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Structural and Thermodynamical Insights into the Recognition of Native Proteins by Anti-Peptide Antibodies

A. A. Armstrong¹, James E. K. Hildreth², and L. M. Amzel^{1,*}

¹Department of Biophysics and Biophysical Chemistry, The Johns Hopkins School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205

²Department of Pharmacology and Molecular Sciences, The Johns Hopkins School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205

Abstract

The mechanism by which antibodies elicited against protein-derived peptides achieve cross-reactivity with their cognate proteins remains unknown. To address this question we have carried out the complete thermodynamic characterization of the association of a monoclonal antibody (260.33.12) raised against a peptide (SNpep) derived from staphylococcal nuclease (SNase) with both eliciting peptide and cognate protein. Although both ligands bind with similar affinity ($K_d = 0.42 \mu\text{M}$ and $0.30 \mu\text{M}$ for protein and peptide, respectively), protein and peptide binding have highly different thermodynamic signatures: peptide binding is characterized by a large enthalpic contribution ($\Delta H = -7.7 \text{ kcal/mol}$) whereas protein binding is dominated by a large entropic contribution ($-\Delta T \Delta S = -7.2 \text{ kcal/mol}$). The structure of the SNpep:Fab complex, determined by x-ray diffraction, reveals that the bound conformation of the peptide differs from the conformation of the corresponding loop region in crystal structures of free SNase. The energy difference, estimated by molecular dynamics simulations between native SNase and a model in which the Ω -loop is built in the conformation of the Fab-bound peptide, shows that the energetic cost of adopting this conformation is compatible with the enthalpic cost of binding the protein vis-à-vis the peptide. These results are compatible with a mechanism by which the anti-peptide antibody recognizes the cognate protein: high affinity is maintained upon binding a nonnative conformation by offsetting enthalpic penalties with reduced entropic losses. These findings provide potentially useful guidelines for the identification of linear epitopes within protein sequences that are well suited for the development of synthetic peptide vaccines.

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*Corresponding author. mamzel@jhmi.edu Phone: 410-955-3955 FAX: 410-955-0637.

Present Addresses: A. A. Armstrong, Janssen Research and Development, LLC, 145 King of Prussia Road, Radnor, PA 19087; J. E. K. Hildreth, College of Biological Sciences, University of California Davis, One Shields Avenue, Davis, CA 95616

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Accession Numbers

Coordinates for the structure of 260.33.12 Fab in complex with SNpep have been deposited in the PDB with accession number **2GSI**

Keywords

antibody; protein crystallography; isothermal titration calorimetry; peptide vaccine

Introduction

Synthetic peptides comprising the sequence of exposed stretches of chain in folded proteins have long been used for eliciting antibodies that recognize the native protein.^{1,2} Uses of this technology include development of artificial vaccines, therapeutic monoclonal antibodies, and reagents for research. Discussions of the process by which anti-peptide antibodies capable of recognizing the folded protein might be generated have considered whether cognate protein recognition is an event of high or low frequency. Early experiments supported the hypothesis that this was a high frequency event leading to the idea that the entire protein surface is antigenic and that little attention need be paid to the selection of the eliciting peptide sequence.² Subsequent work, however, indicated that in many cases the apparent high frequency of native protein recognition was an artifact of the methods employed for detecting binding to the protein.³ With this realization, a number of methods have been developed to identify sequences within a protein that have a higher probability of eliciting antibodies capable of binding the folded protein. Most often these methods employ a sliding window approach in which tabulated values for parameters relating to surface accessibility, hydrophilicity, antigenicity (prevalence of antigenic sequences), and/or relative mobility are summed for each stretch of sequence.⁴ Other methods have considered the antigenicity of amino acid pairs⁵ or employed neural network algorithms in epitope detection.⁶ These phenomenological approaches fail to consider the molecular underpinnings of protein recognition by anti-peptide antibodies.

Short peptides in solution have traditionally been regarded as unstructured, and yet some anti-peptide antibodies exhibit cross-reactivity with the corresponding folded protein. This phenomenon was referred to as the order-disorder paradox by Dyson *et al.*^{7,8} Two structural scenarios are possible: upon interacting with the antibody the protein might be distorted and adopt the conformation of the bound peptide or the bound peptide conformation may approximate a folded conformation of the protein. In fact, examples of both of these cases have been observed. Antibody BI3I2 raised against a peptide derived from the C-helix of myohemerythrin binds peptide and apo-myohemerythrin in similar conformations that differ from the conformation of the corresponding region in the unbound native protein.^{8,9} In contrast, an antibody raised against a peptide derived from influenza virus haemagglutinin binds the antigenic peptide in a conformation very similar to that of the corresponding sequence in the unbound native protein.¹⁰ Formally, a third possibility exists in which the bound conformations of the protein and peptide are fundamentally different. No examples of this situation have been reported to date, and this mechanism seems unlikely (although not impossible) as it would require a new set of fortuitous interactions between the antibody and the protein sequence flanking the eliciting epitope to compensate for the loss in binding energy resulting from the disruption of the network of contacts maintained between the peptide in the alternate conformation and the antibody. Much of the existing thermodynamic

and structural data relating to peptide:antibody interactions have been obtained using antibodies elicited against the cognate protein.

