Teaching Catalytic Antibodies to Undergraduate Students

An Organic Chemistry Lab Experiment

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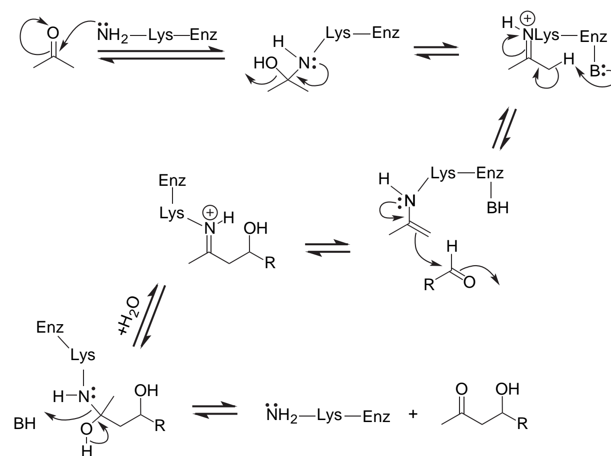
Introduction

It is now 13 years since the first antibody catalysts elicited against transition-state analogs were reported (*1*). A wide variety of chemical transformations have already been successfully catalyzed by antibodies, in some cases with rate accelerations that rival natural enzymes and in other cases with the ability to defy the selection rules of organic chemistry (*2*). Antibodies have successfully catalyzed highly disfavored chemical processes, such as anti-Baldwin cyclizations, exo-Diels–Alder cycloaddition, ketalization in water, regio/stereoselective ketone reduction, and cationic cyclopropanation, to name just a few. The remarkable ability of antibody catalysts to control the rate and stereochemistry along the reaction coordinate is rapidly leading to the synthesis of highly interesting and complex natural products.

No other area of bioorganic chemistry has taught us so much about the use of large combinatorial libraries of molecules in the service of chemistry and about the potential of binding energy to do chemical work. As a chemical discipline, antibody catalysis is fundamentally about design. The detailed chemical instructions given to the antibody molecule are transferred from the well-designed hapten (antigen) to the antibody binding site through the immunization process, as confirmed by structural and mechanistic studies.

The recent introduction of the new concept of reactive immunization represents a major milestone in the field. In reactive immunization one immunizes with highly reactive compounds to create a chemical reaction during the binding of the antigen to the antibody. The same reaction becomes part of the mechanism of the catalytic event. In other words, the antibodies are elicited against a chemical reaction, instead of a stable transition-state analog.

The power of this concept has been nicely demonstrated by eliciting antibodies that catalyze the aldol condensation using the same mechanism as the natural class I aldolase enzymes (*3*). The latter utilize the ε-amino group of a lysine residue in their active site to form a Schiff base with the donor substrate, which activates it as an enamine nucleophile that reacts with the aldol acceptor to form a new C–C bond (Scheme I). The resultant Schiff base is then hydrolyzed to release the aldol product. Hapten **1** was designed to trap the essential lysine residue inside the antibody's active site and at the same time to create the appropriate binding site that will facilitate this bimolecular reaction by overcoming the entropic barrier. The driving force for the reaction of such a hapten with the antibody is the formation of a stable covalent bond between the hapten and the primary amine residue in the binding pocket (Scheme II). The resultant conjugated enamine (vinylogous amide) can be identified using its characteristic absorption at 316 nm. Two anti-**1** antibodies, 38C2 and 33F12, proved to bind covalently to their hapten. Both antibodies catalyze the addition of acetone to aldehyde **2** to produce β-hydroxy ketone **3**, exhibiting Michaelis–Menten kinetics (Scheme III).



