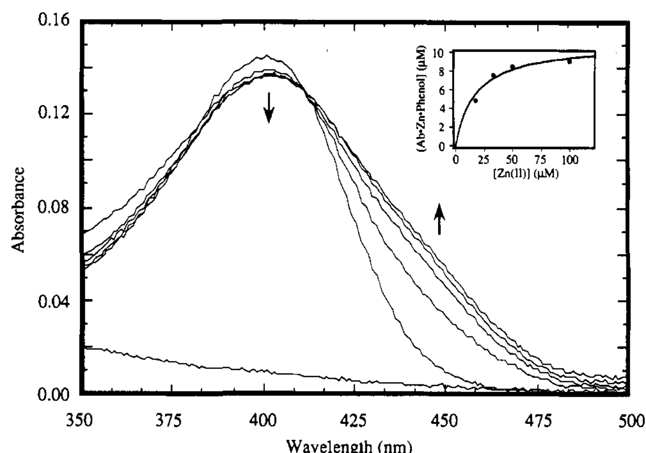

Figure 2. Titration of the N-H33-H, Y-H95-H 43C9 SCA–phenol complex with Zn(II). Absorbance spectra (from bottom): 1, N-H33-H, Y-H95-H 43C9 SCA (35 μM); 2, N-H33-H, Y-H95-H 43C9 SCA (35 μM) plus *p*-nitrophenol (10 μM); 3, As in curve 2, plus 16.7 μM Zn(II); 4, As in curve 2, plus 33.3 μM Zn(II); 5, As in curve 2, plus 50 μM Zn(II); 6, As in curve 2, plus 100 μM Zn(II). Inset: Saturation of the SCA–phenol complex by added Zn(II). The K_D value for Zn(II) binding was determined by numerical simulation of the kinetic binding equation.

The N-H33-H, Y-H95-H 43C9 SCA is an attractive basis for a Zn(II) biosensor. The binding of Zn(II) produces a large signal (an 85% decrease in protein fluorescence), allowing 5×10^{-8} M Zn(II) to be detected easily. Furthermore, the >100-fold selectivity for Zn(II) over other ions commonly found in biological samples makes the method highly specific. The SCA can be produced inexpensively in *Escherichia coli* and is stable at room temperature. Finally, by engineering substrates that contain *p*-nitrophenyl moieties as well as ligands for Zn(II), it may be possible to observe metal ion-assisted catalysis by this antibody, and these studies are currently in progress.

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Supplementary Material Available: A representative fluorescence titration with its associated Scatchard plot (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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