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Model Studies of Metalloenzymes Involving Metal Ions as Lewis Acid Catalysts

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Metal ions of metalloenzymes participate in catalysis either by acting as Lewis acid catalysts or by changing their oxidation states. The most important metal ion that participates as a Lewis acid catalyst in the actions of metalloenzymes is the Zn(II) ion, and virtually all types of organic reactions are catalyzed by Zn(II)-metalloenzymes.[1]

Model studies provide valuable information for understanding the chemistry involved in the action of these metalloenzymes and for designing efficient artificial metalloenzymes. For example, catalytic roles of metal ions that can be utilized in enzymatic reactions are revealed by model studies. Model studies are also undertaken to resolve mechanistic ambiguities concerning a specific target enzyme. In addition, biomimetic catalysts can be synthesized as models of metalloenzymes.

We have performed model studies in three directions for metalloenzymes which involve metal ions acting as Lewis acid catalysts: elucidation of novel catalytic features of the metal ions;[2-15] design of models of a specific target enzyme, carboxypeptidase A (CPA);[16-21] and construction of artificial metalloenzymes based on polyethylenimine (PEI).[22] Discovery of new catalytic features is made through mechanistic analysis of organic reactions catalyzed by metal ions. Models of CPA are designed to reproduce many important characteristics of CPA with small molecules by combining several catalytic factors together. In building artificial enzymes with PEI, molecular recognition of both substrates and transition states is attempted with tight metal centers created on the polymer backbone. These model studies are closely related to one another, because the ability to discover various new catalytic roles of metal ions and to incorporate the catalytic features of metal ions with other catalytic factors is exploited in the preparation of effective biomimetic catalysts.

Novel Catalytic Roles of Metal Ions Acting as Lewis Acid Catalysts

Metalloenzymes use active-site metal ions as well as several organic functional groups as catalytic tools. Catalytic features of metal ions[23,24] acting as Lewis acid catalysts have been investigated much less intensively

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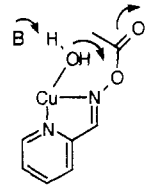
compared with those of organic functional groups[25-28] such as carboxyl, imidazolyl, hydroxyl, phenolic, sulfhydryl, amino, and amide groups. Many mechanistic points must be cleared in order to understand details of the catalytic action of the metal ions of metalloenzymes. What kinds of catalytic roles can be played by the metal ions? What kinds of catalytic features can be manifested by water molecules and hydroxide ions bound to the metal ions? How do the metal ions and the metal-bound water molecules or hydroxide ions cooperate with organic catalytic groups? How is the catalytic efficiency affected by the nature of the metal ion, by the structure of the ligand, or by the configuration around the metal ion? These questions can be answered most effectively with model studies.

The following catalytic roles of metals ions acting as Lewis acids have been revealed previously: activation of electrophiles such as carbonyl,[13,29-35] nitrile,[36,37] and phosphoryl groups;[38,39] activation of various leaving groups such as alkoxide ions,[40-42] hydroxide ion,[43] oxide ion,[43] oximate ions,[2-4,6,9-12,44] amide ions,[15,45-47] sulfur derivatives,[48] and halides;[49] activation of ambient acids such as water,[50,51] alcohols,[52] oximes,[5,53,54] amines,[55,56] and the C_α–H of chelated carbonyl compounds;[57-59] template effects to convert intermolecular processes into intramolecular ones;[23] induction of strain in the reaction center;[13,60,61] and nucleophilic attack by metal-bound hydroxide ions at carbonyl,[43,62-67] nitrile,[68] phosphoryl,[68-70] and olefinic groups.[71] Novel catalytic features of metal ions demonstrated in this laboratory are described below as follows.

