Interactions of Hepatitis B Core Antigen and Peptide Inhibitors

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The core protein (HBcAg) of hepatitis B virus (HBV) has been shown to interact with the large surface antigen during HBV morphogenesis, and these interactions can be blocked by small peptides selected from either linear or constrained phage display peptide libraries. The association of HBcAg with peptide inhibitors was quantitatively evaluated by isothermal titration calorimetry. The thermodynamic data show that the interaction between HBcAg and peptide MHRSLLGRMKGA is enthalpy-driven and occurs at a 3:1 stoichiometry and dissociation constant (K_d) value of 79.4 μM. However, peptide WSFFSNI displays a higher binding affinity for HBcAg with a K_d value of 18.5 μM when compared to peptide MHRSLLGRMKGA. A combinatorial approach using chemical cross-linking and surface-enhanced laser desorption/ionization-time-of-flight-mass spectrometry shows that the Lys of peptide MHRSLLGRMKGA interacted either with D64, E77, or D78 of HBcAg.

Introduction

Hepatitis B virus (HBV[a]) is a major cause of liver cirrhosis and hepatocellular carcinoma in humans. HBV infection still remains a global health problem despite the availability of safe and effective vaccines. Until recently, it has been estimated that 400 million people are chronically infected with HBV, which is responsible for 500 000 to 750 000 deaths a year.[1] The virus belongs to the *Hepadnaviridae* family and has a 3.2 kb long, partially double-stranded DNA encoding the viral envelope, core, polymerase, and X proteins.[2] The viral envelope contains small (S), middle (M), and large (L) surface antigens (HBsAgs), which surround the nucleocapsid.[3] The L-HBsAg plays a central role in the production of infectious virions. Both the PreS and S domains of L-HBsAg form docking sites for the viral nucleocapsid during HBV morphogenesis.[4,5]

The HBV core antigen (HBcAg) is a 183-residue protein that forms the building blocks of the viral capsid. The carboxyl-terminal end of the HBcAg of about 40 residues is highly rich in arginine residues which interact with the viral genome. HBcAg can be expressed efficiently in *Escherichia coli*,[6] where it assembles into icosahedral shell with triangulation number, $T = 3$ (180 subunits) and $T = 4$ (240 subunits).[7] Truncated mutants that lack the Arg-rich C-terminal region can also be produced in *E. coli* and they also assembled into two sizes of particles.[8] Previous work has shown that peptide ligands that were selected from a linear hexapeptide phage display library inhibit the binding of L-HBsAg to HBcAg.[9] The dominant peptide LLGRMKG and some related peptides also inhibit the production of HBV in hepatoma cells and could therefore be interesting lead compounds for therapeutic agents against the virus.[10] Ho et al.[11] demonstrated that a 7-mer peptide with the sequence WSFFSNI, selected from a disulfide-constrained heptapeptide library, inhibits the binding of L-HBsAg to the core protein with a greater inhibitory effect ($IC_{50} = 12 \pm 2$ μM) relative to peptide LLGRMKG ($IC_{50} = 46.2 \pm 7.4$ μM).[12] It is, therefore, of interest to study the binding thermodynamics of these peptide inhibitors to HBcAg by titration microcalorimetry.

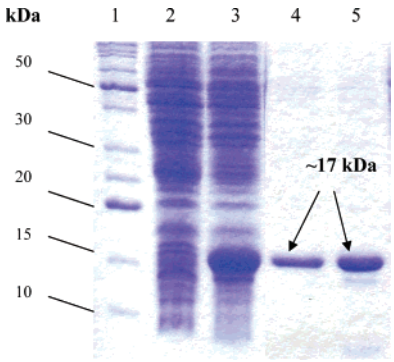

Figure 1. SDS-PAGE of HBcAg. Lane 2 is the lysate of *E. coli* encoding HBcAg before IPTG induction, while lane 3 is after IPTG induction. Lanes 4 and 5 are concentrated HBcAg from HPLC and sucrose gradient preparations, respectively. Lane 1 is protein markers in kDa. The gel was stained with Coomassie Blue. Arrows indicate the purified HBcAg protein.

Electron cryomicroscopy and image analysis indicated that peptide GSLLGRMKGA with an improved affinity ($K_d^{rel} = 1.7 \pm 0.3$ nM)[12] bound to the tips of the spikes of HBcAg particles.[10] A peptide, MHRSLLGRMKGA with a further enhanced affinity ($K_d^{rel} = 0.55 \pm 0.03$ nM)[12] can be cross-linked to HBcAg particles with EDC and sulfo-NHS.[10] Here we map the peptide-binding site for HBcAg by using a strategy that combined chemical cross-linking with surface-enhanced laser desorption/ionization-mass spectrometry (SELDI-MS) analysis. In addition, we established a new purification method for HBcAg particles by using a preparative high-performance liquid chromatography (HPLC).