Scheme I
General mechanism of aldol addition reaction catalyzed by class I aldolases

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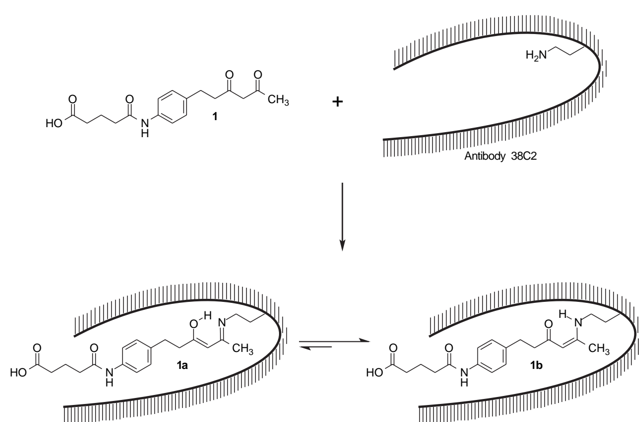
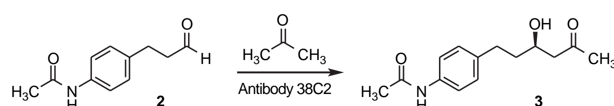


Scheme II



Scheme III

Mechanism of trapping the essential ε-amino group of a lysine residue in the antibody's binding pocket by using the 1,3-diketone hapten **1**

Many universities have already incorporated short courses on catalytic antibodies into their graduate chemistry–biochemistry and even undergraduate teaching programs. For the past three years the Department of Chemistry at the Technion–Israel Institute of Technology has been regularly providing such an undergraduate/graduate course as part of the syllabus of Advanced Organic Chemistry. However, until recently it was virtually impossible to bring the new technology into the undergraduate teaching laboratories owing to unavailability of the novel biocatalysts. This frustrated chemistry professors eager to train new generations of undergraduate students with modern organic chemical methods. The situation changed dramatically when Aldrich Chemical Company announced that antibody 38C2 had become the first commercially available catalytic antibody (*4*).

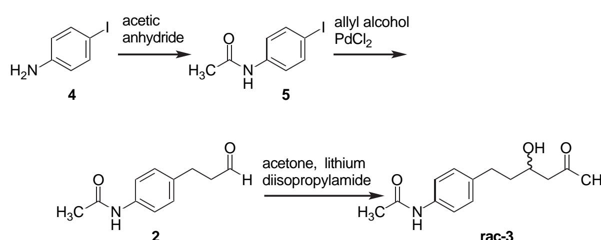
Although antibody 38C2 catalyzes aldol reactions with a broad variety of aldehydes and ketones, for this student lab we chose the reaction of aldehyde **2** with acetone for a pedagogical reason: to demonstrate the close analogy between the substrates of this reaction and hapten **1**. In fact, the conversion of **2** to **3** was the first reaction that was found to be catalyzed by antibody 38C2 (*3*).

The lab project outlined here benefits the student by providing training not only in biocatalysis but in a number of related aspects of chemical and biochemical research, including organic synthesis, mechanistic organic chemistry, and chemical kinetics, and in the use of modern experimental techniques such as UV–vis spectroscopy and reverse-phase high-performance liquid chromatography (HPLC) to monitor chemical reactions and elucidate kinetic parameters. Moreover, students are exposed to concepts and terminology of bioorganic chemistry such as protein structure and function, inhibition, active-site titration, and basic principles of biocatalysis. Above all, students are given a unique opportunity to get a taste of a rapidly developing field at the forefront of scientific research.

This project is designed to fit the time limitation and complexity level of the Advanced Organic Chemistry Lab in the Technion. Every student in this lab is expected to carry out by himself, under the supervision of a teaching assistant, a multistage project over a period of six 8-hour meetings. A shorter version of the project will be incorporated into the regular Organic Chemistry Lab taken by undergraduate students in Biology and Biotechnology. To save lab time in the shortened version of the project, synthesis of substrate and product (following section) can be omitted.

Synthesis of Substrate and Product

The aldehyde substrate **2** and the racemic aldol product **3** (Scheme IV) are prepared in a three-step synthesis from 4-iodoaniline as described below. Acetylation of **4** with acetic anhydride affords 1-acetamido-4-iodobenzene, **5**. Palladium-catalyzed Heck arylation (*5*) of allyl alcohol with **5** produces aldehyde **2**. Finally, aldol condensation of **2** with the lithium enolate of acetone gives **3** in a racemic form. This aldol product is used as an authentic reference compound for the HPLC studies.



