Acta Chemica Scandinavica, 1996: 50: 328–332
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ACTA
CHEMICA SCANDINAVICA
ISSN 0904–213X

Antibody Catalysis of Pericyclic Reactions*

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Ulrich, H. D., Driggers, E. M. G. and Schultz, P. G. 1996. Antibody Catalysis of Pericyclic Reactions. – Acta Chem. Scand. 50: 328–332. © Acta Chemica Scandinavica 1996.

In an effort to increase our insight into the catalysis of pericyclic reactions we have initiated a detailed study of an antibody that catalyzes an oxy-Cope rearrangement. We have determined the stereochemistry of the antibody-catalyzed reaction, and experiments are in progress to determine the conformation of the substrate bound in the antibody combining site. The genes encoding the variable regions of this antibody have been cloned and sequenced, and we have made use of a bacterial expression system to produce this antibody as a Fab fragment in recombinant form, making it amenable to genetic manipulations such as site-directed mutagenesis. The recombinant Fab fragment has been crystallized in the presence of its transition state analog, and we are now in the process of determining its active site structure.

A major goal of chemists and biochemists alike is the design of highly selective catalysts based on the mechanistic principles learned from studies of natural enzymes. One successful approach to this problem has taken advantage of the tremendous molecular diversity of the immune system to isolate antibodies that bind and stabilize the rate limiting transition state on a reaction pathway.[1,2] By designing the appropriate hapten, based on available mechanistic information, it has been possible to generate antibodies that catalyze a large number of reactions, from redox and hydrolytic reactions to disfavored cyclization and elimination reactions.

An important challenge to our understanding of biological recognition and catalysis is the generation of catalysts for which we know of no natural precedent. One such class of reactions for which few enzymatic catalysts have been identified are the pericyclic reactions. Perhaps the best known biological example is the Claisen rearrangement catalyzed by the enzyme chorismate mutase, for which the mechanism remains unclear despite an enormous amount of investigation.[3,4] We chose to ask whether antibodies could be generated that catalyze a related [3,3] sigmatropic reaction, the oxy-Cope rearrangement, which has been used extensively in organic synthesis and has been the subject of much mechanistic work, but has not yet been observed in a biological system.[5,6]

In light of the fact that the uncatalyzed reaction has been shown to occur via a chair-like transition state,[7] Braisted and Schultz generated antibodies to the 1,4-diphenyl-substituted cyclohexane derivative **4** (Fig. 1). It was expected that antibodies generated to **4** would bind substrate **1** in a reactive chair conformation, thereby lowering the entropic barrier for reaction by acting as an entropy trap. Several catalytic antibodies were isolated,[8] the most active of which accelerated the reaction 5300-fold relative to the uncatalyzed unimolecular transformation.

In order to understand better the mechanism of this novel biological catalyst and generate antibodies with improved catalytic efficiencies, we have initiated a series of stereochemical, mechanistic, and structural studies of these antibodies. In addition we have begun to study the effects of variations in hapten structure on the activities of the resulting antibodies. Our initial studies have focussed on the antibody AZ-28, with apparent $k_{cat} = 0.026\ min^{-1}$ and $K_m = 97\ \mu M$, the most active one in the series. As both hapten and substrate were racemic in the original work, an analysis of the stereochemistry of the antibody-catalyzed reaction is necessary.

Although the hapten contains three stereocenters, the two enantiomeric substrates differ only at the secondary alcohol. These enantiomers were readily separated by reversed phase chiral HPLC, and then assayed individually for catalysis by antibody AZ-28. Only one enantiomer was measurably accepted as a substrate by the antibody, the other being converted into product at the background rate in the presence of antibody, even at a concentration ten times the previously reported K_m. The racemic mixture gave a rate identical to that observed

*Contribution presented at the Nobel Symposium on *Catalytic Asymmetric Synthesis*, September 3–7, 1995 at Tammsvik, Bro, Sweden.

