The classical view of immunoglobulin molecules posits two functional domains defined by the variable (V) and constant (C) regions, which are responsible for antigen binding and antibody effector functions, respectively. These two domains are thought to function independently. However, several lines of evidence strongly suggest that C region domains can affect the specificity and affinity of an antibody for its antigen (Ag), independent of avidity-type effects. In this study, we used isothermal titration calorimetry (ITC) to investigate the thermodynamic properties of the interactions of four V region identical monoclonal antibodies (mAbs) with a univalent peptide antigen. Comparison of the binding of IgG1, IgG2a, IgG2b and IgG3 to a 12-mer peptide mimetic of Cryptococcus neoformans polysaccharide revealed a stoichiometry of 1.9 to 2.0 with significant differences in thermodynamic binding parameters. Binding of this peptide to the antibodies was dominated by favorable entropy. The interaction of these antibodies with biotinylated peptides manifested greater enthalpy than for native peptides indicating that biotin labeling affected the types of Ag-Ab complexes formed. Our results provide unambiguous thermodynamic evidence for the notion that the C region can affect the interaction of the V region with an Ag.

Antibody (Ab) binding to its antigen (Ag) is a fundamental step for the development of protective adaptive immune responses. Understanding the biophysical properties of antigen-antibody interactions is essential to comprehend the evolution of the adaptive immune response. Like other protein–protein associations, antigen–antibody complexes arise from non-covalent interactions including electrostatic and van der Waals forces, hydrogen bonds and hydrophobic effects (1,2). A critical condition for Ab-Ag binding is the formation of a specific complex between the Ab and the Ag. Understanding the interaction of these two biological macromolecules requires detailed knowledge of the structure and functional characteristics of the complex. The structure of the Ab-Ag complex can be described using x-ray crystallography and computer generated structural models. The functional activity can be described by the kinetic rate constants, equilibrium constants and thermodynamic parameters of the binding complex.

Historically, it was widely assumed that the antibody heavy constant (C\text{H}) domain determines Ab isotype without directly affecting Ag binding affinity and/or specificity. This concept dates to the discovery that when B cells switch from one C\text{H} region to another they maintain the same variable (V) regions, leading to the inference that the avidity and effector functions of an Ab change without altering the specificity for the antigen (3). Hence, the classical view of Ab function was that of a bi-functional molecule with the V domains being solely responsible for Ab affinity and specificity while the C region was responsible for the biological properties such as complement activation, Fc receptor binding, avidity and serum half-life (4).
However, in recent years this dogma has unraveled with the accumulation of new data, which suggest that the C₃ region can affect V region structure, thereby affecting Ab affinity and specificity (5-13). Perhaps the strongest evidence for this effect comes from surface plasmon resonance (SPR) studies showing that V region identical antibodies differing in C region manifest large differences in binding to univalent antigens (10,11). Those results indicated kinetic and thermodynamic variations that implied diverse Ab-Ag interactions within IgG molecules expressing different C₃ regions but identical V regions.

Although SPR is a very powerful and useful technique for studying protein-ligand interactions, this method is vulnerable to possible artifacts. For example, SPR measurements can be affected by mass transport effects, excluded volume effects, surface concentration and the possibility that protein immobilization affects its affinity for antigen. Furthermore, interpretation of the SPR data required data analysis by fitting to binding models, which introduces additional uncertainty. Consequently, it is important to validate conclusions derived from SPR data by other techniques. In the present work, we used isothermal titration calorimetry (ITC) and a univalent peptide (P1) (Table 1) to investigate the thermodynamic binding properties of the GXM-binding mAb 3E5 (IgG3) and its IgG switch variants. These Abs have identical V regions but differ in their C₃ domains (10). ITC simultaneously and directly determines the enthalpic and entropic contributions, as well as the binding constant and stoichiometry in solution. ITC revealed differences in the binding energetics of V region identical mAbs differing in isotype to a peptide mimetic thus establishing the influence of the C₃ region, in Ag-Ab binding interactions. The results have important implications for Ab engineering and for the use of therapeutic Abs of different isotype.

