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Functional Mimicry of the Active Site of Carboxypeptidase A by a Molecular Imprinting Strategy: Cooperativity of an Amidinium and a Copper Ion in a Transition-State Imprinted Cavity Giving Rise to High Catalytic Activity

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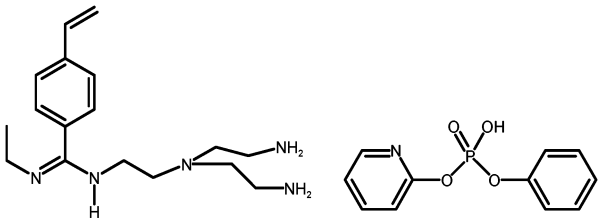
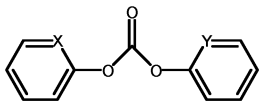
Designing efficient artificial mimics for naturally occurring enzymes has been a challenging area to chemists and biologists.[1] Inspired by the concept of transition-state stabilization in enzyme catalysis,[2] catalytically quite active antibodies have been raised against stable transition-state analogues (TSAs) of the corresponding reaction.[3] Similarly, molecularly imprinted polymers[4] offer an excellent possibility to mimic the active site of natural enzymes since not only can the shape of the transition state be mimicked by imprinting but also at the same time suitable catalytic groups and binding sites can be introduced into the active site in a predetermined orientation.[5−7] However, only recently has strong catalytic activity approaching[6e] or surpassing[7] the activity of the catalytic antibodies been reported.

High activity could be obtained[7] in a model of carboxypeptidase A by a combination of imprinting with a stable transition-state analogue and introducing an amidinium function and a Zn^{2+} binding site in the active cavity in a defined orientation. We report now on the replacement of Zn^{2+} by Cu^{2+} and the use of other substrates which are more similar in structure to the template. These changes result in a dramatic increase in catalytic activity. At the same time, these models show typical enzyme properties such as selectivity, Michaelis−Menten kinetics, competitive inhibition, etc.

In our earlier article on Zn^{2+}-containing catalysts, the optimization showed that the use of functional monomer **1** and template **2**



1 **2**



3 X = Y = C
4 X = C, Y = N
5 X = Y = N

resulted in the most active catalysts. The monomer **1** contains a triamine functionality for binding the metal ion and an amidinium ion. The amidinium group is designated to bind the tetrahedral transition state of carbonate hydrolysis similar to the catalytic role of the guanidinium moiety of Arg 127 in carboxypeptidase A.[8] The enzyme also contains an active Zn^{2+} ion playing an important role

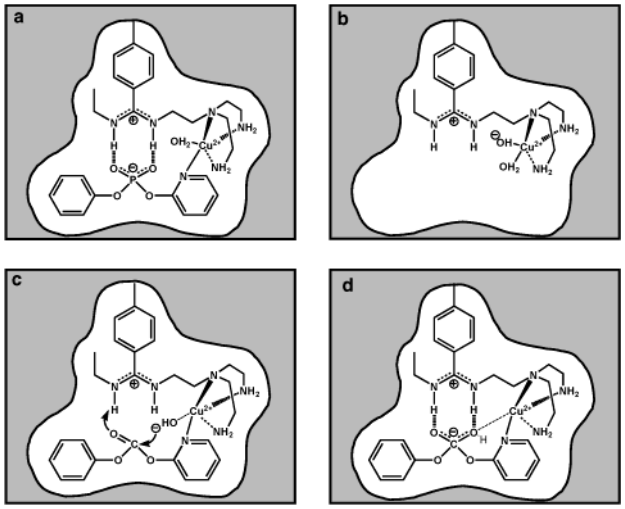


Figure 1. Preparation and Function of Imprinted Catalyst. (a) Molecular imprinting with the template **2** and monomer **1** in the presence of Cu^{2+}. (b) Cavity after removal of the template **2**. (c) Substrate **4** bound in the cavity of b. (d) Intermediate in the catalysis. After release of products (phenol, pyridone, CO_2), structure b is regenerated.

in catalysis. In our present model, a Cu^{2+} center is introduced instead of the Zn^{2+}, although natural carboxypeptidases do not contain copper. Cu^{2+} is expected to form more stable complexes between the triamine part of **1** and the pyridine nitrogen of the stable transition-state analogue **2**,[9] thus providing a better defined conformation of the complex during the imprinting procedure (see Figure 1a). More importantly, Cu^{2+} generates a more nucleophilic OH^- compared to Zn^{2+} for promoting the catalysis.

The polymers have been prepared in bulk by standard procedures (see Supporting Information). The templates were removed to about 75% from the crushed polymer particles (45−125 μm). A control polymer was prepared accordingly but without the template **2**. The catalytic activity of the imprinted polymers was determined by investigating the rate of hydrolysis of different carbonates in HEPES buffer (pH 7.3)/MeCN 1:1. A comparison of the catalytic activity of the corresponding Zn^{2+} and Cu^{2+} catalysts with *diphenyl carbonate* (**3**) as the substrate (see Table 1) showed that the best values of the Zn^{2+} catalyst show an 3260-fold[7] enhancement compared to buffer solution, whereas the new copper catalyst showed a considerably stronger enhancement of 8015-fold.