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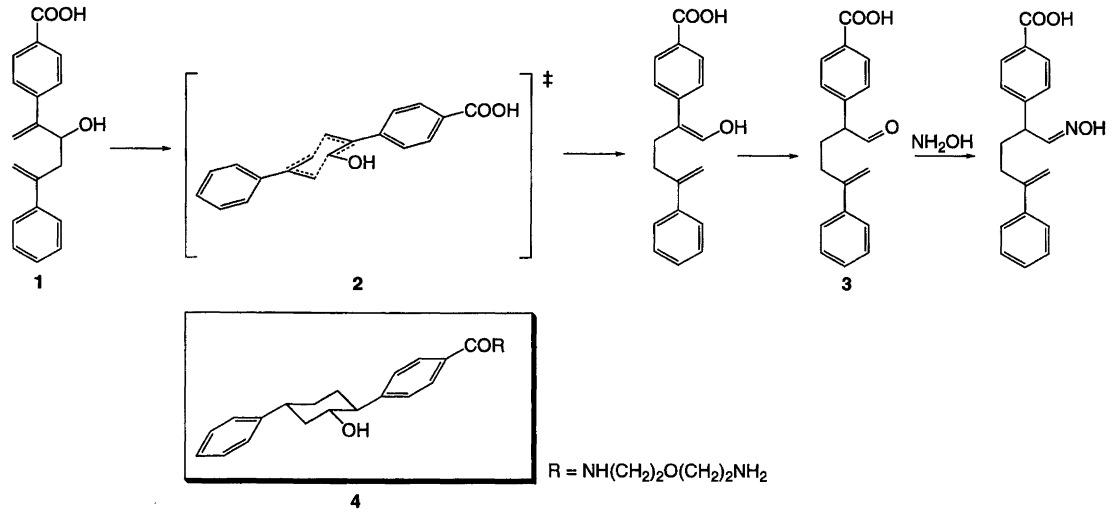

Fig. 1. Antibody-catalyzed Cope rearrangement and corresponding transition state analog.

with the purified enantiomer. In order to determine the absolute stereochemistry of the substrate, the two enantiomeric alcohols of the methyl ester corresponding to the substrate were separated by chiral HPLC. One enantiomer was converted into diastereomeric esters of α-methoxy-α-trifluoromethylphenylacetic acid (MTPA); the other enantiomer was hydrolyzed to the free acid and correlated to the enantiomeric substrates by chiral HPLC. The MTPA esters gave ^{19}F-NMR spectra consistent with an *S* configuration.[9,10] As the remaining *R* enantiomer correlated in activity and retention time to the inactive substrate enantiomer, the antibody was shown to prefer the *S* alcohol. Because only one enantiomer acts as a substrate, while the other appears not to bind, the racemic K_m and k_{cat} can be corrected directly to $K_m = 48.5$ μM, $k_{cat} = 0.026$ min^{-1}. In addition to the above studies, transferred NOE nuclear magnetic resonance (NMR) experiments are in progress to determine whether substrate **1** is bound in a chair-like or boat-like conformation in the Michaelis complex with antibody AZ-28.

A wealth of mechanistic information has come from both structural and mutagenesis studies of enzymes. In order to carry out similar analyses of catalytic antibodies, it is necessary to produce the protein in a recombinant system that is amenable to easy genetic manipulations such as site-directed mutagenesis. The expression of antibodies or their antigen-binding (Fab) fragments in bacterial systems has been demonstrated (reviewed in Ref. 11), but many of the reported systems lack general applicability because of solubility problems encountered with many antibodies. We recently described the application of an expression system that exploits the beneficial nature of human-derived constant regions for the expression of catalytic antibody variable regions in soluble form as chimeric Fab fragments (Fig. 2a).[12,13] Since the variable regions themselves remain unaltered in this system, hapten-binding and catalytic activity should be preserved in the recombinant versions of the cloned catalytic antibodies.

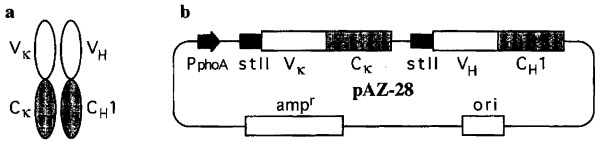

Fig. 2. Generation of chimeric Fab fragments for expression in *E. coli*. **a**, schematic domain arrangement of the chimeric Fab fragment; **b**, expression vector pAZ-28. V_H, V_κ, variable regions of heavy and κ light chain; C_H1, C_κ, constant regions of heavy and κ light chain; P_{phoA}, alkaline phosphatase promoter; stII, bacterial leader sequences; amp^r, gene for β-lactamase; ori, ColE1 origin of replication.

