Using an Enzyme's Active Site To Template Inhibitors**

Régis Nguyen and Ivan Huc*

The identification of substances that have a strong affinity for the surface or the active site of a protein is not a trivial task. Numerous strategies can be followed, ranging from de novo design to the systematic screening of large libraries. One approach makes use of the recognition between the target protein and building block precursors to favor the formation of a complementary substance. Thus, materials capable of selectively recognizing protein surfaces may be prepared by using the molecular imprinting technique. The protein acts as a template to kinetically generate specific recognition sites in a polymer matrix, which is formed irreversibly.[1, 2] In a related approach, namely dynamic combinatorial chemistry, an enzyme's active site may template the assembly of a complementary substance from a mixture of interconverting species (Figure 1). In this case, the precursors combine through reversible linkages. Under thermodynamic conditions, the protein selects and stabilizes the products with the highest affinity,[3, 4] which leads to their amplification and hence their identification.

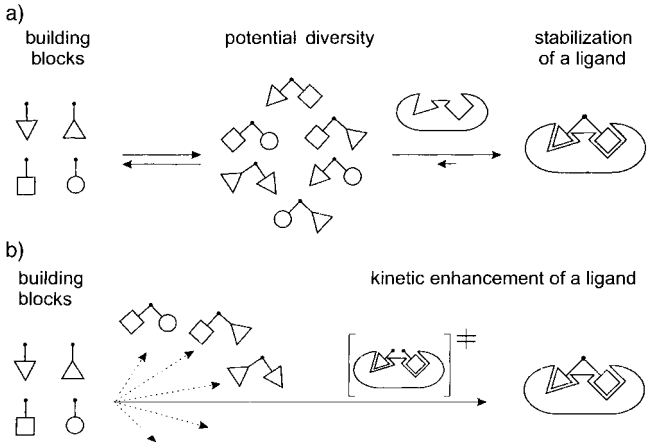
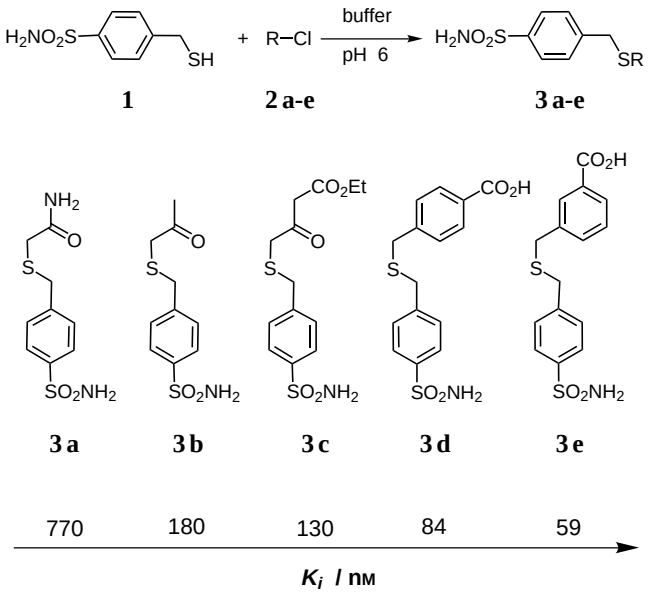


Figure 1. Casting of a ligand for a protein cavity a) from reversibly connecting components (e.g. dynamic combinatorial chemistry), b) by using an irreversible reaction between building block precursors.

Herein we show for the first time that an inhibitor can be cast by using the enzyme's active site as a template in both reversible and irreversible reactions (Figure 1).[5]

We have previously[4c] exploited the well-characterized binding of *para*-substituted aromatic sulfonamides to the Zn^{II} metalloenzyme, bovine carbonic anhydrase (CA II, EC 4.2.1.1).[6] These compounds insert into the active site of CA II, and the nitrogen atom of the sulfonamide group coordinates to the Zn^{II} center. Hydrophobic groups substituted at the *para* position to the sulfonamide group lie on a hydrophobic wall of the protein, which stands above the Zn^{II} pocket.[6, 7]

A series of inhibitors **3a–e** was prepared from α-mercaptotosylamide **1**[8] and various alkyl chlorides **2a–e** (Scheme 1). Their inhibition of the esterase activity of CA II was measured by using a simple spectrophotometric assay that gives a reliable value of the affinity of the sulfonamides for the active site (Scheme 1).[9] The inhibition constants can be ranked from 770 nM for **3a** to 59 nM for **3e** according to an increasing hydrophobicity of the substituent *para* to the sulfonamide group.