Results

Preparation and Purification of HBcAg. The HBcAg has been successfully expressed in *E. coli* as a soluble protein with

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[a] Abbreviations: HBV, hepatitis B virus; HBcAg, hepatitis B core antigen; L-HBsAg, large hepatitis B surface antigen; ITC, isothermal titration calorimetry; SELDI-TOF-MS, surface-enhanced laser desorption/ionization-time-of-flight-mass spectrometry; IPTG, isopropyl-β-thiogalactopyranoside; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; sulfo-NHS, *N*-hydroxysulfosuccinimide; DTT, 1,4-dithiothreitol; BCIP, 5-bromo-4-chloro-3-indoyl phosphate; NBT, nitro blue tetrazolium chloride; PNPP, *p*-nitrophenyl phosphate.

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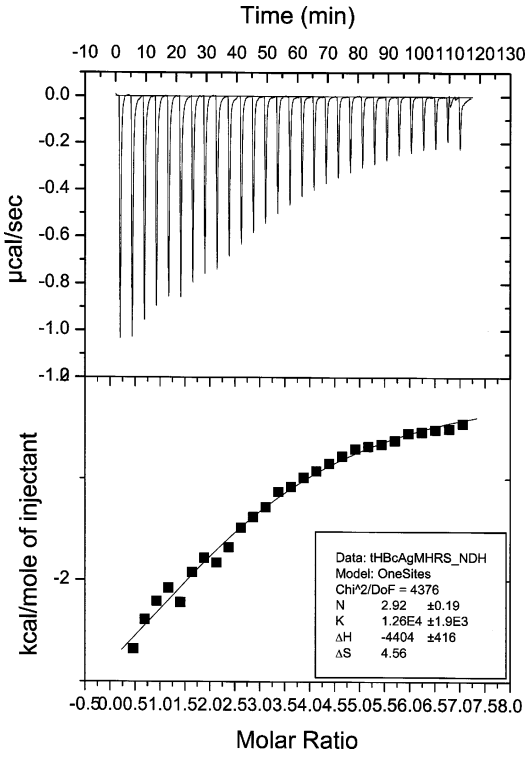

Figure 2. Calorimetric titration of HBcAg with peptide MHRSLLGRMKGA. The cell (1.4 mL) contained 45 μM of HBcAg in 0.2 M Na_2HPO_4 (pH 7.5), meanwhile the syringe contained 1.47 mM of peptide MHRSLLGRMKGA in the same buffer. This experiment was performed at 37 °C. The top panel shows raw data in power versus time. The area under each peak is proportional to the heat produced at each injection. The bottom panel shows data after peak integration, subtraction of blank titration data, and concentration normalization. The solid line is the fit to a single binding site model. The inset shows the results of the curve fitting.

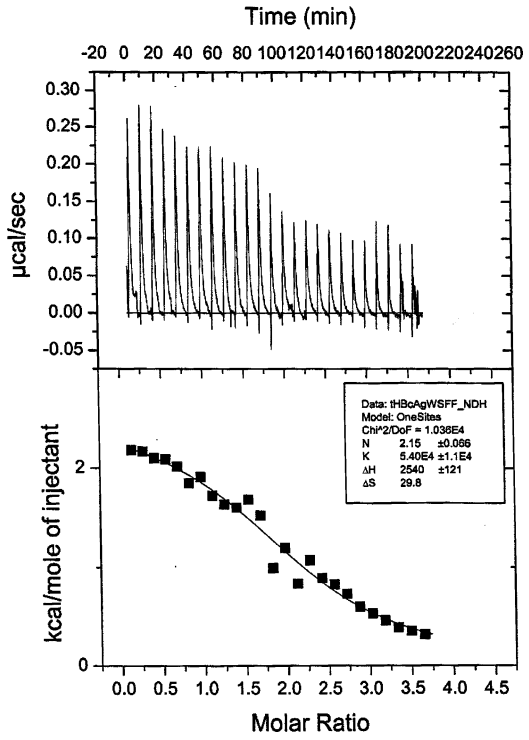

Figure 3. Calorimetric titration of HBcAg with peptide WSFFSNI. The cell (1.4 mL) contained 58 μM of HBcAg in 0.2 M Na_2HPO_4 (pH 7.5), meanwhile the syringe contained 1.11 mM of peptide WSFFSNI in the same buffer. This experiment was performed at 37 °C. The top panel shows raw data in power versus time. The bottom panel shows data after peak integration, subtraction of blank titration data, and concentration normalization. The solid line is the fit to a single binding site model. The inset shows the results of the curve fitting.

a molecular mass of ∼17 kDa upon IPTG induction (Figure 1, lane 3). Purification of the crude HBcAg with HPLC produced a positive peak with retention time 6.2 to 12.5 min. SDS-PAGE analysis of this fraction gave rise to an intense band of ∼17 kDa (Figure 1, lane 4). Table 1 compares HBcAg purified with HPLC and sucrose density gradient centrifugation. The purity of HPLC-purified HBcAg was 98.7%, which is significantly higher than that of the sucrose density gradient centrifugation (88.3%). The yield of HBcAg purified with HPLC was 3-fold higher, and the total time consumed was 6 h faster compared to the sucrose gradient centrifugation. Although the yield of the HPLC-purified HBcAg improved significantly, the sample was found to be polydispersity (Table 1) due to the presence of $T = 3$ and $T = 4$ HBcAg particles. The estimated R_h for this mixture was ∼20 nm. Transmission electron microscopic analysis revealed the presence of two types of particles in the HPLC purified sample (see Supporting Information).