Scheme IV

Experimental Procedure

1-Acetamido-4-iodobenzene, **5**. Methylene chloride (40 mL), 4-iodoaniline (3.28 g, 15 mmol) and triethylamine (6.34 mL, 45 mmol) are added to a 100-mL flask equipped with a magnetic stirring bar and a calcium chloride drying tube. The red-brown solution is cooled using a water/ice bath. Acetic anhydride (4.25 mL, 45 mmol) is added dropwise, the mixture is stirred at 0 °C for 10 minutes, and then it is allowed to warm to room temperature, resulting in a white precipitate. After 45 min at room temperature no starting material can be detected by TLC (hexane–ethyl acetate, 1:1; **4**, R_f = .73; **5**, R_f = .41). Solvent is removed under reduced pressure and the residue is recrystallized from methylene chloride (40 mL). The white precipitate is collected, washed with cold methylene chloride, and dried under high vacuum for 1 h to give **5** (3.15 g, 80.5%) in the form of a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.58 (d, J = 8.7 Hz, 2H), 7.36 (br, 1H), 7.26 (d, J = 8.7 Hz, 2H), 2.14 (s, 3H).

3-(4-Acetamidophenyl)-propanal, **2**. Anhydrous DMF (4 mL)[1] and compound **5** (0.65 g, 2.5 mmol) are added to an oven-dried 25-mL round-bottom flask equipped with a magnetic stirring bar and a condenser, under argon atmosphere. After **5** is completely dissolved, tetrabutylammonium chloride (0.69 g, 2.5 mmol), sodium bicarbonate (0.55 g, 6.5 mmol), and allyl alcohol (0.51 mL, 7.5 mmol) are added. The mixture is stirred at room temperature for 20 min before the addition of $PdCl_2$ (0.11 g, 0.6 mmol). The reaction mixture is then stirred at 80 °C for 3 h until no starting material can be detected by TLC (1:1 hexane–ethyl acetate).[2]

The mixture is cooled to room temperature, crushed ice is added, and the mixture is extracted twice with ethyl acetate. The combined organic phase is washed with water, dried over $MgSO_4$, and filtered, and the solvent is removed under reduced pressure to give crude **2** (0.52 g). The crude product is purified by flash column chromatography using a 2-cm (i.d.) × 30-cm column with 20 g of silica gel[3] and eluted with 1:1 hexane–ethyl acetate. The 10-mL fractions are analyzed by TLC (7:3 ethyl acetate–hexane; **5**, R_f = .3)[4] and combined to give **5** (0.26 g, 54%) in the form of a colorless oil, which solidifies under high vacuum. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.78 (br, 1H), 7.5 (br, 1H), 7.39 (d, J = 8.3 Hz, 2H), 7.1 (d, J = 8.3 Hz, 2H), 2.89 (t, J = 7.2 Hz, 2H), 2.73 (t, J = 7.2 Hz, 2H), 2.13 (s, 3H).

