

Direct selection for a catalytic mechanism from combinatorial antibody libraries

KIM D. JANDA*, CHIH-HUNG L. LO, TINGYU LI, CARLOS F. BARBAS III, PETER WIRSCHING,
AND RICHARD A. LERNER*

Departments of Molecular Biology and Chemistry, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037

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ABSTRACT Semisynthetic combinatorial antibody library methodology in the phage-display format was used to select for a cysteine residue in complementarity-determining regions. Libraries were panned with an α -phenethyl pyridyl disulfide that undergoes disulfide interchange. Out of 10 randomly picked clones, two contained an unpaired cysteine, one of which was studied. The antibody catalyzed the hydrolysis of the corresponding thioester where the electrophilic carbonyl occupies the three-dimensional space that was defined by the reactive sulfur atom during selection. The reaction operates by covalent catalysis. Although the steady-state rate enhancement relative to the activated thiol ester substrate is modest, hydrolysis of the acylated cysteine intermediate is remarkably efficient with a catalytic advantage of about four orders of magnitude. The results suggest that iterative mechanism-based selection procedures can recapitulate the enzymatic mechanisms refined through evolution.

The essential idea underlying antibody catalysis is to anticipate the mechanism of a chemical reaction and then rely on binding energy to lower activation barriers along the reaction coordinate (1). Such catalysis depends upon a correct assumption of mechanistic details, the extent to which the hapten models critical features of the rate-determining transition state, and the manner in which the immune system responds to the antigen. Enzymes generate large rate enhancements through the subtle interplay of many favorable binding interactions refined through the process of evolution. Therefore, one way antibody catalysis could be made even more powerful is by similarly harnessing the diversity of the immune response to control the nature and position of amino acid residues in the active site.

The advent of semisynthetic combinatorial antibody library methodology allows selection for desired functionalities within antibody-combining sites because large numbers of different proteins are displayed on a phage format, thus linking recognition and replication (2–4). In this way, a single antibody that carries out a chemical reaction can be identified and replicated so long as the chemical event distinguishes the phage-bearing antibody from the rest of the population. Here, this concept has been implemented to select for an antibody with a predefined chemical mechanism involving acyl transfer to an active-site sulfhydryl nucleophile to achieve covalent catalysis.

MATERIALS AND METHODS

Selection Procedure for Cysteine Residues. All microtiter plates used in the panning procedure were prepared as follows: The hapten-bovine serum albumin (BSA) conjugate [compound 1 (Fig. 1) is available from Pierce] was diluted to 0.1 μ g/ml, and 25 μ l was added to each well. The plates were

incubated at 37°C overnight. The dried plates were fixed by adding 50 μ l of methanol to each well. After 5 min, the methanol was discarded, and the plate was dried at room temperature. After blocking with phosphate-buffered saline (PBS; 10 mM sodium phosphate/150 mM NaCl, pH 7.4)/1% BSA at 37°C for 30 min, the plates were ready for panning. In the first round of panning, each library was added into six different wells and incubated at 37°C for 2 hr. The phage solution was discarded, 100 μ l of washing buffer Tris-buffered saline (TBS; 50 mM Tris base/150 mM NaCl, pH 7.5)/0.5% Tween/1% BSA was added into each well and incubated at 25°C for 5 min. These wells were washed with 100 μ l of water, 100 μ l of acid solution (0.1 M HCl, pH 2.2, adjusted with glycine), and then water again. The bound phage were eluted twice with 50 μ l of 20 mM dithiothreitol after standing for 5 min. The elutions from each library were combined together and used to infect 10 ml of *Escherichia coli* cells. After 15 min at 25°C, 1 μ l of infected cell culture was diluted to 10^3 , 10^6 , and 10^8 with superbroth and then 1 μ l of each dilution was plated on a LB (Luria–Bertani) plate/carbenicillin to measure the output. Carbenicillin (20 μ g/ml) was added to the cell culture and shaken at 37°C for 1 hr. The concentration of antibiotic was increased to 50 μ g/ml and shaken at 37°C for another hour. The cell culture was diluted into 100 ml of superbroth that contained carbenicillin at 50 μ g/ml, tetracycline at 10 μ g/ml, and 10^{12} plaque-forming units of VCSM13 helper phage (Stratagene). After shaking 2 hr at 37°C, kanamycin (70 μ g/ml) was added and shaken at 37°C overnight. The cells were removed by centrifugation, and 4% PEG and 3% NaCl were added to the supernatant. After 30 min on ice, the phage particles were centrifuged ($9000 \times g$, 30 min, 4°C). The phage were resuspended with TBS/1% BSA and clarified by centrifugation ($16,000 \times g$, 10 min, 4°C). The phage solution was ready for further panning. For the next four rounds of panning, the washing procedures were modified as follows: second round (twice with washing buffer, once with acid solution); third round (five times with washing buffer, once with acid solution for 5 min); fourth round (10 times with washing buffer/3% BSA, twice with acid solution for 5 min); fifth round (10 times with washing buffer/3% BSA, twice with acid solution for 5 min). For each round, the bound phage were eluted twice with 50 μ l of 20 mM dithiothreitol for 5 min. The eluted phage were measured and amplified as described above. After the fifth round of panning, the phagemid DNA was purified, digested with *Spe* I and *Nhe* I, and purified on agarose gel to remove the DNA of gene III. The 4.7-kb fragment was electroeluted, religated, and transformed into *E. coli*. The colonies were picked up and grown until the $OD_{600} = 0.8$, 1 mM isopropyl β -D-thiogalactopyranoside was added, and the culture was incubated at 30°C overnight. The cells were lysed with freeze-and-thaw cycles between –70°C and 37°C in PBS. The supernatant was tested on ELISA plates coated with the