Scheme 1. Alkylation of thiol **1** by using various alkyl halides **2**, to form inhibitors of CA II. The measured inhibition constants of the esterase activity of CA II are specified under each structure.

Subsequently, the ability of CA II to enhance the formation of its inhibitors was assessed in competition assays. Thiol **1** was treated with two alkyl chlorides,[10] first in the absence of CA II, then in the presence of CA II (1 equivalent, so that **1** is bound almost quantitatively by the enzyme). The final proportions of the two thioether products measured by HPLC analysis revealed that CA II strongly favors the best inhibitor (Table 1 and Figure 2). For example, when chloride **2a** competes with **2d**, the proportion of product **3d** shifts from 50% in the absence of CA II to 92% in its presence (Table 1, entry 1). When the products have similar affinities for CA II (Table 1, entry 5), their proportions are hardly affected by the presence of the protein in the reaction medium. Despite the fact that the inhibition constants of **3a–e** extend over only one order of magnitude, the competition experiments give a good estimate of their relative affinities. Based on these results, one can expect even stronger effects for larger differences in affinities.

[*] Dr. I. Huc, R. Nguyen
Institut Européen de Chimie et Biologie
ENSCPB—Av. Pey Berland
33402 Talence Cedex (France)
Fax: (+33) 557-96-22-26
E-mail: i.huc@iecb-polytechnique.u-bordeaux.fr

[**] This work was supported by the Centre National de la Recherche Scientifique and by the Ecole Polytechnique (predoctoral fellowship to R.N.). We thank Prof. Jean-Marie Lehn for stimulating discussions.

  0044-8249/01/11309-1824 $ 17.50+.50/0

Table 1. Reactions[a] between thiol **1** (320 μM) and two competing alkyl chlorides.

Entry	Competing electrophiles	Conc.[b] [equiv]	Products (K_i in nM)	Yield[c] (without additives) [%]	Additives (equiv)	Yield[b] (with additives) [%]
1	**2a** $ClCH_2CONH_2$	50	**3a** (770)	50	CA II (1)	8
	2d *p*-$ClCH_2C_6H_4CO_2^-$	12.5	**3d** (84)	50		92
2	**2a** $ClCH_2CONH_2$	50	**3a** (770)	40	CA II (1)	10
	2e *m*-$ClCH_2C_6H_4CO_2^-$	12.5	**3e** (59)	60		90
3	**2a** $ClCH_2CONH_2$	500	**3a** (770)	46.5	CA II (1)	16
	2b $ClCH_2CCOCH_3$	2	**3b** (180)	53.5		84
4	**2a** $ClCH_2CONH_2$	125	**3a** (770)	15	CA II (1)	2
	2c $ClCH_2COCH_2CO_2Et$	1.08	**3c** (130)	85		98
5	**2b** $ClCH_2CCOCH_3$	25	**3b** (180)	49	CA II (1)	43
	2c $ClCH_2COCH_2CO_2Et$	9.25	**3c** (130)	51		57
6	**2a** $ClCH_2CONH_2$	50	**3a** (770)	50	– (CH_3CN 75%)	70
	2d *p*-$ClCH_2C_6H_4CO_2^-$	12.5	**3d** (84)	50		30
7	**2a** $ClCH_2CONH_2$	50	**3a** (770)	50	CA II (1)	60
	2d *p*-$ClCH_2C_6H_4CO_2^-$	12.5	**3d** (84)	50	+ methazolamide[12] (20)	40

[a] The reactions were carried out at 25 °C in buffered water at pH 6 (sodium phosphate 200 mM), and were complete after 48 h. [b] The initial stoichiometry of the chlorides was adjusted to compensate for large differences in reactivity. [c] The yield was determined by integrating the peaks in the HPLC chromatograms, and comparing them to an internal standard (benzoic acid). These data were reproducible within 15% when the same batch of CA II was used.

