Analysis of the Antigen Binding Site of Anti-Deoxycholate Monoclonal Antibody Using a Novel Affinity Labeling Reagent, Acyl Adenylate†

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ABSTRACT: Large-scale analysis of protein−protein interaction sites is especially needed in the postgenomic era. The combination of affinity labeling with mass spectrometry is a potentially useful high-throughput screening method for this purpose. However, reagents in current use are not ideal as some cause damage to the target molecule and others have poor solubility in physiologic aqueous buffers. In this paper, we describe a novel affinity labeling reagent, acyl adenylate, which is highly soluble in aqueous solutions and reacts in a pH-dependent manner. The adenylate of deoxycholic acid reacts with amino groups on the side chain of a lysine residue and at the N-terminus of proteins/peptides. The reactivity and stability of this reagent were investigated, and it was confirmed that, after formation of a reversible ligand−protein complex under weakly acidic conditions, derivatization with acyl adenylate occurred at the target site under weakly alkaline condition. We further demonstrated the utility of this reagent for affinity labeling using a monoclonal antibody with high affinity for deoxycholic acid. Competitive ELISA indicated that deoxycholic acid was labeled around the antibody ligand binding site, thus enabling the structural elucidation of the ligand−protein interaction. In addition, LC/ESI-MS/MS analysis of the labeled peptide obtained by enzymatic digestion and affinity extraction allowed the identification of the structure surrounding the antigen binding site.

Much work has been focused recently on the elucidation of mechanisms of interaction of ligands with their corresponding proteins such as receptors, transporters, enzymes, and antibodies. For this purpose, X-ray and NMR analyses, which allow the modeling of not only three-dimensional structures around the interaction site but also binding mechanisms, are widely used (*1*, *2*). However, X-ray analysis requires a uniform crystal of the ligand−protein complex, and NMR analysis lacks the sensitivity necessary to visualize noncovalent complexes formed under physiological conditions. Mass spectrometry (MS)[1] with electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) can identify characteristic ions by molecular weight and characteristic fragment ions, indicating the amino acid sequence of proteins and peptides present at the low picomole level. By employing this method, it has become feasible to analyze the mass spectra of reversible ligand−protein complexes, resulting in estimates of binding affinities. The specific enzymatic digestion of stable noncovalent ligand−protein complexes in the presence or absence of deuterium substitution can also be used to probe binding site structure (*3*−*5*).

An affinity labeling method producing a stable covalent bond between the ligand and the protein interaction site can also probe the structure surrounding the binding site (*6*−*8*). Two common reactions, one photochemical (*9*−*13*) and the other chemically active, have been used for this purpose. During the photochemical procedure, the target protein may often be subjected to unexpected damage, but the reaction can be easily controlled by varying the irradiation intensity (*9*). Haloketones (*7*, *14*), epoxides (*6*), and active esters (*15*, *16*) are chemically active to form covalent bonds with proteins. Among these, active ester-type reagents are preferred for labeling target ligands, because the reaction can be controlled by adjusting the pH. However, the active esters developed thus far, including *p*-nitrophenyl ester and *N*-hydroxysuccinimidyl ester, have a low degree of water solubility under physiological conditions (*15*, *16*). The addition of an organic solvent to dissolve the reagents often causes conformational changes in the target protein.

Carboxylic acid is metabolized into coenzyme A thioester, which is the starting material for β-oxidation, fatty acid synthesis, epimerization of α-aryl propionates, and amino acid conjugation. This thioester is biosynthesized through a

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[1] Abbreviations: CDR, complementarity-determining region; α-CHCA, α-cyano-4-hydroxycinnamic acid; DCA, deoxycholic acid; DCA-AMP, deoxycholyl adenylate; ESI, electrospray ionization; HRP, horseradish peroxidase; LC/ESI-MS/MS, liquid chromatography/electrospray ionization tandem mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; TOFMS, time-of-flight mass spectrometry.

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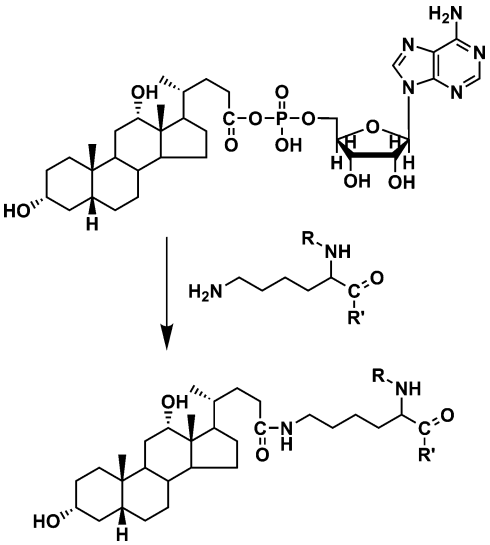

FIGURE 1: Structure of deoxycholyl adenylate and reaction with the lysine ϵ-amino group.

highly reactive acyl adenylate intermediate (*17*−*20*), which produces an amide bond nonenzymatically with an amino group when in solution (*21*). In this report, we utilize highly reactive acyl adenylate as a novel affinity labeling reagent. Deoxycholic acid (DCA), a secondary bile acid expressing a carboxyl group at the C-24 position on the side chain, was selected as the model ligand (Figure 1). The structure of the steroid nucleus of bile acids is very important for binding to proteins such as FXR/BAR (*22*, *23*) and hepatic bile acid acyl glucuronosyltransferase (*24*). The novel affinity labeling reagent demonstrated in this study is expected to be suitable for structural analysis around the binding site, because the bile acid side chain is located far from the protein−ligand binding site.

