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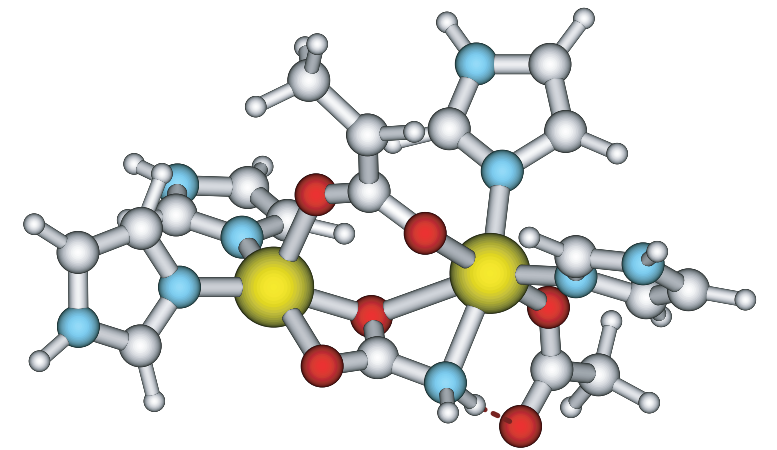
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# Mechanistic Studies of Dinuclear Metalloenzymes - A Model Approach



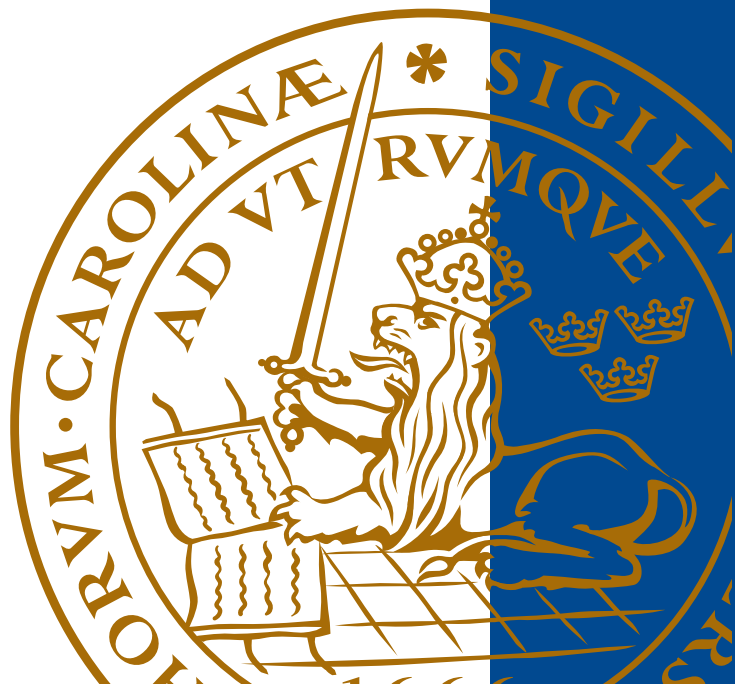
Håkan Carlsson

Inorganic Chemistry  
Lund University, 2003

Håkan Carlsson

Mechanistic Studies of Dinuclear Metalloenzymes - A Model Approach

2003



# **Mechanistic Studies of Dinuclear Metalloenzymes - A Model Approach**

Front cover: Structure of a suggested intermediate in the hydrolytic mechanism of urease generated by Molden.

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# Mechanistic Studies of Dinuclear Metalloenzymes - A Model Approach

Håkan Carlsson



LUND UNIVERSITY


Avhandling för filosofie doktorsexamen  
Naturvetenskapliga fakulteten, Lunds universitet, Lund, Sweden

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<b>Title and subtitle</b> Mechanistic Studies of Dinuclear Metalloenzymes - A Model Approach		
<b>Abstract</b> <p>With the goal to study the mechanisms of dinuclear metalloenzymes, new synthetic and computational models for such proteins were prepared and studied. Multidentate ligands based on a central phenolate and with imidazole- and carboxylate-containing donor moieties were synthesized to simulate carboxylate-rich ligand environments. The two ligands that were found to be the most useful, were the symmetric 2,6-Bis[N-(N-(carboxylmethyl)-N-((1-methylimidazol)methyl)amine)methyl]-4-methylphenolate (BCIMP) and the closely related asymmetric 2-(N-isopropyl-N-((1-methylimidazol)methyl)aminomethyl)-6-(N-(carboxylmethyl)-N-((1-methylimidazol)methyl)aminomethyl)-4-methylphenol (ICIMP).</p> <p>To study the mechanism of the dinickel enzyme urease, complexes containing two nickel ions were prepared from the two ligands. Analysis of the crystal structures of the synthesized model complexes indicated that urea prefers a coordinatively unsaturated metal ion for its initial coordination. Reactivity studies of the complexes showed that an open coordination site is important in facilitating hydrolytic catalytic activity. Computational models, based on the native crystal structure of the enzyme, showed that the first coordination of urea most likely occurs on the coordinatively more unsaturated nickel ion, trans to the carbamylated lysine ligand of the urease active site and that a proposal based on the attack of the bridging hydroxide in the enzyme is unlikely due to the high energy barrier for the formation of the tetrahedral intermediate.</p> <p>When ICIMP is reacted with zinc, a tetranuclear zinc complex is formed. Evidence from mass spectrometry indicates that it dissociates to dinuclear complexes in solution. The structure of the tetranuclear zinc complex is similar to the corresponding nickel complex. In addition, the structure of each dizinc unit in tetranuclear complex is structurally similar to the environment around the active site in the enzyme zinc phosphotriesterase. The (dissociated) zinc complex has also been found to be a potent functional model for hydrolytic enzymes. Homo- and heteronuclear iron complexes were also synthesized. As a useful precursor, a mononuclear iron complex of ICIMP could be isolated and crystallographically characterized. Based on the mononuclear system, four new heterodinuclear iron complexes were isolated. Of these, the FeZn complex is a potential model complex for the FeZn site of plant purple acid phosphatase.</p>		
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*To Christy and Noah*





## List of Papers included in this Thesis

- Paper 1     **Hydrolytically Active Tetranuclear Nickel Complexes with Structural Resemblance to the Active Site of Urease**  
Carlsson, H.; Haukka, M.; Nordlander, E.  
*Inorg. Chem.* **2002**, 41, 4981-4983
- Paper 2     **Nickel Complexes of Carboxylate-Containing Polydentate Ligands as Models for the Active Site of Urease**  
Carlsson, H.; Latour, J.-M.; Haukka, M.; Bousseksou, A.; Nordlander, E.  
Submitted for publication
- Paper 3     **Computational Modeling of the Mechanism of Urease**  
Carlsson, H.; Nordlander, E.  
Manuscript
- Paper 4     **A Structural and Functional Model of the Active Site of Zinc Phosphotriesterase**  
Carlsson, H.; Haukka, M.; Nordlander, E.  
To be submitted for publication
- Paper 5     **Synthesis and Characterization of Dinuclear Fe(III)-M(II) (M=Fe, Zn, Cu, Co) Complexes of Relevance to the Active Sites of Dinuclear Metalloenzymes**  
Carlsson, H.; Thrukhan, V.; Haukka, M.; Bendix Jensen, K.; Nordlander, E.  
Manuscript



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# 1. Introduction

Chemistry has traditionally been divided into different subsections, such as organic, inorganic and physical chemistry. During the 20th century the new area of biochemistry has increased in importance and applications have continuously accentuated the significance of a clearer understanding of how different organisms function on a molecular level. Large opportunities have opened up for interdisciplinary work and new thriving research areas have emerged. Bioinorganic chemistry is such an area.

## 1.1 Bioinorganic Chemistry

Inorganic chemistry has traditionally been focused on the chemistry and properties of metals. Even if organic carbon-based compounds comprise the bulk of the net-weight of living organisms, it has become more and more apparent that metals play very important roles in biochemical systems. The activity of enzymes with metal ions in their active sites is often completely dependent on the presence of the metal. As a result, the field of bioinorganic chemistry has become more and more important and, especially for many traditional inorganic chemists, it has become an exciting area in which to apply their fundamental chemical knowledge. A popular name for the field is "Metals in Biology", which can be found in names of conferences and meetings.

A quick search of the Protein Data Bank (PDB) shows that more than 50% of the structure-content contains one or more non-innocent metal atom(s) (excluding e.g. weakly bound sodium ions).<sup>1</sup> Taking into account that more scientists are interested in metalloenzymes, that such enzymes may be more intensely studied than other proteins, and that the same enzyme may have several entries in the PDB, it is safe to assume that a third of all biological systems contain metal ions which are important for their function. A surprising amount of metals in the periodic table are essential or involved in biological systems. In Fig. 1.1, the elements are gathered into three groups. The first group is found in all living systems, the second is found in most organisms and a the third contains elements that are only found occasionally.<sup>2</sup> Noticeably, most first-row transition metals and even some further down the periodic table are found in the majority of all organisms.

1 H																	1 H	2 He
3 Li	4 Be											5 B	6 C	7 N	8 O	9 F	10 Ne	
11 Na	12 Mg											13 Al	14 Si	15 P	16 S	17 Cl	18 Ar	
19 K	20 Ca	21 Sc	22 Ti	23 V	24 Cr	25 Mn	26 Fe	27 Co	28 Ni	29 Cu	30 Zn	31 Ga	32 Ge	33 As	34 Se	35 Br	36 Kr	
37 Rb	38 Sr	39 Y	40 Zr	41 Nb	42 Mo	43 Tc	44 Ru	45 Rh	46 Pd	47 Ag	48 Cd	49 In	50 Sn	51 Sb	52 Te	53 I	54 Xe	
55 Cs	56 Ba	57 La	72 Hf	73 Ta	74 W	75 Re	76 Os	77 Ir	78 Pt	79 Au	80 Hg	81 Tl	82 Pb	83 Bi	84 Po	85 At	86 Rn	
87 Fr	88 Ra	89 Ac	104 Rf	105 Db	106 Sg	107 Bh	108 Hs	109 Mt	110	111	112		114		116		118	

■ Essential element for life  
■ Required in most organisms  
■ Required in some organisms

Fig. 1.1 Periodic table of biological important elements

Metals participate in several different processes in biological systems. These may be organized into five categories:<sup>1</sup>

1. Structural - forms and holds proteins in place through bringing units together by specific binding.
2. Storage - metal storage, uptake and release of metal ions.
3. Electron transfer - mediation, uptake, storage and release of electrons.
4. Oxygen binding - transport, uptake, storage and release of molecular oxygen.
5. Catalytic - substrate binding, activation and turnover



Of these, group 5 is the most studied and itself the origin of a new set of subgroups and classifications. Since metals are involved in many different transformations in the organism, they are important targets for the pharmaceutical industry. Understanding and subsequent control of a biochemical process yields opportunities to artificially enhance its activity or to try to limit it - this is indeed what most drugs available on the market do. It is also interesting to study the function of metals in biology and apply the knowledge in the area of artificial catalysis. Many enzymes facilitate reactions at normal temperature and pressure. Several of these are processes in which man currently invests much effort and energy to utilize them on an industrial scale. Enzymes are also much more specific than man-made catalysts when it comes to regio- and stereoselectivity. Finally, it is also interesting to understand more about how metal-containing biological systems work for a more academic reason. My personal point of view is that my studies reveal a little of what God so magnificently created and research and studies of the creation are ways for us to learn more about Him.

The field of bioinorganic chemistry can be classified into different areas. Most research activity goes into unveiling and increasing the understanding of the five areas mentioned above. However, the field includes all aspects of metal compounds in biology. The study of what happens when exogenous metals/metal complexes interact with living systems constitutes a large part of bioinorganic chemistry. The use of metal compounds in medicine fits in here.<sup>3</sup> Several pharmaceutical drugs are based on metal-containing compounds. Cisplatin, a drug based on platinum, has been extensively studied and is well-established in cancer treatment.<sup>4</sup> Gadolinium complexes are useful as contrast agents in magnetic resonance imaging.<sup>5</sup> The spread in traditional research backgrounds also strengthens the field. A new generation of researchers, brought up as bioinorganic chemists from the beginning, are now working with members of the community whom are still traditional inorganic chemists or biochemists. Also organic chemists interested in ligand synthesis, theoretical chemists involved in modeling, physicists working on spectroscopical studies, etc. are working in the bioinorganic field.

## 1.2. General Methods Available

Since bioinorganic chemistry has become a rather extensive field with many different areas of interest and scientific backgrounds, it is hard to summarize the different types of tools and methods that are used. However, I will make an attempt, since it might be useful to have a picture of how a bioinorganic problem can be handled. I have divided the methods into two major groups, direct and indirect ones. The former involve direct access and work on the specific enzyme, while the latter uses other techniques to gain information on a system.

### 1.2.1. Direct Methods

When a new system is identified, it is first necessary to learn some basic facts by studying the actual physical system. Structure and other physical properties are of interest together with functional aspects. The first goal is normally to try to obtain a pure sample in sufficient amounts for further investigations. This can now be done with rather sophisticated biochemical methods. Normally the gene for a protein can be cloned and the biosynthesis amplified in a suitable system. Next, different methods are used to purify the crude system, often with the goal of obtaining crystalline material for crystallographic investigations. However, on the way to this goal other properties can be studied and discussed. This was especially true earlier when protein crystallography was in a less advanced state.