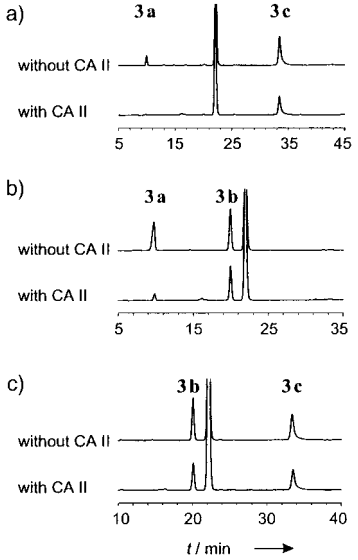


Figure 2. Selected chromatograms showing the proportions of products in reactions between **1** and two competing chlorides **2**: a) **1**+**2a**+**2c** (Table 1, entry 4), b) **1**+**2a**+**2b** (Table 1, entry 3), c) **1**+**2b**+**2c** (Table 1, entry 5). In each case, the top trace is for the reaction performed without CA II, and the lower trace is for the reaction performed with CA II (1 equivalent with respect to **1**). The peak at 22.4 min is that of the internal reference (benzoic acid). See Table 1 and Experimental Section for details.

Several control experiments were performed to establish that CA II actually operates as a template in these reactions. When the concentration of the enzyme was varied, saturation behavior was observed: the ratio of products **3d**/**3a** increased progressively when the amount of CA II was increased from zero to one equivalent, and leveled off at 1.5 equivalents of CA II (data not shown).[11] When CA II was replaced by a protein that has no affinity for **1** (e.g. bovine serum albumin), or when **1** was replaced by a thiol that has no affinity for CA II (e.g. α-mercapto-*para*-toluenesulfonic acid), the proportion of products remained unchanged. More importantly, when methazolamide,[12] a known inhibitor of CA II, was added, it competed with **1** for binding and the effect of CA II on the proportion of the products was very limited (Table 1, entry 7).

These experiments consistently show that the enzyme directs reactions of **1** only when it is bound. The possibility that the nonpolar environment of the active site may selectively enhance some reactions (medium effects or catalysis) was ruled out—when the medium polarity was decreased by adding CH_3CN, the reactions between **1** and benzylic chlorides **2d** and **2e** were clearly disfavored,[13] whilst they are favored in the presence of CA II (Table 1, entries 1, 2, and 6).

Thus, the most likely mechanism for the observed effects is the preassociation of thiol **1** in the Zn^{II} pocket with a hydrophobic chloride against the hydrophobic wall.[7] The reagents are brought into close proximity of each other in a ternary complex with CA II, and their reaction is favored (Figure 1). Nonhydrophobic chlorides are less strongly bound, and their reactions are not favored.

The formation of an inhibitor can be enhanced under kinetic control by the active site of the enzyme. Under thermodynamic control (e.g. when a reversible reaction is used), the reaction products may be stabilized and enhanced in a complex with the target protein (Figure 1).[3, 4] For an irreversible reaction, the enhancement of the products is an indication that the high-energy reaction intermediates are tightly bound, and not necessarily the products. In the S_N2 reactions used herein and in model systems,[14] the products resemble the transition states, which results in a correlation between the amplitude of the template effect and the affinity of the product for the template.

The method described herein may be useful to identify new inhibitors and to evaluate their relative affinities. Further developments include combinatorial assays where multiple reagents compete simultaneously in the reaction.

Experimental Section

In a typical assay, stock solutions of thiol **1**, chlorides **2a**–**c**, benzoic acid (internal standard), and CA II in an aqueous phosphate buffer (200 mM, pH 6) were prepared just before use. The desired quantities of these solutions were mixed in a screwcap vial equipped with a small magnetic stirrer bar. When necessary, the total volume was adjusted to 500 μL. The

stirred reaction mixtures were degased under vacuum, and purged three times with argon. Stirring was stopped, and the solutions were allowed to react at room temperature under an anaerobic atmosphere. After 48 h, a 250-μL aliquot was removed, diluted with CH_3CN (750 μL), and sonicated for 7 min to precipitate the CA enzyme. The suspension was centrifuged on an ultrafree-CL-Biomax membrane (PBCC 5000 UFC4 BCC25). This treatment was also applied when no CA was present. The filtrate was lyophilized and redissolved in H_2O/CH_3CN (1:1, 200 μL). The solution was analyzed by reversed-phase HPLC with detection at 230 nm on a Waters 2690 instrument equipped with a Merck RP-Select B reversed-phase column (5 μm, 250 × 4 mm, flow rate: 1 mL min^{-1}). A ternary solvent gradient (solvent A: 0.1 % trifluoroacetic acid in H_2O; solvent B: 0.08 % trifluoroacetic acid in CH_3CN; solvent C: isopropanol) was optimized so that most of the compounds used in this study have different retention times: C: constant at 2 %; B: 0 % during 3 min, then increased to 80 % over 79 min.