EXPERIMENTAL PROCEDURES

Reagents and Apparatus. The 6−17 fragment of dynorphin A was purchased from American Peptide Co., Inc. (Sunnyvale, CA). A matrix for MALDI-TOFMS, α-cyano-4-hydroxycinnamic acid (CHCA), was supplied by Aldrich Chemical Co. (Milwaukee, WI). Affi-Gel-10 was purchased from Bio-Rad Laboratories (Hercules, CA). A ZipTip C_{18} cartridge was purchased from Millipore (Milford, MA). Deoxycholyl adenylate (DCA-AMP) was synthesized in our laboratory (*21*). Horseradish peroxidase (HRP, EC 1.11.1.7, grade I-C, 263 units/mg) was obtained from Toyobo (Osaka, Japan), and HRP-labeled deoxycholic acid, an enzyme-labeled antigen, was synthesized in our laboratory (*25*). AffiniPure rabbit anti-mouse IgG + IgM antibody was purchased from Jackson ImmunoResearch (West Grove, PA). Distilled and ion-exchanged water was prepared by the CPW-100 Ultrapure water system (Advantec Toyo Co., Tokyo, Japan), and other reagents and solvents were of HPLC grade.

An LC-10Ai system (Shimadzu, Kyoto, Japan) equipped with an SCL-$10A_{VP}$ system controller, LC-10Ai pumps, and a detector, SPD-$M10A_{VP}$, was used to monitor both peptide adduct and residual ligands. MALDI-TOFMS analysis was performed using Voyager DE-STR (Applied Biosystems, Framingham, MA) and AXIMA-CFR (Shimadzu) equipped with an N_2 laser (337 nm). LC/ESI-MS/MS analysis was performed using QSTAR pulser i (Applied Biosystems) combined with a micro 21 LC-01 (Jasco Co., Tokyo) or model 1100 HPLC pump (Agilent Technologies, Palo Alto, CA). The single DCA adduct of the 6−17 fragment of dynorphin A and the affinity-extracted peptides were analyzed on a MAGIC-MS column (0.2 mm i.d. × 50 mm; AMR, Tokyo) using linear gradient elution of mobile phase B concentration from 5% to 50% over 30 min at a flow rate of 4 μL/min or from 10% to 60% over 20 min at a flow rate of 5 μL/min: mobile phase A, water/methanol/formic acid (95:4:1 v/v/v); mobile phase B, water/methanol/formic acid (5:94:1 v/v/v).

Effect of pH on Formation of DCA-Bound Peptide and Hydrolysis of Acyl Adenylate. DCA-AMP (20 nmol) was incubated with the 6−17 fragment of dynorphin A (20 nmol) at 37 °C in 200 μL of 50 mM potassium phosphate buffer under various pH conditions (pH 5.0, 6.0, 7.4, 8.0, 8.5). Aliquots (10 μL) of the reaction mixture were withdrawn at 2, 4, 8, 12, 16, 24, and 48 h. To these aliquots was added 6 μL of 50% acetic acid for reaction termination, followed by 1 μg of luteinizing hormone-releasing hormone as an internal standard. A 5 μL aliquot of the solution was analyzed by HPLC along a linear gradient for the identification of DCA-labeled dynorphin A. The formed peptide adducts were analyzed by HPLC on a Jupiter 5uC18 300 Å (5 μm, 2.0 mm i.d. × 150 mm; Phenomenex, Torrance, CA) at a flow rate of 200 μL/min with a 5−45% linear gradient of mobile phase B over 50 min. Mobile phase A was water/acetonitrile (9:1 v/v) containing 0.08% TFA, and mobile phase B was water/acetonitrile (1:9 v/v) containing 0.08% TFA. An additional 3 μL of the reaction mixture was withdrawn at the same time. To this aliquot was added 3 μL of acetic acid for reaction termination followed by 2 μg of chenodeoxycholic acid as an internal standard. The remaining DCA-AMP and hydrolyzed DCA were quantitated by HPLC on a Capcell Pak C_{18} UG120 column (5 μm, 4.6 mm i.d. × 150 mm; Shiseido, Tokyo) with 20 mM ammonium phosphate buffer (pH 6.0)/acetonitrile (2:1 v/v) as the mobile phase at a flow rate of 1 mL/min. The analytes were monitored by UV detection at 205 nm.

Preparation of Affinity Gel Immobilized Anti-Deoxycholate Monoclonal Antibody. Affi-Gel 10 (1 mL) was sequentially washed with 4 mL of chloroform, 4 mL of 2-propanol, 4 mL of 50% (v/v) methanol, and 4 mL of 10 mM sodium acetate buffer (pH 4.5). The gel was then equilibrated with the coupling buffer, 100 mM HEPES buffer (pH 8.0), and incubated with 10 mg of anti-deoxycholate IgG (*26*) at 4 °C overnight in 1.5 mL of the same buffer. The reaction was terminated by the addition of 100 μL of 1 M monoethanolamine hydrochloride, and the mixture was incubated at 4 °C for 2 h. The residual IgG in the supernatant was measured to calculate immobilized IgG.

