Introduction of Nucleophiles and Spectroscopic Probes into Antibody Combining Sites

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A general chemical strategy has been developed whereby antibody combining sites can be selectively derivatized with natural or synthetic molecules, such as catalytic groups, drugs, metals, or reporter molecules. Cleavable affinity labels were used to selectively introduce a thiol into the combining site of the immunoglobulin A MOPC 315. This thiol acted both as a nucleophile to accelerate ester thiolysis 60,000-fold and as a handle for selectively derivatizing the antibody with additional functional groups. For example, derivatization of the antibody with a fluorophore made possible a direct spectroscopic assay of antibody-ligand complexation. This chemistry should not only extend our ability to exploit antibody specificity in chemical catalysis, diagnostics, and therapeutics, but may also prove generally applicable to the functional modification of other proteins for which detailed structural information is unavailable.

THE ADVENT OF MONOCLONAL ANTIbodies has made possible the generation of homogeneous receptors to a vast array of structurally diverse ligands. The high binding affinity and specificity of antibodies can be exploited in the development of selective catalysts for acyl transfer (*1*), concerted (*2*), photochemical (*3*), and redox reactions (*4*). In each case, antibodies were raised against haptenic groups that were designed to generate combining sites with specific catalytic functions, such as stabilization of a rate-determining transition state. Alternatively, it should be possible to directly introduce catalytic groups into antibody combining sites, either by generating a "cofactor" binding site or by genetic or chemical methods. We report a chemical strategy whereby antibody combining sites can be selectively modified with a wide variety of synthetic or natural catalytic groups. Cleavable affinity labels were used to introduce a nucleophilic thiol into the combining site of the immunoglobulin A (IgA) MOPC 315 to generate an antibody that accelerates ester cleavage 60,000-fold compared with the background rate. The thiol also provides a handle whereby additional chemical functionality, such as enzymatic cofactors, metal-ligand complexes, drugs, or spectroscopic probes, can be selectively introduced into the antibody combining site. For example, derivatization of the thiolated MOPC 315 with the fluorophore-*N*-fluoresceinthiourеido-2-mercaptoethylamine has made possible a direct spectroscopic assay of antibody-ligand complexation.

The antibody MOPC 315 binds substituted 2,4-dinitrophenyl (DNP) ligands with association constants ranging from 5×10^4 to $1 \times 10^6 M^{-1}$ (*5*). Although a three-dimensional structure is not available, the antibody combining site has been characterized spectroscopically (*6*) and by chemical modification (*7–9*), and the primary amino acid sequence of the variable region is known (*10*). Given a crystal structure of the immunoglobulin variable region, one might use oligonucleotide-directed mutagenesis to site-selectively introduce a nucleophilic cysteine into the antibody combining site. In the absence of detailed structural information, affinity-labeling reagents have been used to selectively modify proteins (*8, 11*). We have incorporated cleavable tethers into affinity labels specific for the antibody combining site. Covalent attachment of the label to the antibody, followed by cleavage of the cross-link and removal of free ligand, results in site-specific incorporation of a free thiol into the antibody combining site.

In order to site-specifically introduce a nucleophilic thiol into the combining site of MOPC 315, affinity labels **1** through **5** were synthesized (Fig. 1) (*12*). These labels consist of the DNP group linked to electrophilic aldehyde or α-bromoketone groups through cleavable disulfide or thiophenyl linkages. The geometry of the affinity labels varies with regard to the distance between the DNP group and electrophilic moiety, since the position of a nucleophilic combining site Lys, His, or Tyr side chain is not precisely known. The position of the cleavable linkage was designed to approximate that of the ester in the corresponding substrate, **6** or **7**, in order to ensure that the incipient thiol was positioned appropriately in the combining site to attack the ester. The Fab fragment of MOPC 315 was treated with aldehyde **1** or **2**, followed by reduction with $NaCNBH_3$, or directly with α-bromoketone **3**, **4**, or **5** (Fig. 2). The labeled Fab fragment was purified by affinity chromatography (*13*) in order to remove unlabeled or nonspecifically labeled Fab. With labels **2** and **4**, greater than 90% of label was incorporated (85% yield after chromatography) and less than 10% nonspecific labeling occurred in the presence of 10 m*M* of the competitive inhibitor *N*-DNP-glycine. Cleavage of the label with dithiothreitol (DTT), desalting, and subsequent reaction with 2,2′-dithiodipyridine afforded the *S*-thiopyridyl–derivatized Fab in greater than 90% chemical yield (65% recovery in the case of **2**).