Here we report the complete thermodynamic characterization of the interaction of antibody 260.33.12, raised against a peptide fragment (SNpep) corresponding to residues 44–54 of the protein staphylococcal nuclease (SNase), with both the eliciting peptide and the cognate protein, as well as the crystal structure of the complex of the antibody (Fab) with the peptide. Our data suggest a possible mechanism by which anti-peptide antibodies achieve cross-reactivity with cognate proteins.

Isothermal titration calorimetric experiments reveal that SNase and SNpep bind to 260.33.12 with similar micromolar affinities but with very different thermodynamic signatures: the interaction of 260.33.12 with SNpep is characterized by a highly favorable enthalpic contribution whereas the interaction with SNase is characterized by a highly favorable entropic contribution. Furthermore, the structure of the complex of the 260.33.12 Fab with SNpep shows that the conformation of the peptide recognized by the antibody differs from the conformation of the corresponding loop region in unbound SNase. These data suggest that flexibility of the peptide has two opposing effects: while it allows for highly favorable interactions by accommodating to the antibody binding site, it pays a large entropic cost upon losing the large number of degrees of freedom it has in solution. In contrast either the protein or the antibody pays a large enthalpic cost for adopting a non-native conformation, but since the ends of the loop are fixed, the entropic cost is minimized.

Results

To define possible mechanisms of anti-peptide antibody cross-reactivity with the native protein we set out to identify a protein sequence with the correct characteristics to be used as an antigenic peptide. First, the sequence should be surface exposed to be accessible to the monoclonal antibody. Second, the sequence should contain some large residues to act as defining epitopes and to increase antigenicity. Third, the sequence should be structured but not rigid to increase the chances of successfully developing a cross-reactive monoclonal antibody by allowing recognition of a wider range of possible conformations of the cognate protein. Flexibility was predicted by consideration of B-factors in high resolution crystal structures, spatial constraints as determined by NMR, and stability as predicted by the program COREX.¹¹ Using these criteria, an 11-residue sequence corresponding to residues 44–54 from the Ω -loop within the well-studied protein staphylococcal nuclease (SNase) was selected (sequence TKHPKKGVEKY). The corresponding peptide (SNpep) was synthesized and used to generate a panel of cross-reactive monoclonal antibodies selected to bind native SNase.

Structure of 260.33.12-bound SNpep

Fab 260.33.12 in complex with SNpep crystallized in space group C222₁ with four copies of the complex per asymmetric unit (Figs. 1 and 2; Table 1). Superscripts A–D will be added following the molecule identifier to reference a particular complex in the asymmetric unit. Electron density for SNpep residues 47–54 was present within the combining site of all four Fabs (Fig. 2a; SNpep numbering reflects that of the corresponding SNase sequence). All

copies of the bound peptide were observed to adopt a similar conformation (Fig. 2b) with the largest Ca RMSD among the copies being 0.37 Å. Additional electron density was present for residues 45–46 of SNpep^A and for residues 44–46 of SNpep^B and SNpep^C; however, these residues do not interact significantly with the corresponding Fab fragments and are conformationally heterogeneous (Figs. 2b and 3). The lack of significant interaction between the SNpep N-terminus and 260.33.12 likely stems from an inaccessibility of these residues due to N-terminal coupling of the KLH carrier protein during immunization.

The most prominent feature of the bound conformation is a turn involving residues 48–51 which approximates a canonical type-II tight turn (Fig. 2). Additionally, Pro47 causes an angular deviation of the backbone of residues 44–46 of ~45° away from the opposite strand. This conformation of SNpep is stabilized by a combination of electrostatic, hydrogen bonding, and van der Waals interactions between the antibody CDRs and the peptide (Figs. 1b and 3; Table 2). No interactions are observed between the peptide and the antibody framework residues. Calculations of shape complementarity between SNpep and 260.33.12 yield an S_c value of 0.761 indicating a high degree of complementarity between peptide and protein.¹³ Comparable values have been observed for other antibody:antigen complexes.^{14,15}

Light chain interactions with SNpep are limited to the side chains of L-Thr27D (L-, H-, or pep- precede residue names to identify them as belonging to the 260.33.12 light chain, heavy chain, or SNpep polypeptide, respectively) and L-Tyr32 in CDR L1 and the backbone of residues 91–93 and side chain of L-Arg96 in CDR L3 (Figs. 1b and 3). L-Tyr32 packs against the side chains of pep-Lys48, pep-Val51, and pep-Glu52, and CDR L3 contacts involve the same set of SNpep side chains. No interactions are observed with CDR L2. Of the eight hydrogen bonds between SNpep and 260.33.12 found in 3 or more instances of the complex in the asymmetric unit, only one involves the light chain, occurring between the side chain amino group of pep-Lys48 and the backbone carbonyl of L-Arg92 (Fig. 1b; Table 2).