Affinity Labeling of DCA to Anti-DCA Monoclonal Antibody. To isolate and identify labeled peptide, anti-DCA monoclonal antibody (8.7 nmol/380 μL) and DCA-AMP (17.4 nmol/12.8 μL) were incubated in 50 mM potassium phosphate buffer (pH 6.0) at 37 °C for 2 h. The reaction mixture was adjusted to pH 7.4 by adding 500 mM sodium hydroxide solution (31.5 μL), and the mixture was further incubated at 37 °C for 24 h. The reaction mixture (424 μL) was added into 0.87 mL of 1.65 M Tris-HCl buffer (pH 8.6) containing 7.3 M guanidine hydrochloride and 0.03 M EDTA and incubated for 1 h at 60 °C. The mixture was reduced

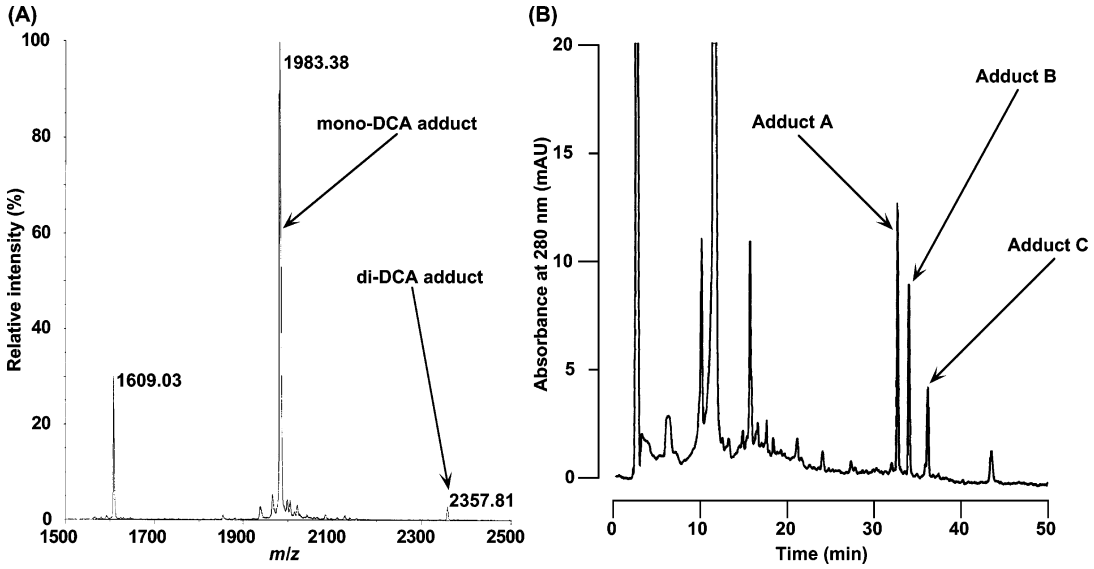

FIGURE 2: MALDI mass spectrum (A) and HPLC chromatogram (B) of the reaction mixture resulting from the incubation of deoxycholyl adenylate with the 6−17 fragment of dynorphin A for 8 h. MS conditions: instrument, Voyager DE-STR (linear mode); matrix, α-CHCA; accelerating voltage, 25 kV; grid voltage, 18 kV; guide wire voltage, 50 V. For HPLC conditions, see Experimental Procedures.

for 3 h at 50 °C using dithiothreitol (0.44 mg), 2.2 mg of iodoacetamide was added to the mixture, and the resultant mixture was incubated for 3 h at room temperature in the dark. After the reduced and carboxyamidomethylated proteins were dialyzed against 50 mM ammonium bicarbonate solution/methanol (95:5 v/v), the proteins were digested with trypsin (the enzyme-to-substrate ratio was approximately 1:20). The obtained peptide fragment mixture (0.5 nmol/1.54 mL) was applied to the anti-DCA antibody-immobilized affinity gel (8.67 nmol/0.13 mL gel) equilibrated with 50 mM sodium phosphate buffer (pH 7.3). After gentle incubation at 4 °C overnight, the gel was washed twice with 2.5 mL of 20 mM ammonium acetate buffer (pH 5.0)/methanol (95:5 v/v), and the labeled peptide was eluted with 2.5 mL of water/methanol (1:1 v/v) containing 0.1% TFA. After concentration, the residue was dissolved in 100 μL of water/acetinitrile (1:1 v/v) containing 0.1% TFA and analyzed by MALDI-TOFMS using α-CHCA as a matrix.