Fab fragments labeled with **2** or **4** were subjected to tryptic digestion and peptide mapping (*14*) in order to determine the selectivity of thiol incorporation (Fig. 3). Greater than 95% of label **2** was incorporated into the heavy (H) chain and greater than 95% of label **4** was incorporated into the light (L) chain. Isolation and sequencing of the radiolabeled tryptic peptides revealed greater than 95% derivatization of Lys^{52} H and Tyr^{34} L with labels **2** and **4**, respectively. These residues are identical to those labeled by Givol and co-workers (*14*) with the reagents bromoacetyl-N^{ε}-DNP-L-lysine and *N*-bromoacetyl-*N*′-DNP-ethylenediamine.

Cleavage of the DNP-coumarin esters **6** and **7** by the thiol-containing Fabs (generated from **2** and **4**) was assayed in the presence of 0.1 μ*M* antibody (*p*H 7.0, 10°C) by monitoring the release of free coumarin spectrophotometrically (*15*). The antibody affinity-labeled with **2** was found to acceler-

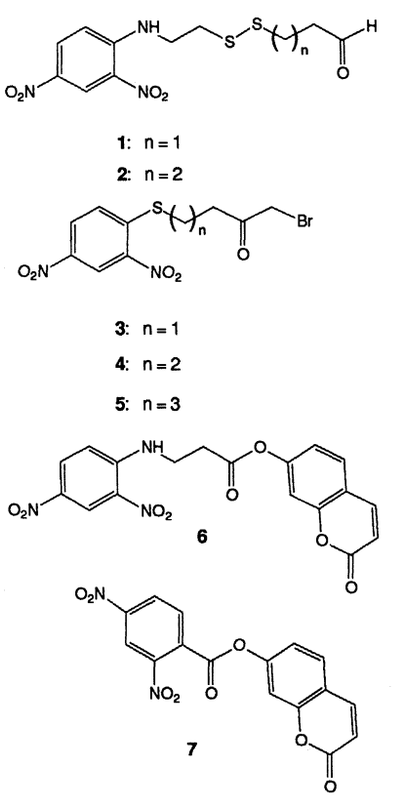

Fig. 1. Affinity labels and substrates.

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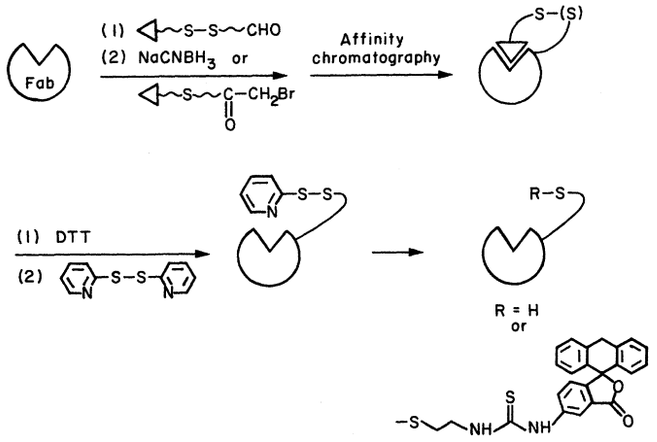