Many analytical methods provide important information which may reveal and be correlated to the structure. They can also be useful for the testing of mechanistic hypotheses once a structure has been determined. The metal content of a protein is usually determined early, as well as the primary structure and possible binding sites for metal ions. Likely substrates can be identified, often by analogy studies in which the primary structure is compared to related systems. Kinetic studies can reveal mechanistic properties of enzyme reactions. The majority of tools, however, are spectroscopic. Many spectroscopic methods can be tuned to give specific information about the metal center and its environment. This is very useful since the protein is a large molecule with masses up to and over 1,000,000 Da and it would otherwise be hard to gain information about a metal ion with a mass around 50. In Tab. 1.1, some common spectroscopic methods are listed.<sup>1</sup>

**Table 1.1.** Spectroscopic methods and the information gained.<sup>1</sup>

<i>Method</i>	<i>Information</i>
Vibrational (Raman and IR)	Ligands around metal center
Electronic absorbance (UV/vis etc)	Ligand field and charge transfer information
Magnetic susceptibility	Number of unpaired electrons, giving magnetic interactions between metal centers.
Mössbauer	Normally <sup>57</sup> Fe. Oxidation and spin state, chemical environment.
Electron Spin Resonance (ESR)	Odd electron systems. Ground state wave function. Ligands connected etc
Electron-Nuclear Double Resonance (ENDOR)	Hyperfine interactions
Electron Spin Echo Envelope Modulation (ESEEM)	Related to ENDOR. Smaller variations in hyperfine interactions.
Nuclear Magnetic Resonance (NMR)	Structure, electronic properties for paramagnetic systems.
Magnetic Circular Dichroism (MCD)	Polarization information to understand geometrical features.
Extended X-ray Absorption Fine Structure (EXAFS)	Structure around metal center.

The structure of the material is most often determined by X-ray crystallography. However, with the continuous improvement of NMR spectrometers and computers, NMR structures of ever larger systems have become available. After the structure has been determined, mutation studies of the systems can often be used to further investigate mechanistic properties.

### 1.2.2. Indirect Methods

It is often complicated to discern information directly from an enzyme. The main problem is normally the lack of a pure, highly concentrated material in a structure/modification that still resembles the native state. Another way to obtain information about the system is to use model systems. Normally, after a model compound has been prepared, certain properties are verified versus the structure, function and spectroscopic data of the native enzyme. In other words, it is useful to have these data available. If the model compound has properties that are similar to the enzyme, the investigations can continue and the properties of the model can with some relevance be applied to the biological system. Model compounds can be classified into the following different types.

### 1.2.2.1. Structural Models

These are models which are designed to resemble the structure of the metal center and its environment as exactly as possible. If we take an active site in an enzyme as an example, the metal ion is normally connected to the protein and other compounds in different ways. The environment changes the properties of the system and a good structural model that emulates this immediate surrounding can thus give some insight into the properties of the real system. Model compounds are especially useful when a structure is not available. If a possible structural model includes many of the features found through spectroscopic studies of the system, the structure of the model might be very similar to that of the metal center in the enzyme. An excellent example is the structure of the oxygenated dicopper site of hemocyanin, which was elucidated by Kitajima and coworkers.<sup>6</sup> The structure of the model compound was later verified through the crystal structure determination of the protein. Bioinorganic structural models are often small inorganic complexes with more or less elaborate ligands.

### 1.2.2.2. Functional Models

The main function of catalysts is to enhance reactivity. In the design of functional models for catalysis, focus may be laid on the preparation of models that facilitate the reaction that the enzyme catalyzes. In this case the structure of the model compound is of less importance. Even though the metal can be different, the model can still be used to generate interesting data. If a complex is both a structural and functional model, this is even more useful.

### 1.2.2.3. Theoretical models

Recently, as faster computers and more refined computational codes have become available, many studies can be performed directly on a computer. The stabilities of different structures can be compared, spectroscopic values can be estimated and reaction mechanisms can be tested. This has shown to be a method that grows in importance, especially if the synthetic models and the native enzyme are unstable for different reasons. Larger systems can now be studied by quantum mechanical methods (QM) and methods to combine the less accurate but cheaper molecular mechanics (MM) with QM are now available.

#### 1.2.2.4. De Novo synthesis

A new and increasingly important technique is to take the modeling a little bit further and make models, which partly contain peptide ligands. The protein is never completely innocent and if the active site is buried far into a hydrophobic protein, it is important to be able to place a possible model in the same environment. The understanding of how to generate helices and helix bundles has come the furthest and is now studied by many groups around the world.<sup>7</sup>

### 1.3. Dinuclear Proteins and the Scope of This Thesis

The aim of this thesis is to shed light on a particular group of enzyme active sites, the dinuclear one, containing two metal centers. For different reasons, several enzymes have developed active sites with two metal ions in close proximity. A large group of the dinuclear enzymes are hydrolytic enzymes, specialized in breaking down a long range of organic molecules.<sup>8</sup> Many of the systems are homonuclear, i.e. have two sites with the same element, while others are heteronuclear. The goal has been to study some dinuclear systems through preparation and subsequent studies of model compounds. Hopefully this will teach us more about the specific systems and also lead to more general conclusions about the nature of dinuclear active sites.

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## 2. Ligand Environment

In the design and synthesis of a new model compound, considerable effort is aimed at tuning the ligand environment to suit the goal of the model. This is especially important for structural models. The ligands in the natural systems are either coordinating groups from the protein backbone or other compounds such as coenzymes or small inorganic ions. The material used for the metal environment can be simple inorganic compounds such as water or ammonia, but also more complex multidentate organic ligands. This means that organic synthesis is an intrinsic part of bioinorganic and biomimetic chemistry.

### 2.1. Coordination Properties in the Natural System

Metal ions are normally bound in biological systems through coordination to the protein backbone (endogenous ligands) or to some other organic or inorganic compound (exogenous ligands).<sup>1</sup> This forces the ion to stay in one location but it also inflicts a stability and order to the protein, especially in the case of structural metal ions. In addition, the ligand environment is important for the catalytic activity of the system. For example, iron is found with several kinds of ligands (as iron/sulfur clusters, hemes etc) and has very different properties depending on the ligand environment.

### 2.1.1. Endogenous Ligands

Ligands suitable for metal coordination provided by proteins are summarized in Tab. 2.1. As seen in the table, there is quite a selection of possible coordination sites in a regularly sized protein. It is important to keep in mind, though, that the system is normally very selective. Only certain areas in the protein have the correct number of donor atoms available in a geometry suitable for metal interaction. This also means that certain metals are favored over others in each case. For catalytic biomolecules, the rate of the catalyzed reaction is most often considerably lowered if the metal ion is exchanged for another.

**Table 2.1.** Summary of endogenous ligands.<sup>1</sup> Donor atoms denoted by asterisks.

<i>Coordinating group</i>	<i>Origin in protein</i>
N-donors	
Amino-amido-amidato (- <sup>*</sup> NH <sub>x</sub> )	Lysine side chain, asparagine side chain, glutamine side chain, N-terminus of any residue
Imidazolyl, imidazolato (-Im or -Im <sup>-</sup> )	Histidine side chain
Guanidine (-NHCH <sub>2</sub> -C(N <sup>*</sup> H <sub>2</sub> )(NH))	Arginine side chain
O-donors	
Carbamate (-NH-COO <sup>*</sup> )	Carbamylated lysine residue
Carboxylate (-COO <sup>*</sup> )	Aspartate side chain, glutamate side chain, C-terminus of any peptide chain
Carbonyl (-CO <sup>*</sup> -)	Asparagine side chain, glutamine side chain, back bone carbonyl of any residue
Phenol, phenolate (-Ph-O <sup>*</sup> )	Tyrosine side chain
Hydroxyl (-CH <sub>2</sub> -O <sup>*</sup> H)	Serine and threonine side chains
Olate (-CH <sub>2</sub> -O <sup>*</sup> )	Serine and threonine side chains
S-donors	
Thioether (-CH <sub>2</sub> -S <sup>*</sup> -CH <sub>3</sub> )	Methionine side chain
Thiol (-CH <sub>2</sub> -S <sup>*</sup> H)	Cysteine side chain
Thiolate (-CH <sub>2</sub> -S <sup>*</sup> )	Cysteine side chain
Disulfide (-CH <sub>2</sub> -S <sup>*</sup> -S-CH <sub>2</sub> -)	Cystine (dicysteine) side chains

### 2.1.2. Exogenous Ligands

In addition to the ligands originating from the protein, a broad range of other compounds can contribute to the ligand environment of the metal center. A common ligand, in the case of active sites in enzymes, is the substrate/product/inhibitor. The native enzyme (the system



without any substrate/product/inhibitor bound) often has an "open coordination site". It has one ligand less than what is normally found in a comparable inorganic complex or alternatively, a loosely bound ligand such as water in that site. During turnover a substrate molecule binds at this open site and subsequently gets manipulated to form the product, which is later released. The nature of this ligation does of course vary from reaction to reaction and from enzyme to enzyme.

Another large group of exogenous ligands, contributing to the ligand environment of metal centers, is small inorganic biomolecules. The most common ones are water, hydroxide, oxide, hydrogen sulfide and sulfide. In addition to these, small inorganic molecules from buffers and ionic media are often observed in crystal structures. In some cases these can act as inhibitors as well. Finally, the coenzymes and cofactors are worth mentioning. In some cases, rather large organic molecules need to be incorporated for the enzyme to become active. A good example is the pterins that are essential for the metal binding and function of several molybdenum- and tungsten-dependent enzymes.<sup>2</sup> These pterins are rather large heterocyclic compounds, which anchor themselves in the protein by H-bonding to surrounding amino acid residues. In some cases, the metal ions are completely held in place by the pterin and have no direct coordination to the protein.

## 2.2. Organic Synthesis of Suitable Model Ligands

To control the ligand environment of the synthetic models, organic ligands are specially designed and synthesized. Since many small monodentate ligands theoretically can yield many possible isomers, larger polydentate ligands are often preferred. However, these also constitute a larger synthetic challenge. As seen in Section 2.1, the most interesting donor atoms in ligands are nitrogen, oxygen and sulfur. This makes nucleophilic substitution a simple, but very useful reaction type during ligand synthesis. The synthetic ligands are commonly designed with donors spaced by saturated carbon chains or phenyl rings. Examples from the project, illustrating how such ligands may be prepared, are described below.

### 2.2.1. Tri- and Tetradentate ligands

In the modeling of urease (*vide infra*), one goal was to synthesize dinucleating carboxylate-containing ligands. The first ligands selected for synthesis were tri- and tetradentate ligands with both nitrogen and carboxylate donors (Fig. 2.1). The idea was to design complexes with two metal centers with one ligand each. The centers were then to be bridged by a carboxylate group.

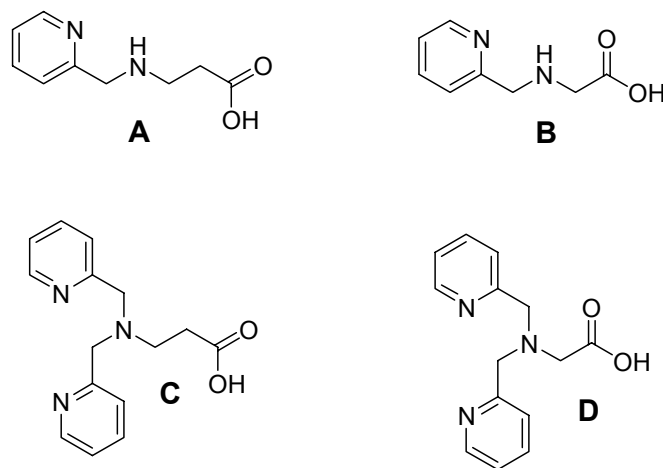


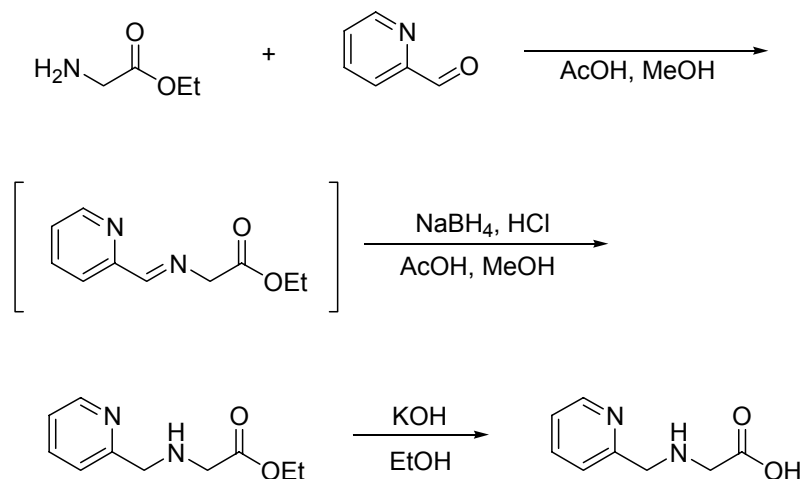
Fig. 2.1. Tri- and tetradentate ligands for urease models.

The compounds A and B are tridentate and based on one pyridine ring, one amine and one carboxylate group. Compounds C and D are tetradentate and have one additional pyridine ring. The spacer between the amine and the carboxylate group is also varied from two methylene groups in A and C to one methylene group in B and D. Unfortunately, so far these ligands have not led to any adequate nickel compounds and the preparations were not published. However, one of the experimental procedures can be used as an example of how such a ligand can be prepared.

**Synthesis of B, 2-aminomethylpyridine-N-acetic acid.** The synthesis is based partly on a previously published synthetic route.<sup>3</sup> Glycine ethyl ester hydrochloride (20.9 g, 0.15 mol), acetic acid (5.7 ml, 0.10 mol) and methanol (200 ml) were combined in a round-bottomed flask. Pyridine-2-carbaldehyde (9.6 ml, 0.10 mol) was added to yield a brownish solution. Sodium borohydride (6.3 g; 0.10 mol) was added to the crude mixture. This yielded gas evolution and a yellow suspension. Hydrochloric acid was added until acidic pH and gas

evolution ceased. The mixture was cleaned by extraction with diethyl ether. Sodium hydroxide was added to neutral pH and the mixture was extracted four times with dichloromethane. The organic phases were combined, dried and evaporated. Distillation at 115°C/5 torr yielded 6.1g (31%). The final product is reached by deprotecting the ester in ethanol and 5% KOH.

The synthesis involves reductive amination. An imide is formed when the aldehyde is reacted with the amine of the protected glycine (Scheme 2.1). The imide is then reduced *in situ* to yield the protected product. These syntheses laid the groundwork for the published ligands (*vide infra*).