Interactions of SNpep with 260.33.12 are heavily biased toward the heavy chain CDRs (Figs. 1 and 3). The backbone of 260.33.12 heavy chain residues 30–32 contacts the side chain of pep-Lys53. 260.33.12 residue H-Tyr33 packs against the SNpep β -turn, interacting with the backbone of residues 48–53, as well as the side chain of pep-Lys53. The side chain hydroxyls of H-Tyr33 and H-Tyr35 participate in hydrogen bond interactions with the backbone carbonyls of pep-Glu52 and pep-Gly50, respectively. The side chains of residues H-Trp50 and H-Glu58 at the base of the CDR H2 loop interact with pep-Lys49 in the β -turn, and H-Asp52 and H-Glu53 at the tip of the CDR H2 loop make contacts with the side chain of pep-Lys53. CDR H3 residues 96–99 interact with SNpep C-terminal residues 52–53; however H-Met99 additionally contacts pep-Pro47. Residues 100 to 100B of CDR H3 could not be modeled in complexes A–C, but they are present in complex D where they do not interact with SNpep.

Calorimetry of SNpep binding to IgG 260.33.12

The results of isothermal titration calorimetry (ITC) experiments performed at 25°C for the titration of antibody 260.33.12 with SNpep are given in Table 3 (see also Fig. 4). At pH 7.4,

the interaction between 260.33.12 and SNpep is characterized by a dissociation constant of $0.30 \mu\text{M}$ ($\Delta G = -8.9 \text{ kcal/mol}$). Furthermore, entropic and enthalpic changes are both favorable: they contribute -1.2 kcal/mol ($-\Delta T \Delta S$) and -7.7 kcal/mol respectively to the binding free energy. Additional experiments at 20°C , 25°C , and 30°C were performed to estimate the change in heat capacity upon binding (ΔC_p), assuming linearity in the heat capacity change over this temperature range (Table 4 and Fig. 5). The value of ΔC_p , -490 e.u ($1 \text{ e.u.} = 1 \text{ cal/K}\cdot\text{mol}$; approximately -45 e.u./residue) falls within the typical range of heat capacity changes observed for peptide-protein interactions. Gomez and Freire report a value of -310 e.u. for the binding of the six residue pepstatin A by endothiapepsin,¹² and Varadarajan *et al.* determined values ranging from -640 to -960 e.u. for the interaction between point mutants of a truncated version of S-peptide and S-protein in the ribonuclease S system.¹⁶

To determine whether peptide binding occurs with the uptake or release of protons,¹⁷ ITC experiments were performed in buffers differing in ionization enthalpy (Table 3). Association occurs with the uptake of 0.5 protons at pH 7.4 and with the release of 0.7 protons at pH 6.0 suggesting that over this pH range at least two titratable groups undergo pKa shifts resulting in proton exchange with the buffer. Because this exchange occurs close to neutral pH, it is likely that at least one of these groups is a histidine side chain. SNpep contains a single histidine residue (His46), and the only histidine present within the 6 CDRs of 260.33.12 is His90 of CDR L3. As detailed above, this CDR has limited interaction with SNpep.

Calorimetry of SNase binding to antibody 260.33.12

ITC experiments involving the titration of SNase into 260.33.12 were carried out in phosphate buffer at pH 7.4 (Fig. 4 and Table 4). SNase binds 260.33.12 with a dissociation constant of $0.42 \mu\text{M}$ ($\Delta G = -8.7 \text{ kcal/mol}$), similar to that of the SNpep:260.33.12. Both enthalpic and entropic contributions to the free energy are favorable, but in contrast to SNpep binding, SNase binding occurs with an entropic contribution ($-\Delta T \Delta S = -7.2 \text{ kcal/mol}$) that is significantly greater than the enthalpic contribution ($\Delta H = -1.5 \text{ kcal/mol}$).

Discussion

An understanding of possible mechanisms by which anti-peptide antibodies achieve cross-reactivity against the parent protein from which the antigenic peptides were derived requires both structural as well as thermodynamic data detailing the binding events. We have obtained these data for one such system involving the protein staphylococcal nuclease (SNase). The crystallographic temperature factors for residues 44–54 comprising an omega loop are among the highest observed in SNase. Nevertheless, a survey of deposited crystal structures indicates that this region is structured; it adopts a tight turn involving residues 46–49 and a type II β -turn involving residues 52–55. The crystal structure of SNpep bound to monoclonal antibody 260.33.12 reveals that SNpep does not adopt this conformation when bound to the antibody (Figs. 1, 2, and 6). Instead the SNpep bound conformation is a type II β -turn involving residues 48–51. As no crystallographic structure of SNase in complex with 260.33.12 is available, a model was constructed for the complex using the SNpep:260.33.12

structure and the structure of unbound SNase (Fig. 6b). The model suggests that the SNase Ω -loop can adopt the bound peptide conformation with minimal conformational disturbance to residues flanking the antigenic sequence upon 260.33.12 binding. This would require significant conformational rearrangement within the antigenic sequence itself; however, the resulting enthalpic penalties would be partially offset by the intrinsic interactions between SNase and Fab, similar to the interactions observed in the peptide:antibody structure, as well as by any heteroclitic interactions occurring in the SNase complex that are not possible for the SNpep complex (Supp. Tables 1–3). The fact that this loop is intrinsically flexible in the SNase structures reported to date would additionally make the enthalpic cost of changing its conformation less onerous. The calorimetrically determined value of the binding enthalpy of -1.5 kcal/mol is 6.2 kcal/mol less favorable than that of binding the peptide, consistent with these structural considerations.