Fig. 2. Introduction of thiol and fluorophore into antibody combining site. Fab fragments were generated from MOPC 315 by treatment of the reduced and alkylated IgA with papain followed by chromatography on Sephadex G-50 and subsequent affinity chromatography on DNP-coupled Sepharose 4B (*13*). The Fab fragment (10 μM) was treated either with 1.5 equivalents of aldehydes **1** or **2** and 7.5 equivalents of $NaCNBH_3$ in 0.2*M* sodium phosphate, *p*H 7.0, at 37°C for 20 hours or directly with 1.3 equivalents of α-bromoketones **3**, **4**, or **5** in 0.1*M* sodium bicarbonate, *p*H 9.0, at 37°C for 16 hours. In both cases, the labeled Fab was purified by chromatography on Sephadex G-50 and subsequent affinity chromatography on DNP-coupled Sepharose 4B (*13*). The extent of derivatization was quantitated spectrophotometrically (for labels **1** and **2**, wavelength of maximum absorbance, λ_{max} = 362 nm, $\varepsilon = 16{,}000M^{-1}\ cm^{-1}$; for labels **3**, **4**, and **5**, λ_{max} = 360 nm, $\varepsilon = 12{,}800M^{-1}\ cm^{-1}$) and additionally by incorporation of tritium from $NaCNB^3H_3$ (labels **1** and **2**). Cleavage of the affinity-labeled Fab (2.0 mg) with 50 m*M* DTT (2 m*M* EDTA and 0.1*M* sodium phosphate, *p*H 8.0) afforded the free thiol. The thiol-containing Fab was collected by Pharmacia fast-desalting chromatography in 0.1*M* sodium phosphate, *p*H 7.3, directly into 1.0 ml of a 4.5 m*M* 2,2′-dithiodipyridine solution (0.1*M* sodium phosphate, *p*H 5.5, containing 15% acetonitrile). This mixture was allowed to react at 20°C for 12 hours after which excess 2,2′-dithiodipyridine was removed by exhaustive dialysis. The thiolated antibody was derivatized in greater than 90% yield (65% recovery in the case of **2**) based on the absorbance of thiopyridone at 343 nm ($\varepsilon = 7060M^{-1}\ cm^{-1}$) (*18*) after cleavage with 10 m*M* DTT, and the protein absorbance ($E^{0.1\%}_{280\ nm}$ = 1.44, molecular weight 50,000 for Fab) (*13*). For fluorescein derivatization, the *S*-thiopyridyl Fab labeled with **2** (9.0 nmol in 0.50 ml of 0.1*M* sodium phosphate, *p*H 7.3) was treated with *N*-fluoresceinthioureido-2-mercaptoethylamine (*17*) (52 nmol in 0.50 ml of 0.1*M* triethylammonium acetate, *p*H 7.5, containing 10% acetonitrile). The reaction was monitored by release of thiopyridone at 343 nm (*18*). After 3 hours, no additional thiopyridone was released, and the mixture was dialyzed exhaustively against assay buffer (50 m*M* sodium chloride, 50 m*M* sodium phosphate, *p*H 7.0).