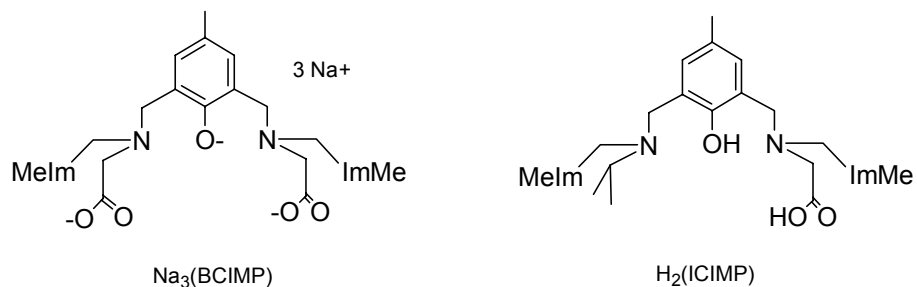


**Scheme 2.1.** Synthesis of 2-aminomethylpyridine-N-acetic acid

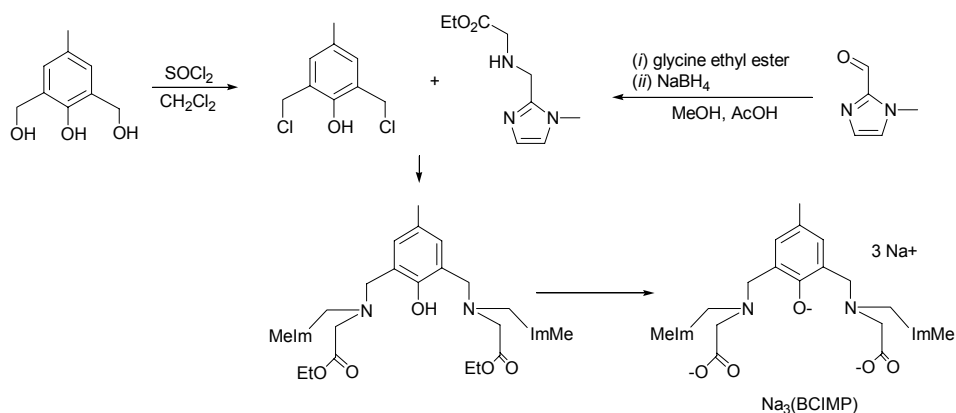
### 2.2.2. Phenolate-Based Ligands, BCIMP and ICIMP

Previous research has shown that if a phenol ring is used to connect the two smaller ligands, the chance of receiving stable dinuclear complexes is increased.<sup>4-8</sup> This observation led to two synthetic goals: the symmetric ligand BCIMP, 2,6-bis[N-(N-(carboxymethyl)-N-((1-methylimidazol)methyl)amine)methyl]-4-methylphenolate, and the asymmetric analog ICIMP, 2-(N-isopropyl-N-((1-methylimidazolyl)methyl)aminomethyl)-6-(N-carboxymethyl-N-((1-methylimidazolyl)methyl)aminomethyl)-4-methylphenol (Fig. 2.2). Imidazole was believed to be a better synthetic model for the desired histidine. Otherwise, the synthesis of the two side arms was accomplished by reductive amination analogous to the smaller ligands

in section 2.2.1. In the case of BCIMP, the side arms were then connected to an activated dimethyl phenol (Scheme 2.2).

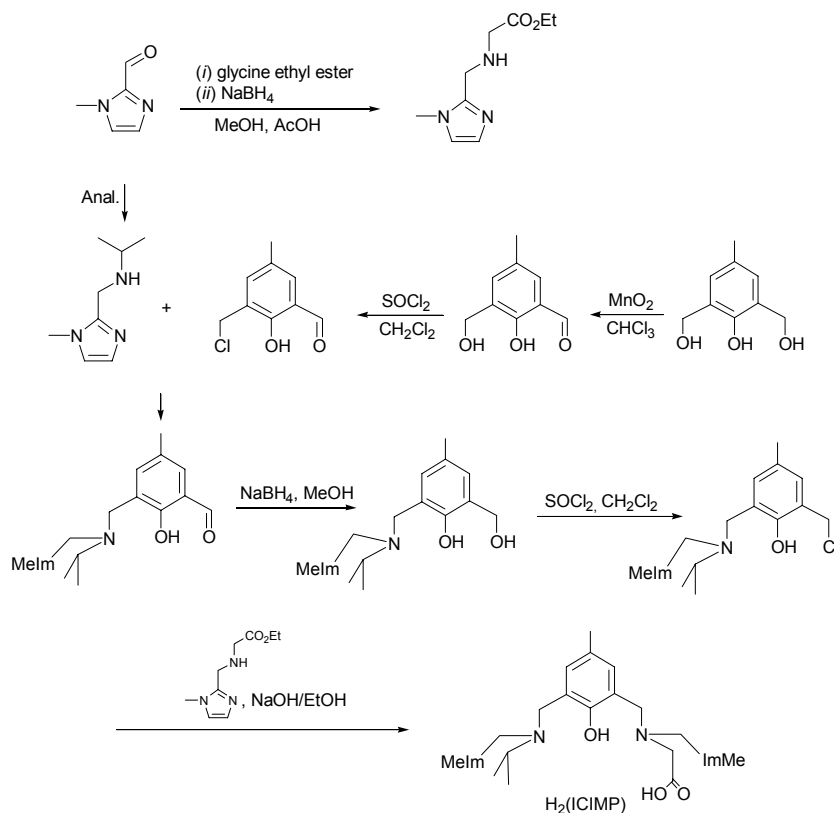


**Fig. 2.2.** Schematic structures of  $\text{Na}_3(\text{BCIMP})$  and  $\text{H}_2(\text{ICIMP})$



**Scheme 2.2.** Synthesis of  $\text{Na}_3(\text{BCIMP})$

For ICIMP, the synthetic route was slightly more complicated. To ensure a pure asymmetric result, the different side arms had to be added stepwise.<sup>9</sup> Attempts to add both at once and then separate the products were unsuccessful. The asymmetry was generated by careful oxidation of 2,6-hydroxymethyl-4-methyl-phenol to yield 2-(hydroxymethyl)-6-carbaldehyde-4-methyl-phenol. It was then possible to add the two arms stepwise (Scheme 2.3).

Scheme 2.3. Synthesis of H<sub>2</sub>(ICIMP).

## 2.3. References

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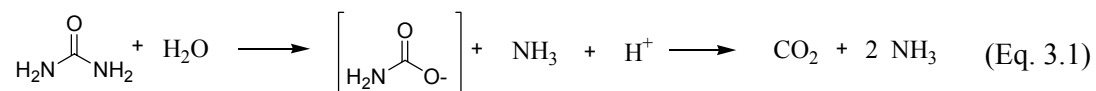


# 3. Urease - A Dinickel Enzyme

The observation of nickel in biological systems is a quite new discovery.<sup>1-4</sup> The common oxidation state for biochemical nickel is +II, but +I, +III and +VI have been observed. In the Ni<sup>2+</sup> state, nickel is normally tetra-, penta- or hexacoordinate. This is useful as the changes in coordination number and geometry can allow for open coordination sites to occur and, in turn, be filled. To date, nickel has been found in six different enzymes. These are urease (*vide infra*), NiFe hydrogenase<sup>5</sup>, methyl-CoM reductase<sup>6</sup>, CO dehydrogenase<sup>7</sup>, acetyl-CoA synthase<sup>8</sup> and Ni superoxide dismutase<sup>9</sup>. The diversity of catalyzed reactions shows that nickel, although rare, is a versatile element in biological systems. Accordingly, the structures of the nickel active sites also show a rich diversity.<sup>10</sup>

## 3.1. Biochemical Background to Urease

Urease is a hydrolytic enzyme, which catalyzes the breakdown of urea to ammonia and carbamate (Eq. 3.1).<sup>11-13</sup> Under hydrolytic conditions, carbamate is an unstable molecule and



decomposes rapidly to ammonia and carbon dioxide. Urease is also generally active towards small amides, which are hydrolyzed to their corresponding carboxylic acids.<sup>14</sup> The enzyme

can be found in a wide range of organisms.<sup>15</sup> It was first isolated from jack beans by Sumner,<sup>16</sup> and was the first enzyme ever to be crystallized. In 1975, it also became the first enzyme to be found to contain nickel.<sup>17</sup> Urease has attracted attention because of its importance in human health and agriculture. It has proven to be central to the virulence of *Helicobacter pylori*, which has been found in connection with ulcers in humans.<sup>11</sup> In agriculture urease is involved in the destruction of urea, which is a common source of nitrogen in fertilizers.<sup>18</sup>

Early data predicted that the active site of urease contained two nickel ions.<sup>17</sup> Data from magnetic measurements and EXAFS have shown that the ions are together in the active site, are weakly antiferromagnetically coupled and that the two ions coordinate histidines and oxygen donors.<sup>20,21</sup> Several crystal structures have been published.<sup>19,22-25</sup> They depict a dinuclear nickel site bridged by an oxygen donor (hydroxide) and a carbamylated lysine, i.e. a lysine residue reacted with one molecule of carbon dioxide (Fig. 3.1). The discovery that the enzyme requires carbon dioxide for its function occurred at approximately the same time as the crystal structure was solved.<sup>26</sup> Each nickel ion also coordinates two histidine residues and

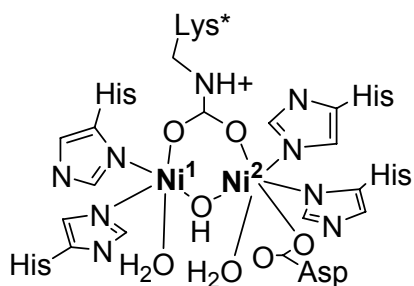


Fig. 3.1 Schematic depiction of the structure of the active site of *Bacillus pasteurii* urease.<sup>19</sup>

one water molecule, while one of the ions (normally termed Ni2) in addition has aspartate side chain in its coordination sphere. This means that the nickel centers are non-equivalent, which may be important for the catalysis. The nickel-nickel distance is about 3.5Å.

A number of mechanisms for the hydrolysis have been suggested.<sup>14,19,27,28</sup> They are all in agreement when it comes to the first part of the catalytic cycle, in which the carbonyl oxygen of urea is coordinated to the open coordination site on Ni1. The origin of the attacking oxygen donor is being debated. A terminal water/hydroxide on Ni2 or a bridging hydroxide have been suggested. The latter hypothesis has been inferred from the fact that urea analogues which function as inhibitors bind at the active site in a tetrahedral fashion, bridging the two nickel atoms.<sup>19</sup> Other suggestions include e.g. a cyanate intermediate.<sup>28</sup>



A fair number of structural and functional models for urease have already been published.<sup>28-42</sup> Some of these are asymmetric<sup>31,32,39</sup> and some have succeeded in incorporating urea.<sup>35,43,44</sup> However, no symmetric or asymmetric systems seem to have incorporated both imidazole and carboxylate ligands in a dinickel urease model prior to the present investigation.

## 3.2. Structural Models of Urease

After the efforts of making stable complexes with tri- and tetradentate ligands (cf. chapter 2.2.1), the focus of the research was shifted to the phenolate-based ligands BCIMP and ICIMP (Fig 2.2).

### 3.2.1. Nickel Complexes with the Symmetric Ligand BCIMP

Two nickel complexes were synthesized based on BCIMP; one with the co-ligand acetate (**1**) and one with diphenyl acetate (**2**) (Fig 3.2). The general synthesis involved dissolving 1 eq. of

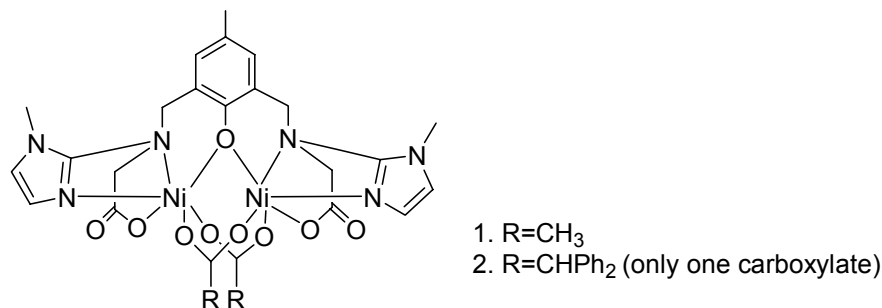


Fig. 3.2. Schematic structure of complex **1** and **2**.

Na<sub>3</sub>(BCIMP) in methanol and then adding methanolic solutions of 2 eq. of nickel(II)perchlorate and 2 eq. of carboxylate subsequently. In the synthesis of complex **1**, 1 eq. of NaBr was added for crystallization purposes. The reaction is more or less immediate, as evidenced by the rapid color change from the less intense green nickel(II)(aq) to the more intense blue complex. This is demonstrated by Fig. 3.3, which shows the titration of nickel perchlorate with aliquots of 0.2 eq. BCIMP and diphenyl acetate. In each step, the reaction is already complete before the cell is transferred into the spectrophotometer. Another feature of the spectrum is that the gap from trace to trace diminishes until an overlap occurs after 2 eq.

(10 aliquots), indicating that the stable stoichiometry of the product complex is dinuclear. Analysis of mass spectra suggests that compound **1** is  $\text{Na}_2[\text{Ni}_2(\text{BCIMP})\text{Ac}_2]\text{Br}$  and compound **2** is  $[\text{Ni}_2(\text{BCIMP})(\text{Ph}_2\text{Ac})]$  and that the steric hindrance of the diphenyl acetate prohibits coordination of a second co-ligand. However, one can not exclude that further coordination of the Ni ions is possible even when the diphenyl acetate is coordinated; it is thus possible that a product mixture may exist and this may be one of the reasons for the difficulties in crystallizing complex **2**.