The variable region genes of the antibody AZ-28 were cloned from the original hybridoma cell line by extraction of mRNA, reverse transcription, and amplification by polymerase chain reaction (PCR) with antibody-specific sets of primers. The PCR products were cloned into the expression vector p4xH,[12] yielding pAZ-28 (Fig. 2b), and the primary structure of the AZ-28 variable regions was determined by sequencing several independent clones. The sequences are given in Fig. 3.

Using a promoter induced by limiting phosphate (phoA), the recombinant AZ-28 Fab fragment was produced in shake flasks in the *Escherichia coli* strain 25F2 with yields of 1.6 mg l^{-1}. High-density fermentation in a 2.0 l Bioflow III fermentor (New Brunswick Scientific) yielded up to 100 mg. After passage over a Protein G affinity column the protein was obtained in greater than 90% purity. The Fab fragment showed full catalytic activity as well as antigen-binding inhibitable by free hapten.

In an effort to determine the three-dimensional structure of the active site hapten complex the recombinant Fab fragment has been crystallized in the presence of its hapten. Using an incomplete factorial approach[14] followed by optimizations, final conditions were found for

Light chain variable region:

```
E  L  V  L  T  Q  S  P  S  S  M  Y  A  S  L  G  E  R  V  T  I  T
GAGCTCGTGCTCACCCAGTCTCCATCTTCCATGTATGCATCTCTAGGAGAGAGAGTCACTATCACT
C  K  A  S  Q  D  I  N  S  Y  L  N  W  F  Q  Q  K  P  G  K  S  P
TGCAAGGCGAGTCAAGACATTAATAGCTATTTAAACTGGTTCCAGCAGAAACCAGGGAAATCTCCT
K  T  L  I  Y  R  T  N  R  L  V  D  G  V  P  S  R  F  S  G  S  G
AAGACCCTGATCTATCGTACAAACAGATTGGTAGATGGGGTCCCCTCAAGGTTCAGTGGCAGTGGA
S  G  Q  D  Y  S  L  T  I  S  S  L  E  Y  E  D  M  G  I  Y  Y  C
TCTGGGCAAGATTATTCTCTCACCATCAGCAGCCTGGAATATGAAGATATGGGAATTTATTATTGT
L  Q  Y  D  E  F  P  Y  T  F  G  S  G  T  K  L  E  I  K
CTCCAGTATGATGAGTTCCCGTATACGTTCGGCTCGGGGACAAAGCTTGAAATAAAA
```