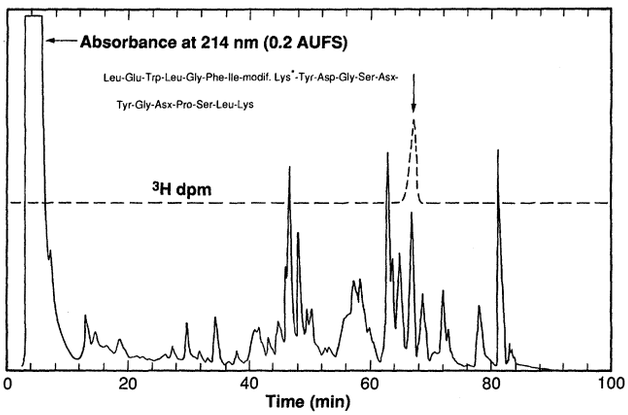

Fig. 3. High-performance liquid chromatography (HPLC) profile of the tryptic digest of the heavy chain of Fab labeled with **2**, monitored by ultraviolet absorbance (solid line) and radioactivity (dashed line). Fab was affinity labeled in the presence of label **2** and $NaCNB^3H_3$ (12 mCi/nmol) or with tritium-labeled **4** (4 mCi/mmol) as described above. The labeled Fab (1.0 mg/ml) was then denatured in 8*M* urea and 0.1*M* tris-HCl, *p*H 8.0, reduced with 20 m*M* DTT (1 hour at 37°C) and alkylated with 60 m*M* iodoacetamide (1 hour at 37°C). After dialysis against 7*M* urea and 20 m*M* tris-HCl, *p*H 8.0, the heavy and light chains were separated by anion-exchange chromatography on a Pharmacia Mono Q fast protein liquid chromatography column. Separation was achieved at 1.0 ml/min in 7*M* urea and 20 m*M* tris-HCl, *p*H 8.0, with a linear gradient of 40 to 200 m*M* sodium chloride for a 30-min period. In the case of Fab labeled with **2**, the heavy chain was found to contain over 95% of the incorporated tritium; with Fab labeled with **4**, over 95% of the tritium label was on the light chain. In each case, the radiolabeled chain was dialyzed against 2*M* urea–100 m*M* ammonium bicarbonate, *p*H 8.2, and then treated with trypsin [1:25 (w/w) trypsin: Fab] in the presence of 0.1 m*M* $CaCl_2$ at 37°C in the dark. After 8 hours, the reactions were quenched with 10% (v/v) acetic acid. The radiolabeled peptides were purified by reversed-phase HPLC with a 70-min linear gradient from 0 to 50% acetonitrile in water at 1.0 ml/min; [0.1% and 0.06% (v/v) trifluoroacetic acid were added to the water and the acetonitrile, respectively]. Peptides were detected by their absorbance at 214 nm, fractions were collected at 1.0-min intervals, and aliquots were counted for radioactivity. Fractions containing radioactivity were rechromatographed with a 70-min linear gradient from 0 to 50% 2-propanol in water at 0.75 ml/min. (The water and the 2-propanol contained 0.1% and 0.06% trifluoroacetic acid, respectively.) The amino acid sequences of the pure peptides were determined on an Applied Biosystems 477A Protein Sequencer.

ate the cleavage of ester **6** by a factor of 6×10^4 over the cleavage reaction with 0.1 μM DTT. The reaction kinetics are consistent with the formation of a Michaelis complex: the kinetic constants k_{cat} and K_m for the reaction are 0.87 ± 0.08 min^{-1} (mean ± SD) and 1.2 ± 0.1 μM, respectively. The thiolysis reaction was competitively inhibited by *N*-DNP-glycine with an inhibition constant (K_i) of 8 ± 1 μM. Neither the uncleaved affinity-labeled antibody nor the iodoacetamide-alkylated antibody accelerated the rate of the thiolysis reaction above the background rate. The stoichiometry of product release corresponded to 1.0 ± 0.1 coumarin to 1.0 Fab-SH. Upon reaction with **6**, the esterified antibody, after fast-desalting chromatography, did not have a titratable thiol. However, subsequent treatment with hydroxide led to the release of one free thiol. The introduction of both a thiol and general base into the label may lead to a catalytic system (*16*). Interestingly, thiolated Fab labeled with bromoketone **4** did not hydrolyze esters **6**, **7**, or DNP-acetate. Fluorescence quenching experiments revealed that the derivatized Fab did not bind the substrate with appreciable affinity, presumably as a result of steric congestion in the antibody combining site.

In order to demonstrate that the thiolated Fab can be further derivatized with catalysts or reporter molecules, *N*-fluoresceinthioureido-2-mercaptoethylamine (*17*) was selectively introduced through a disulfide exchange reaction (Fig. 2). The resulting ad-

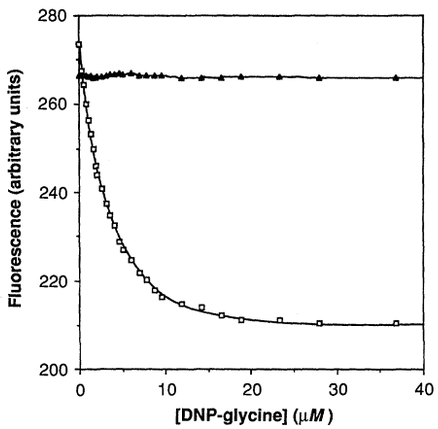

Fig. 4. Fluorescence quenching binding assay for *N*-DNP-glycine with the fluorescein-Fab adduct (□) versus control with fluorescein + underivatized Fab (▲). Fluorescence quenching experiments were carried out at 10°C with 492 nm for excitation and measuring emission at 521 nm. The fluorescein-Fab adduct was diluted with 50 m*M* sodium chloride, and 50 m*M* sodium phosphate, *p*H 7.0 to 0.10 μM. Aliquots of *N*-DNP-glycine were added and, after mixing, the fluorescence observed. Free *N*-fluoresceinthioureido-2-mercaptoethylamine and underivatized Fab, each at 0.10 μM, were treated in a similar experiment.