**EXPERIMENTAL PROCEDURES**

*Monoclonal Antibodies and Peptides.* GXM-binding mAb 3E5 (IgG3) was produced by a hybridoma isolated from a mouse immunized with a GXM-Tetanus Toxoid conjugate vaccine (14). The IgG1, IgG2a, and IgG2b switch variants were generated in *vitro* by sib selection (15). The mAbs 3E5 family share identical V₃ and V₁ sequences (12). All mAbs were purified by protein A or G affinity chromatography (Pierce) from hybridoma culture supernatants, and dialyzed against PBS. MAb concentration was determined by ELISA and Bradford protein measurements. All Abs were tested by PAGE to verify their integrity and correct molecular weight. The MAbs of the 3E5 family were analyzed by MALDI-TOF mass spectrometry at the Laboratory for Macromolecular Analysis at the Albert Einstein College of medicine to confirm the absence of mAb aggregates (12). Peptide mimetic of GXM P1 (SPNQHTPPWMLK) (Table 1) (12) was synthesized and biotinylated by the Rockefeller University Proteomics Resource Center.

**Isothermal Titration Microcalorimetry.** ITC experiments were performed using a VP-ITC instrument from Microcal, Inc. (Northampton, MA). Injections of 6 µl of peptide solution were added from a computer-controlled microsyringe at an interval of 4 min into the sample solution of antibody (cell volume = 1.43 ml) with stirring at 350 rpm. An example of an ITC experiment is shown in Figure 1 for IgG2a binding to peptide P1 at 27 °C. The concentration range of the antibodies was 7 µM to 20 µM and the peptide was 1 mM to 2 mM. Titrations were done at pH 7.2 using 20 mM PBS. The experimental data were fitted to a theoretical titration curve using software supplied by Microcal, with ΔH (binding enthalpy kcal mol⁻¹), Ka (association constant) and n (number of binding sites per monomer), as adjustable parameters. The quantity c = Ka Mt(0), where Mt(0) is the initial macromolecule concentration, is of importance in titration microcalorimetry (16). All experiments were performed with c values 1 < c < 200. The instrument was calibrated using the calibration kit containing ribonuclease A (RNase A) and cytidine 2'-monophosphate (2'-CMP) supplied by the manufacturer. Thermodynamic parameters were calculated from the Gibbs Free Energy equation, ΔG = ΔH - TΔS = -RT lnKa, where ΔG, ΔH and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively. T is the absolute temperature and R = 1.98 cal mol⁻¹ K⁻¹.
RESULTS AND DISCUSSIONS:
Our previous serological studies (8,12) revealed that 3E5 IgG3 and its switch variants IgG1, IgG2a and IgG2b possessed different fine specificities for GXM. Specificity differences among these variants with identical variable (V) domains reinforce the notion that the constant (C) region of an antibody plays important roles in determining its specificity. We studied the kinetics and thermodynamics of binding of a 12-mer peptide mimetic (P1) to these variants by employing surface plasmon resonance (SPR) to examine whether quantitative data support this view (10). This study shows that Ab switch variants with identical V domains bound to P1 with different kinetics and thermodynamics. Molecular modeling suggests that amino acid sequence polymorphisms in the C_{H} region influence the secondary structure of the antigen-binding site leading to specificity differences (10). However, the thermodynamic data determined by SPR are indirect and may be affected by certain experimental conditions and intrinsic limitations of the technique. Therefore, in this study, we used isothermal titration calorimetry (ITC) to determine the thermodynamic binding parameters of 3E3 IgG3 and its switch variants IgG1, IgG2a and IgG2b for P1. ITC can directly determine the association constant, stoichiometry, enthalpy and entropy of binding in a single experiment. This method measures binding parameters in solution and is free of the limitations associated with solid phase binding techniques such as SPR.