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Abbreviation: BSA, bovine serum albumin.

*To whom reprint requests should be addressed.

haptens. The positive colonies were grown again, and the phagemid DNA was purified and sequenced.

Sequence Analysis of Isolated Clones. Both clones were derived from the κ 10/F library (5, 6) in which the codons for eight residues of the heavy-chain CDR3 and six residues of the light-chain CDR3 had been randomized (boldface type = randomized position) (32-7: HCDR3/GGRDEF \mathbf{G} CDY, LCDR3/QQYKR \mathbf{G} L \mathbf{L} ST; 32-11: HCDR3/GIYQCTKADP, LCDR3/QQYQR \mathbf{M} SWLT). Based on probability alone, where the synthesis protocol produces 32 codons (one encoding cysteine) and using an average of 8 codons for the number of randomized residues, the unselected, naive library should contain 20% of the $\approx 10^8$ clones with an unpaired cysteine; $[1/32 \times (31/32)^7] \times 8 = 0.20$. Experimentally, sequencing of a small sample population gave a value of 23%. After selection, also $\approx 20\%$ of the clones contained cysteine. Other noncysteine-containing phage-Fab that survived the panning process were apparently very tight binding and only released when dithiothreitol destroyed the Fab structure. The lack of sequence homology demonstrates the vast diversity available to generate a common binding site. Amino acids are denoted by single-letter codes.

Antibody Expression and Purification. The phagemid DNA was transformed into *E. coli* and grown in 100 ml of superbroth with 1% glucose overnight. The cells were spun down, washed with fresh superbroth several times, and then resuspended into 10 l-liter batches of superbroth with carbenicillin at 50 $\mu\text{g}/\text{ml}$ and 20 mM MgCl_2 . The culture grew at 37°C until $\text{OD}_{600} = 0.8$; then isopropyl β -D-thiogalactopyranoside was added to a final concentration of 2 mM. The induction temperature was dropped to 25°C overnight. The cells were collected, resuspended into Tris-EDTA, pH 8.0, and lysed by lysozyme at 1 mg/ml and then French press [18,000 psi (1 psi = 6.9 kPa)]. A fast protein liquid chromatography affinity column was prepared from GammaBind G Sepharose (Pharmacia) and goat anti-human $\text{F}(\text{ab}')_2$ IgG (Pierce) as in ref. 7. Two different pH solutions were used for affinity chromatography: solution A, 0.05 M citric acid/0.5 M NaCl, pH 2.1; solution B, 0.1 M sodium phosphate/0.5 M NaCl, pH 9.2. The column was equilibrated with 10-column volumes of 87.2% solution B (final pH = 7.4), then washed with 3 vol of 10.8% solution B (final pH = 2.3), and finally equilibrated with 87.2% solution B. The sample was loaded directly onto the column followed by several column volumes of washing solution until the 280-nm reading returned to baseline; then the pH was dropped by changing the solution to 10.8% solution B. The eluted fractions were collected, neutralized, concentrated, and dialyzed with 100 mM Mops/0.5 mM EDTA, pH 7.4. The Fab was further purified on an ion-exchange column (Pharmacia Hi-Trap SP) by using a gradient salt buffer. The purity of the Fab was confirmed by SDS/PAGE with or without reducing agent and on ELISA. The pure Fab was concentrated and stored in 100 mM Mops/0.5 mM EDTA, pH 7.4 at 4°C. The final concentration of Fab was estimated from OD_{280} by using $\epsilon = 1.24$, which was estimated from the amino acid sequence $[(5700 \times N_{\text{Trp}} + 1300 \times N_{\text{Tyr}})/\text{molecular weight of Fab}]$.