duct was isolated in 84% overall yield with greater than 90% incorporation of fluorophore. Addition of the ligand *N*-DNP-glycine to the fluorescein-Fab adduct resulted in a decrease in fluorescence, providing a direct assay of ligand binding (Fig. 4). The association constant of *N*-DNP-glycine to the fluorescein-Fab adduct was determined to be $(3.0 \pm 0.5) \times 10^5 M^{-1}$, almost identical to the K_A of *N*-DNP-glycine to underivatized MOPC 315, $2.0 \times 10^5 M^{-1}$ (*5*). Addition of DNP-glycine to a solution of one equivalent free *N*-fluoresceinthioureido-2-mercaptoethylamine and Fab resulted in no detectable fluorescence change.

These experiments show that it is possible to develop selective catalysts that combine the high binding specificity of the immune system with the diverse and efficient catalytic groups available from synthetic chemistry (*19*). In addition, the chemical strategy described here may find application to the selective modification of other proteins for which the three-dimensional structures are unavailable.

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19. Recent unpublished observations demonstrate that derivatization of the thiolated antibody (label **2**) with 4-mercaptomethylimidazole results in a semisynthetic antibody which catalyzes ester hydrolysis 10^3-fold above background.

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Multiple Principal Sigma Factor Homologs in Eubacteria: Identification of the "*rpoD* Box"

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Genes for the principal sigma factor (*rpoD* genes) of various eubacteria were identified with a synthetic oligonucleotide probe corresponding to a conserved sequence in *rpoD* gene products of *Escherichia coli* and *Bacillus subtilis*. Multiple *rpoD* homologs were found in the strains of *Micrococcus*, *Pseudomonas*, and *Streptomyces*, whereas single genes were detected in *E. coli*, *B. subtilis*, and *Staphylococcus aureus*. The four *rpoD* homologs of *Streptomyces coelicolor* A3(2) were cloned and sequenced. A homologous portion with 13 amino acids was found in the *rpoD* genes of *S. coelicolor* A3(2), *E. coli*, and *B. subtilis* and was named the "*rpoD* box."

THE RNA POLYMERASE HOLOENzymes of *Escherichia coli* and *Bacillus subtilis* contain the principal sigma factors (*rpoD* gene products) that play a central role in the initiation of transcription (*1*, *2*). Several other sigma factors, categorized as minor sigma factors, substitute for the *rpoD* gene products under special cir-

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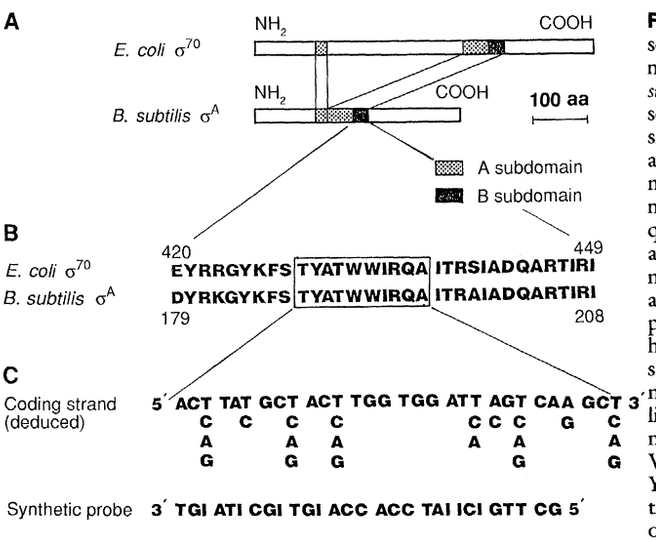

Fig. 1. (**A**) Schematic representation of the principal sigma factors of *E. coli* and *B. subtilis*. The two highly conserved regions are shown by shaded boxes (A subdomain) and filled boxes (B subdomain) (*1*, *2*, *6*). (**B**) Alignment of the B subdomain sequences. The one-letter amino acid notation is used. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine. The boxed portion was used to design the oligonucleotide probe. (**C**) The design of the oligonucleotide containing inosines at degenerated codon positions, including the nucleotide sequence of the coding strand deduced from the amino acid sequence boxed in (B) and the oligonucleotide synthesized.