1. Nucleophilic Attack by Metal-Bound Water Molecules. When a metal ion is bound to a protein, water molecules and hydroxide ions coordinated to the metal ion can play important catalytic roles. The nucleophilic attack by a metal-bound water molecule has been demonstrated in the Cu(II)-catalyzed hydrolysis of the acetyl ester of 2-pyridinecarboxaldoxime.[3,4] Here, two reaction paths were observed: the rate of one path was proportional to hydroxide concentration and that of the other independent of pH. The apparent rates for the hydroxide and the water paths were enhanced by 2.2×10^7 and 1.1×10^4 times, respectively. Structure–reactivity analysis indicated nucleophilic attack by the metal-bound water molecule at the carbonyl carbon of the bound ester (1), instead of the kinetically equivalent attack by an external water molecule at the ester linkage bound by the metal ion. The basicity and nucleophilicity of water would decrease upon coordination to metal ions. Efficient nucleophilic attack by the Cu(II)-bound water in 1 is attributable to the general-base assistance from another water molecule and to the efficient intramolecular reaction between the nucleophile and the ester. In metalloenzymes, metal-bound water molecules may act as effective nucleophiles in cooperation with general bases. This has been proposed for CPA as shown below.



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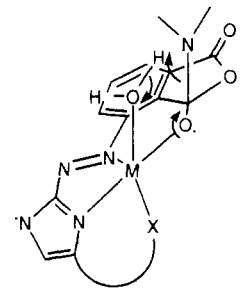

(X = H or CH_2COO^-, M = Cu(II) or Ni(II))

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2. General-Acid Catalysis by Metal-Bound Water Molecules. Upon coordination to a metal ion,

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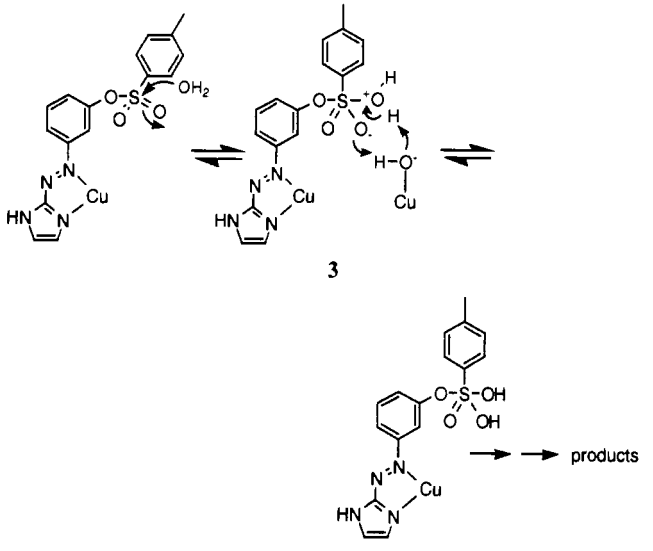
water becomes a weak acid and, therefore, may act as a general acid. This catalytic role has been demonstrated in the hydrolysis of an alkyl amide cocatalyzed by a metal ion and carboxylate anion in dimethyl sulfoxide (DMSO) containing 5% (v/v) water (**2**).[19-21] When an amine leaves from a tetrahedral intermediate, protonation of the nitrogen atom is required in order to avoid expulsion of the highly unstable amide anion.[17,72-74] In the reaction of **2**, the metal-bound water is the only proton donor available and acts as a general acid to protonate the leaving nitrogen atom. This reaction was studied as a model of CPA as described below. Metalloenzymes, therefore, may utilize metal-bound water molecules, in addition to acidic organic functional groups, as general acids.

3. General-Base Catalysis by Metal-Bound Hydroxide Ions. Because metal-bound hydroxide ions are weak bases, they can act as general-base catalysts. This catalytic role has been demonstrated in the Cu(II)-catalyzed hydrolysis of *m*-(2-imidazolylazo)phenyl *p*-toluenesulfonate.[14] Saturation behavior was observed for the dependence of rate constant on [Cu^{II}]. The saturation behavior agreed with the shift of rate-determining step between the formation and the breakdown of an intermediate upon increase in [Cu^{II}]. Analysis of the pH dependence of various kinetic parameters indicated that hydroxocopper(II) ion participates as a general base in the proton transfer (**3**) between addition intermediates. In the action of metalloenzymes, general-base catalysis may be achieved by using metal-bound hydroxide ions as well as basic organic functional groups.