Isothermal Titration Calorimetry. The binding energetics of peptide inhibitors to HBcAg were analyzed by ITC at 37 °C. The binding of peptide MHRSLLGRMKGA to HBcAg was exothermic as indicated by negative peaks (Figure 2). The hyperbolic titration curve demonstrates that the binding site of HBcAg was saturated with the corresponding peptide. The binding enthalpy (ΔH) of the peptide to HBcAg was -4.4 ± 0.4 kcal/mol, whereas the entropy changes $-T\Delta S$ was −1.4 kcal/mol. The fit yielded a binding affinity (K_a) of $1.26 \pm 0.19 \times 10^4$ M^{-1} ($K_d = 79.4$ μM) and binding stoichiometry of about 3. The ITC experiment clearly indicates that the binding of peptide WSFFSNI was endothermic as characterized by the positive peaks (Figure 3). The sigmoidal titration curve indicates the saturation of HBcAg by corresponding peptide has been achieved. The ΔH of the peptide to HBcAg was $+2.5 \pm 0.1$ kcal/mol, with $-T\Delta S$ of −9.2 kcal/mol, $K_a = 5.40 \pm 1.10 \times 10^4$ M^{-1}, and $K_d = 18.5$ μM. The binding stoichiometry was calculated to be approximately 2, indicating that this peptide binds to a dimer of HBcAg capsid. The binding energetics of peptides MHRSLLGRMKGA and WSFFNI to HBcAg at 37 °C are summarized in Table 2.

Table 1. Comparison of HBcAg Prepared from Sucrose Gradient Centrifugation and HPLC System

output	sucrose gradient	HPLC
amount of HBcAg from 1 L culture (mg)	24	72
purity (%)	88.3	98.7
time consumed (h)	7	1
R_h[a] (nm)	20.3	20.06
C_p[b] (nm)	11.3	6.540
C_p/R_h[c] (%)	55.6	32.6

[a] R_h is the hydrodynamic radius. [b] C_p is the coefficient of polydispersity. [c] C_p/R_h is the relative polydispersity. The particles can be considered to be virtually identical in size or monodisperse when the percent polydispersity is less than 15%. The particle population can be considered to contain significantly different sizes or polydisperse when the percent polydispersity is greater than 30%.

Chemical Cross-Linking. A band shift corresponding to ∼1 kDa was observed on SDS−polyacrylamide gel (Figure 4a, lane 3) when cross-linkers EDC and sulfo-NHS were added to HBcAg in the presence of peptide MHRSLLGRMKGA. These coupling reagents covalently linked the Lys from the peptide to a neighboring Asp or Glu from HBcAg (see Supporting Information), causing its molecular weight to increase. The relative amount of the cross-linked HBcAg monomer (∼18 kDa)

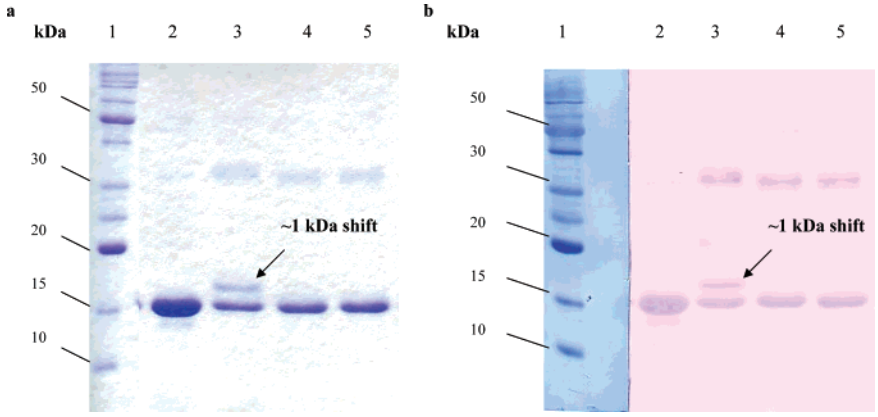

Figure 4. SDS-PAGE and Western blot of cross-linking products. (a) SDS-polyacrylamide gel stained with Coomassie Blue. Lane 2 is purified HBcAg. Lane 3 is HBcAg plus the peptide MHRSLLGRMKGA and cross-linkers. Lane 4 is HBcAg plus cross-linkers served as a control. Lane 5 is HBcAg plus the peptide WSFFSNI and cross-linkers. Lane 1 is protein markers in kDa. Arrow indicates a shifted band equivalent to ~1 kDa. (b) Western blot of HBcAg and cross-linking products. Lane 2 is purified HBcAg. Lane 3 is HBcAg plus the peptide MHRSLLGRMKGA and cross-linkers. Lane 4 is HBcAg plus cross-linkers. Lane 5 is HBcAg plus the peptide WSFFSNI and cross-linkers. Lane 1 is protein markers in kDa. The nitrocellulose membrane contained separated protein marker was stained with Coomassie Blue, whereas the bands were developed with BCIP/NBT. Arrow indicates a shifted band equivalent to ~1 kDa.