6-(4-Acetamidophenyl)-4-hydroxyhexan-2-one, **3**. Anhydrous THF (1.5 mL)[5] and acetone (40 µL, 0.54 mmol)[6] are added under argon atmosphere to a 10-mL round-bottom flask equipped with a magnetic stirring bar and a rubber septum. The mixture is cooled to −78 °C; a solution of lithium diisopropylamide (LDA) (2.0 M, 0.3 mL, 0.59 mmol)[7] is added using a 1-mL syringe, and the mixture is stirred at the same temperature for 30 min. A solution of aldehyde **2** (93 mg, 0.48 mmol) in anhydrous THF (1.1 mL) is added using a 2-mL syringe and the mixture is stirred at −78 °C. The progress of the reaction is monitored by TLC (ethyl acetate; **3**, R_f = .45; **2**, R_f = .75). The reaction is quenched after 2.5 h at 80–90% conversion by addition of saturated aqueous ammonium chloride. Water is added and the mixture is extracted three times with ether and twice with ethyl acetate. The combined organic layer is dried over $MgSO_4$ and filtered, and the solvents are removed under reduced pressure to give a yellowish oil. Purification by flash column chromatography using a 2-cm (i.d.) × 30-cm column[3] with 20 g of silica gel and eluted with ethyl acetate affords **3** (53 mg, 40%) in the form of a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (br, 1H), 7.36 (d, J = 8.4 Hz, 2H), 7.07 (d, J = 8.4 Hz, 2H), 3.99 (m, 1H), 2.95 (br, 1H), 2.75–2.5 (m, 4H), 2.13 (s, 3H), 2.10 (s, 3H), 1.73 (m, 1H), 1.61 (m, 1H).

HPLC Characterization of the Antibody-Catalyzed Reaction

The HPLC technique with reversed-phase columns is currently the most widely used method to monitor antibody catalyzed reactions. As detection by UV–vis is sensitive enough to detect chromophores at low concentrations (10^{-5}–10^{-8} M), an HPLC system equipped with a UV detector is particularly useful for analyzing substrates and products that contain a UV-active chromophore. This is the case for both substrate **2** and product **3**.

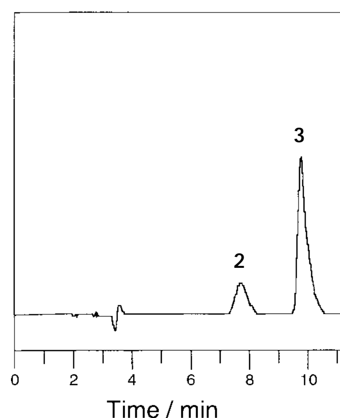


Figure 1. HPLC chromatogram of aldehyde **2** (R_t = 7.74 min) and aldol **3** (R_t = 9.80 min).

To obtain accurate and reproducible results while monitoring the antibody-catalyzed reactions by HPLC, students should prepare concentrated stock solutions of **2** and **3** in acetonitrile and use them to prepare dilute solutions in phosphate buffered saline (PBS), pH 7.4, prior to their experiments. Before carrying out the antibody-catalyzed reactions the retention times of **2** and **3** should be determined using these solutions. Both **2** and **3** are conveniently separable with an isocratic solvent system, allowing for the employment of standard, relatively inexpensive HPLC systems. To save lab time, the exact separation conditions may be determined by the teaching assistant. Alternatively, determination of the separation conditions may be carried out by the students, and they can thereby be trained in the theory and practice of the HPLC techniques.

At this stage, the student is ready to perform the antibody-catalyzed reaction. Catalysis by this antibody is known to be inhibited by various 1,3-diketones, including acetylacetone, via formation of a vinylogous amide as discussed above and illustrated in Scheme I. Three reaction mixtures are prepared: A, an antibody-catalyzed aldol condensation; B, the same antibody-catalyzed reaction inhibited by acetylacetone; and C, a background reaction in PBS in the absence of the catalyst. To maximize the observed catalytic efficiency, reactions are performed at relatively high antibody concentration and low substrate concentration.

Experimental Procedure

Stock solutions. The following concentrated solutions are prepared.

a. Aldehyde **2** (0.1 M) in acetonitrile[8]
b. Aldol **3** (0.01 M) in acetonitrile
c. Acetylacetone (0.01 M) in acetonitrile
d. Phosphate buffered saline (PBS), pH 7.4[9]
e. Antibody 38C2 (1 mg/mL) in PBS, pH 7.4[10]

HPLC analysis.[11] The students search for separation conditions (baseline separation of the peaks) by injecting dilute solutions[12] of **2** and **3** (10 µL each) into the HPLC instrument using various proportions of water and acetonitrile. Good separation conditions for these two compounds have been achieved (**2**, R_t = 7.74; **3**, R_t = 9.80 min) using a C18 reverse-phase column[11] with 15% acetonitrile in water[13] containing trifluoroacetic acid (0.1% v/v) at flow rate of 1.0 mL/min and detection at 254 nm (Fig. 1).