Heavy chain variable region:

```
Q  V  Q  L  L  E  S  G  A  E  L  M  K  P  G  A  S  V  K  I  S  C
CAGGTTCAGCTGCTCGAGTCTGGAGCTGAGCTGATGAAGCCTGGGGCCTCAGTGAAGATATCCTGC
K  A  T  G  Y  T  F  S  S  F  W  I  E  W  V  K  Q  R  P  G  H  G
AAGGCTACTGGCTACACATTCAGTAGCTTCTGGATAGAGTGGGTAAAGCAGAGGCCTGGACATGGC
L  E  W  I  G  E  I  L  P  G  S  G  G  T  H  Y  N  E  K  F  K  G
CTTGAGTGGATTGGAGAGATTTTACCTGGAAGTGGTGGTACTCACTACAATGAGAAGTTCAAGGGC
K  A  T  F  T  A  D  K  S  S  N  T  A  Y  M  Q  L  S  S  L  T  S
AAGGCCACATTCACTGCAGATAAATCCTCCAACACAGCCTACATGCAACTCAGCAGCCTGACATCT
E  D  S  A  V  Y  Y  C  A  R  G  H  S  Y  Y  F  Y  D  G  D  Y  W
GAGGACTCTGCCGTCTATTACTGTGCAAGAGGACATAGTTACTATTTTTACGACGGAGACTACTGG
G  Q  G  T  S  V  T  V  S  S
GGTCAAGGAACCTCGGTCACCGTCTCCTCA
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Fig. 3. Nucleotide and amino acid sequences of AZ-28 light chain and heavy chain variable regions. Complementarity determining regions (CDRs) are given in italics.

the hanging drop method at room temperature with 12 mg ml^{-1} protein, 2 mM hapten, 100 mM sodium acetate, pH 4.6, 200 mM ammonium sulfate, 200 mM cadmium chloride, and 18–20% (w/v) polyethylene glycol 1000. The resulting crystals are elongated plates with approximate dimensions 1.0 mm × 0.2 mm × 0.1 mm. Preliminary oscillation pictures collected on a Rigaku r-axis II have shown that these crystals are monoclinic and diffract to ca. 2.5 Å. Experiments are now in progress to optimize conditions for cryo-crystallography in order to obtain a complete data set with the use of synchrotron radiation as well as to crystallize the antibody in the absence of hapten and in the presence of substrate analogs.

Completion of the structure determination will enable us to address more detailed questions about the mechanism of this antibody using site-directed mutagenesis. Specifically, we would like to discern the factors that contribute to the observed rate acceleration, i.e., the relative contributions of conformational and electronic factors. The oxy-Cope reaction is facilitated by electron-rich substituents in the 3 position which lower the dissociation energy of the adjacent 3–4 carbon–carbon bond due an anionic substituent effect.[15,16] It is conceivable that an antibody with a general base in the active site might provide a corresponding rate acceleration by polarizing the hydroxy group in the 3 position of substrate **1**. Thus, it will be interesting to see whether AZ-28 contains an appropriately positioned residue that might act as a general base. On the other hand, if no basic group is found, it might be possible to introduce a corresponding residue by mutagenesis based on the crystal structure.

As a parallel approach, we have begun to generate

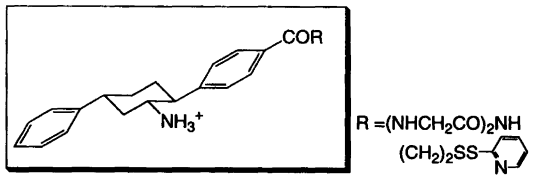

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Fig. 4. Alternative hapten with amino-substituent for eliciting a general base in the antibody combining site.

antibodies against structural variants of hapten **4**. For example, antibodies are being generated to hapten **5** (Fig. 4), which contains a positively charged amino group at the position that corresponds to the hydroxy group of the substrate. A complementary carboxylate in the combining site of the antibody might act to deprotonate the substrate hydroxy group partially and thereby accelerate the rearrangement further.

Finally, we are determining the sequences of the other active antibodies with lower rate accelerations as well as some inactive clones in this series. Combined with the structural information from AZ-28 their sequences might provide additional insight into specific residues important for catalysis.

Experimental

General. Proton NMR spectra were recorded on a Bruker AM-400 (400 MHz) or a Bruker AM-500 (500 MHz) Fourier transform NMR spectrometer. HPLC purification was carried out on a Rainin system equipped with 2 HPXL pumps and a solvent mixer; peaks were detected by UV absorbance on a Rainin UV-1 spectrophotometer. Thin layer chromatography was performed on 0.25 mm

silica gel plates (Merck Fertigplatten, 60F-254, Art. 5765). Pyridine was distilled under N_2 from CaH_2 immediately prior to use. MTPA-Cl was purchased from Aldrich and used without further purification.

Isolation of enantiomers. Substrates were synthesized as previously described.[8] The enantiomeric alcohols were separated by chiral reversed-phase HPLC on a 1 cm Chiraltech OD column, eluted isocratically at 35% CH_3CN (0.1% TFA)–65% H_2O (0.1% TFA). Retention times were 110 (*R*) and 121 (*S*) min.