Radiolabeling Experiments. The radiolabeled compound was prepared from compound 1 and an ethanolic solution of unlabeled and [^{14}C]methylamine hydrochloride (American Radiolabeled Chemicals, St. Louis; specific activity 55 mCi/mmol; 1 Ci = 37 GBq). The product was purified by preparative thin-layer chromatography ($R_f = 0.35$, 75/25 ethyl acetate/hexane) to give compound 4 in nearly quantitative yield (specific activity 2.88 mCi/mmol). When labeling Fab, reactions were carried out at room temperature, and dialyses were done at 4°C (100 mM Mops/0.5 mM EDTA, pH 7.4). It was necessary to also count the dialysis membrane because of the small scale involved and the propensity of the protein to adhere to the tubing.

Stopped-Flow Kinetics. The reactions were done by using a Hi-Tech stopped-flow spectrophotometer: a 0.2-ml cell, 1-cm path length, 0.2-ml stop volume, 412 nm, filter time 33 msec. Syringe 1 was charged with 4 μM 32-7 Fab in the variable pH buffer system (Mops and Bicine for pH range 7–9) with 10% dimethylformamide/dioxane. Syringe 2 was charged with 400 μM Ellman's reagent in the same solvent. When using conventional spectrophotometry, only the final 10% of the reaction course of 3 or Ellman's reagent could be recorded.

Steady-State Antibody Kinetics. The reactions were performed in 100 mM Mops/0.5 mM EDTA, pH 7.4, or 100 mM Bicine/0.5 mM EDTA, pH 8.5/8% dimethylformamide/2% dioxane in the presence of substrate and with or without antibody at 25.0°C in 0.2-ml (1-cm) cuvettes or in ELISA microtiter plates. The rates were monitored by observing the increase in absorbance at 343 nm (Shimadzu spectrophotometer) or at 340 nm (Molecular Devices ELISA plate reader) due to the formation of thiopyridone. The extinction coefficient of thiopyridone was calibrated on both instruments ($\epsilon = 7.50 \times 10^{-3} \mu\text{M}^{-1}\text{cm}^{-1}$ for spectrophotometer; $\epsilon = 5.36 \times 10^{-3} \mu\text{M}^{-1}\text{cm}^{-1}$ for plate reader; pH values 7–9). A typical procedure using the spectrophotometer was as follows. The cuvettes were filled with 180 μl of buffer, and then the appropriate amounts were removed and replaced with Fab 32-7 stock solution. Dimethylformamide (16 μl) was added to the cuvette, and the solutions were mixed. The absorbance was "auto-zeroed," and the reaction was initiated by the addition of 4 μl of 10 mM substrate in dioxane with mixing. The total time from substrate addition to the start of reaction was ≈ 30 sec. The ELISA reader reactions were done in a total of 100 μl . A 90- μl portion of buffer was added into the wells, and then an amount of buffer was removed and replaced with Fab 32-7 stock solution. The absorbance at 340 nm was read after adding and mixing 8 μl of dimethylformamide. The kinetic mode was initiated after adding 2 μl of substrate. The data were recorded every 30 sec.