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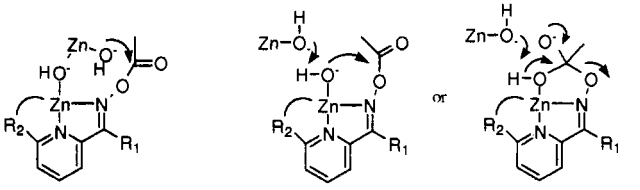
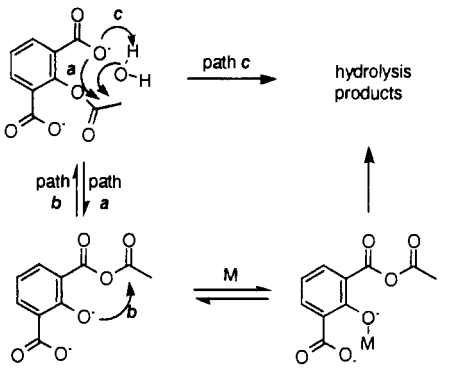
4. Catalysis by Binuclear Metal Ions. The Zn(II) ion-catalyzed hydrolysis of the acetyl esters of 2-acetylpyridine ketoxime and 6-carboxy-2-pyridinecarboxaldoxime involves participation of two Zn(II) ions.[4,7] In the hydrolysis of the 6-carboxy derivative, the reaction was apparently accelerated by about 10^4 times by 0.01 M Zn(II) at pH 7 whereas catalysis was not achieved with Cu(II). Comparison of the kinetic data obtained with these and other related esters in the presence of Zn(II) and Cu(II) ions indicated the participation (**4**) of binuclear Zn(II) ions as the catalytic unit, instead of the kinetically equivalent participation (**5**) of two separate Zn(II) ions.[7] In addition, catalysis



4 ($R_1 = CH_3$, $R_2 = H$; $R_1 = H$, $R_2 = COO^-$) 5

by mononuclear Zn(II) ion was not observed at all in the hydrolysis of the acetyl ester of 6-carboxy-2-pyridinecarboxaldoxime. The equilibrium concentration of the binuclear Zn(II) ion must be very low compared with that of the mononuclear species. The efficient catalysis by the binuclear Zn(II) species is apparently due to the geometry of the transition state. Some other metal-catalyzed reactions involve two metal ions in the transition state.[11,18] In these reactions, binuclear metal ions might be involved as the catalytic units, although the exact mechanisms are not known. It is not very likely to find binuclear metal ions as catalytic units in metalloenzymes. Nevertheless, binuclear metal ions can be exploited in designing effective artificial enzymes.

5. Catalysis by Blockade of Inhibitory Reverse Paths. The hydrolysis of 3-carboxyaspirin is accelerated by Fe(III) or Al(III) ion (about 100 times by 1 mM Fe(III) or 0.05 M Al(III) at pH 2.5–2.6 and 25 °C).[8] More important to note than the degree of rate acceleration[75] is the change of reaction mechanism upon addition of the metal ions. In the anhydride intermediate (**6**) formed by the nucleophilic attack (path a) of the carboxylate group at the ester linkage, the reverse attack (path b) of the phenolate anion at the anhydride



6 (M = Fe(III) or Al(III)) 7

linkage is very effective. In the absence of the metal ions, therefore, hydrolysis of the substrate occurs through an alternative route, via general-base catalysis (path c) by the intramolecular carboxylate group. When Fe(III) or Al(III) ion is added, the metal ion binds the anhydride intermediate (**7**) at the salicylate portion,

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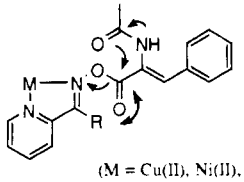
(75) Since the reaction mechanism changes upon addition of the metal ions, the degree of acceleration achieved for the nucleophilic path by the metal ions is ≫100.

blocking the phenolate anion. In the presence of the metal ions, the substrate is hydrolyzed through the anhydride intermediate as evidenced by the trapping of the intermediate. The catalysis is, therefore, solely due to the blockade of an inhibitory reverse path by the metal ion.

A great number of enzymatic reactions involve covalent intermediates.[76] When the enzymatic reaction is a substitution reaction, the leaving group of the substrate remains in the vicinity of the reaction site after it is cleaved by the attack of the enzymatic group. The reverse attack of the leaving group at the resultant intermediate, however, also should be very efficient if the leaving group remains in close proximity to the reaction site. Since this retards the overall reaction, the enzyme must separate the leaving group from the reaction site or block the reactivity of the leaving group. Thus, metal ions of some metalloenzymes might participate in catalysis simply by blocking inhibitory reverse paths through binding the leaving groups.