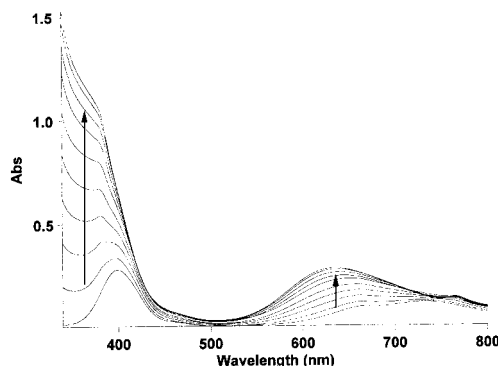


Fig. 3.3. Results from titration of BCIMP and diphenyl acetate to a nickel perchlorate solution (0.05 M).

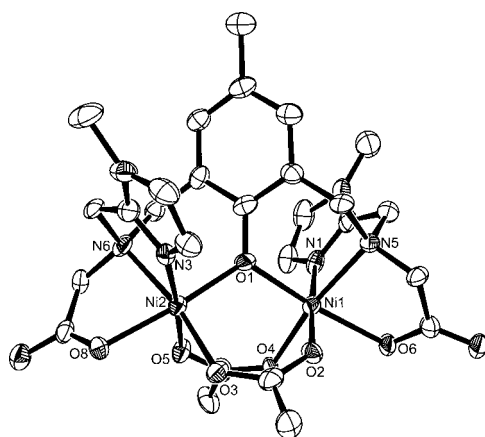


Fig. 3.4. An ORTEP representation of the molecular structure of one dimer of compound **1**.

$\text{Na}_{1.5}[\text{Ni}_2(\text{BCIMP})\text{Ac}_2]\text{Br}_{0.5}\cdot\text{MeOH}\cdot 0.75\text{H}_2\text{O}$ . The structure includes two different complexes with similar structures. The main difference is that one complex has one sodium ion associated with it while the other one has two. The overall structure is similar to the predictions above (Fig. 3.4). The phenolate bridges the two nickel ions and the rest of the ligand wraps around the unit and is coordinated to each ion via an imidazole

nitrogen, the amine nitrogen and a carboxylate oxygen in a terminal fashion. In addition, the two acetate ligands bridge the ions to give two hexacoordinate nickel centers. The octahedral geometries are slightly distorted, which may be due to geometrical restrictions in the ligand. As an example the angle between the amine nitrogen atoms and their axial counterpart is approximately  $168^\circ$  instead of  $180^\circ$  for a regular octahedral geometry. The strain also induces a slightly longer nickel-amine bond (approx.  $2.13 \text{ \AA}$ ) than expected in a relaxed structure (approx.  $2.09 \text{ \AA}$ )<sup>45-47</sup>. The nickel-nickel distances are  $3.396(2) \text{ \AA}$  (Ni(1)-Ni(2)) and  $3.415(2) \text{ \AA}$  (Ni(3)-Ni(4)), which is comparable to the  $3.5 \text{ \AA}$  seen in urease. The relatively short Ni-Ni distance is probably due to the presence of three metal-bridging ligands (BCIMP and the two acetates).

### 3.2.2. Nickel Complexes with the Asymmetric Ligand ICIMP

After these positive results using the BCIMP ligand, it was natural to continue to improve the ligand and the complexes. The asymmetric ligand ICIMP became the ligand of choice. By adding the asymmetry feature and thus an potentially open coordination site and a more open structure, a more applicable model was expected. The ICIMP chemistry has so far yielded four different structures. In all cases, the relatively bulky diphenyl acetate was used to prevent the coordination of two coligands. Compounds **3-5** are tetranuclear, while **6** is dinuclear. Compound **3** is believed to be the "native" compound, while complex **4** and **5** have DMF and urea, respectively, bound in the open coordination site (Fig. 3.5)

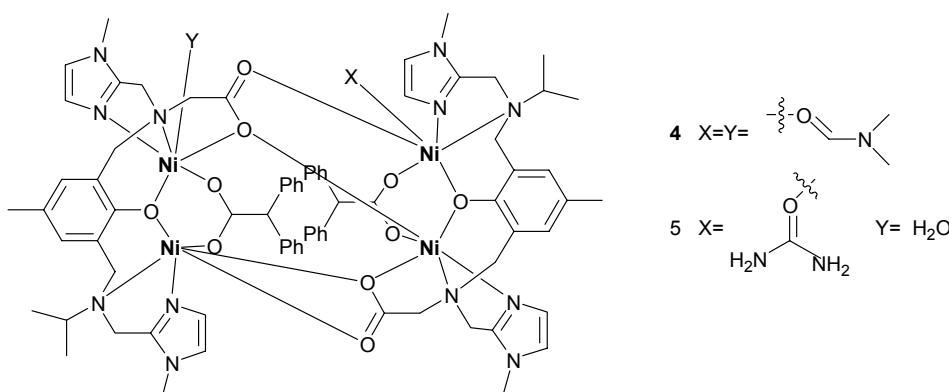


Fig. 3.5. Schematic representation of complex 4 and 5.

In the synthesis of the tetranuclear structures, 2 eq of nickel (II) perchlorate was dissolved in ethanol. The reason for the use of a different solvent compared to the BCIMP syntheses is that  $\text{Na}_3(\text{BCIMP})$  is not soluble in ethanol. Next, 1 eq of  $\text{H}_2\text{ICIMP}$ , 2 eq. of diphenyl acetate and sodium methoxide were dissolved and slowly added to the mixture. This gave a clear green-blue solution. Within 30 minutes, a thick greenish precipitate was formed, which was filtered, and dried. If compound **3** was dissolved in DMF and left under an atmosphere containing *t*-butyl-methyl-ether, beautiful blue crystals (**4**) appeared. If instead urea was added to the supernatant from the reaction and the solution was left for a while, then small blue crystals (**5**) appeared.

The structure of compound **4** has been elucidated and it was found to be an example of the "stickiness" of carboxylates. The overall molecular structure of compound **4** is  $[\text{Ni}_4(\text{ICIMP})_2(\text{Ph}_2\text{Ac})_2(\text{DMF})_2][\text{ClO}_4]_2 \cdot 2.5\text{DMF}$  (Fig. 3.6a). The open coordination sites on the Ni1 and Ni2 centers are in both cases occupied by DMF, which is a strongly coordinating solvent. The coordination is probably a major reason for the specific solubility in DMF. One of the two added equivalents of diphenyl acetate is incorporated just like previously seen in compound **2**. This leaves two open coordination sites per dinickel unit in addition to the one left vacant by the ICIMP ligand. These have been occupied by a terminal carboxylate from a neighboring complex, which replaces the water molecules in the native urease structure (*vide supra*). The nickel-nickel distances within the Ni2 units are 3.470(2) Å (Ni(1)-Ni(2)) and 3.513(4) Å (Ni(3)-Ni(4)). As predicted these are slightly longer than seen in the symmetric structure and more or less identical with the distance found in urease (3.5 Å).

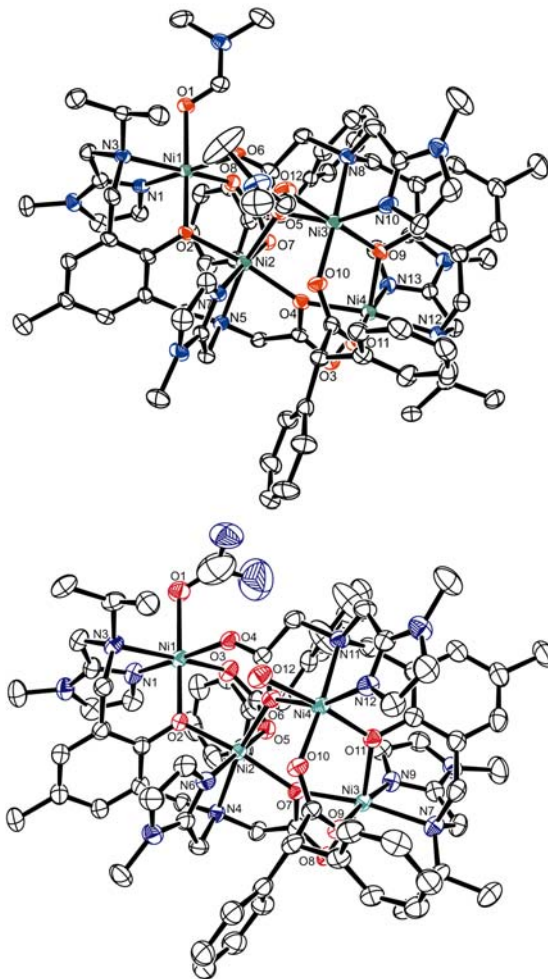


Fig. 3.6 (a) ORTEP representation of the crystallographic data for compound **4**. (b) ORTEP representation of the crystallographic data for compound **5**.

A nice artifact is that the two dinickel units in **4** are not symmetrically oriented. One of the neighboring ligands bridge the two nickel ions (Ni(1) and Ni(2)). This case resembles well the structure of urease, if the water analogy is used. In the other dimer, the carboxylate for the other dimer instead chelates one of the nickel atoms and in urease replaces one of the water molecules together with the open site. This leaves the open site at the other nickel ion instead. In other words we have a model with two distinct dinickel units. One ion has an open

coordination site on a nickel ion similar to Ni1 in urease, while the other ion has an open coordination site on Ni2 (on which the internal terminal carboxylate ligand can be found). The two orientations of the specific carboxylate represent a "carboxylate shift".<sup>48</sup>

It is also important, when the applicability of the tetranuclear complexes to urease is discussed, to mention that the closest nickel-nickel distance for nickel ions from different Ni2 units is 3.810(1) Å (Ni(2)-Ni(3)) and the two dimers are thus very much in contact. This complicated the evaluation of the magnetic data. Another feature that contributes to the packing is the phenyl ring interactions contributed by the diphenyl acetates. This gives connectivity between the tetramers.

The next goal was to study what would happen if urea were added to the Ni<sub>4</sub> complex. This was a somewhat complicated task since the complex did not dissolve in non-coordinating solvents. When coordinating solvents were used (such as DMF), no urea was incorporated. Fortunately, the spontaneous precipitation of complex **3** in the synthesis is not complete, as indicated by the yield. When urea was added to the supernatant after precipitation and the solution was left for about two to three weeks, small crystals of the urea-containing complex **5** formed. When the crystals were studied by X-ray crystallography, a complex similar to **4** was found. The overall formula was [Ni<sub>4</sub>(ICIMP)<sub>2</sub>(Ph<sub>2</sub>Ac)<sub>2</sub>(urea)(H<sub>2</sub>O)][ClO<sub>4</sub>]<sub>2</sub>·0.5EtOH·H<sub>2</sub>O and we can see that urea is coordinated to the Ni1 model site, while a water molecule is coordinated to the Ni2 site. Otherwise the structure is more or less identical to compound **4** (Fig. 3.6). The preferential coordination of urea to the model site that represents Ni1 in the urease enzyme is interesting. This might indicate that the first coordination of urea to the urease active site is indeed at Ni1. DFT calculations on the stability of the two Ni<sub>2</sub> units also show that the Ni1 model dimer of complex **4** is the more stable configuration.

If the same reaction as in the synthesis of the tetramers was performed with a fourfold excess of diphenyl acetate, the precipitate formed more slowly and had a more greenish color. The solubility of the product was also better. The mass spectrum indicates that the product is dinuclear with a vacant coordination site. If the compound is suspended in alcohol, in which it is normally insoluble, the solubility drastically increases when a unidentate ligand with nonpolar side chains, such as triphenyl phosphine oxide, is added. Unfortunately no structure of **3** is yet available.

### 3.2.3. Conclusions from the Structural Modeling of Urease

Five new nickel complexes have been synthesized. The "dinucleating ability" of the shorter tri- and tetradentate ligands does not seem to be sufficient for generation of stable dinuclear complexes. Bridging phenolate-based ligands had previously shown to be dependable and were successfully used in our case as well. The symmetric mixed-imidazole-carboxylate ligand BCIMP yields dinuclear complexes with nickel, which might have a potential as structural models for urease. In the model, the two histidine residues, coordinated to each of the metal centers in the urease active site, are modeled by one methyl-imidazole and one amine nitrogen. One of the acetates models the carbamylated lysine, while the other one replaces the water molecules. The Ni<sup>2+</sup> aspartate is modeled by one of the terminal carboxylates from the ligand, while the other carboxylate blocks the open coordination site on Ni<sup>1</sup>. The nickel-nickel distance is similar, although a bit shorter, in the model vs. urease. If diphenyl acetate is used as a co-ligand instead of acetate, the steric bulk prevents a second carboxylate from coordinating, making the number of bridging ligands more similar to urease.

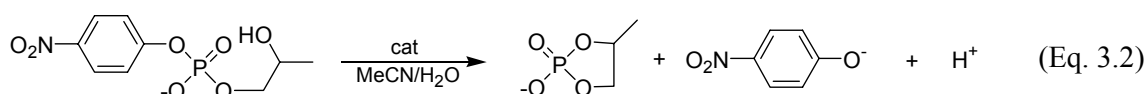
In ICIMP, the ligand carboxylate, blocking the open coordination site, is replaced by an inert isopropyl group. If an excess of the co-ligand is used in the synthesis, a dinuclear compound is formed. The mass spectrum indicates that two carboxylates are coordinated. If 2 eq of carboxylate is used, the carboxylate concentration appears to be too low and tetramers with only one carboxylate per dimer are formed. The Ni<sub>2</sub> units dimerize by coordination of the terminal carboxylate of a neighboring unit. This gives relatively stable tetrameric structures. Due to the asymmetry in the dimerization of the units, the structure can be used to predict the primary coordination structure of the catalytic cycle, which is indicated to involve Ni<sup>1</sup> and the carbonyl oxygen of urea.

### 3.3. Functional Models of Urease

To widen our understanding of the urease mechanism, a study of the functional properties of the model complexes was made. A study of the catalytic ability of two of the structural models above (complex **2** and **3**) was performed. If catalytic properties could be

demonstrated, then it would strengthen the claim that these complexes are suitable models. Possible differences in activity might be related to structural differences between the models.