The assay described above was optimized to limit side reactions such as disulfide formation, alkyl chloride hydrolysis, and trialkyl sulfonium formations. Some of these side products have been identified on the chromatograms and are mentioned below. The products **3a**–**e** were synthesized and characterized separately to validate their assignments on the chromatograms.

The following retention times and absorption coefficients were measured: **3a** (10 min, $\varepsilon_{230} = 12000$ cm^{-1}M^{-1}), **3b** (20 min, $\varepsilon_{230} = 16000$ cm^{-1}M^{-1}), **3c** (33 min, $\varepsilon_{230} = 15000$ cm^{-1}M^{-1}), **3d** (57 min, $\varepsilon_{230} = 23000$ cm^{-1}M^{-1}), **3e** (57 min, $\varepsilon_{230} = 23000$ cm^{-1}M^{-1}), **2d** (40 min), **2e** (40 min), 4-hydroxymethylbenzoic acid (14 min), 3-hydroxymethyl benzoic acid (14 min), benzoic acid (22 min), α,α-tosylamide disulfide (61 min, $\varepsilon_{230} = 23000$ cm^{-1}M^{-1}).

Received: October 30, 2000
Revised: January 8, 2001 [Z16018]

[1] For reviews on molecular imprinting, see: K. Mosbach, O. Ramström, *Bio/Technology* **1996**, *14*, 163–170; G. Wulff, *Angew. Chem.* **1995**, *107*, 1958–1979; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1812–1832; B. Sellergren, *Angew. Chem.* **2000**, *112*, 1071–1078; *Angew. Chem. Int. Ed.* **2000**, *39*, 1031–1037.

[2] a) H. Shi, W.-B. Tsai, M. D. Garrison, S. Ferrari, B. D. Ratner, *Nature* **1999**, *398*, 593–597; b) S. Hjertén, J.-L. Liao, K. Nakazato, Y. Wang, G. Zamartskaia, H.-X. Zhang, *Chromatographia* **1997**, *44*, 227–234; c) M. Kempe, M. Glad, K. Mosbach, *J. Mol. Recognit.* **1995**, *8*, 35–39; S. Mallik, S. D. Plunkett, P. K. Dhal, R. D. Johnson, D. Pack, D. Shnek, F. H. Arnold, *New J. Chem.* **1994**, *18*, 299–304; d) M. Glad, O. Norrlöw, B. Sellergren, N. Siegbahn, K. Mosbach, *J. Chromatogr.* **1985**, *347*, 11–23.

[3] For reviews on dynamic combinatorial chemistry, see: I. Huc, R. Nguyen, *Comb. Chem. High Throughput Screening* **2001**, *4*, 53–74; A. V. Eliseev, J.-M. Lehn, *Curr. Top. Microbiol. Immunol.* **1999**, *243*, 159–172; J.-M. Lehn, *Chem. Eur. J.* **1999**, *5*, 2455–2463.

[4] a) O. Ramström, J.-M. Lehn, *ChemBioChem* **2000**, *1*, 41–48; b) B. A. Katz, J. M. Clark, J. S. Finer-Moore, T. E. Jenkins, C. R. Johnson, M. J. Ross, C. Luong, W. R. Moore, R. M. Stroud, *Nature* **1998**, *391*, 608–612; c) I. Huc, J.-M. Lehn, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 2106–2110; d) S. Sakai, Y. Shigemasa, T. Sasaki, *Tetrahedron Lett.* **1997**, *38*, 8145–8148; e) P. G. Swann, R. A. Casanova, A. Desai, M. M. Frauenhoff, M. Urbancic, U. Slomczynska, A. J. Hopfinger, G. C. Le Breton, D. L. Venton, *Biopolymers* **1996**, *40*, 617–625; f) B. A. Katz, J. S. Finer-Moore, R. Mortezaei, D. H. Rich, R. M. Stroud, *Biochemistry* **1995**, *34*, 8264–8280.

[5] An early report already opened up such a possibility: D. Rideout, *Science* **1986**, *233*, 561–563.