6. Cooperation of Metal Ions with Organic Functional Groups. The catalytic efficiency of metal ions is enhanced when other catalytic factors participate in cooperation with metal ions. Cooperation between metal ions and organic functional groups becomes very effective when both the organic catalytic group and the metal-binding site are located near the reaction center. Models of CPA presented in the next section can be considered as the most efficient model system ever designed for cooperation between metal ions and the carboxyl group. In addition, cooperation between metal ions and amide oxygen atoms has been observed in the cleavage of aryl ester bonds (e.g., 8; 5×10^7 and 1×10^6 times acceleration when M was Cu(II) and Ni(II), respectively).[12,77] In the reaction of 8, the amide oxygen makes a nucleophilic attack at the ester linkage while the metal ion activates the leaving oximate anion.[12] It is possible that some primitive forms of metallohydrolases utilize the amide groups of polypeptide backbones, instead of other polar organic functional groups, as nucleophiles in cooperation with the active-site metal ions.[12]



(M = Cu(II), Ni(II), or Zn(II);
R = H or CH_3)

8

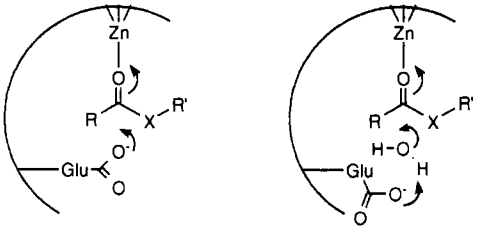
Unlike most of the organic catalytic groups, metal ions often perform several catalytic roles simultaneously. The repertories of metal ions in the ester hydrolysis represented by 4,[7] for example, include the template effect of the metal ion, participation of a binuclear metal ion, activation of the leaving oxime by the metal ion, enhanced ionization of water upon coordination to the metal ion, and nucleophilic attack by the metal-bound hydroxide ion.

Metal ions are superior to organic functional groups in terms of the variety of catalytic roles. In metalloenzymes, metal ions become more effective by cooperation with organic catalytic groups. In addition, metal ion catalysis can become more effective through adjustment of the structures of the chelating sites on the protein. The catalytic repertories of metal ions listed above can be used as a guide both in understanding the catalytic behavior of specific metalloenzymes and in designing effective artificial metalloenzymes.

Models of a Specific Target Enzyme, Carboxypeptidase A

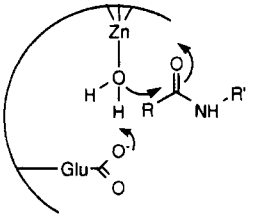
In model studies of a specific target enzyme, it is attempted to reproduce a greater number of characteristics of the enzyme with small organic molecules. In addition, the model studies are undertaken to resolve mechanistic ambiguities concerning the target enzyme. Sometimes, information obtained directly with the target enzyme is reevaluated with model compounds. Such model studies are especially useful for metalloenzymes, since few effective physical tools are available at present for investigation of the catalytic roles of the active-site metal ions.

The mechanism of CPA,[78-82] a Zn(II)-metalloprotease, has been the center of controversy in spite of intensive investigation. In one (9) of the most often proposed



(X = O, NH)

9 10



11

mechanisms, the Glu-270 carboxylate makes a nucleophilic attack at the carbonyl carbon of the substrate. In support of this anhydride mechanism, we reported accumulation of intermediates during the CPA-catalyzed hydrolysis of an ester.[83,84] In addition, we were able to show that the accumulating intermediate is a productive one leading to product formation, instead

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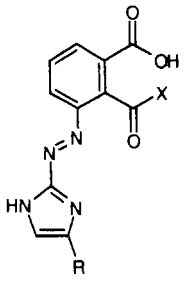
of being a side-equilibrium product.[85] Several lines of evidence supported that the accumulating intermediate is the anhydride formed by nucleophilic attack of Glu-270,[83-86] although positive identification of the structure of the accumulating intermediate is needed in order to settle the mechanistic controversy.[82] In another widely proposed mechanism, the Glu-270 carboxylate acts as a general base to assist the attack of water at the substrate (**10**).[81,87] Recently, nucleophilic attack by the Zn(II)-bound water molecule at the substrate with general-base assistance from Glu-270 has been proposed (**11**).[82] This mechanism is based on the results of X-ray crystallographic studies on unproductive and static complexes of CPA formed with pseudosubstrates or inhibitors.