Peptide mimetics of GXM bind to the antigen-binding site of an antibody and are useful tools for determining the fine specificities of mAbs with identical V regions (Valadon et al 1996). The present study was done with P1 (Table 1), a peptide mimetic of GXM representing a sequence isolated from the screening of peptide-expressing phage library with 3E5 IgG3. The phage expressing P1 showed differences in its binding reactivity with the 3E5 switch variants (12).

Binding Stoichiometry of the Antibodies for the Peptide P1:
ITC can directly and unambiguously determine the number of binding site (n) of a macromolecule. The n values of mAb 3E5 and its switch variants range from 1.9 to 2.2 (Table 2). These values indicate that the antibodies possess two binding sites for the peptides and each intact antibody molecule can bind to two molecules of P1, as would be expected for an IgG. The excellent correlation between the predicted and experimentally determined binding stoichiometries for the mAb 3E5 variants provides an important internal control for the high quality of the immunoglobulin preparations. Furthermore, the near-2.0 ratio of peptide to immunoglobulin molecule indicates that each V region is interacting with a single ligand, ruling out avidity effects. Anti-p24 (HIV-1) monoclonal antibody CB4-1 shows similar stoichiometry for four epitope related and three library derived peptides (17).

Peptide Binding Affinities of 3E5 and its Switch Variants:
ITC data show that the association constants Ka (affinity) of 3E5 IgG3 and its variants for P1 peptide are different (Table 2). The Ka (M^{-1}) values of mAb 353 IgG3 and its switch variants IgG1, IgG2a and IgG2b for non-biotinylated P1 peptide were 360 x 10^{6}, 300 x 10^{4}, 156 x 10^{4} and 270 x 10^{4}, respectively at 27°C. The Ka (M^{-1}) values obtained with biotinylated P1 in similar conditions were 280 x 10^{4} (3E5 IgG3), 440 x 10^{4} (IgG1) and 190 x 10^{4} (IgG2a). The affinities of the mAbs determined by ITC are greater than those previously determined by SPR, while the differences in the affinities of the individual isotypes are smaller in magnitude. Certain intrinsic limitations associated with SPR might have contributed to the underestimation of the affinities of the antibodies. Different antibodies show a range of Ka values for different peptides. For example, the affinities of the anti-p24 (HIV-1) mAb CB4-1 for a series of peptides vary from 5100 x 10^{4} M^{-1} to 14 x 10^{3} M^{-1} (17). Sigella flexneri Y O-antigen specific antibody SYA/J6 binds to an octapeptide, a functional mimic of the O-antigen, with a Ka value of 25 x10^{3} M^{-1}. A constituent pentasaccharide of the O-antigen binds with comparable affinity to the same binding site of the antibody (18). The functional oligosaccharide analog of P1 is unknown, therefore, we are unable to compare the affinity of the oligosaccharide epitope to that of its mimetic peptide P1. Furthermore, in contrasting the similarities and differences between SPR and ITC
it is noteworthy that the former method employed immobilized antibody binding to soluble peptide while the latter measures the interaction of both entities in solution. Despite differences in the magnitude of effects measured by SPR and ITC, both techniques show that V region identical antibodies manifest differences in the energetics of binding to a 12-mer peptide that must reflect subtle changes in Ag-Ab interactions imposed by isotype-related differences in CH1 domain sequences.