The enantiomeric substrate esters were eluted from the same column with retention times of 132 (*R*) and 141 (*S*) min on a gradient of 30–50% CH_3CN (0.1% TFA)–H_2O (0.1% TFA) over 60 min followed by isocratic elution at 50% for 120 min.

Synthesis of MTPA esters. The substrate esters were converted into the MTPA derivatives and purified according to the method of Dale and Mosher.[10] The methyl ester was hydrolyzed with 1 N NaOH in dioxane at 0 °C and purified by preparative silica gel TLC (5:1 hexanes–ethyl acetate, 0.5% acetic acid) to give the free acid. Further purification for assays was by reversed-phase HPLC as described.[8]

Cloning of the AZ-28 variable regions. The general strategy as well as the expression vector p4xH have been described.[12] Restriction sites in oligonucleotides are indicated in bold type. Total RNA was isolated from ca. 10^8 cells by guanidine thiocyanate extraction and enriched for mRNA by affinity chromatography on oligo-dT cellulose (Pharmacia). Reverse transcription was performed with equimolar mixtures of the J region specific primers J_H1–J_H4 and $J_\kappa1$–$J_\kappa5$[12] for heavy and κ light chain sequences, respectively. PCR conditions with Taq polymerase (Promega) were examined in parallel reactions with the mixtures of J region primers combined with each one of the variable (V) region specific primers H1–H9 or L3–L7[17] and found optimal with H2 (5′-AGGTCCAGCTG**CTCGAG**TCTGG-3′) and L3 (5′-CCAGTTCC**GAGCTC**GTGCTCACCCAGTCTCCA-3′). The PCR products were digested with the appropriate restriction enzymes (*Sac*I and *Hin*dIII for the light chain, *Xho*I and *Bst*EII for the heavy chain) and inserted into the vector p4xH. Several independent clones of each chain were sequenced to exclude PCR errors. The J region sequence corresponding to the primer J_H4 (5′-TGAGGAGAC**GGTGACC**GAGGTTCCTTGACCCCA-3′) was found in all heavy chain clones. However, multiple J_k primers had served to amplify the light chain variable regions. The correct J region (corresponding to $J_\kappa4$: 5′-TTTTATTTC**AAGCTT**TGTCCCCGAGCCGAACGT-3′) was confirmed by reverse transcription of the mRNA with L9[17] (5′-GCGCCG**TCTAGA**ATTAACACTCATTCCTGTTGAA-3′) and subsequent PCR with L9 and L3 to amplify the complete Fab fragment. The PCR product was directly sequenced in PCR-based reactions using a ^{32}P-labeled primer and Vent Polymerase (New England Biolabs).

Expression and purification of the recombinant Fab fragment. Shake flask expression and high density fermentations as well as Protein G affinity chromatography were performed as described.[12] For crystallographic purposes the Fab fragment was further purified by ion exchange chromatography on an FPLC system (Pharmacia) using a MonoS 5/5 or 10/10 column in 50 mM MES, pH 5.5, with a slow gradient of 5–100 mM NaCl and a flow rate of 1 or 4 ml min^{-1}, respectively. The purified protein was concentrated to 15–20 mg/ml and exchanged into 20 mM Tris–HCl, pH 7.2, 100 mM NaCl, 0.5 mM EDTA, 1 mM methionine using a Collodion membrane (Schleicher and Schuell). Immediately before crystallizations were set up, the protein was passed over a Superose 12 gel filtration column (Pharmacia) in the same buffer.

Crystallization and data collection. Crystallization conditions were examined in 24 well Linbro plates using the hanging drop method, combining 2 µl of protein solution containing 2 mM hapten with 2 µl of mother liquor over a reservoir of 1 ml. Initial screens were performed at room temperature and 4 °C by incomplete factorials according to Jancaric and Kim,[14] followed by optimizations. Final conditions were found at room temperature with 12 mg ml^{-1} protein in 100 mM sodium acetate, pH 4.6, 200 mM ammonium sulfate, 200 mM cadmium chloride, 18–20% (w/v) PEG 1000. Initial data were collected on a Rigaku r-axis II area detector, exposing over 15 min with 1° oscillations.

Acknowledgments. We thank Prof. Ray Stevens, U.C. Berkeley, for help with the crystallography experiments. This work was supported by the Director, Office of Energy, Office of Basic Energy Sciences, Division of Materials Sciences, and also by the Division of Energy Biosciences of the U.S. Department of Energy under contract no. DE-AC03-76SF00098. P.G.S. is a Howard Hughes Medical Institute Investigator. H.D.U. is supported by a National Science Foundation Predoctoral Fellowship in Biological Sciences.

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Received October 28, 1995.