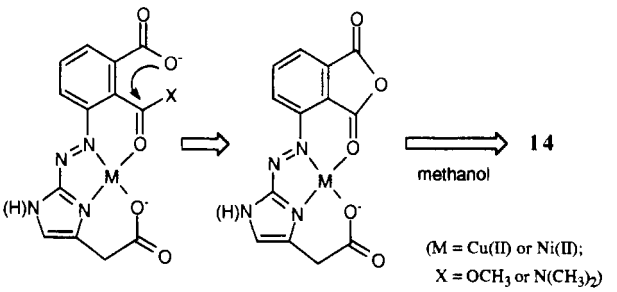
The effective nucleophilic attack by metal-bound hydroxide ions at amide bonds[62-65] and the nucleophilic attack[3] (**1**) by a metal-bound water at a bound ester may be considered as models supporting the nucleophilic attack (**11**) by the Zn(II)-bound water in the CPA action. In view of the proposed anhydride mechanism (**9**) of CPA, several attempts have been made to achieve cooperative catalysis by metal ions and carboxyl group in ester or amide hydrolysis. These attempts, however, have not been successful in most of the model studies.[16,17,65,88-90]

Efficient cooperation between metal ions and carboxyl group in the hydrolysis of both alkyl ester and alkyl amide linkages has been achieved by using the 2-imidazolylazo moiety as a metal chelating site (**12–15**).[18-21] When DMSO containing 5% (v/v) water was employed as the reaction medium, the Cu(II) or Ni(II) ion-catalyzed deacylation of **12–15** manifested the catalytic features of CPA such as efficient cleavage of alkyl ester and alkyl amide bonds (half-life; ca. 10 min for **14** and **15** in the presence of Cu(II) ion and 40–100 min for **14** and **15** in the presence of Ni(II) ion at 50 °C); cooperation among metal ion, carboxyl group, and medium in catalysis; and optimum reactivity attained when the catalytic carboxyl group is in the anionic state.[19-21]



12: X = OCH_3, R = H
13: X = $N(CH_3)_2$, R = H
14: X = OCH_3, R = CH_2COOH
15: X = $N(CH_3)_2$, R = CH_2COOH

Reactions with model compounds **14** and **15** that contain a carboxymethyl group as an extra chelating site led to accumulation of an anhydride intermediate (**17**) formed by the nucleophilic attack of the catalytic carboxylate anion at the scissile ester or amide linkage.[21] The structure of the anhydride intermediate was further confirmed by trapping of the intermediate with methanol leading to the formation of **14**. The Cu(II)- or Ni(II)-catalyzed deacylation of **14** and **15**, therefore, proceeds through nucleophilic attack by the carboxylate ion (**16**) and the general-acid catalysis by the metal-bound water molecule (**2**) in the breakdown of the tetrahedral intermediate.



16 **17**

The rate constant measured at 50 °C for amide **15** complexed to Cu(II) (1×10^{-3} s^{-1}) or Ni(II) ion (1×10^{-4} s^{-1}) is greater than or comparable to that for ester **14** complexed to Cu(II) (8×10^{-4} s^{-1}) or Ni(II) ion (3×10^{-4} s^{-1}).[91] The leveling of reactivity toward the amide and the ester is noteworthy in view of the much greater stability of amide bonds compared with ester bonds. This is another important feature of CPA reproduced by the model.

The carboxylate anion of **14** or **15** acts as a nucleophile in the metal ion-catalyzed deacylation as proposed by mechanism **9** for the CPA action. Cooperation of carboxylate anion with metal ions is required for both the model and CPA. In addition, 95% (v/v) DMSO resembles the microenvironment of the active site of CPA.[92] Results of the model study suggest that, if ester substrates are hydrolyzed through the anhydride mechanism (**9**) in the CPA action, peptide substrates might be equally well hydrolyzed by the same mechanism.