Relative Contributions of Enthalpy and Entropy to the Binding Free Energy:
Binding of P1 to all antibodies is associated with favorable entropy (TΔS = 1.3 to 3.6) and favorable enthalpy (ΔH = -5.2 to -7.2) (Table 2). The absolute values of ΔH and TΔS varied among the antibodies, however the overall trends are comparable. The favorable entropic values indicate that the binding may be dominated by hydrophobic interactions and are consistent with the displacement of water molecules from the antibody binding site and/or peptide hydration shell. The ΔH values in Table 2 suggest the formation of a modest number of hydrogen bonds in the P1-antibody complexes. As discussed below, insights from the crystal structure of the comparable mAb 2H1-peptide PA1 complex (19) support these notions. The favorable entropic contribution in P1 binding is somewhat unique compared to some other antibody-peptide interactions. For example, in another system, binding of peptides to mAbs CB4-1 and SYA/J6 is mostly enthalpy driven and is significantly compensated by unfavorable entropic effects (17,18). However, it is interesting to note that binding of a pentasaccharide and other oligosaccharides corresponding to O-polysaccharide to SYA/J6 is associated with favorable entropy (TΔS = 2.3 to 5.9) and hydrophobic interactions make significant contributions to binding (20). Similar observations were made in the binding of a trisaccharide epitope of a Salmonella O-antigen to mAb Se155-4 (21).

Insight from the Crystal Structure of mAb 2H1-PA1 Complex:
We have previously solved the crystal structures of mAb 2H1 (another mAb to <i>C. neoformans</i> polysaccharide) complexed with a 12 amino acid residue peptide PA1 (GLQYTPSWMLVG) (Table 1) (19), mAb 2H1 is highly homologous (>90% sequence identity) with mAb 3E5 and the binding site residues of these two antibodies are highly conserved (10). On the other hand, PA1 and P1 (SPNQHTPPWMLK) (Table 1) belong to a group of peptides that possess a common motif TPXWM/LM/L. The presence of a common motif indicates that these residues are essential for binding the peptides to the antibody. Indeed, X-ray crystallographic studies have shown that PA1 interacts with mAb 2H1 through Thr, Pro, Trp, Met and Leu (19). These residues are also present in the motif region of P1. Since mAbs 2H1 and 3E5 are highly homologous antibodies and PA1 and P1 possess a common motif, structural insights gained from 2H1-PA1 complex may help explain some of the thermodynamic binding data obtained with mAb 3E5 and P1.

As observed in other linear peptides, PA1 contains some secondary structure. Due to the presence of a β-turn between Ser<sup>P7</sup> and Leu<sup>P10</sup> of the peptide, the entire motif consisting of residues between Thr<sup>P5</sup> and Leu<sup>P10</sup> is compressed into a tight cluster that, in turn, place the motif residues deeply buried into the combining site of mAb 2H1. Due to its structural resemblance with PA1, P1 may show similar feature that can facilitate its binding to mAb 3E5. The combining site of mAb 2H1 is essentially uncharged and is composed of residues from both the light (Val<sup>HI47</sup> and Trp<sup>H155</sup>) and heavy (Phe<sup>H33</sup>, Leu<sup>H44</sup>, Thr<sup>H50</sup>, Ile<sup>H51</sup>, Asn<sup>H52</sup>, Lys<sup>H54</sup>, Thr<sup>H56</sup>, Arg<sup>H59</sup> and Leu<sup>H100</sup>) chains. In general, a β-turn maximizes the total buried surface area of a peptide bound to an antibody and results in a better fitting in the combining site (22). The combining site of mAb 2H1 is predominantly hydrophobic in nature, therefore, peptides with bulky hydrophobic residues such as Trp and Met would most complementarily interact with the binding site. The β-turn containing Trp<sup>P8</sup> and Met<sup>P9</sup> is a crucial structure for the peptide to bind most efficiently to the antibody. Indeed, the residues of the peptide located within the β-turn that point into the binding site are highly conserved. In addition, two highly hydrophobic cavities are formed between Met<sup>P9</sup> of the PA1 and Phe<sup>H33</sup>, Ser<sup>H35</sup>, Thr<sup>H50</sup>, Arg<sup>H59</sup>, Val<sup>H54</sup>, Leu<sup>H44</sup> and Tyr<sup>H58</sup> of 2H1. P1 may potentially be involved in
hydrophobic interactions with mAb 3E5 through the Trp and Met residues of the peptide. The favorable TAS (entropy) values associated with P1 binding to mAb 3E5 and its switch variants strongly support this possibility. Hydrophobic interaction through Met and Trp residues of the peptide has also been reported for an octapeptide binding to mAb SYA/J6 (18).