Initial detection of catalysis. Three reaction mixtures (50 µL each) are prepared in 1.5-mL microcentrifuge tubes, each containing 1 µL of the stock solution of **2** (0.001 M in PBS, pH 7.4) and 3 µL of acetone,[14,15] and one of the following:

A. Antibody 38C2 (46 µL solution of 10 mg/mL in PBS, pH 7.4)
B. Antibody 38C2 (43.5 µL solution of 10 mg/mL in PBS, pH 7.4), acetylacetone (2.5 µL of a 0.01 M solution in acetonitrile)
C. No antibody, 46 µL of PBS (pH 7.4)

The mixtures are mixed in the tubes using a vortex stirrer. The reactions are allowed to stand for about 3 h and then

analyzed by HPLC. In our hands the following results were obtained:

A. Antibody-catalyzed reaction, t = 175 min, 40% conversion
B. Inhibited reaction, t = 187 min, 2% conversion
C. Background reaction, t = 199 min, no product detected

Titration of the Antibody Active Sites

To correctly elucidate the kinetic parameters of the antibody (next section), it is essential to know the actual concentration of catalytic sites in the reaction mixture. Theoretically, each antibody molecule contains two active sites. Although the commercially available antibody has been purified, partial denaturation of this protein could account for slight reduction in the actual concentration of the catalyst. Thus, titration of the active sites may show that the actual number of active sites per antibody molecule is smaller than two.

In the case of antibody 38C2, active site titration can be easily carried out by taking advantage of the fact that various 1,3-diketones, including acetylacetone, are inhibitors of this antibody. Moreover, as illustrated in Scheme II, 1,3-diketones such as **1** react with the antibody's lysine residue to produce a conjugated enamine having a characteristic absorption at 316 nm. Thus titration of the commercially available antibody with acetylacetone is easily monitored using a simple UV–vis spectrophotometer. We found that the actual number of active sites per antibody molecule is 1.9. The value of this experiment extends beyond the mere quantitative analysis. It demonstrates clearly the concept of reactive immunization by showing that the antibody actually reacts with 1,3-diketone to produce a covalent product—a conjugated enamine (vinylogous amide). The absorption of this enamine at 316 nm has been used for screening the library of antibodies in order to select those having a lysine residue in the active site (*3*).

Experimental Procedure

A PBS solution (pH 7.4, 80 µL) is placed in a 100-µL quartz ultramicro cuvette in a UV–vis spectrophotometer[16] to define the baseline in the range of 250–350 nm. The cuvette is cleaned and dried and a solution of antibody 38C2 (20.2 mg/mL, 80 µL)[17] is placed in it using a pipettor. Aliquots of a solution of acetylacetone in acetonitrile (9.54×10^{-4} M, 1 µL each) are added to the cuvette. After each addition the cuvette is gently stirred using a vortex mixer, and its absorbance at 316 nm is recorded (Fig. 2).

The absorbance (OD) at 316 nm is measured in each spectrum and these values are displayed as a function of the concentration of acetylacetone (Fig. 3).[18,19] The presaturation and saturation regions of this graph are separately linearly regressed. The two linear equations are solved and the acetylacetone concentration at the crossing point is found. In our hands this value was found to be 195.4 µL. Using a triangular ratio the volume of solution displaying this concentration was calculated to be 102.3 µL, which corresponds to 1.999×10^{-8} moles of acetylacetone. Thus the amount of antibody in the solution is 1.077×10^{-8} moles[20] and the ratio between the two numbers is 1.9 active sites per antibody.