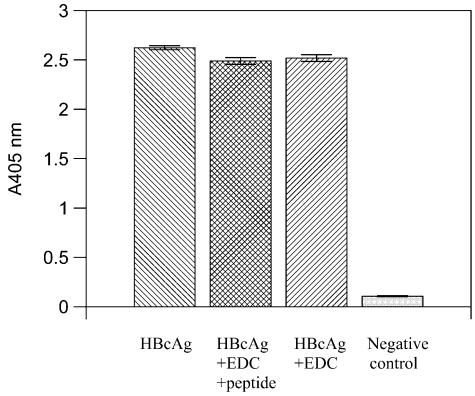

Figure 5. ELISA of HBcAg and cross-linking products. Purified HBcAg (0.84 mg/mL), HBcAg plus the peptide with cross-linker (0.84 mg/mL), and HBcAg plus cross-linkers (0.84 mg/mL) and 10% milk diluent (negative control) were immobilized on wells. Anti-core mAb C1-5 was added to interact with HBcAg and cross-linking products. Then goat anti-mouse antibody conjugated with alkaline phosphatase was added followed by PNPP substrate. A_{405} was determined after 15 min of incubation. Assays were performed in triplicates and the error bars represent the standard deviation from the arithmetic mean.

and dimer (~34 kDa) was about 50%. Peptide WSFFSNI, which lacks a Lys residue for cross-linking, did not give rise to a band shift (Figure 4a, lane 5). When the protein and cross-linking products were immunoblotted with mouse monoclonal antibody (mAb) C1−5, which recognizes a linear epitope located between residues 78−83,[13] the shifted band was also detected (Figure 4b, lane 3). Furthermore, ELISA shows that the cross-linked HBcAg does not affect the binding of mAb C1−5 to native HBcAg particles (Figure 5).

SELDI-TOF-MS Mass Analysis of the Trypsin-Digested Peptide. The amino acid sequence of truncated HBcAg and its corresponding trypsin-digested fragments are shown in Figure 6a, meanwhile the trypsin cleavage sites for peptide MHRSLLGRMKGA are shown in Figure 6b. A comparison between the experimental and the theoretical monoisotopic $[M + H]^+$ masses of the trypsin-digested HBcAg along with their modifications is listed in Table 3. A total of 6 out of 11 unmodified fragments were detected by the SELDI-TOF-MS. These included fragments 2, 3, 4, 6, 9, and 10. The undetectable fragments were 1, 5, 7, and 8. However, the modified fragments 1, 4, and 5 were observed. In the presence of cross-linker and

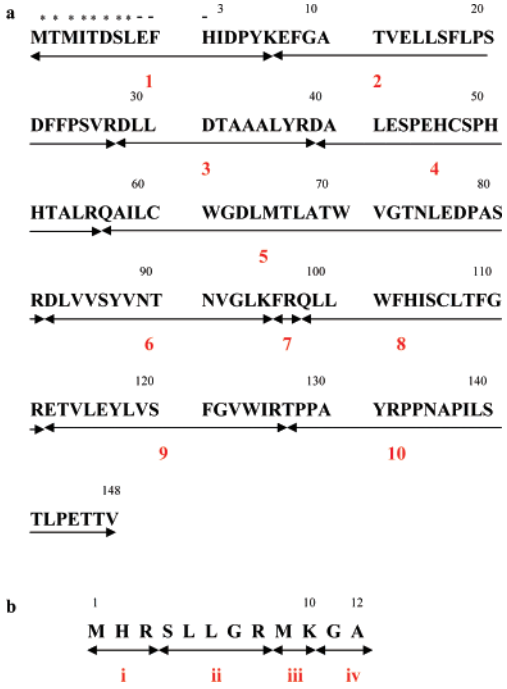

Figure 6. Trypsin digested fragments of HBcAg and peptide MHRSLLGRMKGA. (a) HBcAg sequence. Arrows indicate trypsin-digested HBcAg fragments. aa 1 to 8 (∗) are from the N-terminal of β-galactosidase, the linker aa 9 to 11 (−) are derived from the cloning strategy and aa 12 to 157 are from HBcAg (aa 3 to 148). The positions of HBcAg residues are indicated by the numbers on top of the sequence. (b) Peptide sequence. Arrows indicate trypsin-digested peptide MHRSLLGRMKGA fragments i (aa 1 to 3; 443.2 Da), fragment ii (aa 4 to 8; 545.3 Da), fragment iii (aa 9 to 10; 278.2 Da), and fragment iv (aa 11 to 12; 147.1 Da).