RESULTS AND DISCUSSION

The racemic reagent 1 was coupled to BSA and used as a probe for cysteine groups in antibody-binding sites through the process of disulfide interchange (8, 9) (Fig. 1). The bioconjugate 2 was immobilized on microtiter plates and used to pan six separate semisynthetic libraries, each containing $\approx 10^5$ copies each of $\approx 10^8$ distinct transformants (5, 6). These phage displayed Fab fragments containing randomized complementarity-determining region 3 (CDR3) sequences in either a heavy chain, a light chain, or both. After binding to compound 2, the phage-Fabs were sorted by using a series of three separate elutions. The last elution used dithiothreitol to release phage anticipated to be covalently attached through a disulfide bond (Fig. 2). Out of 10 clones picked at random, two were shown by sequence analysis of phagemid DNA to have the codon for an unpaired cysteine in the heavy-chain CDR3.

One clone, 32-7, was chosen for further investigation. The Fab was overexpressed in *E. coli* and isolated by using affinity chromatography followed by ion-exchange chromatography, affording a protein fraction that was judged to be $>98\%$ pure Fab on inspection of native and denaturing gel analyses. As hoped, Fab 32-7 reacted with compound 3, liberating thiopyridone, which could be followed spectrophotometrically (10). Based on the estimated total protein concentration and the molar equivalents of thiopyridone produced, the Fab 32-7 consisted of $50 \pm 10\%$ functional Fab. The remainder is likely to be improperly folded Fab structures. The existence of a covalent antibody complex was further substantiated using ^{14}C -labeled compound 4. Radioactive protein was isolated after exhaustive dialysis and contained an amount of label corresponding to the quantity of

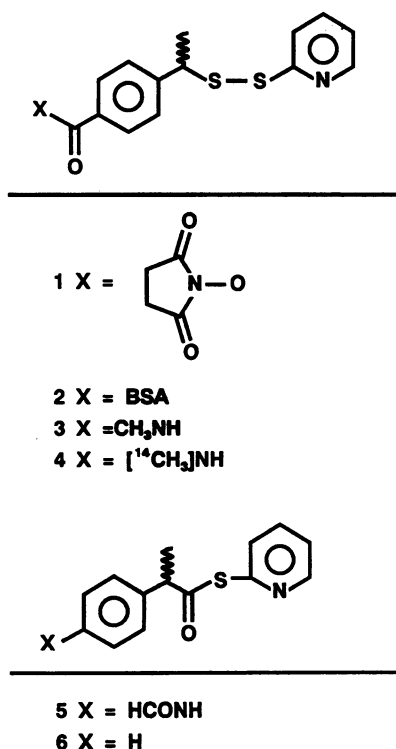


FIG. 1. Structures and numerical assignments of compounds under discussion.

functional Fab determined spectrophotometrically. As for most enzymes that possess an active cysteine, Fab 32-7 was also prone to disulfide interchange with Ellman's reagent (3-carboxy-4-nitrophenyl disulfide) (11). Under pseudo-first-order conditions at pH 7.4, stopped-flow spectroscopy indicated a half-life for the antibody of 35 sec (Fig. 3A). Interestingly, this is nearly 30 times slower than the exchange reaction involving free cysteine ($t_{1/2} = 1.3$ sec). Kinetic titration using Ellman's reagent provided a $pK_a = 8.25$ for the active-site sulfhydryl. This value is reasonable for a cysteine that behaves as an "isolated" thiol rather than as part of an interactive system (13, 14). Remarkably, Fab 32-7 remained stable for months (100 mM Mops/0.5 mM EDTA, pH 7.4; 4°C) with no apparent loss in activity and required no exogenous thiol for activation. The resistance to oxidation, taken together with the kinetic data, suggests the cysteine may be sequestered. Inactive papain, for example, consists of

protein with incorrect disulfide bonds or cysteines as sulfenic acids (15). Even though the Fab molecule contains five cysteine linkages, inherent structural motifs or thermodynamic considerations likely preclude intramolecular disulfide shuffling. A buried cysteine would also make protein-S-S-protein formation difficult.