To study the catalytic efficiency, an assay reaction is needed. Different assays have been used for urease activity.<sup>49,50</sup> Since it is complicated to monitor the urease reaction, it was decided to simplify the investigation by using a general assay for hydrolytic reactions, the transesterification of 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP) (Eq 3.2). The reaction is performed in 50:50 water:acetonitrile and the formation of *p*-nitrophenoxide is monitored



by UV/vis spectrophotometry at 400 nm. The substrate HPNP decomposes spontaneously and slowly and it is important to refrigerate the compound prior to use and take the autocatalytic activity into account when the rates are discussed.<sup>50</sup>

Mass spectra of the reaction solution of compound **3** indicate that the tetramer breaks up during catalysis. The spectrum was very similar to the dinuclear compound **6**. A closer look shows that the tetramer is intact until both substrate and water is added and then rapidly

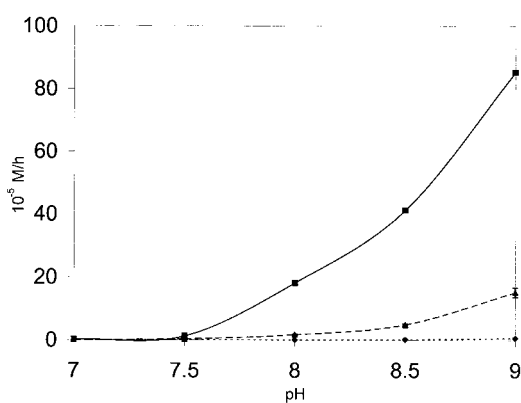


Fig. 3.7. Dependence on the initial rates of hydrolysis of HPNP at different pH for compound **2** (▲), compound **3** (■) and uncatalyzed reaction (●).

breaks down to the dimers. This is important since we now have an ideal model for urease at the point of catalysis. Compounds **2** and **3** were studied in the pH range 7 to 9. The results can be seen in Fig. 3.7. Both compounds are catalytically active and enhance the hydrolysis by approximately two orders of magnitude. Compound **3** is on average ten times more efficient than compound **2**. Tabulated results can be seen in table 3.1.

proceeds via coordination to the nickel ions modeling Ni1. However, other pathways, most likely involving the water sites, from which coordinated carboxylate has dissociated, are possible. In any case, the nature of the site is important.

### 3.4. Computational Models of Urease

In order to learn more about the urease mechanism, but also to deepen my understanding of theoretical calculations, computational modeling of the

hydrolytic reaction was undertaken. The computational program suite ADF<sup>51</sup>, a DFT code already in use in the group, was chosen. Previously, calculations at a relatively low level have been performed on the urease mechanism.<sup>52,53</sup> The work was, in a first step, focused on finding a suitable model of the resting structure. Next the initial coordination of urea to the nickel site was studied in detail. The likelihood that the bridging oxygen donor is involved in the attack of urea, which has been suggested, was also investigated (*cf.* section 3.1).<sup>19</sup>

#### 3.4.1. Finding a Suitable Model

The first goal was trying to find a suitably sized model system for the task. After attempting several larger systems, all based on the protein crystal structures 2UBP and 3UBP,<sup>19</sup> a comparison of three different size models was run. The result indicated that the calculations using a smaller model give very similar results to the more comprehensive systems. The system in Fig. 3.8 was finally chosen. The histidine residues were modeled as imidazoles, the aspartate residue was capped as a methyl group at the  $\beta$ -carbon while the carbamylated lysine

**Tab. 3.1.** Initial rates of hydrolysis of HPNP by some published catalysts<sup>25</sup> in comparison to complex **2** and **3** in MeCN/buffer (0.01 M CHES, pH 8.5) and [HPNP]=0.82 mM.

Catalyst	Initial rate ( $10^{-5}$ M/h)	Rate ratio vs. uncat.
2	0.49	27
3	4.1	230
[ZnFe(BPMOP)] <sup>4+</sup>	2.1	112
[ZnFe(BPMP)] <sup>4+</sup>	1.0	54
[Fe <sub>2</sub> (BPMOP)] <sup>4+</sup>	3.0	11
[Fe <sub>2</sub> (BPMP)] <sup>4+</sup>	0.059	3
Uncatalyzed (this work)	0.01783	1
Uncatalyzed (published)	0.01917	1



was terminated at the  $\epsilon$ -carbon. The rest of the metal-bound ligands were left unchanged. The crystal structure 2UBP, which is the native structure of urease from *Bacillus Pasteurii*, gave most of the information needed to construct the starting structure for the work. However, since hydrogen atoms normally do not appear in X-ray density maps, calculated starting values for the location of the protons were generated. The nature

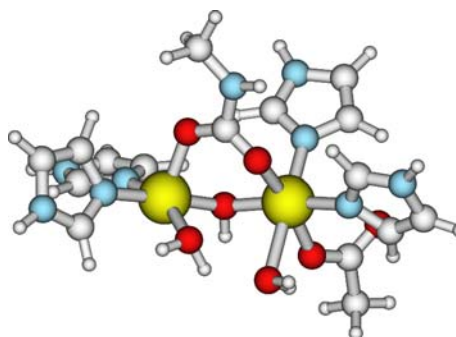


Fig. 3.8. Calculated geometry of the "resting structure" with chosen model.

of the bridging oxygen donor was therefore uncertain, but this was resolved by comparing the energies for the three different systems including  $\text{H}_2\text{O}$ ,  $\text{OH}^-$  or  $\text{O}^{2-}$ . The calculations showed that the hydroxide ion was more stable by at least 40 kJ/mol.

### 3.4.2. Urea Coordination

To generate the best starting structure for the optimization, urea was introduced to the model by a stepwise approach. Urea was placed about 5.5 Å away from the nickel atoms at a fixed distance. After a few iterations, the fixed distance was shortened and the system was reoptimized. This procedure was reiterated until a bond had been established. The qualitative result was that urea stayed in a relatively centered position during the approach but as it came closer to the active site, it migrated towards  $\text{Ni}_1$ , where it finally settled in a binding interaction with nickel. Urea was aligned by coordination of its oxygen atom to the *trans* position to the carbamylated lysine. Comparisons were made with other possibilities, but the appearing structures were always less stable than one referred to above.

### 3.4.3. The Source of the Attack

The rest of the theoretical investigation was focused on one of the proposed mechanisms, which was studied in more detail. The idea was to start with some different intermediates. Empirical activation energies for urease are published (approx 50 kJ/mol). If the calculated intermediates are found to possibly be a part of the urease mechanism, staying within the empirical energy, the intermediates can be refined to find out more exactly what the energy would be for the overall activation energy structure. The starting structures were prepared the

same way as in 3.4.2. A step-wise move of one of the urea nitrogen towards Ni<sup>2</sup> resulted in a weak bonding interaction. This Ni<sup>2</sup>-N coordination, as described in the literature, actually has a lower energy than the urea coordinated one. The next approach was more energy demanding as the bridging hydroxide was brought closer to the carbonyl carbon of urea. The final structure was found to be about 120 kJ/mol more energetic than the ground state. The structures following protonation of the non-coordinated nitrogen atoms and subsequent release of ammonia are also more than 50 kJ/mol higher in energy than the calculated "resting structure". This indicates that a reaction mechanism in which the bridging hydroxide acts as the nucleophile is not very likely.

### 3.5. Conclusions and Mechanistic Implications

In general the inorganic model studies and the theoretical calculations agree well. The first step in the mechanism is most likely the coordination of urea to the *trans* position of the carbamylated lysine on Ni<sup>1</sup>. Both complex **5** and the urea bound theoretical model indicate that this is the case. The nature of the subsequent reactions is still not clear. However, both the calculations and the functional model studies suggest that the bridging hydroxide does not act as a nucleophile in the hydrolysis. An attacking terminally bound hydroxide is less desirable in some ways, mostly because of the relatively low concentration it can be found at the pH at which urea is the most active. However, it does certainly look more promising at this point.

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## 4. Zinc Phosphotriesterase - A Dizinc Enzyme

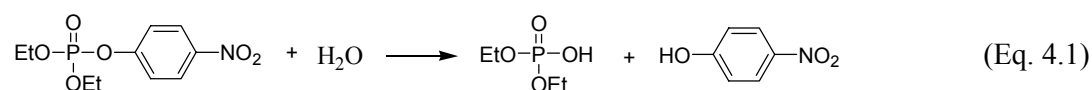
Zinc shares several properties with nickel (chapter 3) even though zinc, with its filled d shell, is a post-transition metal. As in the case of nickel, divalent zinc is the common oxidation state. In the case of zinc, it is also the only one found in biological systems. The divalent ions have similar ionic radii, 0.69 Å for nickel vs. 0.74 Å for zinc. Zinc is the second most abundant metal ion in biological systems (next to iron). It is involved in structural applications such as zinc fingers,<sup>1</sup> but it is also very common in enzymes.<sup>2,3</sup> Being redox inert, it still finds applications in catalysis. This is often because of its ability to act as a Lewis acid. Some well-known zinc enzymes are carbonic anhydrase, which assists in the hydration of carbon dioxide,<sup>4,5</sup> CuZn superoxide dismutase, which scavenges superoxide in the organism<sup>6,7</sup> and alcohol dehydrogenase, which removes alcohol from our blood<sup>8</sup>

Zinc is also involved in several metalloenzymes with polynuclear sites, such as  $\beta$ -lactamase<sup>9</sup>, aminopeptidases<sup>10</sup>, alkaline phosphatase<sup>11</sup> and zinc phosphotriesterase. Many zinc enzymes are hydrolytic, where the capacity of zinc to stabilize hydroxo groups at physiological pH due to its Lewis acidity makes it an excellent catalytically active metal.

## 4.1. Biochemical Background to Zinc Phosphotriesterase

This chapter is devoted to the enzyme zinc phosphotriesterase (ZPTE). The reason for this is mainly its structural similarities to urease, but also that the efficiency of transesterification catalysis by the nickel complexes (section 3) indicates that the ligands BCIMP and ICIMP can be useful for modeling of phosphoesterases.

In the middle of the 70's, an interesting discovery was made. Two different strains of soil bacteria (*Pseudomonas diminuta* and *Flavobacterium sphaeroides*.) were shown to hydrolyze organophosphates.<sup>12</sup> This brought some attention since many potent poisons such as the war agents sarin, VX and several insecticides are organophosphates. The discovery showed potential for methods of detoxification. Since organic phosphotriesters are fairly uncommon in nature, it is still not completely clear if there is a natural substrate for the enzyme.<sup>13</sup> Organophosphates were not widely used as insecticides until after World War II. It is unlikely that the ability to break down the poison instead of being destroyed, as other organisms are, develops during such a short time span. Until a natural substrate is found, the insecticide paraoxon (Eq. 4.1) is often used as an example of a wide range of phosphotriesters identified as substrates.



As mentioned above, the structure of the active site of ZPTE shows many similarities to the active site of urease. A number of crystal structures of the enzyme have been published.<sup>14,15</sup> They also include some metal exchanged complexes. Among others, a Cd<sub>2</sub> substituted ZPTE has been used for a number of spectroscopic studies.<sup>16</sup> As in urease, the two metal atoms, zinc in the case of ZPTE, are bridged by a carbamylated lysine residue (Fig. 4.1). Urease and ZPTE are the only two examples of this feature found up until now. A hydroxo group also bridges the zinc ions. Each ion has two histidine residues in its coordination sphere. In addition, one of them is coordinated by a monodentate aspartate. In the crystal structure, the other zinc ion has a labile oxygen donor coordinated, which completes the pentacoordinate geometry for both centers. The metal-metal distance was found to be a little bit shorter than in urease, but still comparable (3.4 Å in ZPTE vs. 3.5 Å in urease).

When it comes to the mechanistic considerations, three main proposals have been made.<sup>17</sup> The first step is similar in all cases, *viz.* the coordination of the free phosphate oxygen to the coordinatively unsaturated Zn ion (Zn1), replacing the labile donor. It demonstrates the need for a vacant/labile coordination site, but not necessarily a symmetric coordination sphere. In the next

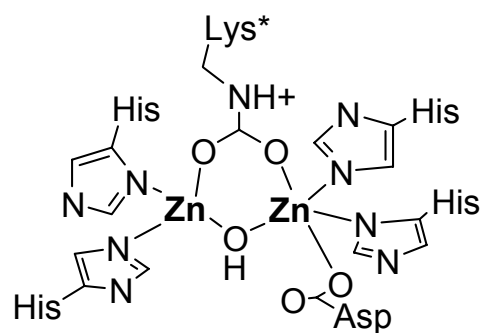


Fig. 4.1. Schematic presentation of the dinuclear site of zinc phosphotriesterase.<sup>14,15</sup>

step either a terminal hydroxo group on Zn2, or a water molecule activated by the same metal, attacks the phosphate phosphorus and releases the product alcohol. This leads to a bridging or terminal phosphate group, which is subsequently released. A similar mechanism involving the bridging hydroxo group as nucleophile has been discussed.

Dinuclear complexes with alkoxide or phenoxide bridges have previously been used in the modeling of active sites.<sup>18-21</sup> No dinuclear zinc complex, in which imidazole and carboxylate ligands together constitute a suitable ligand environment, as a model for the histidine and aspartate-ligated active site of ZPTE, seem to have been described before the current work.

## 4.2. Structural Models of Zinc Phosphotriesterase

Because of the structural similarities between ZPTE and urease (Chapter 3), the mixed carboxylate-imidazole ligands BCIMP and ICIMP (Fig. 2.2) were again found suitable as ligands for structural modeling. The structural feature of the open coordination site is better modeled by the ICIMP ligand. However, the BCIMP models are still interesting and useful for comparison.

### 4.2.1. Zinc Complexes with the Symmetric Ligand BCIMP

Two different complexes were made from zinc and BCIMP, one with acetate as co-ligand,  $\text{Na}[\text{Zn}_2(\text{BCIMP})\text{Ac}_2]$  (**7**), and one with diphenyl acetate,  $[\text{Zn}_2(\text{BCIMP})(\text{Ph}_2\text{Ac})]$  (**8**). The general synthetic procedure involved dissolving 2 eq. of zinc perchlorate in methanol.