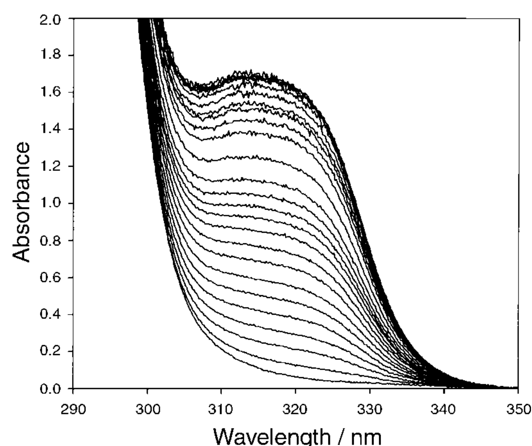


Figure 2. Twenty-four readings taken by the spectrophotometer in the titration of antibody 38C2 with acetylacetone.

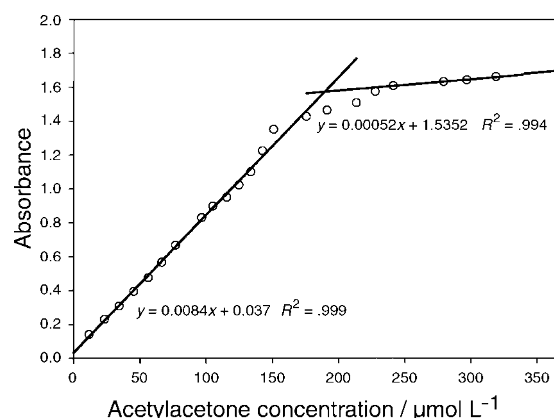


Figure 3. The titration curve and the regressed lines of the presaturation and saturation regions of the titration according to the reading at 316 nm.

Table 1. Setting Up the Antibody-Catalyzed Reactions

Reaction	Substrate[a] Concn/µM	Substrate[a] Vol/µL	Acetone Vol/µL	38C2 Vol/µL[b]	PBS 7.4 Vol/µL	t/min[c]
1	20	2	5	85	8	196
2	30	3	5	85	7	212
3	40	4	5	85	6	226
4	50	5	5	85	5	239
5	60	6	5	85	4	253
6	70	7	5	85	3	266
7	80	8	5	85	2	281
8	100	10	5	85	0	298
9	150	3[d]	5	85	7	314
10	200	4[d]	5	85	6	328
11	250	5[d]	5	85	5	342
12	350	7[d]	5	85	3	359

[a] 1×10^{-3} M solution of aldehyde **2** in PBS.
[b] 1 mg/mL solution of antibody 38C2.
[c] Time from beginning of reaction to injection time.
[d] 5×10^{-3} M solution of aldehyde **2**.

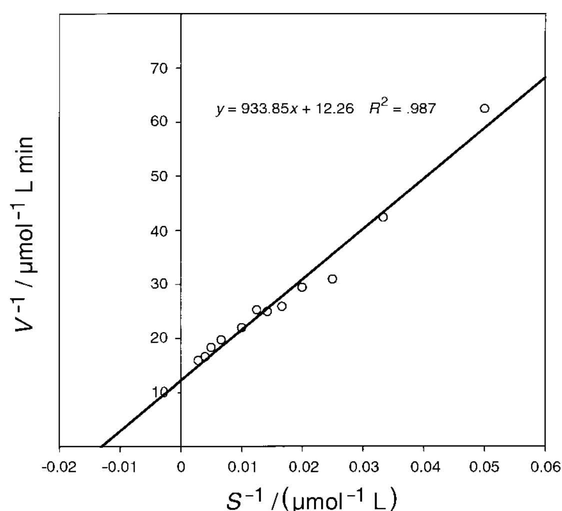


Figure 4. Lineweaver–Burk plot relating to 38C2 antibody kinetics and its kinetics parameters; v_{max} = 0.082 μM/min, K_m = 76 μM.