At this point, the active site is known to contain a nucleophilic thiol poised to react with an electrophile at a position in three-dimensional space defined by the reactive sulfur atom of compound 3. Hence, the congruent thioester 5, where a carbonyl group supplants this atom, was tested and found to be a substrate for the antibody. In retrospect, the choice of a disulfide for panning is in many ways ideal, not only because of its chemoselectivity for cysteine groups, but also because the reactive sulfur atom has tetrahedral geometry with two lone pairs of electrons. This condition may help select for combining sites that promote, or at least allow, formation of the tetrahedral intermediate and attract critical hydrogen bonds that may participate during the catalytic event. When the racemic ester 5 was added to various concentrations of the antibody, burst kinetics indicative of the formation of a covalent intermediate were observed (16). An analysis of progress curves (product inhibition was not significant) showed the amplitude of the burst to be proportional to $50 \pm 10\%$ of the total antibody concentration comparable with the results using disulfide reagents and suggesting full accumulation of the acyl-antibody (Fig. 3B). The reaction could be completely inhibited with compound 3 or the thiol-labeling reagent methyl methanethiolsulfonate (17). The antibody also utilized compound 6 (*R* or *S* methyl group) as substrates with a rate similar to compound 5 without demonstrating appreciable stereospecificity. Apparently, binding interactions in the region of the methyl group are not significant. The first-order rate constant for approach to the steady-state was $k = 0.044 \text{ min}^{-1}$. This rate is $\approx 10^4$ times slower than reactions of papain with amino acid *p*-nitrophenyl esters but similar to guinea pig liver transglutaminase, an enzyme invoking cysteine rather than an imidazolium-thiolate pair (18). A less than ideal orientation of the carbonyl carbon relative to the sulfur nucleophile may be responsible for the sluggish acylation of Fab 32-7. The Fab underwent multiple turnovers, and the dependence of the initial velocity on substrate concentration followed simple saturating kinetics ($k_{\text{cat}} = 0.030 \text{ min}^{-1}$, $K_m = 100 \mu\text{M}$; 100 mM Mops/0.5 mM EDTA, pH 7.4, 25°C); this afforded a 30-fold steady-state rate enhancement over the background hydrolysis of compound 5. The fact that the catalyst turns over at all is encouraging because an unactivated thiol ester is an

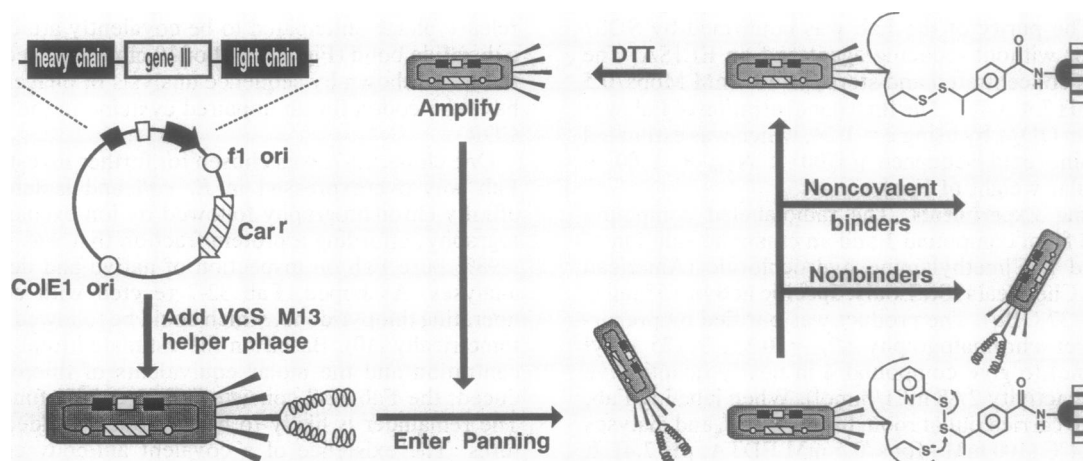


FIG. 2. Scheme illustrating the phage-display of Fab fragments to select for antibody-combining sites containing an unpaired cysteine. DTT, dithiothreitol; Car^r , carbenicillin-resistance gene.