ELISA for Quantification of Labeled DCA Bound to Antibody. To demonstrate specific labeling to the binding site, anti-DCA monoclonal antibody possessing two interaction sites (0.43 nmol) and DCA-AMP (0.09, 0.43, and 0.85 nmol) were incubated in 75 μL of 50 mM potassium phosphate buffer (pH 6.0) at 37 °C for 2 h. The reaction mixture was adjusted to pH 7.4 with a 500 mM sodium hydroxide solution, and the mixture was further incubated at 37 °C for 24 h. ELISA was performed to determine the amount of residual DCA.

AffiniPure rabbit anti-mouse IgG + IgM antibody diluted 1:400 with 50 mM sodium phosphate buffer (pH 7.3) containing 0.9% (w/v) sodium chloride (0.63 μg/100 μL) was applied to each well of EIA/RIA plates and incubated overnight at 4 °C. After the wells were washed with 50 mM sodium phosphate buffer (pH 7.3) containing 0.9% (w/v) sodium chloride and 0.05% (v/v) Tween 20 (buffer A), 10% (w/v) skim milk in 50 mM sodium phosphate buffer (pH 7.3, 200 μL) was applied for 1 h at room temperature to block nonspecific binding. The wells were washed three times with buffer A, and the reaction mixture diluted 1:10000 with 100 μL of 50 mM sodium phosphate buffer (pH 7.3) containing 0.1% (w/v) gelatin and 0.9% (w/v) sodium chloride (buffer B) was then added. After incubation at room temperature for 1 h, the solutions were aspirated, and the wells were washed three times with buffer A. HRP-labeled antigen (DCA coupled to HRP, 4 ng) dissolved in 100 μL of buffer B was then added, and the samples were incubated overnight at room temperature. After the wells were washed with buffer A, bound enzyme activity was measured colorimetrically using 100 μL of 25 mM citrate−50 mM sodium phosphate buffer (pH 5.0) containing 0.04% (w/v) *o*-phenylenediamine dihydrochloride to which 60 μL of 30% (w/v) H_2O_2 was added. After incubation at room temperature for 1 h, 100 μL of 1 M H_2SO_4 was added to terminate the enzymatic reaction, and absorbance at 492 nm was measured using an MRP-A4I microplate reader (Tosoh, Tokyo).

RESULTS

In order for cross-linking reagents to be useful for later structure determination, they should be readily soluble in physiologic aqueous buffers. The acyl adenylate derivative DCA-AMP is a newly described reagent that meets this criterion (*21*). The reactivity of acyl adenylate in physiologic aqueous solutions without organic solvent was investigated. The 6−17 fragment of dynorphin A (RRIRPKLKWDNQ, 100 nmol/mL) was incubated with equimolar DCA-AMP at 37 °C in 50 mM potassium phosphate buffer (pH 7.4). Aliquots of the reaction mixture were analyzed by HPLC and MALDI-TOFMS (Figure 2); a peak corresponding to the 6−17 fragment of dynorphin A derivatized with one DCA molecule was observed after 1 h, and an ion corresponding to the peptide modified with two DCA molecules appeared 8 h after in the MALDI-MS spectra. The peaks corresponding to the single DCA adducts were analyzed with ESI-MS/MS, and the product ion spectra of $[M + 3H]^{3+}$ were illustrated in Figure 3. There appeared a b_1 ion with a mass shift of 374 at *m*/*z* 531.49 as the doubly charged ion in Figure 3A (adduct A), and other b-ion series were clearly observed, indicating that DCA was coupled to the N-terminus. In the similar manner, we identified both adduct B and adduct C as the dynorphin A fragments coupled to DCA