peptide MHRSLLGRMKGA, three modified fragments (1, 4, and 5) and two unmodified fragments (3 and 10) were observed. However, fragments 2, 6, 7, 8, and 9 were undetectable, probably caused by the hindrance of cross-linked peptide on the tryptic digestion. Interestingly, an unknown fragment with a peak at *m*/*z* 3147.5 was detected, indicating that peptide MHRSLLGRMKGA has been cross-linked to one of the trypsin-digested HBcAg fragments. This peak is not present in either HBcAg mass map or HBcAg plus cross-linker mass map (Figure 7). Because the Lys of peptide MHRSLLGRMKGA took part in the cross-linking reaction, the extra peak can be explained

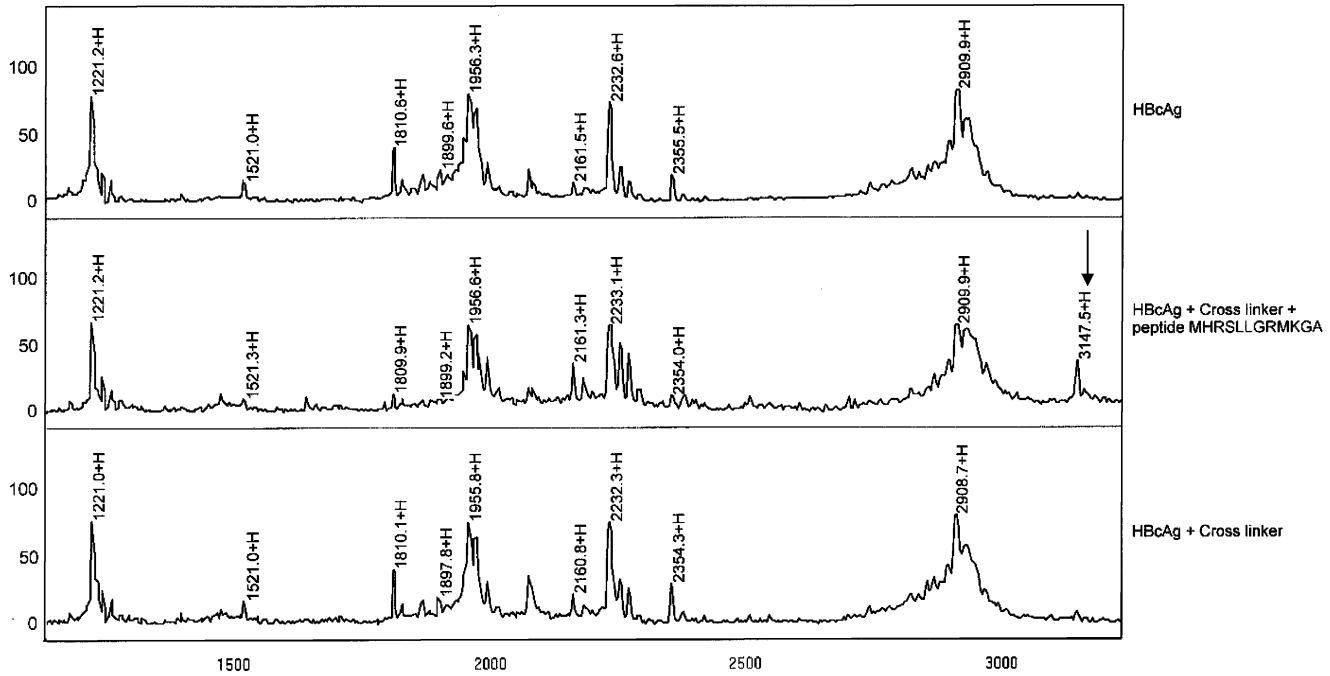

Figure 7. SELDI-TOF-MS spectra of trypsin-digested HBcAg and cross-linking products. The *m*/*z* values are indicated as abscissa axis; ion abundances are indicated as ordinate axis. Arrow indicates extra peak at *m*/*z* 3147.5 detected for HBcAg cross-linked with peptide MHRSLLGRMKGA.

Table 2. Thermodynamic Parameters Obtained upon Titrating HBcAg with Various Peptides at 37 °C

peptide	ΔG (kcal/mol)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	K_a ($\times 10^4\ M^{-1}$)	K_d (μM)	n
MHRSLLGRMKGA	−5.8	−4.4	−1.4	1.26 ± 0.19	79.4	2.92 ± 0.19
WSFFSNI	−6.7	+2.5	−9.2	5.40 ± 1.10	18.5	2.15 ± 0.06

Table 3. Expected and Observed $[M + H]^+$ Values for the Peptides Generated from the Trypsin-Digested HBcAg and Cross-Linking Products in the Presence of a Reducing Agent