Separately, a methanolic solution containing 1 eq. of Na<sub>3</sub>BCIMP and 2 eqs. of the appropriate carboxylate co-ligand was prepared and added to the zinc solution, yielding a clear, almost colorless solution. After stirring, diethyl ether was used to precipitate a white solid in good yields. Unfortunately, the Zn(BCIMP) complexes did not yield good enough crystals to allow X-ray analysis. However, because of the similarities in ionic radii and chemistries of nickel and zinc, it is reasonable to assume that the two complexes **1** and **7** have similar structures. This means two hexacoordinate zinc centers with two bridging acetate ligands. Pentacoordination is however not impossible.

A nice feature in the zinc case is that the diamagnetic metals permit the use of NMR as a spectroscopic tool. The NMR spectra of the complexes are similar to the uncoordinated ligand, but slightly perturbed. The signals originating from the protons closest to the metal centers are more shifted than the protons further away. Integration shows that there is only one diphenyl acetate in complex **8**, which was assumed already in the case of complex **2** in chapter 3. The reason for this is probably the steric hindrance of the bulky co-ligand (*i.e.* diphenyl acetate) already coordinated. A study of the IR spectra indicated that there are two different carboxylates in the complexes, one bridging and one terminally bound. This would be consistent with the structure of complex **1**.

#### 4.2.2. Zinc Complexes with the Asymmetric Ligand ICIMP

In order to achieve the synthesis of water soluble complexes, three different Zn(ICIMP) compounds were prepared. A sharp difference in solubility between the acetate complex [Zn<sub>2</sub>(ICIMP)Ac<sub>2</sub>] (**9**) vs. the pivalate, [Zn<sub>4</sub>(ICIMP)<sub>2</sub>(Me<sub>3</sub>Ac)<sub>2</sub>][ClO<sub>4</sub>]<sub>2</sub> (**10**), and the diphenyl acetate, [Zn<sub>4</sub>(ICIMP)<sub>2</sub>(Ph<sub>2</sub>Ac)<sub>2</sub>][ClO<sub>4</sub>]<sub>2</sub> (**11**), respectively, was noted. Complex **9** could be synthesized in water without any precipitation, while complex **10** and **11** precipitated as soon as the co-ligand was added, if synthesized in aqueous solution.



Complex **10** and **11** were prepared by adding a ligand solution containing 1 eq. of  $\text{H}_2\text{ICIMP}$ , 2 eq. of co-ligand in ethanol to a solution of zinc perchlorate. In both cases, precipitation occurred within an hour. The precipitate could be filtered, dried and analyzed. NMR studies again showed perturbed ligand features. Integration indicated that one co-ligand was coordinated for each ligand unit. Also the pivalate is large enough to prevent a second co-ligand to enter. The vibrational spectra show two different carboxylates in the structure. At this point the stretch for the two signals are rather low. This would indicate that the terminal carboxylate in the ligand has a partly bridging nature. An explanation for this is revealed in the mass spectrum of complex **10** and the X-ray structure of complex **11**, which show that the complexes are tetranuclear. In analogy with the nickel complexes **3-5**, the single coordinated co-ligand and the open coordination site generated by ICIMP creates a suitable situation for dimerization of the two  $\text{Zn}_2$  units. The "terminal" carboxylate ligand of ICIMP assumes a bridging position between the  $\text{Zn}_2$  units, which would explain the IR data.

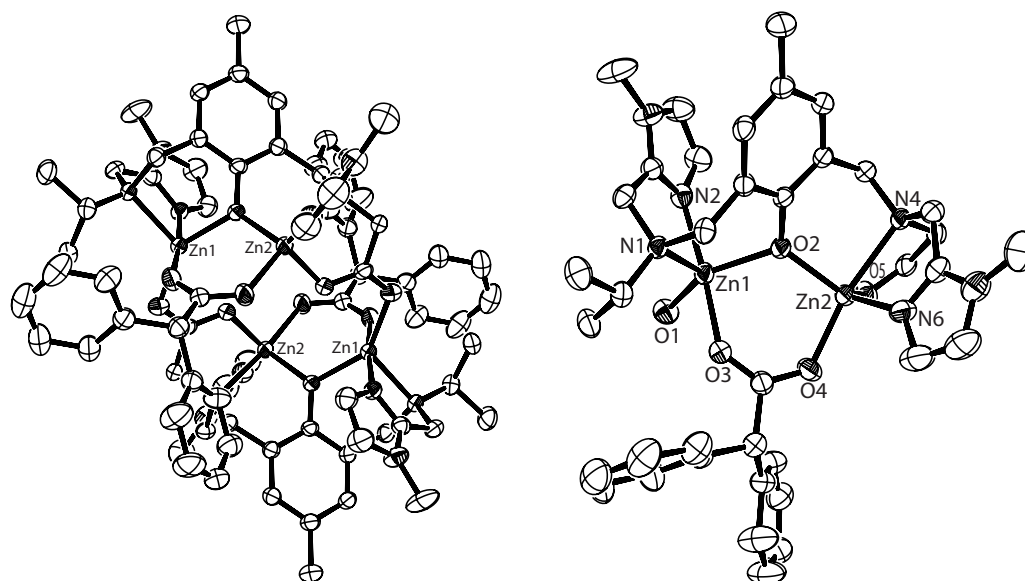


Fig. 4.2. ORTEP representations of the molecular structure of the complete compound **11** and the asymmetric identity respectively.

The X-ray structure of complex **11** (Fig. 4.2) reveals a structure similar to the X-ray structure of **3** but with some striking differences: First of all, no solvent is present in the crystal structure of **11**. In addition, the neighboring carboxylate ligand is only monodentately coordinated to each dizinc unit. This leaves both zinc ions pentacoordinate in distorted trigonal bipyramidal geometries. The distortion seems to be due to the limited distance from

each of the pendant arms to the bridge point amine nitrogen. Even if the distances were a bit short, one additional spacer methylene has shown to yield structures with worse distortions associated with a long spacer.<sup>22</sup> The second main difference is that the dimerization of the dizinc units is symmetrical due to the monodentate coordination of the neighboring ligand, so that the Zn<sub>2</sub> units are crystallographically equivalent. The solution structures of complex **10** and **11** are not necessarily tetrameric and the tetramer is believed to dissociate easily. In solution no tetrameric structure could be detected in the mass spectrum of complex **11** and in the case of complex **10**, the tetrameric peaks are very small compared to the dimeric ones.

### 4.2.3. Conclusions from the Structural Modeling of Zinc Phosphotriesterase

The results of the structural modeling of ZPTE give us some new insights to the modeling chemistry of BCIMP and ICIMP and the active sites they depict. As in the nickel case (chapter 3), the more sterically demanding carboxylates, diphenyl acetate and pivalate, prevent the association of a second carboxylate. This yields a better model for the active site since there is only one bridging carboxylate-like unit at the enzyme site and that is the carbamylated lysine. In the case of BCIMP, the open coordination sites generated in these mono-carboxylate complexes seem to destabilize the crystal packing and make the growing of good crystals more difficult.

In the case of ICIMP, where an additional weakly coordinated site is enforced by the ligand, the case becomes even more intricate. When three open sites become available, the driving force to coordinate other ligands becomes sufficiently high to initiate dimerization of the Zn<sub>2</sub> units. This generates rather unstable tetrameric structures, which appear to be easily separated in solution. The tetramerization also seems to facilitate crystallization. This is probably due to the fact that the open coordination sites are blocked and the risk of exchanging ligands, inhibiting the crystal growth, is reduced. The crystal structures are in turn very useful in the evaluation and application of the model.

Each Zn<sub>2</sub> unit in the tetrameric complex **11** is a very suitable structural model for the active site of ZPTE. The interatomic distances in the structure are similar to the distances in the

active site. The active site has two pentacoordinate zinc centers and the same is reflected in the model.<sup>14</sup> The additional aqua ligands, which are present in the urease active site, are not seen in ZPTE. The equivalent is reflected in the tetranuclear Zn model through the unidentate coordination of the neighboring carboxylate to each dimer. In the Ni complex **4**, the corresponding carboxylate coordinates in both bridging and chelating modes at two different sites.<sup>23</sup>

### 4.3. Functional Models of Zinc Phosphotriesterase

When Zn complexes of both BCIMP and ICIMP had been prepared, the focus was changed to the catalytic activity of the compounds. Comparisons between the complexes showing the effect of the open coordination site, but also the effect of other metals and ligands, would be interesting. In the zinc case two different functional assays were used. One was the transesterification of (HPNP) (Eq 3.2) and the other the hydrolysis of double-stranded DNA.

#### 4.3.1. Hydrolysis of 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP)

Even though the forte of ZPTE lies in the hydrolysis of phosphotriesters, hydrolysis of HPNP became the assay of choice. The reasons for this are several: The phosphotriesters are normally extremely toxic. They inhibit the enzyme acetylcholinesterase, which is critical in nerve function, and failure of the enzyme is lethal to a wide range of cell types.<sup>13</sup> The HPNP assay was also readily available in the lab, it is potentially interesting as an RNA hydrolysis model and interesting comparisons are available both from

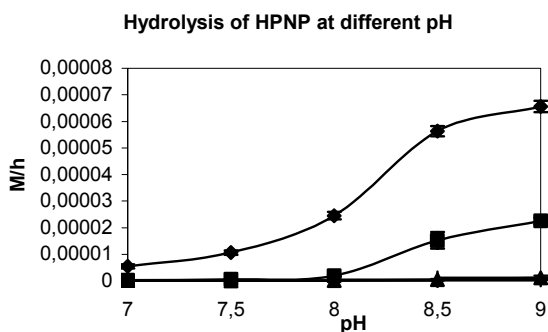


Fig. 4.3. Dependence on the initial rates of hydrolysis of HPNP at different pH for Zn<sub>4</sub>(ICIMP)(♦) and Ni<sub>4</sub>(ICIMP)(■) catalyzed and uncatalyzed reaction(▲).

the nickel work and previous publications.<sup>24</sup> The HPNP hydrolysis is also an example of a rather facile hydrolysis, which can be contrasted with the hydrolysis of DNA (chapter 4.3.2).

The following samples were studied: complex **8** and **11** from the structural modeling, zinc acetate hexahydrate and one sample batch was run uncatalyzed as a control experiment. In all cases except for zinc acetate, the pH was scanned between 7 and 9. The result is shown in Fig 4.3. An accelerated trend is seen when the pH is increased in all cases. This would suggest that the availability of hydroxide ion is a vital part of the mechanism. In analogy with the nickel data, the ICIMP complex (**11**) comes in ahead of the BCIMP complex (**8**). The open coordination is important for the reaction, however not essential. An interesting feature is that free zinc acetate catalyzes with about the same rate as complex **8**. This may suggest that the BCIMP activity originates from mononuclear catalysis, which certainly would be a possibility in complex **8**, where one open site is available on each zinc ion due to the fact that only one co-ligand is coordinated. Finally, it is also noteworthy that there is an activity even if the bridging hydroxide is incapacitated and can not act as an attacking group. This might suggest that the attacking group is a terminal hydroxide or activated by such a group.

### 4.3.2. Reactivity with DNA

In addition, a study of how our zinc complexes is involved in DNA hydrolysis was performed. The experiment was performed using complex **9**, since it closely resemble the dinuclear active site and the acetate complex was the only complex to show to be water-soluble. To ensure that this complex was hydrolytically active, the complex was sampled in the HPNP reaction and the activity was found comparable to the tetranuclear complexes.

Plasmids are small circular DNA pieces, which are useful in several biochemical techniques. Just like regular DNA, the stable structure of a plasmid is often supercoil. This helps packing DNA in the nucleus. When the supercoiled plasmid is nicked, it allows the structure to relax and become circular. The supercoiled and circular forms can easily be separated and quantified using DNA electrophoresis, which makes the use of plasmids a suitable tool in studying the amount of DNA hydrolyzed in a reaction.

The hydrolytic activity of complex **3** was compared to an uncatalyzed system and also to a sample where a Fenton reagent was used to show a high hydrolytic activity comparison. Free

zinc was also used to give another frame of reference. The result of the experiment showed that the activity of **3** on DNA hydrolysis to be very low, comparable or even lower than free zinc. The reaction was repeated several times with the same result. It was concluded that the model complex is inactive in DNA hydrolysis.

When considering the reason for the low activity, some possible reasons come to mind. The first one comes from the structural comparison between HPNP and DNA. In the case of HPNP, the intramolecular reactive nucleophile is close proximity. This makes HPNP a good analog for RNA. In DNA hydrolysis, the nucleophile must come from water. This is one of the reasons to the useful feature of RNA being much less stable than DNA. Our catalyst can be dependent on the proximity of the nucleophile, which would indicate it to be a less useful model for triphosphoesterase, which show no dependency on an intramolecular nucleophile. Another reason can be that DNA might be too bulky to be hydrolyzed by **3**. This would partly explain why free zinc ion shows a low, but slight effect, which is not noticed in HPNP hydrolysis. A way to test this would be to study the hydrolysis of paraoxon, which I will try when it can be performed in a safe way.

## 4.4. Conclusions and Mechanistic Implications

Some conclusions can be drawn from the studies. Similarly to the nickel system, it is again apparent that the open coordination site on one of the zinc sites is close to essential for its hydrolytic function. This would indicate a direct coordination of the substrate to the coordinatively unsaturated zinc ion. Since free zinc has a low activity compared to the models, at least in the hydrolysis of HPNP, the second zinc ion seems to be important for the activity. Again it is important to also point out that the bridging hydroxo group is unavailable for hydrolytic attack, since it is modeled using a phenolate group. All this would suggest that the mechanism is similar to the one suggested for urease. The phosphate coordinates to the coordinatively unsaturated zinc ion. Next a terminal hydroxide on the other zinc ion attacks the phosphorous, which leads to products. A reason to the increased activity of the zinc complexes *vs.* the nickel would be the Lewis acidity of zinc, which would better stabilize the terminal hydroxide.