Artificial Metalloenzymes Based on Polyethylenimine

Host molecules capable of tight binding of metal ions, complexation with organic substrates by molecular recognition, and acceleration of transformations within the resultant supramolecular complexes would lead to efficient artificial metalloenzymes. Tight binding of a metal ion may be achieved by employing a multiaza macrocyclic complex as a part of the catalytic unit. In addition, multiaza macrocyclic metal complexes[93-95]

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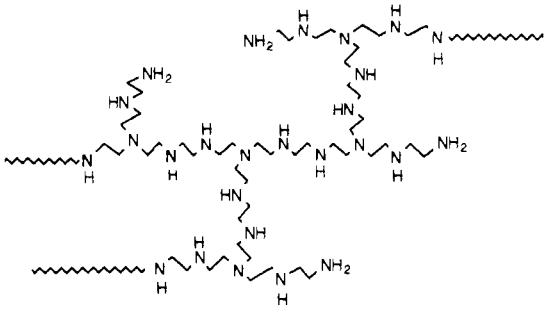
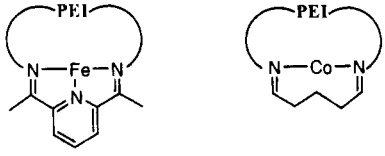
manifest catalytic ability in aldehyde hydration,[66,96] ester hydrolysis,[96,97] and phosphate hydrolysis,[98] as well as molecular recognition of small organic molecules.[99]

Many multiaza macrocyclic metal complexes are prepared by the condensation of carbonyl compounds with multiamines in the presence of metal ions.[93-95] In this regard, PEI (18) can be used as a synthon of macrocyclic complexes as well as the backbone of polymeric macrocycles. In addition, several derivatives of PEI have been investigated as synthetic enzymes, demonstrating their ability to form complexes with organic compounds and to catalyze several types of organic reactions.[100]



18

Structures 19–22 depict typical macrocyclic complexes built on PEI.[22] Each of these structures shows the nature of the metal ion and the dicarbonyl compound used in the condensation, instead of the exact structure of the complex. For example, 22 is prepared by the Ni(II)-template condensation of PEI with butanedione.



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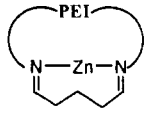

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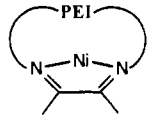
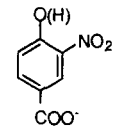


22

The macrocycle-containing PEIs possess fixed metal centers that are not removed by repetitive dialysis. The polymeric macrocycles exhibit greater affinity toward anion 23 (K_d = 1.8 × 10^{-4} M for 21) compared with unmodified PEI ($K_d \gg 5 \times 10^{-3}$ M). Recognition of 24 (K_m = ca. 3 mM) by 19–22 and the consequent complex formation are reflected in the saturation behavior for the dependence of pseudo-first-order rate constants on polymer concentration. Deacylation of anionic ester 24 is ca. 100 times faster than that of neutral ester 26 in the presence of 19–22. Deacylation of 24 inactivates the reaction centers, indicating that the amine nitrogen atom located close to the metal center attacks the bound ester on the acyl carbon (26). Thus, creation of tight metal centers, recognition of anions, and acceleration of deacylation of the bound anionic ester are achieved by the macrocycle-containing PEI derivatives.[22]



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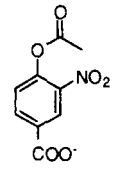

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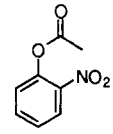

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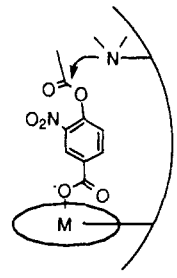

26

Construction of effective artificial metalloenzymes on PEI backbones is at an early stage. The macrocyclic metal centers of 19–22 are used primarily to anchor anionic molecules, and catalytic turnover is not observed in the deacylation of 24. Upon elaboration of the structure of the PEI-based artificial metalloenzymes, the metal centers would be able to perform various catalytic roles discussed above, catalyzing various types of organic reactions as metalloenzymes do in biological systems.