		HBcAg		HBcAg + cross linker		HBcAg + cross linker + peptide		
fragment	expected mass	observed mass	deviation[a] (%)	observed mass	deviation[a] (%)	observed mass	deviation[a] (%)	artificial modification(s)[b]
1	1940.9							
1	1972.9	1970.3	99.87	1969.7	99.84	1970.3	99.87	MSO: *,*
2	2358.2	2355.5	99.88	2354.3	99.83			
3	1221.6	1221.2	99.97	1221.0	99.95	1221.2	99.97	
4	1899.8	1899.6	99.99					
4	1956.8	1956.3	99.97	1955.8	99.95	1956.6	99.99	Cys_CAM:48
5	2861.4							
5	2877.4							MSO: 66
5	2918.4	2909.9	99.71	2908.7	99.67	2909.9	99.71	Cys_CAM:61
6	1520.8	1521	100.01	1521.0	100.01			
7	322.18							
8	1720.8							
8	1777.9							Cys_CAM:107
9	1810.9	1810.6	99.98	1810.1	99.96			
10	2235.2	2232.6	99.88	2232.3	99.86	2233.1	99.91	
unexpected fragment						3147.5		
trypsin autolysis	2163.3	2161.5	99.92	2160.8	99.88	2161.3	99.91	

[a] The percentage deviation was taken from a ratio of observed mass divided by expected mass multiplied by 100. [b] Cys_CAM represents alkylation of Cys residues to *S*-carboxymethylcysteine; MSO represents oxidation of Met residues. The modified amino acid positions are shown in Figure 6a.

as the sum of two tryptic fragments: fragment iii (MK) from peptide MHRSLLGRMKGA and an unknown HBcAg fragment. Subtraction from the mass of fragment iii (278.2) and a H_2O yields a fragment mass of 2851.3, which matches fragment 5 of trypsin-digested HBcAg, which suggests that the tryptic digest (MK) from peptide MHRSLLGRMKGA was cross-linked to the tryptic digest (57QAILCWGDLMTLATWVGTNLED-PASR82) from HBcAg. Because Lys of peptide MHRSLLGRMK-GA was hypothesized to cross-link to a negatively charged residue of fragment 5, D64, E77, or D78 of HBcAg was involved directly in the binding of the peptide. This is in agreement with the point mutation analysis of HBcAg, suggesting that E77 and D78 are important for the interaction.[10]

Discussion

A method for the purification of HBcAg particles with HPLC has been established. This method was found to be better than the conventional sucrose density gradient centrifugation in terms of yield, purity, and time consumption. However, the size-exclusion column containing zirconium-stabilized silica, with a pore size of 300 Å, could not separate $T = 3$ and $T = 4$ HBcAg particles. So far these two particles can only be separated by

two or more rounds of sucrose density gradient ultracentrifugation,[14] which is laborious and time-consuming. Therefore, it remains a challenge to separate these two particles by other means.

ITC allows a simultaneous determination of the association constant (K_a), stoichiometry (n), as well as the enthalpy (ΔH) and entropy (ΔS) changes associated with the binding of a ligand to a macromolecule.[15] This information can provide an insight of the dominant forces associated with binding. The association between MHRSLLGRMKGA and HBcAg is primarily driven by enthalpy with an unfavorable entropic contribution. The dominant negative enthalpy suggests that there are a large number of favorable hydrogen bond contacts or van der Waals interactions[16] between HBcAg and the peptide. The negative Gibbs energy (ΔG) indicates that the binding of peptide MHRSLLGRMKGA to HBcAg is spontaneous at all temperatures. On the other hand, peptide WSFFSNI is significantly more hydrophobic and its binding to HBcAg at 37 °C was dominated by large solvation entropy and an unfavorable enthalpy change. The entropy contribution could be originated from the release of water molecules upon binding (solvation entropy) and due to the loss of conformational degrees of freedom[16] by the peptide and some residues in the protein.

Peptide WSFFSNI displayed a higher binding affinity for HBcAg when compared to peptide MHRSLLGRMKGA. The K_d obtained from ITC for peptide WSFFSNI and MHRSLLGRMKGA were about 3776-fold and 144 364-fold weaker than the K_d^{rel} phage particles bearing the same fusion peptide sequence as determined by the phage titration method.[17] This could be due to the fact that the peptides were fused to a gpIII protein, which might play a role in the binding. Furthermore, each phage contains three to five copies of gpIII proteins. Attempts to study the thermodynamic profiles of the fusion phage with HBcAg particles using ITC were not successful because the interactions between the two macromolecules requires more than 5 h to reach equilibrium.[5,17] Peptide WSFFSNI competed with mAb C1−5[11] for binding to the immunodominant region (amino acids 78−83) of HBcAg located at the tip of the capsid spike.[18] In this study, the ITC data show that this peptide binds to a dimer, therefore, it is likely that only a single copy of this peptide interacts with either one of the immunodominant regions located at the spike tip.