Given the large change in loop conformation required for binding that is suggested by the model, the energetic cost of SNase adopting such a conformation was estimated using a series of short molecular dynamics simulations. A 50,000 femtosecond simulation was first performed on the modeled SNase conformation following energy minimization. Importantly, the loop conserved the bend where modeled but in the absence of contacts with the Fab was drawn closer to the bulk of the SNase molecule. To estimate the energetic cost of adopting the bound loop conformation, the potential energy averaged over the production phase of this simulation was compared to values obtained from simulations performed under similar conditions for unbound SNase. Crystal structures deposited in the PDB under accession codes 1KAA and 1STN were used as starting models for these simulations. These two structures align with a main chain RMSD of 0.19 angstroms, and are nearly identical with the exception of differences in omega loop backbone torsions involving primarily residues 49 and 50. It was found that the potential energy difference between bound and unbound conformations was 6.7 kcal/mol when using 1KAA and 18.9 kcal/mol when using 1STN (Table 5). The experimentally determined enthalpic change upon SNase binding of -1.5 kcal/mol additionally includes favorable interactions between SNase and Fab that are not reflected in the energies calculated from the simulation. It is important to note, however, that the differences in energy between unbound and bound conformations of SNase, 6.7 kcal/mol when starting from 1KAA and 18.9 kcal/mol when starting from 1STN, are slightly less than and slightly more than, respectively, the difference in energies between the simulations employing different starting conformations of unbound SNase (12.2 kcal/mol). These differences are on the order of 1% of the potential energy of the system. Furthermore, the difference in energy between bound and unbound SNase simulations results primarily from non-bonded vdw and electrostatic terms rather than from the bonded terms that might reflect strained geometries. The increased energies arising from the non-bonded terms might be expected due to reduced interaction between the loop and SNase body in the bound conformation, and would be offset by interactions with the Fab in the complex.

Despite this validation of our structural model, alternative modes of binding cannot be completely ruled out. For example, slight differences in the interactions between protein and peptide complexes not reflected in our model of the protein complex might account for the observed differences in binding enthalpy. The bound SNase loop may also adopt a conformation other than that of the bound peptide. In this case the antibody CDRs must

undergo major conformational adjustments to accommodate this second pose of the same sequence. By necessity, the sets of interactions between antibody and peptide and antibody and protein would be very different, and thus the same CDRs would have to function in dual roles in order to engage both protein and peptide with reasonably high affinity. To our knowledge there are no examples of an antibody that recognizes the same sequence in both the context of an unstructured peptide and a folded protein in different conformations. Formally, it is also possible that the SNase epitope is a site other than the Ω -loop. This represents the most unlikely scenario as it would necessitate the existence of an entirely new set of specific interactions between SNase and 260.33.12.

Although 260.33.12 binds SNase and SNpep with similar affinity, the two interactions occur with different thermodynamic signatures. The driving force for peptide binding is a large favorable enthalpic contribution (-7.7 kcal/mol) with entropy contributing favorably but to a lesser degree (-1.2 kcal/mol). In contrast, the interaction of SNase with 260.33.12 is entropically driven. These data support a limited number of possible mechanisms by which this anti-peptide antibody may recognize the native protein. If 260.33.12 binds SNase in a non-native conformation an affinity comparable to that of the eliciting peptide can be maintained through an offset of the enthalpic penalties associated with conformational rearrangements in the protein by comparatively small entropic losses when binding the protein relative to those involved in peptide binding. Alternatively, the mechanism of cross-reactivity employed in this antibody-peptide/antibody-protein pair might not involve rearrangement of the protein epitope but rather rearrangement of the antibody CDRs to accommodate the native protein conformation. In this case, the antibody would recognize the same sequence in two distinct conformations. The reduced favorable enthalpic contribution to binding would stem from a penalty imposed for CDR rearrangement, suboptimal recognition of the protein sequence, or a combination thereof. Similar studies will undoubtedly reveal additional thermodynamic signatures resulting from other possible mechanisms of cross-reactivity employed by anti-peptide antibodies.

Our study provides one possible interpretation of the structural studies involving anti-peptide and anti-protein antibodies recognizing epitopes on the V3 loop of the HIV-1 envelope glycoprotein gp120.^{18,19} Antibody 447-52D presumably recognizes a native loop conformation as it was generated as part of the antibody response in an HIV-1 infected individual. Solution and crystal structures of V3_{MN} peptides bound by 447-52D reveal a peptide conformation that differs from those observed in crystal structures of V3_{MN} peptides in complex with anti-peptide antibodies directed against the V3 loop that are capable of neutralizing HIV-1 infection.^{18,19} These anti-peptide antibodies may employ a mechanism of cross-reactivity similar to that supported by this work.

The observations discussed above have important implications for the development of peptide vaccines. In some cases flexible peptides may be preferable to constrained peptides (*e.g.* through cyclization) for eliciting antibodies that recognize the complete protein. Furthermore, in addition to their accessibility, loop regions might serve as ideal targets because they are able to accommodate structural rearrangement with relatively minor enthalpic penalties. Enthalpic losses can be almost entirely recovered by entropic gains

given the limited conformational freedom of the loop in the parent protein relative to that of the eliciting peptide.