[6] a) A. M. C. Bunn, R. S. Alexander, D. W. Christianson, *J. Am. Chem. Soc.* **1994**, *116*, 5063–5068; b) *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics* (Eds.: S. J. Dogson, R. E. Tashian, G. Gros, N. D. Carter), Plenum, New York, **1991**; c) T. Mann, D. Keilin, *Nature* **1940**, *146*, 164–165.

[7] For human carbonic anhydrases B and C, it has been shown in series of homologous sulfonamides that an increase in the binding constant to CA is mainly a result of an increase in the association rate constant. The effect of a hydrophobic group substituted at the *para* position to the sulfonamide group is not a direct stabilization of the coordinated complex (decrease in the dissociation rate), but the stabilization of an intermediate uncoordinated complex that probably involves the hydrophobic wall; see: R. W. King, A. S. V. Burgen, *Proc. R. Soc. London B* **1976**, *193*, 107–125.

[8] Thiol **1** was synthesized from thiourea and α-bromotosylamide (R. L. Colescott, R. R. Herr, J. P. Dailey, *J. Am. Chem. Soc.* **1957**, *79*, 4232–4235) by following a standard procedure: M. Bellas, D. L. Tuleen, L. Field, *J. Org. Chem.* **1967**, *32*, 2591–2595.

[9] J. McD Armstrong, D. V. Myers, J. A. Verpoorte, J. T. Edsall, *J. Biol. Chem.* **1966**, *241*, 5137–5149.

[10] The reaction between the cysteine thiol of a protein and an alkyl halide has been shown to proceed smoothly in neutral water: a) A. Planas, J. F. Kirsch, *Biochemistry* **1991**, 8268–8276; b) S. B. Smith, F. C. Hartman, *J. Biol. Chem.* **1988**, *263*, 4921–4925.

[11] An increase in the concentration of CA II also causes a non-specific oxidation of **1** into the corresponding disulfide, despite the anaerobic conditions (see Experimental Section). When more than 1.5 equivalents of CA II is present, the oxidation is significant and product analysis becomes inaccurate.

[12] G. S. Ponticello, M. B. Freedman, C. N. Habecker, P. A. Lyle, H. Schwam, S. L. Varga, M. E. Christy, W. C. Randall, J. J. Baldwin, *J. Med. Chem.* **1987**, *30*, 591–597.

[13] This is presumably a result of more polar intermediates for S_N2 reactions with benzylic chlorides.

[14] One consequence is product inhibition: no turnover is expected, and a stoichiometric amount of template is neccesary to maximize product enhancement; see: a) R. J. Pieters, I. Huc, J. Rebek, Jr., *Chem. Eur. J.* **1995**, *1*, 183–192; b) D. W. J. McCallien, J. K. M. Sanders, *J. Am. Chem. Soc.* **1995**, *117*, 6611–6612; c) C. J. Walter, H. L. Anderson, J. K. M. Sanders, *J. Chem Soc. Chem. Commun.* **1993**, 458–460.

Nonhazardous Direct Oxidation of Hydrogen to Hydrogen Peroxide Using a Novel Membrane Catalyst

Vasant R. Choudhary,* Abaji G. Gaikwad, and Subhash D. Sansare

Hydrogen peroxide is a clean oxidizing agent that is useful for converting organic compounds into value-added products (bulk and fine chemicals), as well as for industrial and municipal waste-water treatment, and water disinfection. However, because of the high cost of its production by the standard anthraquinone process,[1] hydrogen peroxide cannot be used for the production of bulk organic chemicals or for water treatments. Moreover, the anthraquinone method is not a green process. Hence, it is of great practical importance to develop an environmentally friendly process based on the direct oxidation of hydrogen to hydrogen peroxide. Although the formation of hydrogen peroxide in the palladium-catalyzed liquid-phase oxidation of hydrogen has been known since 1914, and several patents have been issued since then,[2–11] this process could not be put into practice. This is mostly because of its highly hazardous nature (the explosive limits of hydrogen/oxygen gas mixtures are very wide and are further widened with increasing pressure), and/or poor

[*] Dr. V. R. Choudhary, Dr. A. G. Gaikwad, Dr. S. D. Sansare
Chemical Engineering Division, National Chemical Laboratory
Pune 411008 (India)
Fax: (+91) 20-5893041/(+91) 20-5893355
E-mail: vrc@ems.ncl.res.in, vrc@che.ncl.res.in

  0044-8249/01/11309-1826 $ 17.50+.50/0