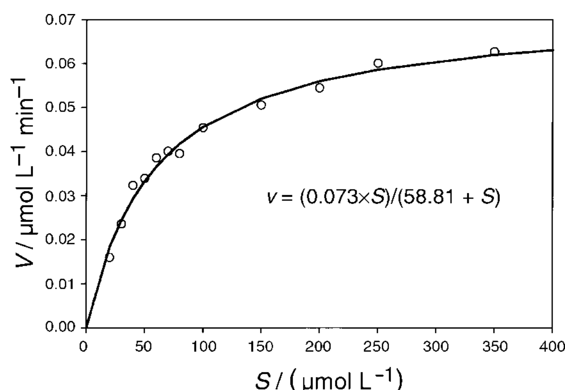


Figure 5. A Michaelis–Menten plot of the 12 antibody-catalyzed reactions and its kinetic parameters; V_{max} = 0.073 μM/min, K_m = 59 μM.

Table 2. Results of Antibody-Catalyzed Reactions

No.	[S]/μM	*t*/min	Conversion (%)	*v*/ μM min^{-1}	v^{-1}/ min μM^{-1}	[S]$^{-1}$/μM^{-1}
1	20	196	15.683	0.01600	62.488	0.05000
2	30	212	16.669	0.02359	42.394	0.03333
3	40	226	18.241	0.03229	30.974	0.02500
4	50	239	16.215	0.03392	29.479	0.02000
5	60	253	16.265	0.03857	25.925	0.01667
6	70	266	15.226	0.04007	24.957	0.01429
7	80	281	13.900	0.03957	25.270	0.01250
8	100	298	13.550	0.04547	21.993	0.01000
9	150	314	10.588	0.05058	19.771	0.00667
10	200	328	8.939	0.05451	18.347	0.00500
11	250	342	8.228	0.06015	16.626	0.00400
12	350	359	6.440	0.06279	15.927	0.00286

Analyzing the Kinetics of the Antibody-Catalyzed Reaction

Antibody-catalyzed reactions follow the Michaelis–Menten kinetics characteristic of natural enzymes. In this part the students determine the kinetic parameters of the antibody (K_M, k_{cat}, and v_{max}). Special attention and care should be given to the precision required when performing such small-scale reactions.

Experimental Procedure

A set of 12 antibody-catalyzed reactions are carried out at different substrate concentrations using 1.5-mL microcentrifuge tubes, as detailed in Table 1. Acetone is used here in saturating concentration (5% v/v). After approximately 3 h, a 10-μL sample is withdrawn from each tube and injected into the HPLC instrument[21] beginning with the reaction with the lowest substrate concentrations and continuing on to the one with the highest concentration.

Starting material **2** and product **3** have similar chromophores and therefore their peak areas correspond to their actual concentration in solution. Hence, it is easy to obtain the conversion ratio (in percent) by dividing the peak area of **3** by the sum of the peak areas of **2** and **3**. The conversion ratio is divided by the reaction time and multiplied by the starting substrate concentration [S] to give the initial velocity v at that concentration. The experimental conditions required ca. 3–4 h for 10% conversion of substrates to product. This analysis is repeated for all 12 reactions. Using data-processing software (see Table 2) the results are plotted in the form of $1/v$ vs 1/[S] to give a linear correlation, known as the Lineweaver–Burk plot. From this graph[22] students can calculate the v_{max} and K_m values of the antibody catalyst (Fig. 4).

An alternative and often more accurate procedure for determining these kinetic parameters is the direct Michaelis–Menten plot. The velocity v is plotted vs the substrate concentration [S] and a curve fit is executed according to the Michaelis–Menten equation (see Fig. 5). The execution of this curve fit produces the kinetic parameters directly.

The value of k_{cat} is calculated by dividing the value of v_{max} by the concentration of active sites. This concentration is obtained by active-site titration (see above). In our hands, with antibody concentration of 5.67 μM and 1.9 active sites per antibody molecule, k_{cat} was found to be either 0.0076 min^{-1} (using the Lineweaver–Burk plot results) or 0.0068 min^{-1} (using the Michaelis–Menten plot).