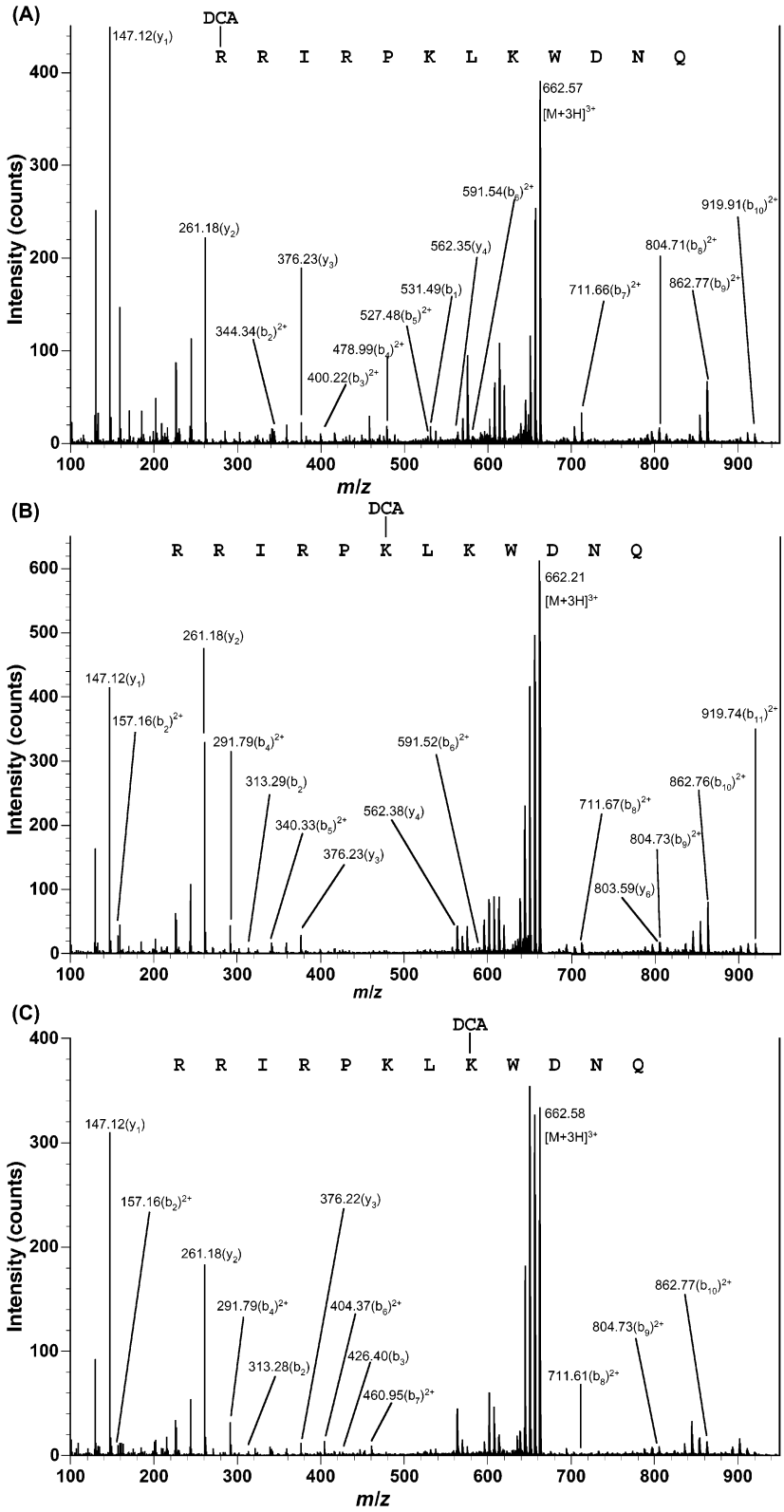

FIGURE 3: Product ion mass spectra of the $[M + 3H]^{3+}$ ion of one DCA molecule adduct to the 6−17 fragment of dynorphin A at *m*/*z* 662.2: (A) adduct A; (B) adduct B; (C) adduct C. Conditions: instrument, QSTAR pulsar i system equipped with micro 21 LC-01; column, MAGIC-MS (0.2 mm i.d. × 50 mm); flow rate, 4 μL/min; mobile phase A, water/methanol/formic acid (95:4:1 v/v/v), and mobile phase B, water/methanol/formic acid (5:94:1 v/v/v); linear gradient program, mobile phase B concentration from 5% to 50% over 30 min; declustering potential, 60.0 V; collision energy, 50.0 V; collision gas, 5 units (N_2).

through Lys6 and Lys8, respectively. Those data clearly indicated that DCA-AMP reacts with free amino groups located at both the lysine residues and N-terminus. Since the peak area ratio of adducts on the HPLC chromatogram shown in Figure 2B agrees well with the relative distribution of label among amine groups, the ratio of adducts A, B, and C is 3:2:1.

To be useful for structural analyses of protein−ligand interactions, affinity labels should be allowed to react following the binding of the ligand of interest to its receptor. Modula-

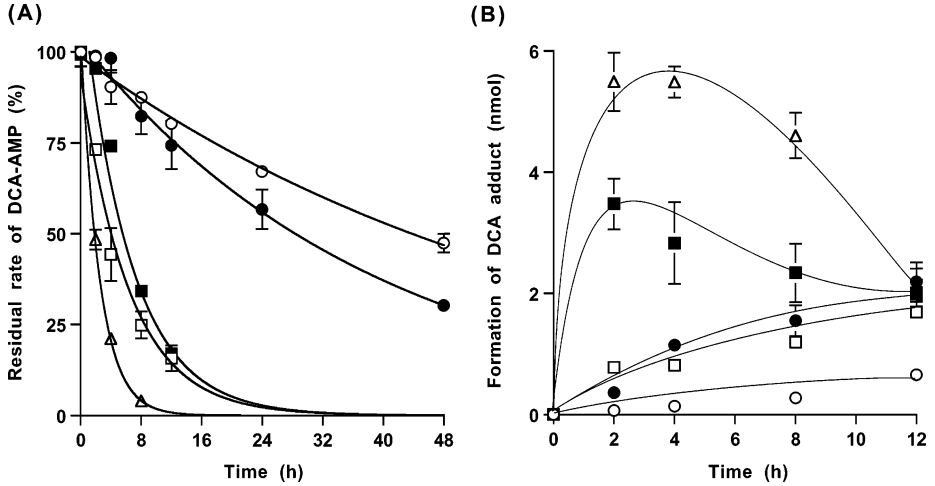

FIGURE 4: Effect of pH on hydrolysis of deoxycholyl adenylate and the formation of DCA−peptide adducts. (A) Time course of hydrolysis of deoxycholyl adenylate under the various pH conditions. (B) Time course of formation of the DCA adduct under the various pH conditions: pH 5.0 (open circle), pH 6.0 (filled circle), pH 7.4 (open square), pH 8.0 (filled square), and pH 8.5 (open triangle).

tion of the labeling reaction is essential to reduce nonspecific binding, and pH modification is a common means to control such reactions. Therefore, the stability of DCA-AMP under various pH conditions was investigated. The rate of DCA-AMP hydrolysis was greatly influenced by pH, and it was completely hydrolyzed after 24 h at pH 7.4 or above (Figure 4A). Conversely, nearly 50% of the DCA-AMP remained after 48 h at pH 5.0, and greater than 80% of the starting DCA-AMP remained after 8 h at pH 5.0 and 6.0.