Materials and Methods

Peptide Synthesis and Purification

SNpep (TKHPKKGVEKY; residues 44–54, SNase numbering) was synthesized on an Applied Biosystems 430 large scale peptide synthesizer using standard Fmoc chemistry. Crude SNpep determined to be ~75% pure by mass spectrometry was used without further purification in hybridoma development. SNpep used in calorimetry and crystallization experiments was purified by reverse-phase HPLC to >95% purity.

SNase Expression and Purification

E. coli strain AR120 transformed with a SNase overexpression plasmid was a gift from Dr. Bertrand Garcia-Moreno (Johns Hopkins University, Department of Biophysics). SNase expression was induced by IPTG addition to log phase cultures. Cells were pelleted by centrifugation and resuspended in 100 mL of ice-cold Extraction Buffer 1 (EB1; 6 M urea, 25 mM Tris, pH 8.0, 2.5 mM EDTA) per 200 mL original volume of cell culture. Following a 20 minute incubation at 4°C on an orbital shaker, cells were re-pelleted and subsequently resuspended in 50 mL of ice-cold Extraction Buffer 2 (EB2; 6 M urea, 25 mM Tris, pH 8.0, 2.5 mM EDTA, 200 mM NaCl) per 200 mL original volume of cell culture. Resuspended cells were incubated on ice for 30–40 minutes on an orbital shaker. Cell debris was cleared by centrifugation. Contaminants were precipitated by addition of an equal volume of ice-cold ethanol followed by incubation at –20°C for 2.5 to 5 hours and removed by centrifugation. SNase was precipitated by adding an additional equal volume of ice-cold ethanol followed by incubation at –20°C for 30 minutes. SNase was pelleted and resuspended in 10 mL of ice-cold EB1 per 200 mL of original culture volume. SNase was further purified by cation exchange chromatography (Source 15S; GE Healthcare), and eluted from the column with a gradient from EB1 to EB2. SNase-containing fractions were pooled and dialyzed against the different buffers required for the different experiments.

Hybridoma Development and Screening

Balb/c mice were immunized with an SNpep-KLH (KLH: keyhole limpet hemocyanin) conjugate using standard protocols.²⁰ A biotin-SNase capture assay was employed to screen for hybridomas actively secreting antibody capable of recognizing folded SNase. For this assay the wells of an ELISA plate were coated with Fc-specific rabbit anti-mouse F(ab)₂ fragments and blocked with 3% BSA in PBS. Culture supernatants containing secreted IgG were subsequently added and incubated for 1 hr. After washing with PBS containing 0.05% Tween 20, biotinylated SNase (2 µg/mL in PBS containing 5% normal goat serum) was added, and the plate was incubated at room temperature for 1 hour. Wells were emptied and washed as above prior to the addition of a streptavidin-peroxidase conjugate. A 30 min room temperature incubation and wash step preceded addition of the TMB (tetramethyl benzidine) substrate (0.2M Na acetate, 0.1% H₂O₂). The reaction was allowed to proceed for 5 min and stopped with 0.5 M sulfuric acid. Extent of reaction was measured by determining the absorption at 450 nm. For positive wells, the corresponding hybridomas were expanded,

rescreened as necessary, and subcloned by limiting dilution on normal Balb/c splenocyte feeder cells. Peptide binding was assayed by direct ELISA. Structural and calorimetric results reported herein pertain to the antibody produced by one of the subcloned hybridoma lines. This monoclonal IgG is referred to as 260.33.12.

Antibody Production and Purification

260.33.12 hybridoma cells were injected into the peritonea of Balb/c mice and ascites fluid was periodically collected. Cleared ascites was diluted 1:3 with 60 mM sodium acetate, and the pH adjusted to 4.8. Contaminating proteins were precipitated by addition of caprylic acid (0.4 mL per 10 mL of original ascites volume), and removed by centrifugation. The supernatant was dialyzed against PBS buffer, and IgG was precipitated by the addition of ammonium sulfate to 55% saturation at 4°C. The IgG was collected by centrifugation, redissolved in PBS buffer, and dialyzed extensively against PBS. The variable domain sequence of monoclonal antibody 260.33.12 was determined by the method of Orlandi *et al.*²¹

Fab Production and Purification

Antibody 260.33.12 (~2 mg/mL in 185 mM NaCl, 0.9 mM EDTA, 9 mM cysteine) was digested by papain at a ratio of 120:1 (w/w) followed by incubation at 37°C for 10 hours. Cleavage was quenched by the addition of iodoacetamide, and the sample dialyzed against 20 mM Tris, pH 8.0. Cleavage products were resolved with a mono-Q anion exchange column (GE Healthcare). Fractions containing Fabs of similar isoform composition as assessed by isoelectric focusing (IEF) gels were pooled, dialyzed against PBS, and concentrated.