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# 5. Dinuclear Iron Enzymes

Iron has been known as an element for a very long time. Already Tubal-Cain, seven generations removed from Adam in the Bible was "an instructor of every craftsman in bronze and iron".<sup>1</sup> Iron is the most abundant transition metal in nature and a very frequent building block in living organisms. An adult human contains about 5 g of iron. The two most common oxidation states of iron are Fe(II) and Fe(III), but higher oxidation states have been shown to be important in several enzyme mechanisms. The versatile metal ion is involved in electron transfer (Fe/S clusters), oxygen transport (hemoglobin), oxidation (P450) and hydrolysis (dinuclear iron enzymes). Many iron enzymes are heme-based and iron proteins are therefore normally divided into two general groups: heme and non-heme sites. The dinuclear iron enzymes, which will be discussed here, fall naturally in the latter category.

## 5.1. Iron-Containing Dinuclear Enzymes

Several well-studied iron-containing enzymes contain dinuclear active sites. Even if they are diverse in their reactivity, they are still sufficiently similar in structure to be considered as a general group of enzymes. In this work, three iron enzymes will be in focus, *viz.* soluble methane monooxygenase (sMMO), ribonucleotide reductase (RNR) and purple acid phosphatases (PAP). In addition,  $\Delta^9$  desaturase,<sup>2</sup> involved in the introduction of double bonds in stearoyl-CoA, and hemerythrin,<sup>3</sup> which is an important oxygen transport protein, are also dinuclear proteins.

### 5.1.1. Methane Monooxygenase (sMMO)

The enzyme sMMO is one of the key enzymes for the methanotrophs. Methane is used by these bacteria as their only source of carbon.<sup>4</sup> Methane is taken up by the organism and oxidized by sMMO to methanol, using dioxygen as the oxidant. One oxygen atom is incorporated into the substrate and the other oxygen atom oxidizes NADH to produce NAD<sup>+</sup> and water (Eq 5.1).



Methanol can then be further oxidized to formaldehyde and eventually be transformed into energy and biomass. The oxidation of methanol, which is difficult due to the strength of the C-H bonds, is performed at atmospheric pressure and room temperature in the enzymatic process. This fact and the interest in trying to understand how methane, which is normally hard to transport for energy purposes, can be converted into methanol and thus be easier to handle, has made sMMO research a relatively hot topic.

The crystal structure of sMMO has been known for the last 10 years.<sup>5</sup> The two iron ions are surrounded by four glutamate ligands, two histidines and two hydroxide bridges. One glutamate and the hydroxide ions bridge the metals. One iron has an additional two terminal glutamates and one histidine in its coordination sphere, while the other has one glutamate, one histidine and one water molecule. A carboxylate shift<sup>6</sup> has been suggested as a part of the mechanism, as one of the glutamate residues swings over to become bridging in a structure recorded on a reduced form.

### 5.1.2. Ribonucleotide Reductase (RNR)

Another enzyme with a similar structure is ribonucleotide reductase.<sup>7</sup> The iron-containing analog is responsible for the oxidation of ribonucleotides to deoxynucleotides, which allow DNA to be synthesized *de novo*. It was the first enzyme to be found to contain a stable free organic radical, which also has been suggested to be important for the mechanism.<sup>8</sup>



The active site contains two iron ions. A chelating aspartate, a histidine and a water molecule coordinates one of the irons. The other ligates two terminal glutamates, one histidine and one water. Together one glutamate and one oxygen donor bridge the ions. In proximity to the site a tyrosine residue is present, which is also the site for the radical

### 5.1.3. Purple Acid Phosphatases (PAP)

Maybe the most relevant dinuclear enzymes for this work are the purple acid phosphatases, since one of the achievements has been the development of a site-specific route to heteronuclear FeM model complexes. At present, the PAP enzymes are the only ones to known to contain heterodinuclear iron sites,<sup>9</sup> both iron-zinc and iron-manganese have been found. The purple acid phosphatases catalyze hydrolysis of phosphoesters at acidic pH,<sup>10</sup> and a number of them have been crystallographically characterized.<sup>11-14</sup> The structures of the active sites of the purple acid phosphatases isolated from plants are closely related and contain one iron and one zinc ion about 3.0-3.4 Å apart. The metals ligate histidines, tyrosines and carboxylate-containing amino acid residues and are bridged by one of the carboxylate oxygens of an aspartate and a water molecule or a hydroxide ion (Fig. 5.1).

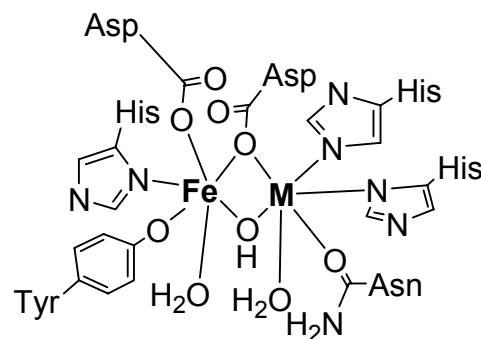


Fig. 5.1. Schematic representation of the active site in purple acid phosphatases.

## 5.2. Stepwise Synthesis of Dinuclear Model Complexes

The iron complexes were by far the most difficult to study. Their stability and the synthetic conditions required were somewhat different from the zinc and the nickel complexes. After many attempts at preparing dinuclear complexes with Fe(III) or Fe(II), the discovery of a stable mononuclear compound was made. This was at first discouraging, but was soon found to be quite useful.

### 5.2.1. A Mononuclear Iron Complex of ICIMP

One stable compound emerged in the syntheses using iron(III) precursors. If 1-2 equivalents of iron(III) perchlorate in methanol is added to a methanolic solution of ICIMP, a deep purple solution is generated. If no free carboxylates were added and the solution was left to evaporate slowly, crystals of a mononuclear iron complex could be grown and analyzed. Studies by mass spectrometry showed high purity. Analysis by X-ray crystallography showed an interesting complex with the overall formula  $[\text{Fe}(\text{H}_2\text{ICIMP})(\text{H}_2\text{O})\text{Cl}][\text{ClO}_4]_4$  (**12**). Two mononuclear complexes build up the structure. The iron(III) ion coordinates phenolate, one amine, one imidazole and the terminal carboxylate. To complete the octahedral coordination, one solvent molecule (water or methanol) and one chloride ligand are present (Fig. 5.2a). Iron coordinates to the site with the most donor atoms, most likely due to the chelate effect. Even if no dinuclear system was obtained, the fact that a mononuclear compound still favors coordination in one of the original sites in a dinucleating ligand would suggest that the ligand directs the entering metal ion in a predictable way, thus avoiding the formation of byproducts.

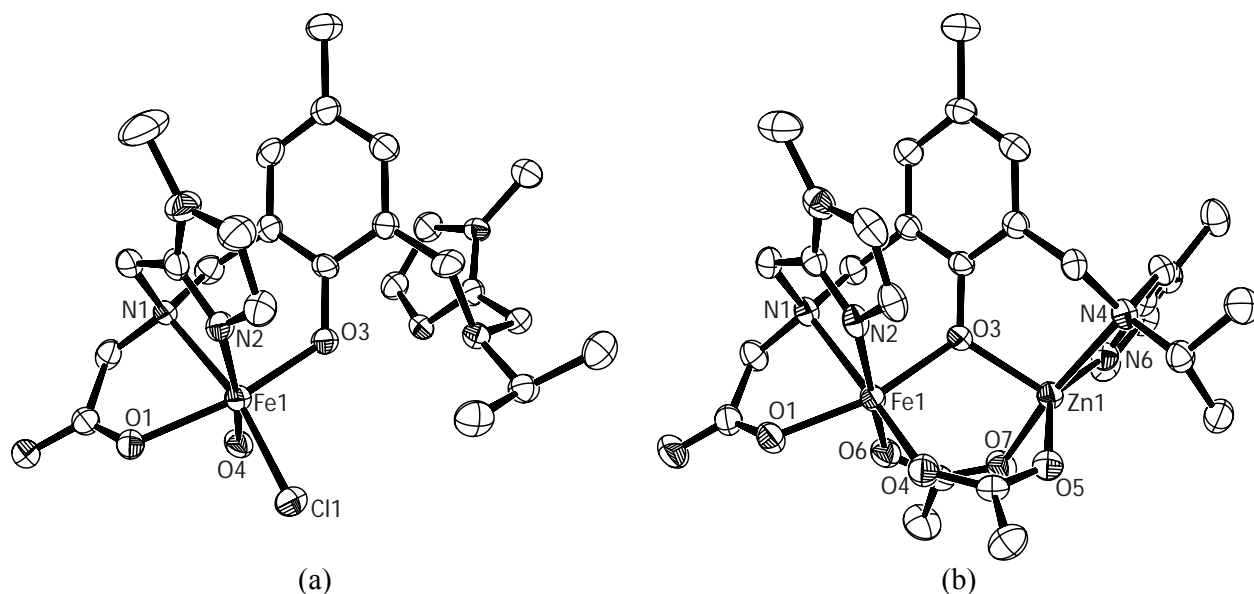


Fig. 5.2. (a) ORTEP representation of one half of complex 1. (b) ORTEP representation of compound 2.

**Tab. 5.1.** Relevant bond distances for compound **1** and comparisons to similar complexes.

<i>Distance</i>	<i>Compound 1</i>	<i>[FeL]<sub>2</sub></i>	<i>[FeZn(BPBPMP)Ac<sub>2</sub>]<sup>+</sup></i>	<i>[FeZn(BPMP)Ac<sub>2</sub>]<sup>2+</sup></i>
Fe-N(amine)	2.226(4)	2.327(12)	2.214(3)	2.193(4)
Fe-N(imidazole)	2.080(4)	2.112(13)	2.182(3)	2.118(3), 2.146(3)
Fe-O(phenolate)	1.929(3)	1.907(10)	2.006(3)	1.982(3)
Fe-O (term. carbox)	2.070(3)	-	-	-

The ligand donor atom-to-metal distances were compared to distances in similar dinuclear complexes (Tab. 5.1). This comparison showed to be useful in understanding more about the nature of the ligand. The terminal ligands (phenolate and imidazole) had a closer binding distance, while the central amine had a longer one. Earlier observations have shown that the amine metal bonds always are rather long in these phenolate bridged polydentate complexes, probably due to strain in the ligand. The distances in this complex support this prediction. Due to the missing effect of the second ion, the complex is less strained and the terminal ligands can bind closer to the metal center. However, this coordination reveals the strain in the ligand and the metal-amine distance needs to increase in order to relieve the strain. One way of doing so would be to synthesize ligands with longer “side arms”, but attempts to do so have not been successful.

The origin of the chloride ion was studied and first after a chlorine analysis of the ICIMP precursors, it was settled that it must originate from a non-stoichiometric amount of chlorine present in the ligand preparation. The strong color of the complex was attributed to phenolate to iron charge transfer, which can also be seen in purple acid phosphatases, which partly accounts for the name.

### 5.2.2. Stepwise Synthesis of an FeZn Complex

The structure of the mononuclear complex **12** suggested that it might be used as a “synthon” for making dinuclear complexes, especially heteronuclear iron species. One similar isolated mononuclear complex has been reported in literature.<sup>15</sup> It was not presented as a possible building block for further synthesis and its structure did not resemble that of a dinuclear complex. The synthetic efforts were focused on FeZn complexes because of their applications to plant purple acid phosphatases. Complex **12** was dissolved in a methanol/acetonitrile solution and methanol solutions of zinc(II) acetate (1 equiv.) and sodium acetate were added

to the solution. This yielded a red solution, from which red-brown crystals of  $[\text{FeZn}(\text{ICIMP})(\text{Ac})_2][\text{ClO}_4]\cdot 2\text{CH}_3\text{OH}$  (**13**) could be grown (Fig. 5.2b).

The crystal structure of **13** showed a very similar structure to complex **12**. The iron(III) ion is still located in the ligand carboxylate site and the zinc has been introduced into the previously open neighboring site. The metal-to-donor atom distances around iron also remain rather similar. This indicates that the zinc ion does not affect the structure very much. This is in contrast to what has been observed in other complexes. The reason for this is most likely that ICIMP is only tridentate towards the zinc, while the comparison above refers to complexes with tetradentate zinc sites. The internuclear distance is very short, 3.339(1) Å, which is just a little bit longer than the FeZn distance in kidney bean purple acid phosphatase (kbPAP).<sup>16</sup> This can be compared to other published structures of FeZn models in the range of 3.44-3.81 Å.<sup>17-20</sup> The distance is normally due to the bridging ligands in the structure. In kbPAP two single-atom bridges are present, one hydroxide and one  $\mu$ - $\kappa^2$  terminal aspartate. In the models, the ligand phenolate and the two carboxylates bridge, which pushes the metal ions apart. The span in distance depends on the nature of the carboxylate, and acetate is a carboxylate with a relatively narrow bite angle.

The strong purple color is bleached when zinc is added. Assuming that the absorbance depends on Fe-O(phenolate) transitions, they are naturally affected by the shift from  $\kappa^1$  to  $\mu$ - $\kappa^2$  coordination modes. The color persists in the enzyme, since it has a terminally bound phenolate, not in close proximity to zinc.

### 5.2.3. Other Fe(III)M(II) Complexes

To further examine the properties of complex **12**, its reactivity towards other possible metal ions was studied. The general synthesis remained the same as for complex **13**. With the addition of Fe(II) perchlorate, a Fe(III)Fe(II) complex (**14**) can be detected using mass spectrometry. The ligand seems to be much more convenient with the mixed oxidation state site. Heterodinuclear complexes with copper(II) (**15**) and cobalt(II) (**16**) were also generated. At this point, attempts to crystallize these complexes for structural studies are in progress. Preparative work aiming at the synthesis of heteronuclear complexes with other metals (nickel and manganese) is also under way.