Next, we investigated the reactivity of DCA-AMP under various pH conditions employing the 6−17 fragment of dynorphin A as a model peptide as shown in Figure 4B. In the MALDI mass spectrum, the signal intensity of a single DCA molecule adduct increased with time up to 4 h at pH 7.4. Under more alkaline conditions (pH 8.0 and 8.5), the reaction rate was increased, and after 4 h, the amount of a single DCA molecule adduct was decreased due to the generation of peptide modified by two DCA molecules. In contrast, adduct formation at pH 5.0 was less than 1% of that at pH 8.0 for 2 h. Mass spectrometry also revealed that, at pH 5.0, DCA bound almost exclusively to the N-terminal amino group. These results strongly indicate that the reaction of DCA-AMP can be controlled by adjusting the pH.

Antibodies have a high affinity for their specific antigens, and affinity labeling of antibodies is important for the identification and characterization of the binding site, as well as for clarification of the binding mechanism (*27*, *28*). Pseudo-ligand−antibody complexes prepared by affinity labeling have also been used as antigen for producing anti-meta-type and anti-idiotype antibodies (*16*, *29*, *30*). We have previously prepared an anti-DCA monoclonal antibody, which has a high affinity and specificity for the DCA molecule, especially its steroid nucleus (*26*). We therefore used DCA-AMP to specifically label the interactive site on the anti-DCA monoclonal antibody molecule. Following incubation of DCA-AMP with antibody, competition with a gross excess of DCA-HRP (HRP-labeled antigen) will displace noncovalently associated DCA-AMP and hydrolyzed DCA. The amount of antibody-associated HRP should be inversely related to the amount of DCA-AMP-derivitized antibody. Anti-DCA monoclonal antibody was incubated with various concentrations of DCA-AMP in 50 mM sodium phosphate buffer (pH 6.0) at 37 °C for 1 h to produce specific noncovalent ligand−antibody complexes. After the pH was adjusted to 7.4 by adding the proper volume of 500 mM sodium hydroxide, antibody affinity labeling was carried out. After the addition of DCA-HRP (HRP-labeled antigen), the bound enzymatic activity was determined as described in the Experimental Procedures. The results obtained by the competitive ELISA are shown in Table 1. Enzymatic activity was inversely related to the antibody:DCA-AMP molar ratio. The results clearly indicate the covalent attachment of DCA to the antigen binding groove of the anti-DCA monoclonal antibody.

Table 1: Residual Binding of DCA−HRP to Anti-Deoxycholate Antibody

molar ratio (antibody:DCA-AMP)	residual binding of DCA-HRP (%)
1:0	100.0 ± 2.22
1:0.1	85.9 ± 5.33
1:0.5	60.7 ± 7.38
1:1	44.5 ± 1.59

The affinity labeling of anti-DCA antibody should only occur on a portion of the antibody molecule. Consequently, enzymatic digestion of DCA−antibody complexes was carried out followed by affinity purification on an anti-DCA antibody column. The obtained peptides were then subjected to mass spectrometry analysis. Fractions containing target peptides were measured by MALDI-TOFMS using α-CHCA as a matrix, and the obtained mass spectrum was compared to the results of unlabeled samples. A unique peak was clearly observed at *m*/*z* 2128.3 in the affinity labeling samples (Figure 5). To determine the amino acid sequence of this peptide fragment, it was injected into a capillary LC/ESI-MS/MS system using the hybrid Q-TOF mass analyzer. The nomenclature for the fragment ions is based on that proposed by Roepstorff and Fohlman (*31*), as modified by Biemann (*32*). The ESI full-scan mass spectrum showed an abundant doubly charged ion at *m*/*z* 1063.6, which was selected as the precursor ion for the MS/MS analysis. As shown in Figure 6, the product ion scan of the above doubly charged ion exhibited doubly charged ions corresponding to both one and two water-eliminated molecules at *m*/*z* 1055 and 1045,