Four residues of PA1 form six hydrogen bonds with mAb 2H1 of which four H-bonds are made by two motif residues namely Thr\textsuperscript{P5} and Met\textsuperscript{P9}. H-bonding interactions of Thr\textsuperscript{P5} are comparable to those found in antibody-polysaccharide complexes (23). All the higher affinity peptides possess Thr at position 5 (24). The Thr and Met of P1 can potentially form similar H-bond with mAb 3E5. The modest ∆H values obtained with P1 (ΔH = -5.2 to -7.2) (Table 2) are consistent with the formation of a limited number of H-bonds between P1 and mAb 3E5. In contrast, the octapeptide is connected through 20 (6 direct and 14 water mediated) H-bonds to SYA/J6 Fab resulting in a higher negative binding enthalpy (ΔH = -16.9 kacal.mol\(^{-1}\)) (18).

**Effect of Peptide Biotinylation on Binding Thermodynamics:**
Experimental techniques such as ELISA that assay antibody-binding properties often use biotinylated ligands to immobilize antigens on solid surfaces. For example, we have previously used biotinylated peptides to assay their antibody binding activities (12). We wanted to examine how the biotin molecules attached to peptides (in this particular case P1) influenced the affinity and other binding properties. Interestingly, all biotinylated P1 peptides tested in this study showed higher ∆H values compared to corresponding biotin-free P1 (Δ∆H = -0.9 to -2.0) (Table 2). The higher ∆H values probably resulted from additional hydrogen bonding through the biotin molecules. However, this enthalpic gain was compensated by unfavorable entropic effect. As a result, the biotinylated P1 did not show any significant changes in affinity compared to biotin-free P1.

**Enthalpy-entropy Compensation:**
Enthalpy-entropy compensation plots have previously been observed during carbohydrate binding to lectins (25,26) and antibodies (21,27,28), and are attributed to the unique properties of water (26). A change in binding enthalpy is always compensated by the change of binding entropy. When an enthalpic interaction such as a hydrogen bond or a salt bridge is lost, motional freedom increases. As a result, certain amount of entropy is gained. On the other hand, when new bonds are formed (enthalpic gain) motional freedom is restricted (loss of entropy). When binding interactions occur in aqueous solution, solvent displacement plays a major role in enthalpy-entropy compensation. Water molecules bind to the surface, especially the hydrophobic surface of the solutes (such as the binding sites of a protein and its ligands) through hydrogen bonds resulting in enthalpic gain. When ligand binds to the combining site of a protein, these surface bound water molecules are stripped off and a certain amount of enthalpy is lost. This loss is compensated by favorable entropy. When a group of ligands interact with their receptors through the same mechanism, the enthalpy and entropy show a linear relationship (28).

A plot of the -ΔH versus -T∆S values at 300 °K for P1 binding to the mAbs in Table 2 shows that it is also compensatory (Figure 2). The plot shows a linear relationship with a slope of 0.87 and the correlation coefficient of 0.98 (Figure 2A). Interestingly, the enthalpy-entropy plot in Figure 2 is similar to those reported earlier for other antibody-carbohydrate interactions in that their slopes are less than unity (21,28), in contrast to lectin-carbohydrate interactions where the slope is often greater than unity (29,30). A slope greater than unity means that the free energy of binding is predominantly driven by enthalpy, while a slope less than unity indicates dominant entropy contributions. The slope value of the enthalpy-entropy compensation plot indicates that the binding of P1 to mAb 3E5 and its switch variants is dominated by entropy. When the data obtained with biotinylated P1 were included (Figure 2B), the slope value increased from 0.87 to 0.95 due to the relatively higher -ΔH values of the biotinylated peptideP1. However, the value is still less than unity.