## 5.3. Reactivity Studies

Unfortunately, the iron complexes have not been found to be predictable as functional models so far. Initial studies with complex **13** were promising. Some problems with precipitation were encountered, but after a fair amount of experimental work it was found that these could be avoided by running the experiments anaerobically. Attempts to verify the speciation by electron spray mass spectrometry showed clearly that the iron-zinc complex was stable in acetonitrile. However, as soon as water was added, the complex decomposed and was replaced by the dizinc complex, similar to complex **9** (*cf.* chapter 4) and the hydrolytic activity could be attributed to the dizinc complex.

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# 6. Concluding Remarks

Several enzymes with a wide range of metals and activities have been discussed in this thesis. The common theme has been the structures of the active sites. The architecture of two metal ions in close proximity has been useful in many systems. Here are some summarizing remarks.

## 6.1. Classifying Mechanisms in Enzymes with Dinuclear Active Sites

When researching dinuclear enzymes and its literature the question "Why two ions?" often comes up.<sup>1,2</sup> The answer varies drastically from enzyme to enzyme. The field would benefit from a classification focusing on this issue. For instance, dinuclear enzyme mechanisms might be classified into four groups depending on "the division of labor" between the ions. The different classes chosen are referred to as partners, collaborators, facilitators and spectators. Hopefully such classification might be useful in future discussions about dinuclear enzyme mechanisms.

The partner group embraces mechanisms where the two ions contribute to the activity on an equal basis. This group includes enzymes with active sites in which substrates are bound and manipulated as a bridging intermediate. Examples include the mechanisms suggested for RNR and MMO.<sup>3,4</sup> Both of these involve a bridging oxygen, which is stepwise converted in preparation for the organic substrate. One of the mechanisms proposed for urease, in which one of the urea nitrogen atoms is coordinated to Ni<sup>2+</sup> and the bridging hydroxy group is attacking, would certainly also qualify for this group.<sup>2</sup>

### 6.1.2. Collaborators

This group of mechanisms includes pathways, in which the two metal centers have different mechanistic roles. For example, one metal interacts with one substrate, while the other one interacts with another. The urease mechanism, in which urea first is coordinated to Ni<sup>1</sup> and then sequentially attacked by hydroxide on Ni<sup>2</sup>, fits well into this family.<sup>5</sup> The division of tasks between the two metal centers is clear.

### 6.1.3. Facilitators

This group involves mechanisms, in which one of the active site metal centers carries out the tasks of binding substrate and performing reaction by itself. The second ion is still essential for the mechanism, e.g. through the removal or supply of electrons or because of its structural influence. As an example of this group, the mechanisms suggested for the dimanganese enzyme catalase can be mentioned.<sup>3</sup> The peroxide substrate is only bound to one of the manganese ions, which also contributes to the oxidation reactivity. The other ion is responsible for redox activity and displays a structural role.

### 6.1.4. Spectators

The final group contains mechanisms in which the activity is independent of one of the two metal ions. This spectator ion is not involved in any part of the mechanism and can basically



be removed from the site without losing activity. An example of this group would be metallo- $\beta$ -lactamases, dinuclear Zn enzymes, which also exist in active mononuclear forms.<sup>1</sup>

## 6.2. Scientific Summary of the Thesis

At this point, it is appropriate to make a brief summary of some of the main results from the work described in this thesis. This work began with organic synthesis. As the need for more and more complex ligand environments increases in bioinorganic chemistry, the more organic synthesis gains importance. The line of ligands which showed to be most useful in this work was ligands with mixed carboxylate/imidazole functionalities, especially BCIMP and ICIMP (Chap. 2). A ten-step synthesis of ICIMP was developed as a part of the project. The lack of previous asymmetric ligands featuring terminal carboxylate functionalities made ICIMP especially interesting. Most of the dinuclear enzyme active sites discussed in the thesis rely on at least one carboxylate ligand originating from the protein.

The first enzyme, to which the newly synthesized ligands were applied, was urease (Chap. 3). Early in the project a symmetric model complex was prepared (**1**). Although this ligand was an important step in the modeling of urease, the synthesis of complexes based on the asymmetric ICIMP ligand added important features to the models. Four different Ni complexes based on ICIMP were synthesized (**3-6**). One of them was dinuclear and clearly the best model for the active site of the enzyme protein. The other models were tetranuclear. Analysis of the crystal structures indicated that the urea prefers metal sites resembling Ni1 for its initial coordination. From kinetic studies comparing the asymmetric complexes with the symmetric ones it became apparent that an open coordination site is important in facilitating hydrolytic catalytic activity.

An alternative model study of the urease mechanism was performed using theoretical calculations (Chap. 3.4). Based on the native crystal structure, a series of possible intermediates were optimized. The conclusions of the calculations were that the bridging oxygen donor in the resting structure is a hydroxide, that the first coordination of urea most likely occurs on Ni1 *trans* to the carbamylated lysine ligand and that the published proposal based on a "partner mechanism" (*vide supra*) involving the attack of the bridging hydroxide is

less likely than the proposed "collaborator mechanism" due to the high energy barrier for the formation of the tetrahedral intermediate.

When ICIMP is reacted with zinc, a tetranuclear compound (**11**) is formed (chap. 4). It can easily be dissociated into dinuclear complexes. The structure of the complex resembles the tetranuclear nickel model, but is more symmetrically organized in terms of the interaction of the two dimers. The complex has also been found to be very potent as a functional model for hydrolytic enzymes. Also, in this case, the open coordination site shows to be important, making the reaction significantly faster with the ICIMP ligand compared to BCIMP, lacking the open coordination site.

Finally, homo- and heteronuclear iron complexes were synthesized (Chap. 5). As a useful precursor, a mononuclear complex (**12**) could be isolated and crystallographically characterized. Based on complex **12**, four new complexes with the general structure  $[\text{Fe(III)M(II)(ICIMP)(RCO}_2)_2]^+$  (M=Fe, Zn, Cu and Co) were generated and isolated.

## 6.3. Future Possibilities and Challenges

This brings us to the future. The coming possibilities and challenges can be discussed on three levels; the immediate progress of this work, challenges from new models and finally some comments about model chemistry in general.

### 6.3.1. Immediate Future of This Project

As a part of the completion of this project, some work remains to be finished. The syntheses of two more iron complexes, FeMn and FeNi, have been undertaken. These complexes have shown to be more difficult to isolate than the related complexes mentioned above. Magnetic measurements have been carried out on the FeZn complex (**13**) and remain to be completely analyzed. The other area which will be advanced is the computational modeling. The major mechanistic proposal for the urease mechanism, the "collaborator mechanism" in which urea is attacked by a terminal hydroxide, will also be studied in detail, so that the mechanisms can be fully compared on a theoretical basis. Other proposed mechanisms may also be considered.

This will hopefully lead to a new complete proposal for the most likely urease mechanism based on calculations.

### 6.3.2. New Model Complexes

There are always new challenges in inorganic modeling of metallo-biosites. The drawback is that they often require new organic ligands and thus a fair amount of preparatory work. The model complexes for urease included in this thesis are not too bad, however. Complex **3** is a very good structural model for the active site of urease, at least in terms of connectivity. One improvement that should be tried is to better model the configuration of the site. Ideally an open coordination site should be located *trans* to the ligand modeling the carbamylated lysine, one of the two bridging carboxylates. This is not the case in the current models. Another possible development of the modeling approach described would be the incorporation of the carboxylic side arms in dinucleating ligands, which would provide for more adequate modeling of methane monooxygenase and ribonucleotide reductase.

### 6.3.3. The Future of Model Chemistry

The future success of model chemistry is often a topic of discussion. The rapid developments in protein X-ray crystallography have reduced the need for structural models, although such models still play a role in the definition of accurate bond parameters and coordination modes. On the other hand functional models, which are useful in elucidating reactivity and dynamics, are becoming increasingly important. Despite this, the synthetic model chemistry is not the area with the strongest growth at this point.

One area that has advanced significantly in recent years is theoretical calculations and especially those of biological systems. As computers get faster, even high-level calculations can be made on larger systems. With even more accurate calculations on even larger systems, which will be available soon, the area will continue to grow in importance.

The interest in a more detailed mechanistic understanding of enzyme reactions remains high. The fact that more crystal structures are available has allowed researchers from all different areas to start thinking about how enzymes work in more detail. Most notably researchers in pharmaceutical and other industry are using the knowledge of mechanisms in

their work. This probably means that model chemistry will remain important for many decades to come.

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# 7. Populärvetenskaplig sammanfattning på svenska

Detta kapitel har som mål att sammanfatta avhandlingen på ett lite mer lättillgängligt sätt. Förhoppningsvis kan sammanfattningen ge en bättre förståelse av vad avhandlingen handlar om även om vissa förenklingar kan vara lite väl allmänna.

## 7.1. Att göra modeller baserade på aktiva säten av enzymer

Enzymer är viktiga ämnen i naturen. Vi talar då och då om att vi förbränner vår mat. Trots detta känner vi oss oftast inte särskilt varma, i alla fall inte så att det bränns. Visst bränner vi vår mat. Socker omvandlas till koldioxid och vatten precis som om vi hade eldat upp det utanför kroppen. Skillnaden är att kroppen omvandlar sockret långsammare och mer kontrollerat. Istället för att enbart värma omgivningen med reaktionen, så tas en stor del av energin tillvara, vilket faktiskt är själva anledningen till att vi äter. Förvandlingen från socker till koldioxid sker skegvis och enzymer är ämnen som deltar i och kontrollerar varje steg. De ser till att reaktionerna går till på rätt sätt och i lagom grad. Enzymer är komplexa ämnen. Det ställe i enzymet, där själva reaktionen sker, kallas enzymets aktiva säte. Där kommer ämnen in, reaktionen genomförs och från detta släpps nya ämnen ut.

Denna avhandling handlar om aktiva säten och enzymer. Även om enzymen är komplexa, så kan vi försöka förstå hur de fungerar. Detta kan i långa loppet t.ex. ge oss nya läkemedel eller billigare bränsle. Till vår hjälp har vi många moderna tekniker. Vi kan lysa med röntgenstrålning på vårt enzym och få fram strukturen och vi kan se hur fort enzymet låter reaktionen gå och vi kan få fram detaljer om hur reaktionen är organiserad. Problemet kan dock ofta vara att vi inte har tillräckligt rent enzymprov. Det kan vara oerhört svårt och ta många år att få fram ett rent enzym. Ett alternativt sätt att förstå mer om enzymet i fråga kan vara att göra en modell. Modellen kan vara enkel eller komplicerad. Den kan vara en liten modell, som motsvarar precis strukturen hos de aktiva sätet eller den kan till och med bara finnas enbart i en dator. Om modellen är bra och liknar det aktiva sätet väl, så kan den leda till mycket information på vägen mot fullständig förståelse av hur enzymer fungerar.

## 7.2. Tvåkärniga modeller

Jag har i denna avhandling försökt berätta om hur jag har gjort modeller för några olika enzym och vad vi kan lära oss från dessa modeller. Ett gemensamt tema för alla de modeller jag har tillverkat är att de alla innehåller minst två metalljoner. De två jonerna kommer från flera olika grundämnen och kan vara lika eller av inbördes olika slag. För att få dessa båda joner att på ett ordnat sätt hålla sig nära varandra, så har jag gjort organiska ämnen som har speciella förutbestämda platser för jonerna. De organiska ämnena har varit en stor del av det arbete jag utfört och avhandlingen beskriver hur jag gjorde för att få fram dem. Detta då de är helt nya ämnen, specialtillverkade för ändamålet och inte går att köpa.

Det enzym som jag arbetat mest med heter ureas och har två nickeljoner i sitt aktiva säte. Ureas bryter ner urinämne, som finns i vår urin och som är ett vanligt gödselmedel. Om vi kunde förstå ureas bättre så kanske vi skulle kunna minska vår gödselanvändning och spara vår natur. Jag har tittat närmare på hur ureas fungerar. Till min hjälp har jag haft de modeller jag har gjort. Jag har också använt en del datorberäkningar. Mina slutsatser är flera. Vi vet nu ganska väl hur urinämne fastnar på ureas och vi har kunnat motbevisa en speciell teori om hur resten av omvandlingen sker. Det sätt som vi föreslår att urinämne omvandlas på har jag valt

att kalla en "samarbetesreaktion" (collaborator reaction) med tanke på de olika uppgifter som de två nickeljonerna har och hur de samarbetar för att få jobbet slutfört.

Den organiska förening som jag använt för lära mig mer om ureas fungerar också bra som bas för en modell med två zinkjoner. Med dessa på plats, så har vi en användbar modell för ett enzym som heter zinkfosfotriesteras (ZFT). Detta enzym bryter ner organiska fosfater och är också av intresse. ZFT delar till stor del samma aktivasätestruktur med ureas, men i ZFT finns två zinkjoner istället för nickel. Med hjälp av två olika organiska ämnen har vi kunnat visa att det är viktigt att det finns en öppen plats på en av zinkjonerna för att reaktionen ska gå relativt snabbt. Detta har vi gjort även för ureas med samma resultat. En annan likhet mellan de två enzymen är vi kommit fram till att den speciell grupp av atomer som hittats mitt emellan metallatomerna (OH-grupp) i de strukturer som finns tillgängliga, troligtvis inte har någon speciell betydelse i reaktionen.

Jag har också arbetat med järn. En modell med två järnatomer har tillverkats tillsammans med flera modeller med blandade joner, t.ex. järn/zink och järn/koppar. Dessa modeller kan användas i studier av bl.a. purple acid fosfater, som är ansvarigt för nedbrytningen av fosfater i sura miljöer.

### 7.3. Framtida användningsområden

Mitt mål med avhandlingen och det arbete som lett fram till den har varit att göra enzymmodeller som kan vara användbara i sökandet efter en bättre förståelse av de olika enzymerna. Mina resultat kommer att publiceras i internationella tidskrifter och även om vi inte löst alla gåtor om de olika enzymerna redan nu, så kommer kunskapen att föras vidare. I framtiden är det inte omöjligt att det vi lärt oss om enzymen i fråga kan vara en del i arbetet mot en ny medicin eller kanske ett nytt gödselpreparat.





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