Conclusion

The experiments described above introduce students to catalytic antibodies, part of the ever-expanding interface between chemistry and biology. They emphasize "chemistry" as the central science for the design, synthesis, and dissection of biological catalysts. Using a commercially available catalytic antibody, students learn basic principles of biocatalysis through multidisciplinary experiments involving transition metal–based synthetic organic chemistry, mechanistic organic chemistry, and enzyme kinetics. Wet experimental techniques and the use of UV–vis and HPLC instrumentation provide a challenging and exciting setting for chemistry instruction. The experiments provide a foundation for further discussion of chemical catalysis, the evolution and mechanism of enzymes, and the potential of designed biocatalysts in the chemistry of the next century.

Notes

1. Anhydrous DMF was purchased from Aldrich Chemical Co.

2. A small sample of the black reaction mixture was added to a small vial containing water and diethyl ether (0.5 mL each). The etheric phase showed no traces of starting material by TLC.

3. The column was packed with silica gel–hexane slurry. The silica gel was purchased from Malinckrodt Baker Inc. (#V150-23; 230–400 mesh; 60 Å)

4. Special attention should be paid to the purification of both substrate **2** and product **3**. Both are used as references when analyzing the catalyzed reaction by HPLC. Therefore, at least a small amount of each compound should be attained in high purity. Our recommendation is that during the purification of those compounds by flash chromatography, a small number of fractions of whose purity the student is certain (the middle fractions) should be put aside for use as HPLC reference. The other fractions containing the compound can be collected for the determination of yield and for the continuation of the synthesis.

5. THF was freshly distilled over Na/benzophenone.

6. Small volumes were taken using a Pipetman pipettor (Gilson Co.) and pipettor tips purchased from Fisher Scientific.

7. A 2.0 M LDA solution in THF–pentane–ethylbenzene purchased from Aldrich Chemical Co. was used. LDA can also be prepared from butyl lithium and diisopropylamine using well-known procedures.

8. Owing to the limited stability of aldehyde **2** in aqueous solutions, it is recommended that **2** be kept in an organic solvent. Fresh solutions of **2** in buffer should be prepared before each experiment. HPLC-grade solvents are highly recommended.

9. The PBS solution is prepared by mixing 50 mL of solution A (500 mM Na_2HPO_4 and 500 mM NaCl) and 13 mL of solution B (500 mM NaH_2PO_4 and 500 mM NaCl), diluting it by a factor of 10 and then adjusting the pH with 1 N HCl using a pH meter.

10. One milliliter of the stock solution (1 mg of antibody) is sufficient for one student. All antibody solutions must be refrigerated when not in use.

11. The system in use was a Hitachi HPLC with L-4200 UV-Vis detector, L-6200 Intelligent Pump, D-6000 Interface, and HPLC Manager software. Data can also be acquired by a simpler chromato-integrator. A pre-column must be used to avoid protein contamination of the column. The column used was a Microsorb-MV C18, 5 µm, 300 Å.

12. Solutions of **2** and **3** (5×10^{-4} M) can be prepared by diluting the acetonitrile stock solutions with PBS buffer pH 7.4.

13. HPLC-grade solvents must be used.

14. The dispensing of solutions by a pipettor is best performed by placing the tip on the tube's inner wall at about half its height and tilting the pipettor at an angle of ca. 30°. The solution can then be dispensed into the tube while insuring that no solution has been left in the tip.

15. Owing to the high volatility of acetone, this reactant is added quickly as the last component and the reaction tube is closed immediately after its addition.

16. We used a Varian Cary bio 3 spectrophotometer.

17. Smaller concentrations of antibody, even 6 mg/mL, will also provide satisfactory results.

18. Any data analysis software such as Kaleidograph or Excel is appropriate.

19. The concentration of acetylacetone must be calculated for each reading keeping in mind the dilution effect.

20. The molecular weight of an antibody molecule (IgG) is 150,000 g/mol.

21. The exact time from beginning of reaction to the injection time must be measured.

22. A detailed explanation of this procedure can be found in any biochemistry textbook.

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