The binding stoichiometry of the 12-mer peptide (MHRSLLGRMKGA) to HBcAg determined by ITC was 3, but not 2, as demonstrated by Böttcher et al.[10] using cryoelectron microscopy and image reconstruction. This could be due to the fact that there are four and seven possible different classes of dimer and monomer, respectively, in a mixed population of $T = 3$ and $T = 4$ capsids. These various configurations might have different affinities for the peptide. It is likely that the peptide may not interact with some of these configurations at all. Point mutation of two acidic residues (E77 or D78) at the tip of HBcAg capsid spike reduced the binding affinity of fusion phage bearing the peptide RSLLGRMK 1000-fold and 160-fold, respectively.[10] This suggests that both of the negatively-charged residues involved in the binding of the peptide and most likely E77 plays a more important role compared to D78 based on the mutational impact on affinity. In this study, the cross-linked peptide MHRSLLGRMKGA did not affect the binding of mAb C1−5 to its epitope (residues 78−83); either it was in denatured form (Western blotting) or native conformation (ELISA). This implies that D78 of HBcAg may not play an important role in the binding of the peptide. The immunodominant region (residues 78−83) lies very close to the tips of the dimer spike, but the binding of mAb C1−5 was not inhibited by the cross-linked peptide, thus implies that the peptide binds to a cleft located at the tip of the spikes. This result is consistent with the finding of Böttcher et al.[10] who employed cryoelectron microscopy and image reconstruction to demonstrate that the octapeptide GSLLGRMKGA lies over the cleft between the two α-helical hairpins and extends a short way down the side of the dimer spike.

SELDI-TOF-MS mass analysis of the trypsin-digested products of the cross-linked reaction indicates that the Lys of the peptide interacts with D64, E77, or D78 of HBcAg. This approach further confirmed the important of residue E77 in the binding to peptide MHRSLLGRMKGA.

Conclusions

In this study, interactions between HBcAg and peptide inhibitors were thermodynamically characterized by using microcalorimetry, which provides an insight of the dominant forces associated with the interactions. In addition, the combined use of trypsin digestion, peptide mass fingerprinting by SELDI-TOF-MS and ELISA have proven as an effective and rapid means for identification of a peptide binding site.

Materials and Methods

Expression and Purification of HBcAg. The truncated HBcAg (amino acids 1−157)[19] was produced in *E. coli* strain W3110IQ, and expression was induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 18 h. Cells were harvested by centrifugation at 4000 × g for 15 min at 4 °C and then washed with lysis buffer [50 mM Tris (pH 8.0), 0.1% (v/v) Triton]. The cell extract was recovered by centrifugation at 11 000 × g for 30 min at 4 °C, and the protein was precipitated by ammonium sulfate (35% saturation). The protein was then purified either by sucrose gradient ultracentrifugation as described[9,20] or by HPLC system (Agilent 1100 Series, U.S.A.). Purification of the crude HBcAg by HPLC was achieved with a Zorbax Bio Series GF-450 column (250 nm length × 9.4 mm I.D.) using 0.2 M Na_2HPO_4 buffer (pH 7.5) over 20 min at a flow rate of 1.0 mL/min, with detection at 250 mm. The collected fractions were subsequently analyzed by SDS-PAGE[21] and the Bradford assay.[22] The purity of HBcAg was measured as relative percentage with the Quantity One Quantification Software (Bio-Rad, U.S.A.) as described by Ng et al.[23] The formation of HBcAg particles was confirmed by transmission electron microscope as described by Ho et al.[11]

Dynamic Light Scattering Measurement. The dispersity of HPLC purified HBcAg was analyzed by a light scattering machine (DynaPro-MS/X, Proterion Corporation). The instrument was calibrated with BSA standard (Pierce, U.S.A.) prior to sample loading. Purified HBcAg (0.5 mg/mL, 100 μL) was loaded into the cell and measurements were conducted at 37 °C. The sample was illuminated by a miniature state laser at a power of 54 mW and at a wavelength of 824.8 nm. An average of 20 readings were recorded for each measurement. Light scattered at a 90° angle to the incident beam was detected and analyzed with the autocorrelation function to deduce the translational diffusion coefficient (D_T) of the molecules in the sample cell. The hydrodynamic radius (R_h) of the molecules was calculated from D_T through the Stokes−Einstein equation: $R_h = k_B T/6\pi\eta D_T$, where k_B was the Boltzman's constant, T was the absolute temperature in Kelvin, and η was the viscosity of the water, $\eta = 1.019 \times 10^{-3}$ Nsm^{-2}. Meanwhile, the polydispersity coefficient (C_p) and molecular weight (MW) of the sample were measured with the Dynamics V5 software (Proterion Corporation).

Preparation of Synthetic Peptides. Peptides were synthesized based on the sequence of the selected fusion phage and their ability to inhibit the binding of L-HBsAg to HBcAg.[10,11] The lyophilized peptide MHRSLLGRMKGA (Genemed Synthesis, U.S.A.) was dissolved in H_2O, whereas peptide WSFFSNI was dissolved in H_2O containing DMSO [10% (v/v)]. The peptides were further diluted

with 0.2 M Na_2HPO_4 buffer (pH 7.5) to the appropriate concentrations for the ITC experiment. For the cross-linking experiment, these peptides were further diluted with H_2O to give a concentration of 10 mM.