Perspectives

A large number of metal atoms including lanthanides and actinides are available as Lewis acid catalysts in organic reactions. When different oxidation states for each metal atom are considered, the number of metal species becomes greater. Mechanistic data are available only for a small fraction of the metal ions and for a small number of organic reactions.

Remarkable changes in catalytic efficiency have been observed in metal-catalyzed organic reactions with changes in the metal ions or the ligands of the catalytic metal centers.[11,68,70,101,102] This has been explained sometimes in terms of the Lewis acidity of the metal ion, strains in the transition state, or changes in reaction mechanisms. Effects of the changes in metal ions and their ligands on catalytic efficiency, however, are unaccountable and unpredictable in many cases. In the Cu(II)-catalyzed hydrolysis of 14 and 15, for example, catalysis is observed only when the imidazolyl N–H is ionized.[19-21] Apparently, the increase in electron density

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on the imidazolyl ring enhances the catalytic activity of the central metal ion. This is related to the carboxylate–imidazole–Zn(II) triad[103] of many Zn(II)-metalloenzymes, which suggests that the properties of the Zn(II) ion are modified through interaction of the imidazole ligand with the distant carboxylate anion. The reason why the increase in the electron density of the imidazole ligand raises reactivity in both the metalloenzymes and the models is not understood. Many novel aspects of catalysis by metal ions as Lewis acids, especially the effects of the nature of metal ions and their ligands, therefore, will be revealed as investigation progresses further in this area.

Incorporation of many catalytic features together in small molecules is needed to improve models of specific target enzymes. As for the models of CPA, the next goal is to raise the reaction rate of the models to the level exhibited by CPA. In addition, reproduction of other characteristics of CPA such as enantioselectivity and ability to form complexes with substrates will be pursued. Both CPA and CPA models are investigated in this laboratory. Such a dual mechanistic approach will provide valuable clues to elucidation of the details of CPA action.

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Reproduction of the major characteristics of enzyme catalysis such as complexation, acceleration, and specificity is pursued intensively by using synthetic or semisynthetic molecules and antibodies.[104-107] In the design of catalysts mimicking metalloenzymes, knowledge of the catalytic roles of metal ions and the ability to combine the catalytic features of metal ions with those of organic catalytic groups are also needed. Artificial metalloenzymes based on PEI may be improved by introducing additional catalytic or binding sites in planned positions close to the macrocyclic centers. This might be achieved by the site-directed modification of PEI backbones using the macrocyclic centers as anchors.

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On the Molecular Mechanisms of the Solar to Electric Energy Conversion by the Other Photosynthetic System in Nature, Bacteriorhodopsin

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Natural Photosynthesis: Chlorophyll vs Bacteriorhodopsin Photosynthesis

Photosynthesis is a process by which nature converts solar energy into the chemical energy that is required for fueling the different living processes on earth. There are two main different photosynthetic systems in nature: the older (~3 billion years) and much more developed chlorophyll system present in green plants, and the much younger (millions of years) bacteriorhodopsin (bR) system present in *Halobacterium halobium*.

As complex chemical changes usually occur on a long time scale, it is fortunate that nature converts solar energy first into electric energy. In order to store most of the photon energy, the initial process occurs extremely rapidly (much faster than 10^{-9} s, the time it takes excited electrons to re-emit the photon energy), thus insuring that the solar energy captured by the absorption process can be stored for later conversion from electric to chemical energy. In both the chlorophyll and bR systems, the solar to electric energy conversion is completed in a few steps, involving charge separation and leading to the creation of proton gradients. The proton gradients (electrochemical gradients) live sufficiently long to drive the metabolic process which converts this form of electric energy into chemical energy in the form of adenosine triphosphate (ATP).

The first and fastest step of the charge-separation process occurs extremely rapidly (on the picosecond time scale). In chlorophyll, the first step involves an electron transfer between one chlorophyll molecule and the other within the special pair in the reaction center. This leads to the formation of an ion pair. This is followed by further charge separation by transferring the electron from the anion of the ion pair to a pheophytin that is distant from the initially formed ion pair

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