Crystallization and Structure Determination of the SNpep:260.33.12 Complex

SNpep was added to 260.33.12 Fab in 9-fold excess. Crystals were grown at 18°C by the hanging drop vapor diffusion method, mixing equal volumes of protein and reservoir solution comprising 18% PEG 2000, 16% isopropanol, 100 mM sodium citrate pH 5.0. The crystals were determined to have four Fab/SNpep complexes in the asymmetric unit. Diffraction data were collected at NSLS beam line X25. Intensity data were indexed and scaled using HKL2000.²² Phases were determined by molecular replacement using the programs AMoRe²³ and Molrep^{24,25} and PDB 1F11 as the search model. Maps were subsequently improved by replacing the initial model with the structures of different V_H (1CR9), V_L (1F58), C_L (2AJU), and C_H1 (2AJU) domains determined at higher resolution and having greater sequence similarity to 260.33.12. The structure underwent rounds of rebuilding and refinement using the programs O²⁶ and Refmac5,^{25,27} respectively. SNpep was built into 2mF_o-DF_c density present at the antigen combining site. TLS (translation, libration, and screw rotation) parameters were refined in latter stages of refinement. Stereochemical quality of the model was assessed using the programs Procheck²⁸ and Molprobtity.²⁹ The programs NCONT²⁵ and HBPlus³⁰ were used for contact analysis, SC¹³ was used for shape complementarity calculations, and PyMol³¹ was used to generate structural figures.

Modeling of the SNase:260.33.12 Complex

A model for the SNase:260.33.12 Fab complex was made using the crystallographic structures of unbound SNase (PDB 1STN) and the structure of the SNpep:260.33.12 Fab complex making the reasonable assumption that the SNase Ω -loop adopts the bound conformation of SNpep. As the N-terminal three residues of SNpep were not observed to interact with 260.33.12 the corresponding SNase residues were allowed the conformational freedom necessary for residues 47–54 to adopt the bound peptide conformation while attempting to minimize the conformational deviations for residues flanking the region 44–54. Using the program O,²⁶ the C α of SNase residue 54 was first positioned on the C α of SNpep residue 54 in the SNpep:Fab structure, and SNase was then rotated about this anchor point to alleviate steric clash between SNase and the Fab and to minimize the distance between C α atoms of residue 44 in SNase and SNpep. The coordinates for SNase residues 47–54 were then replaced with those for SNpep, and the disconnect between SNase residues 46 and 47 was repaired using a combination of manual movements, coordinate replacements with preferred C α and side chain conformations, and stereochemical refinements over the SNase residue range 43 to 47.

The energetic cost of SNase adopting the conformation of the loop observed in the bound peptide was estimated by molecular dynamics (MD) calculations. CHARMM³² version 35b1 with the CHARMM 35b1 force field was used in the computations with a distance dependent dielectric constant. The original coordinates were optimized by minimizing the energy with 1,000 cycles of conjugate gradient minimization followed by 1000 cycles of adopted-basis Newton-Raphson minimization. NVE MD simulations were run for 50,000 femtoseconds. The average value of the energy and its fluctuations during the last 40,000 femtoseconds were calculated with an in-house written program.

Isothermal Titration Calorimetry

Thermodynamic parameters for the binding of SNpep and SNase to 260.33.12 were determined by isothermal titration calorimetry (ITC) using a VP-ITC (MicroCal, LLC, Northampton, MA). For SNase:260.33.12 binding experiments, SNase and 260.33.12 were dialyzed against the same volume of PBS. For SNpep:260.33.12 binding experiments, IgG was dialyzed against the chosen buffers, and buffer from the final dialysis step was used to reconstitute lyophilized SNpep. All experiments were performed using a reference power of 5 μ cal/s, stirring speed of 300 rpm, and time between injections ranging from 220 to 300 seconds. The cell concentration of 260.33.12 was 5–10 μ M for SNpep experiments and 40–50 μ M for SNase experiments with the syringe concentrations of SNpep and SNase being 150–300 μ M and 600–1,300 μ M, respectively. Each experiment consisted of a single 2 μ L injection of SNpep or SNase into 260.33.12 followed by 24 to 31 injections of 10 μ L each. Data analysis was performed with the software package Origin 5.0. The integrated heats were fit using a single-site binding model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

SNase	staphylococcal nuclease
SNpep	peptide derived from SNase sequence
K_d	dissociation constant
Fab	antigen-binding fragment
NMR	nuclear magnetic resonance
RMSD	root-mean-square deviation
KLH	keyhole limpet hemocyanin
CDR	complementarity determining region
L1/2/3, H1/2/3	first, second, and third hypervariable loops of the light and heavy chains, respectively
ITC	isothermal titration calorimetry
C_p	constant pressure heat capacity change
e.u.	entropy unit
IPTG	isopropyl beta-D-1-thiogalactopyranoside
PBS	phosphate buffered saline
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
EB1/2	extraction buffers 1/2
ASA	accessible surface area
PDB	Protein Data Bank

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Highlights

- A system was devised to study the mechanisms by which anti-peptide antibodies react with native proteins.
- Antibody 260.33.12 was raised against a peptide derived from a loop in staphylococcal Nuclease (SNase).
- Structure determination shows that 260.33.12 binds peptide in a SNase non-native conformation.
- 260.33.12 binds peptide and protein with similar affinities but different enthalpies.
- When binding SNase, relative entropic gains offset enthalpic penalties.

Figure 1.