Isothermal Titration Calorimetry Experiment. Both purified HBcAg particles (macromolecule) and peptides (ligand) were prepared in 0.2 M Na_2HPO_4 buffer (pH 7.5) and then degassed for 5 min prior to sample loading. ITC experiments were performed by using a VP-ITC microcalorimeter (MicroCal, LLC., Northampton, MA). HBcAg particles were loaded into the sample cell, whereas the peptides, MHRSLLGRMKGA and WSFFSNI, were loaded into the injection syringe. The calorimeter was first equilibrated at 37 °C, and the baseline was monitored during equilibration. Control experiments were performed by titrating the peptide into buffer and also buffer into HBcAg. The total observed heat effects were corrected for these small contributions. All titration data were subsequently analyzed using the Origin 7 software (MicroCal, LLC.). The binding isotherm was fitted by a nonlinear least-square regression using the One Set of Sites model. The binding Gibbs free energy (ΔG) was calculated from enthalpy changes (ΔH) and association constant (K_a) through the equation: $\Delta G = -RT\ln K_a$, where R was the gas constant and T was the absolute temperature in Kelvin. Equilibrium dissociation constant (K_d) was calculated as the reciprocal of K_a. Meanwhile, the stoichiometry (n) of the interaction was determined from the One Set of Sites model.

Cross-Linking, Western Blotting, and ELISA. The purified HBcAg (15 μg) was incubated in buffer (30 μL) containing KH_2PO_4 (25 mM, pH 7), NaCl (150 mM), EDC (1.8 mM, Pierce, U.S.A.),[24] and sulfo-NHS (1.8 mM, Pierce, U.S.A.)[25] in the presence or absence of the peptide MHRSLLGRMKGA (1 mM) at room temperature for 18 h.[10] This experiment was repeated for peptide WSFFSNI (1 mM). The cross-linking products were analyzed by 15% (w/v) SDS-PAGE,[21] Western blotting[26] and ELISA.[27] For Western blotting, anticore monoclonal antibody (mAb) C1−5 (1:5000 dilution; Chemicon, U.S.A.) was added, followed by incubation of goat antimouse antibody conjugated with alkaline phosphatase (1:5000 dilution; Chemicon, U.S.A.) at room temperature for 1 h. After washing, color development was obtained using BCIP/NBT substrate (Promega, U.S.A.). For ELISA, PNPP (Sigma, U.S.A.) was added and absorbance at 405 nm was measured after 15 min of incubation at room temperature.

In-Gel Digestion. The protein bands of interest (thickness ~1 mm^2) were excised from destained SDS-polyacrylamide gel. The gel pieces were then incubated in ammonium bicarbonate buffer [0.2 M (pH 8.0); 300 μL] containing acetonitrile [50% (v/v)] at 30 °C for 30 min, followed by DTT (0.02 M) incubation at 32 °C for 1 h and iodoacetamide (0.05 M, Sigma) incubation at room temperature for 20 min. The solution was removed and the gel pieces were incubated with acetonitrile [100% (v/v); 50 μL] at room temperature for 15 min. All solvent was removed and the gel pieces were dried in a SpeedVac at 4 °C for 15 min. Ammonium bicarbonate buffer [0.025 M (pH 8.0); 40 μL] containing trypsin (0.02 μg/μl; 10 μL) was applied to the dried gel pieces at 4 °C. The mixture was finally incubated at 32 °C for 18 h.

SELDI-TOF-MS Mass Analysis of the Trypsin-Digested Peptide and Database Search. Both SELDI-TOF mass spectrometer and ProteinChip Array, including the ProteinChip software, were from the Ciphergen Biosystems, Inc. (Fremont, CA). The mass spectrometer was externally calibrated using the $[M + H]^+$ ion peaks of standard human angiotensin I at 1296.7 m/z, human β-endorphin at 3465.6 m/z, and bovine insulin at 5733.6 m/z to ensure a higher mass accuracy before peptide mass fingerprinting. The NP 20 ProteinChip was initially prewet with HPLC grade water and allowed to air-dry. The trypsin-digested sample (1 μL) was spotted onto the chip and allowed to bind for 10 min at room temperature. The spots were then washed with HPLC grade water to remove unbound peptides and impurities. After drying, a matrix solution of α-cyano-4-hydroxy-cinnamic acid (Ciphergen) in acetonitrile supplemented with 1% (v/v) trifluoroacetic acid was applied to each spot and allowed to air-dry. Captured peptides were detected using a Ciphergen PBS II ProteinChip reader. The instrument was operated in a positive ion mode with ion acceleration potential of 20 kV and a detector gain voltage of 2.9 kV. The mass range investigated was from 0 to 5 kDa. Laser intensity was set to 150 and detector sensitivity was set to 9. Peak intensities were normalized according to total ion current after background subtraction. Mass accuracy was normalized to the calibrated standard peaks. The monoisotopic peptide masses with modification of cysteines and oxidation of methionine were initially calculated using the PeptideMass software available online at http//www.expasy.ch. Trypsin-digested fragments were analyzed by comparing the experimental mass values with the theoretical mass values.

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Supporting Information Available: Electron micrograph of hepatitis B particles and a schematic representation of the chemical cross-linking reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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