**mAb 3E5 and its Switch Variants Possess Different Binding Thermodynamics for P1:**
ITC data in Table 2 reveal that the C region of the antibodies significantly contributes to their
binding thermodynamics. Despite having identical V domains, the enthalpy (AH) and entropy TΔS of binding of 3E3 IgG3 and its variants IgG1, IgG2a and IgG2b are very different for P1. The range of ΔH was -5.2 to -7.2 whereas TΔS ranged from 1.3 to 3.6 (Table 2). Thermodynamics of lectin-carbohydrate interactions have been reported to be influenced by the alteration of amino acids that are located far away from the binding site (31). Molecular modeling revealed that there are three regions of minor structural differences within the C\textsubscript{H1} domain of 3E5 IgG isotypes (10). Amino acid residues in these regions of different isotypes may be engaged in differential electrostatic and hydrophobic interactions with other interchain residues and solvent molecules that, in turn, could influence the structure and the microenvironment of the combining site of the antibody. The minor structural differences shown by homology modeling may not be detectable by X-ray crystallography. However, our findings establish that these minor differences can alter the binding thermodynamics of the isotypes, which are in turn detectable by ITC. Hence, ITC provides unambiguous thermodynamic evidence for differences in Ag-Ab interactions caused by constant region contributions.

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FOOT NOTES
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The abbreviations used are: H, heavy chain; L, light chain; GXM, glucuron-oxyxylomannan; Ab, antibody; mAb, monoclonal antibody; Ag, antigen; C, constant region; V, variable region; Ka, association constant; \( \Delta G \), Gibbs free energy; \( \Delta H \), enthalpy; T\( \Delta S \), entropy; PBS, phosphate-buffered saline.

FIGURE LEGENDS
Figure 1. ITC binding profile of IgG2a with peptide P1 at 27°C. Top panel shows data obtained for automatic injections, 6\( \mu \)l each, of P1. The bottom panel shows the integrated curve showing experimental (■) points and the best fit (—).

Figure 2. Enthalpy-entropy compensation plots for the binding of biotin free P1 (A) and biotinylated and free P1 (B) to mAb 3E5 and its switch variants. In (A) the slope is 0.87 and the correlation coefficient is 0.98. In (B) the slope is 0.95 and the correlation coefficient is 0.97.
Table 1 Amino acid sequence of P1 and PA1. The conserved motif is shown in bold.

| Peptide | Sequence          |
|---------|-------------------|
| P1      | SPNQHTPPWMLK      |
| PA1     | GLQYTPSWMLVG      |
Table 2. Thermodynamic binding parameters of mAb 3E5 and its switch variants obtained with P1 at pH 7.4, 27 °C

| Antibody          | Ka (M⁻¹ x 10⁻⁴) | -ΔG (kcal/mol) | -ΔH (kcal/mol) | -TΔS (kcal/mol) | n   |
|-------------------|-----------------|----------------|----------------|----------------|-----|
| **Peptide with biotin** |
| 3E5 IgG2a         | 190             | 8.6            | 8.1            | -0.5           | 1.93|
| 3E5 IgG3          | 280             | 8.8            | 8.5            | -0.3           | 2.04|
| 3E5 IgG1          | 440             | 9.1            | 7.5            | -1.6           | 1.98|
| **Biotin free peptide** |
| 3E5 IgG2a         | 156             | 8.5            | 7.2            | -1.3           | 2.2 |
| 3E5 IgG1          | 300             | 8.8            | 5.7            | -3.1           | 1.92|
| 3E5 IgG3          | 360             | 8.9            | 6.5            | -2.4           | 2.08|
| 3E5 IgG2b         | 270             | 8.8            | 5.2            | -3.6           | 1.97|

Errors in Kd range from 1-7%, Errors in ΔG are less than 3%, Errors in ΔH are 1-5%, Errors in TΔS are 1-7%, Errors in n values are less than 5%.
Figure 1.
Figure 2.
Isothermal titration calorimetry reveals differential binding thermodynamics of variable region-identical antibodies differing in constant region for a univalent ligand

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