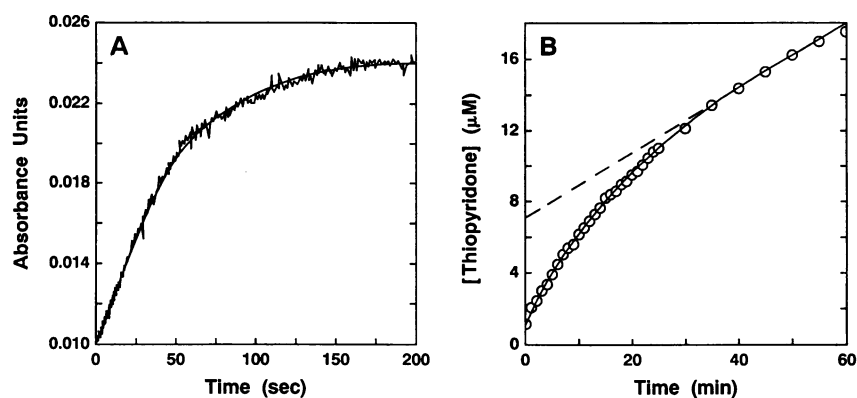


FIG. 3. (A) Time course for complete disulfide interchange of Fab 32-7 with Ellman's reagent. The reaction was followed by measuring the release of 3-carboxy-4-nitro-thiophenol with a stopped-flow spectrophotometer. The conditions were 100 mM Mops/0.5 mM EDTA, pH 7.4/10% total cosolvent (8% dimethylformamide/2% dioxane) in the presence of approximate final concentrations of 2 μM antibody and 200 μM Ellman's reagent. The absorbance change corresponds to 1 μM and proceeds with $k = 0.020 \text{ sec}^{-1}$. (B) A typical progress curve for the liberation of thiopyridone during the reaction of Fab 32-7 with ester 5. The conditions were similar to those in A using 12 μM antibody and 200 μM compound 5. The data were fitted by an expression of the form $[P] = At - B(1 - e^{-kt})$ to evaluate k (12). The dotted line extrapolates to the y -intercept "burst" formation of 7 μM thiopyridone.

extremely stable species under the assay conditions (19, 20). The first-order rate constant for spontaneous hydrolysis of thiol esters at physiological pH is $\approx 10^{-7} \text{ min}^{-1}$, whereas the value for the activated ester 5 was found to be $1.0 \times 10^{-3} \text{ min}^{-1}$ under assay conditions. Interestingly, the antibody slowly catalyzed hydrolysis of the corresponding *p*-nitrophenyl ester of compound 5. However, this reaction may occur on the surface of the protein and with noncysteine residues because it could not be inhibited with methyl methanethiol-sulfonate. *A priori*, it would not have been surprising for the antibody to undergo a stoichiometric reaction with compound 5 and become inactivated (21). Evidently, the microenvironment of the active site provides a means, perhaps attributable to the stereoelectronic features of the sulfur atom targeted during selection, to accelerate the hydrolysis of the intermediate. In this regard, it can be estimated that the antibody affords a catalytic advantage of $\approx 10^4$ relative to the decomposition of an acylated cysteine in solution. Improved catalysis would require more efficient transition-state stabi-

lization for acylation and deacylation or activation of water molecules for the deacylation step. Although details remain to be elucidated, the results are consistent with the minimal three-step mechanism of substrate binding, acyl-intermediate formation, and breakdown fundamental to cysteine and serine proteases (22).

The approximate position of the cysteine residue can be ascertained using model building by comparison to Fabs of homologous sequence and known structure (Fig. 4). Not surprisingly, the group resides in a deep cleft, itself sheltered from the external environment but accessible to certain molecules. Most important, the model represents a specific binding site featuring a precise arrangement of residues that can orchestrate catalysis. In the present case, one is constrained by the requirement for the concerted interaction between a nonoxidized sulfhydryl, sufficient binding energy to overcome the entropic barrier in orienting the nucleophile and electrophile, and a mechanism for hydrolysis of the acyl-intermediate. The probability of randomly accessing