Structure of SNpep in complex with 260.33.12 Fab. **a**, 260.33.12 Fab is shown in surface representation. CDRs are colored as follows: H1 (green), H2 (blue), H3 (cyan), L1 (red), L2 (yellow), L3 (orange). Bound SNpep is shown in stick. **b**, Stick representation showing the interactions between SNpep and 260.33.12 Fab. SNpep is colored pink, and residues from the 260.33.12 Fab light and heavy chains making significant interactions with SNpep are shown in dark blue and light blue, respectively. SNpep residues 44–46 make few contacts with 260.33.12 and are shown in ribbon representation. Intermolecular hydrogen bonds are

shown as black dashed lines and SNpep intramolecular hydrogen bonds are shown as red dashed lines.

Figure 2.

Electron density and conformation for bound SNpep. **a**, SNpep is shown in pink stick representation. mFo-DFc omit map for SNpep contoured at 1.5σ is shown as a blue mesh. **b**, Overlay of all copies of SNpep in the crystallographic asymmetric unit showing N-terminal divergence of the peptide conformation. The same copy of SNpep shown in **a** is shown in pink stick representation. Other copies are shown as black lines.

Figure 3.

Contacts between SNpep and 260.33.12. Schematic summarizing the contacts between bound SNpep and 260.33.12 Fab. Heavy chain residues are positioned above the SNpep sequence (labeled), and light chain residues are positioned below. 230.33.12 residues are grouped according to CDR (labeled). Lines connect two residues if at least one atom in each residue is separated by a distance of 5 Å or less. Red, blue, green, and black lines indicate that a contact between residues is observed in 1, 2, 3, or 4 instances of the complex in the crystallographic asymmetric unit. Blue, green, and black coloring of the SNpep sequence indicates the respective residues are modeled in 2, 3, or 4 instance of the complex in the asymmetric unit.

Figure 4.

Representative isothermal titration calorimetry curves. (left) Titration of SNpep into 260.33.12 in PBS buffer, pH 7.4 at 20°C, (middle) titration of SNpep into 260.33.12 in borate buffer, pH 9.0 at 25°C, and (right) titration of SNase into 260.33.12 in PBS buffer, pH 7.4 at 25°C. The top panel shows heat signal as a function of time. The bottom panel shows the integrated heats as a function of the molar ratio of SNpep or SNase to IgG.

Figure 5.

Temperature dependence of thermodynamic parameters for SNpep:260.33.12 interaction. Entropy differences are plotted in the top panel. In the bottom panel free energy and enthalpy differences are plotted as circles and diamonds, respectively. All data series were fit to a line. The near zero slope of the fit to the free energy reflects the entropy-enthalpy compensation present in this system. The slope of the fit through the observed enthalpies is a measure of the change in heat capacity at constant pressure (C_p) for the binding event and has a value of -490 e.u.

Figure 6.

Model of 260.33.12 bound SNase. **a**, Cartoon representation of a model for SNase interaction with 260.33.12. The model was constructed as described in Methods and Methods. SNase is colored green, and 260.33.12 light and heavy chains are colored dark blue and light blue, respectively. 260.33.12 V_H, V_L, C_{H1}, and C_L domains are labeled. **b**, (left) Overlay of a crystal structure of SNase (PDB 1STN; magenta; labeled) on the model of 260.33.12 bound SNase (green; labeled) showing the extent of Ω-loop (labeled) deformation required for binding. (right) Crystal structure of 260.33.12:SNpep complex shown for comparison to the model of bound SNase. SNpep is colored pink and labeled. SNpep amino- (N) and carboxy- (C) termini are labeled.

Table 1

Crystallographic statistics

Data Collection	
Source	X25
Wavelength (Å)	1.1
Space group	C222 ₁
Cell dimensions	
a,b,c (Å)	105.7, 131.0, 291.3
α,β,γ (°)	90, 90, 90
Resolution (Å)	50–2.8 (2.9–2.8)
Completeness (%)	84.6 (83.6)
Number of reflections	42035 (4113)
Redundancy	4.4 (4.8)
R _{sym} ^a (%)	12 (49)
<I>/<σ(I)>	24.1 (4.9)
Refinement	
Resolution (Å)	48.7–2.8 (2.9–2.8)
Number of reflections	41984 (2918)
R _{work} ^b /R _{free} (%)	22 (30) / 29 (42)
Number of atoms	13378
Protein	13309
Water	62
Other	7
B-factors (Å ²)	37.70
Protein	37.74
Water	29.62
r.m.s.d.	
Bond lengths (Å)	0.015
Bond angles (°)	1.39
Molprobit ^c	
Favored (%)	92.9
Allowed (%)	5.9
Outliers (%)	1.2
Clash score	74 th Percentile
Molprobit score	53 rd Percentile
PDB Code	
	2GSI

Statistics for highest resolution shell are shown in parentheses

$$^a R_{\text{sym}} = \sum hkl \sum i |I(hkl)_i - \langle I(hkl) \rangle| / \sum hkl \sum i \langle I(hkl) \rangle$$

$$^b R_{\text{work}} = \sum hkl |F_o(hkl) - F_c(hkl)| / \sum hkl |F_o(hkl)|$$

^c Calculated with the program Molprobit (ref. 29)