To have substrates that are more similar to the template, the new substrates *phenyl-2-pyridyl-carbonate* (**4**) and *di-(2- pyridyl)-carbonate* (**5**) were used. Both substrates are inherently more reactive toward water than **3**. They are better bound in the cavity compared to **3** since the pyridyl ring interacts with the Cu^{2+} center similarly to the template (see Figure 1c). Investigation of the catalytic hydrolysis showed much stronger rate enhancements of

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Table 1. Pseudo-First-Order Kinetics of the Hydrolyses of Different Carbonates (**3**−**5**) in Presence of Catalytic Imprinted Polymers

imprinted polymer[a]	substrate	k_{impr}(min^{-1})[b]	k_{impr}/k_{soln}	k_{impr}/k_{contr}
PZn1,2[c]	**3**	0.00235	3264	61.5
PCu1,2	**3**	0.00571	8015	49.0
PCu1,2	**4**	0.41	15700	76.9
PCu1,2	**5**	13.0	76570	80.1

[a] The imprinted polymers were prepared from 6.3% of a 1:1 complex of **1** and **2** in the presence of either Zn^{2+} or Cu^{2+}, 83.3% of ethylene dimethacrylate, and 10.4% of methyl methacrylate in the presence of the same volume acetonitrile/DMSO 1:1 (v/v). [b] Hydrolyses of carbonates **3**, **4**, or **5** in a solution of 50 mM HEPES buffer (pH 7.3)/MeCN 1:1 at 20 °C. (HEPES = 2-[4-(2-hydroxy-ethyl)-1-piperazine] ethanesulfonic acid). There are 2 mM of available active sites in relation to 1 mM substrate. k_{impr} is the pseudo-first-order rate constant in the presence of the polymer, k_{contr} is the rate constant in the presence of the control polymer, and k_{soln} is the rate constant in the HEPES buffer (pH 7.3)/MeCN 1:1 solution. [c] Data from ref 7. For experimental details, see Supporting Information.

Table 2. Comparison of Michaelis−Menten Kinetics of Carbonate Hydrolyses with Imprinted Polymers and Control Polymer **CPCu1**

polymer[a]	substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/k_{uncat}	k_{cat}/K_m[b] (min^{-1} M^{-1})
PCu1,2	**5**	28.0	0.58	110000	48200
CPCu1	**5**	0.37	6.10	1450	61
PCu1,2	**4**	2.86	0.65	75700	4400
CPCu1	**4**	0.035	4.25	946	8.2
PZn1,2[c]	**3**	0.035	2.01	6900	17.4

[a] The control polymer **CPCu1** was prepared in the same manner as **PCu1,2**, but only the template **2** was omitted. [b] Data of the Michaelis−Menten kinetics were obtained from a plot of initial velocities of the reaction versus the substrate concentration (see Supporting Information). [c] Data from ref 7.

15 700- and 76 570-fold compared to the reaction in buffer/MeCN solution. Also, the imprinting factors[5a] (i.e., the ratio of the catalysis by the imprinted compared to the control polymer) are rather high, with 76.9 and 80.1 (see Table 1). These catalytic enhancements are the highest values reported until now for catalysts prepared by molecular imprinting.

The better catalysis can be explained by better binding of the substrate and a more efficient catalysis as the data from the Michaelis−Menten kinetics show (see Table 2). Remarkable turnover numbers of k_{cat} = 28.0 (**5**) and 2.86 (**4**) min^{-1} are obtained for the imprinted polymers. k_{cat} is higher than k_{impr}, which is determined for only one ratio of catalyst to substrate. k_{cat}/k_{uncat} ($k_{uncat} = k_{soln}$) is used to express the catalytic activity of antibodies and natural enzymes; it shows in our case values of up to 110 000, a figure that is by far the highest obtained for molecularly imprinted catalysts. These values are even higher by more than 2 orders of magnitude compared to those for catalytic antibodies for which k_{cat}/k_{uncat} = 810 has been reported for carbonate hydrolysis.[10] The Michaelis constants K_m show a considerably better binding in the imprinted polymers compared to the control polymers. Both effects sum up to a much better catalytic efficiency k_{cat}/K_m (min^{-1} M^{-1}) for the imprinted polymer compared to the control by factors of 790 and 536. These differences are remarkable since the control also contains the catalytic functional group of **1**, and the excellent catalysis relates to a very efficient imprinting procedure.

The pH rate profile for the carbonate hydrolysis in the presence of **PCu1,2** is quite different from that of **PZn1,2**. The Zn-containing catalyst shows a strong increase in rate with the pH having an inversion point at pH 7.5;[7] the copper-containing one shows a bell-shaped profile with an optimum at pH 7.2 (see Supporting Information). This clearly shows a unique bifunctional nature of the catalysis similarly as discussed, for example, by Breslow for cyclodextrin-type catalysts for the enolization of ketones.[1c] The maximum rate is obtained when the copper is in the catalytically active *aqua hydroxy* form[1d] (Figure 1b). Suh and co-workers[1e,11] investigated carefully similar catalysts with Cu^{2+} and guanidinium ions for their peptidase activity. These catalysts correspond to our nonimprinted control systems such as **CPCu1**.

The bifunctional catalysis proceeds via a binding as shown in Figure 1c and an activation of the carbonyl group by the protonated amidinium ion followed by hydroxyl attack. The reaction is further accelerated by the preferred binding of the tetrahedral transition state (compared to the substrate). In summary, catalysts with very high catalytic activity and efficiency have been obtained. The high activity and selectivity, together with strong chemical, mechanical, and thermal stability, give these catalysts a real advantage compared to catalytic antibodies and also provides a good alternative compared to natural enzymes.

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Supporting Information Available: More data on the synthesis of monomers and polymers as well as on kinetic investigations. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Binding of **1** and **2** in the presence of Cu^{2+} is rather strong. Copper complexes are prepared in MeCN/DMSO 1:1 (v/v) for solubility reasons, though in this solvent lower association of phosphate with the amidinium ion is seen compared to pure MeCN. The Cu^{2+} ion complex of **1** showed a very high association constant of log *K* 15.8 compared to the Zn^{2+} complex with log *K* 9.3.

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