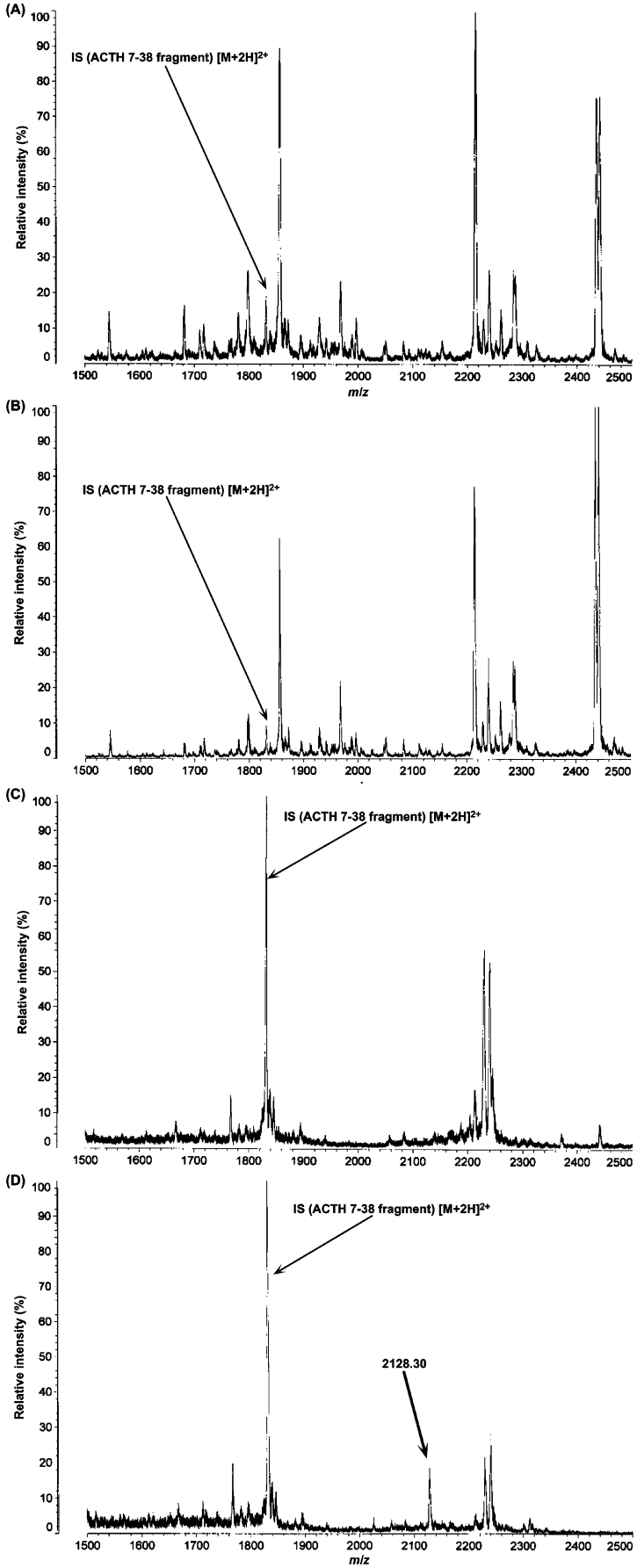

FIGURE 5: MALDI mass spectra of the tryptic digests of anti-DCA monoclonal antibody without both labeling and affinity extraction (A), with labeling and without affinity extraction (B), without labeling and with affinity extraction (C), and with both labeling and affinity extraction (D). MS conditions: instrument, AXIMA-CFR (linear mode); matrix, α-CHCA; accelerating voltage, 25 kV; grid voltage, 18 kV; guide wire voltage, 50 V.