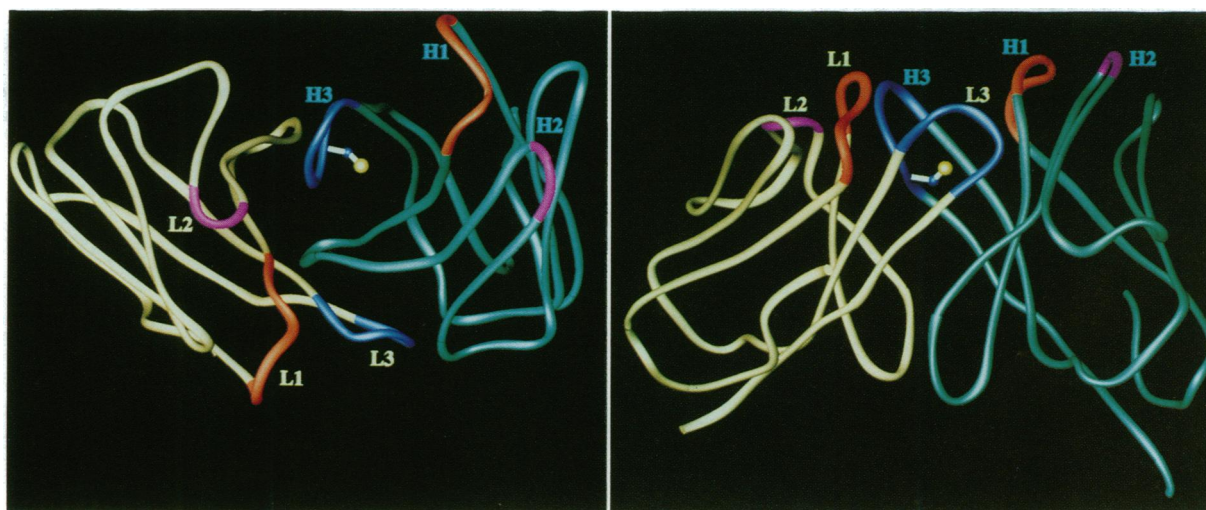


FIG. 4. Molecular model of the antigen-binding region of antibody 32-7. The predicted structure was created by homology modeling (23) using the coordinates of the human antibody Fab 3D6 (24) for the light-chain variable region (yellow) and heavy-chain variable region (cyan) framework regions. The hypervariable loops L1, H2, and H3 were modeled based on the analogous loops from the murine catalytic antibody 1F7 (25), L2 was based on Fab McPC603 (26), H1 was from Fab 3D6, and L3 was from a conformational-loop search. In the predicted structure, the sulfur atom of Cys^{100B} is located $\approx 12 \text{ \AA}$ below the upper rim of a deep groove formed by the hypervariable loops. Although the H3 loop is notoriously difficult to predict (23), the position and orientation of Cys^{100B} can be compared to the structurally equivalent residue in eight other Fab fragment crystal structures containing 10 H3 residues that reveals an average deviation of only 1.7 \AA (0.48–3.4 \AA) for the β -C atom.

such complexity from the naive pool can be expected to be exceedingly low. Yet, the pressure applied through selection has generated a subpopulation consisting of 1 in 10 members that have the desired catalytic activity. Interestingly, a monoclonal antibody elicited by immunization with a transition-state analog was previously shown to operate through an acyl-intermediate, already demonstrating that the interplay between transition-state stabilization and covalent catalysis is within the repertoire of the antibody-combining site (27).

The procedure used here adds to the armamentarium of antibody catalysis in that it allows one to directly select for a chemical event that can be included in the mechanistic plan for the reaction to be catalyzed. Mechanism-based inhibitors have been used in enzymology to inhibit enzymes where a mechanism is known or to give evidence for a proposed mechanism (28). In one sense, the compound we used to select our antibodies can be considered a mechanism-based inhibitor, but here the process has been reversed, and instead of probing a catalytic mechanism it has been used to select for a protein exhibiting a mechanism from an otherwise random system. In principle, any mechanism-based inhibitor can be used to select for at least part of a chemical mechanism. The mechanism can be refined and evolved by iterative procedures of selection using transition-state analogs or other inhibitors to add various nucleophiles, general acids, or bases to improve catalysis. At each iteration, the union between chemistry and combinatorial selection serves as a powerful tool for defining the three-dimensional space of the active site. The two-chain nature of the antibody molecule should be a further advantage because each chain can bring different functionalities into the active site. The order of the selective steps used in such an iterative procedure is probably important, and it would seem best to begin with a transition-state analog to give the selection process at the outset the maximum benefit of chemical insight.

Finally, a significant feature of the present experiment deserves emphasis in that it may shed light on the evolution of catalytic mechanisms. Although there was no direct selection for catalysis initially, the active site when selected for a simple chemical transformation had a specificity that allowed catalysis with appropriate substrates. This may be a general principle where one chemical event may be accompanied by additional chemistry of sufficient utility to afford the catalyst a selectable advantage.

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