Table 2

Hydrogen bonds between 260.33.12 and SNpep

SNpep Residue (Atom)	260.33.12 Residue (Atom) ^a	Chain W ^b	Chain X ^b	Chain Y ^b	Chain Z ^b
K48 (NZ)	L:R92 (O)	3.0	2.6		3.1
K49 (NZ)	H:E58 (OE2)	2.5			
G50 (N)	H:Y33 (OH)				2.8
G50 (O)	H:Y35 (OH)	2.9	2.7	3.0	2.5
V51 (O)	H:I97 (N)	3.3	3.3	2.8	2.8
E52 (O)	H:Y33 (OH)	3.2	2.9	3.1	
E52 (OE1)	L:Y32 (OH)	2.7			
K53 (N)	H:I97 (O)	2.7	3.1	3.1	
K53 (O)	H:M99 (N)	3.2		3.3	2.9
K53 (NZ)	H:K30 (O)	3.0		3.1	3.1
K53 (NZ)	H:Y32 (O)			2.8	
K53 (NZ)	H:D52 (OD1/2)	2.8	2.8	2.8	3.1
K53 (NZ)	H:E53 (OE1)	3.0			3.1

Hydrogen bonds calculated with the program HBPlus (ref. 30)

^aLight and heavy chains are denoted with prepended letters L and H, respectively.

^bSNpep copy. Distances given in Å.

Table 3

Thermodynamic parameters describing SNpep:260.33.12 interaction at 25°C

pH	Buffer	N _{H+}	H ^o _{ion} ^a (kcal/mol)	H ^o _{obs} ^b (kcal/mol)	H ^o ₀ (kcal/mol)	G ^o _{obs} (kcal/mol)	S ^o _{obs} (e.u.)	S ^o ₀ ^c (e.u.)
6.0	Cacodylate	-0.65	-0.7	-3.2	-3.7	-9.4	20.6	19.0
6.0	MES	-0.65	3.5	-6.9	-4.6	-9.1	7.6	15.3
6.0	BisTris	-0.65	6.8	-7.5	-3.1	-8.7	4.1	18.9
	Average ^d				-3.8 +/- 0.7	-9.1 +/- 0.3		17.7 +/- 1.9
7.4	Phosphate	0.53	0.9	-7.0	-7.4	-8.0	3.5	1.9
7.4	HEPES	0.53	5.1	-5.3	-8.0	-9.3	13.6	4.5
7.4	Imidazole	0.53	8.8	-2.8	-7.4	-8.8	20.1	4.5
	Average ^d				-7.7 +/- 0.4	-8.9 +/- 0.6		4.0 +/- 1.9
9.0	Borate	-0.02	3.3	-6.6	-6.6	-9.4	9.3	9.5
9.0	Bicine	-0.02	6.3	-5.0	-4.9	-9.2	14.1	14.5
9.0	TAPS	-0.02	9.7	-6.6	-6.4	-9.0	8.0	8.6
9.0	Ethanolamine	-0.02	12.1	-7.1	-6.9	-8.5	4.7	5.5
	Average ^d				-6.2 +/- 0.9	-9.0 +/- 0.4		9.5 +/- 3.9

^aStandard enthalpies of ionization are those tabulated in Goldberg *et al.* (ref 33) for reactions proceeding at 298.15 K.

^bH₀ is the experimental enthalpy corrected for the buffer enthalpic contribution.

^cS^o₀ was calculated using the corrected enthalpy (H^o₀) according to the relationship G^o= H^o-T S^o.

^dAverage values and estimated standard deviations reported for each pH are calculated over individual experiments.

Table 4

Thermodynamic parameters for interaction of SNpep and SNase with 260.33.12 at varying temperatures

	Temperature (°C)	$K_{a,obs}$ (M^{-1})	G°_{obs} ($kcal \cdot mol^{-1}$)	H°_{obs} ($kcal \cdot mol^{-1}$)	S°_{obs} (e.u.)
SNpep	20	6.7×10^5	-7.8	-4.4	11.5
SNpep	25	7.6×10^5	-8.0	-7.0	3.5
SNpep	30	9.6×10^5	-8.3	-9.4	-1.0
SNase	25	2.6×10^6	-8.7	-1.5	24.5
SNase	30	1.5×10^6	-8.6	-1.3	24.2

Experiments were performed in PBS, pH 7.4.

Table 5

Average energies from molecular dynamics simulations of SNase

	Model	+/-	PDB ISTN	+/-	$E_{\text{Model}} - E_{\text{ISTN}}$	PDB 1KAA	+/-	$E_{\text{Model}} - E_{\text{1KAA}}$
Potential	1221.59	23.63	1202.67	22.41	18.92	1214.85	23.21	6.74
Bond	372.00	14.39	372.02	15.39	-0.02	370.82	14.11	1.18
Angle	977.43	21.02	979.69	20.14	-2.26	975.11	21.31	2.32
Dihedral	565.95	13.20	561.03	13.54	4.92	568.75	14.21	-2.80
Improper	66.49	5.50	66.13	5.47	0.36	66.20	5.61	0.29
van der Waals	-629.13	17.38	-635.72	16.09	6.59	-634.23	16.29	5.10
Electrostatic	-269.31	8.81	-279.60	9.74	10.29	-268.20	9.40	-1.11

All energies are reported in kcal/mol. +/- values represent fluctuations over the course of the simulation.