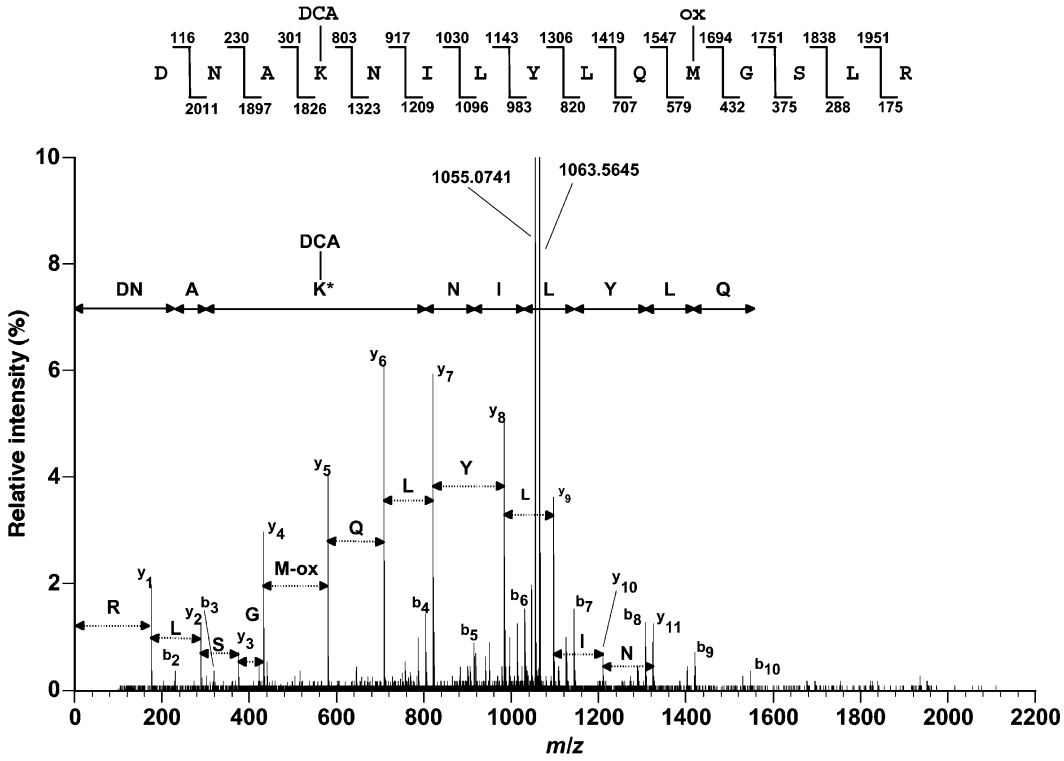

FIGURE 6: Product ion mass spectrum of the $[M + 2H]^{2+}$ ion of the DCA adduct to the anti-deoxycholic acid monoclonal antibody at *m*/*z* 1063.6 extracted from the reaction mixture by affinity extraction gel. Conditions: instrument, QSTAR pulsar i system equipped with an Agilent 1100 pump; column, MAGIC-MS (0.2 mm i.d. × 50 mm); flow rate, 5 μL/min; mobile phase A, water/methanol/formic acid (95:4:1 v/v/v), and mobile phase B, water/methanol/formic acid (5:94:1 v/v/v); linear gradient program, mobile phase B concentration from 10% to 60% over 20 min; declustering potential, 60.0 V; collision energy, 35.0 V; collision gas, 5 units (N_2).

respectively, with a ΔM value of 9 Da. Those fragmentations may be due to the elimination of 3α- and 12α-hydroxyl groups at the steroid nucleus. The tryptic-digested peptide fragment was oxidized at a methionine residue, which was clearly demonstrated by the mass shift from the y_5 ion to the y_4 ion. In the y-ion series, ions y_1 to y_{11} were detected, and the derived sequence was NILYLQM(ox)GSLR. In contrast, the detected b-ion series included ions b_2 to b_{10}. The mass shift from b_4 to b_3 was 502 Da, which corresponded to a lysine residue coupled with one molecule of DCA.

DISCUSSION

We have previously reported some characteristics of a unique affinity labeling reagent, acyl adenylate, and in this paper we have shown the ability of this compound to react with the ϵ-amino group of lysine residues neighboring specific binding sites of proteins by a nucleophilic displacement reaction (Figure 1). Because this reaction is able to be controlled by slight pH changes, protein−ligand interactions are able to be carried out at a near neutral pH in aqueous buffers. Thus, solvent- or pH-induced conformational changes can be minimized during this important step.

Immunoglobulin G, a glycoprotein of 150 kDa composed of two heavy and two light chains covalently associated via disulfide bonds, is frequently used in immunoassays. Both the heavy and light chains possess variable regions, V_H and V_L domains, and constant regions, C_H and C_L domains. The antigen binding site of IgG is comprised of the combination of V_H and V_L domains, and the characteristics, specificity and affinity, of the antibody are determined by the amino acid sequence in this region. Antigen binds to the specific site formed by three complementarity-determining regions,

V_H domain

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1           11          21          31          41
EVQLVESGGD LIQPGGSLKL SCAVSGFTLN YYGMSWVRQT PDRRLEWVAT
                                    CDR1
51          61          71          81          91
IIGGLTYYPA SVKGRFTISR DNAKNILYLQ MGSLRSEDTA MYFCARRGYG
 CDR2
101         111
HHFDYWGQGT ALTVSS
 CDR3
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V_L domain

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1           11          21          31          41
QAVVTQESAL TTSPGETVTL TCRSSTGTVT TSNYANWVQE KPDHLFTGLI
                                CDR1
51          61          71          81          91
GGTNNRVPGV PARFSGSLIG DRAALTITGA QTEDEAIYFC GLWFSNHLVF
 CDR2                                        CDR3
101
GGGTKLTVLG
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FIGURE 7: Amino acid sequences of the V_H and V_L domains of the anti-deoxycholic acid antibody single-chain Fv fragment.

CDR1, CDR2, and CDR3, on the heavy chain and the light chain of the variable domains. The combination of various CDR structures can create a virtually limitless number of specific binding sites for various ligands (*33*, *34*). The determination of binding site structure is, therefore, very important for understanding the mechanism of specific binding at the molecular level. Kobayashi et al. performed DNA sequencing analysis of the heavy and light chains of variable domains (*35*), and the amino acid sequence is shown in Figure 7. The anti-DCA monoclonal antibody used in this study, which is of the IgG isotype, was prepared by immunization of DCA, which conjugates to the ϵ-amino group on bovine serum albumin through the carboxyl group at the C-24 position of its side chain (*26*). This antibody can clearly recognize the steroid nucleus and capture DCA more efficiently than a

DCA−lysine conjugate, suggesting that the side chain of DCA interacts with the antibody binding groove. Consistent with this model, it was found that the DCA acyl adenylate reacts with the ϵ-amino group at Lys74 on the V_H domain of the anti-DCA monoclonal antibody, which is located between CDR2 and CDR3.

In conclusion, acyl adenylate is a novel affinity labeling reagent developed in this study that has a high degree of aqueous solubility and high pH-sensitive reactivity toward nucleophiles located around the binding site of target proteins. These observations indicate that acyl adenylates should be very useful for clarifying ligand−protein interactions related to protein function.

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