On the origin of the broad-band selectivity of bovine-lens-leucine-aminopeptidase

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This article is dedicated to Ernst Anders on the occasion of his 65th birthday. In addition to being a renowned heterocylic chemist, he loves figuring out how nature manages to outwit thermodynamic and kinetic constraints by employing zinc-based metalloenzymes such as carbonic anhydrase

Abstract

Detailed DFT (B3LYP) calculations on the mode of action of bovine lens leucine aminopeptidase (*bl*LAP), a binuclear zinc enzyme that hydrolyzes the N-terminal residue of a peptide chain, provide first explanations for the unusual coordination sphere at Zn(1) as well as the presence of a second water channel. The unusual architecture of the active site promotes the spontaneous generation of a water sluice that continually regenerates a series of species capable of acting as active nucleophiles. We now demonstrate that this sluice mechanism is coupled with substrate docking. Both possibilities lead to a complicated hypersurface in which a multitude of stable intermediates and possible nucleophilic species are generated which are in thermodynamic equilibrium with each other. The hypersurface is characterized by very low barriers for interconversion between the intermediates – all of which possess similar stabilities - and thus provides a first explanation for the broad-band activity of *bl*LAP which is capable of hydrolyzing all amino acids except for proline and arginine. Most steric and electronic constraints in the substrate can be overcome by simply selecting a different pathway on the hypersurface. First calculations indicate that a "classical" two-step hydrolysis over a *gem*-diol/diolate intermediate has a prohibitively high barrier and is thus a probable dead-end on the hypersurface. Alternatively, a non-classical mechanism in which the nucleophilic attack on the carbonyl bond as well as the C-N bond cleavage occurs simultaneously over a chair-like ring transition structure is postulated.

Keywords: Binuclear zinc hydrolases, peptide hydrolysis, bovine lens leucine aminopeptidase, biomimetic models; DFT

Introduction

With the borderlines between organic chemistry and biochemistry becoming increasingly blurred, many traditional organic chemists are currently beginning to investigate reactions taking place in biological systems. Exemplary efforts in this new field of chemical biology are currently being published in the broad field of metalloenzymes. Their unparalleled selectivity as well as the increasing number of solid state structures available in worldwide databases has triggered an immense amount of interdisciplinary research in the last few years. Studies are beginning to shed light on the oft surprisingly complex mode of action[1] employed by nature in order to outwit thermodynamic and/or kinetic constraints and thereby effect the controlled catalysis of reactions with a large thermodynamic gradient (large stability difference between educts and products) – processes which often have notoriously high activation energies in the absence of a catalyst. Many of these reactions appear quite trivial – until one realizes that nature is managing to catalyze them – often with a sheer unbelievable stereospecificity *and* a diffusion limited rate constant - under conditions where such reactions normally do *not* occur (pH 7, 25°C, 1 atm and in water).

It would, of course, be wonderful if we could manage the same under laboratory conditions. The design and synthesis of robust organometallo catalysts that function well in aqueous or nonaggressive media and are finely tunable yet highly selective has thus become a very tempting goal. This is contingent, however, upon an in depth understanding of just how nature manages to make such catalysts "work". Investigations performed directly on enzymatic systems are unfortunately hampered by the very fact that they are *fast*, so fast that most experimental methods are not capable of following them. Furthermore, these biomolecules are generally *huge* in the eyes of an organic chemist (and often available only in minute amounts). These facts demonstrate the necessity of simplifying matters – which basically involves studying model systems instead of the enzymes themselves.

Metalloenzymes usually have a well-defined pocket (active site) in which one or more metal ions is/are bound. Due to the template effect of the metallocenter, the chemical activity is often (but not always) limited to a small area of space (a few Å in diameter) in the immediate neighborhood of the metal ion(s). As a consequence, model systems need only, in a first approximation, to accurately reproduce the structural and electronic properties of the active site. Currently, there are two different ways of obtaining such model systems. One can attempt to synthesize a small biomimetic metallocomplex which hopefully exhibits much the same activity as the enzyme – a task which presents quite a challenge, especially in the fields of ligand design and organometallic chemistry.[2] Or one can perform computational studies on models based on the solid state structure of the active site. Advances in computer technology, and especially the development of density functional theory (DFT)[3] now allows for routine calculations on transition metal complexes containing up to ca. 500 atoms.[4] If proper care in the selection of the DFT method and basis set is exercised, not only structural but also thermochemical accuracy (± 1-3 kcal/mol) can be reached. In the more recent past, a joint approach in which calculations and experiments mutually support each other is being increasingly employed for the study of biomimetic systems. An example of this is the extensive work performed by E. Anders *et al.* (recently summarized in a review article[5]) on the fixation and

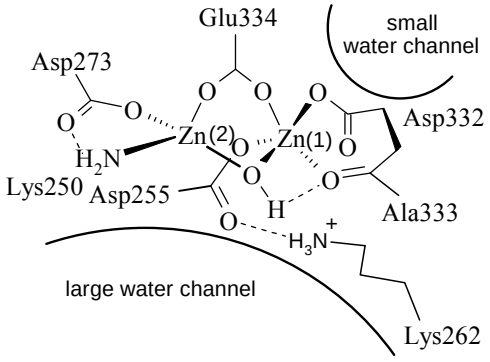
activation of CO_2 and related cummulenes by simple zinc-complexes – a theme bioinspired by the enzyme carbonic anhydrase[6] which contains a single Zn^{2+} ion in its active site.[7,8]

Countless biochemical processes in the metabolism of living systems involves the fixation and activation (usually hydrolysis) of small compounds containing carbonyl groups with the help of metalloenzymes. Due to its fundamentally flexible coordination sphere[9] (4 ↔ 5 ↔ 6 changes usually cost less than 2-3 kcal/mol), d^{10}-configuration (lack of redox activity) and natural abundance is Zn^{2+} often the metal of choice for these enzymes. Enzymes with one, two or even three zinc ions bound in the active site are known.[10] A current review of mechanistic investigations on the mode of action of these bi- and trinuclear zinc enzymes is available[11] and progress in the development of synthetic biomimetic models for monozinc enzymes has also been recently reviewed.[12]

Continuing in the mechanistic tradition of E. Anders, one of the focal points of our research is to compute the mode of action of binuclear zinc enzymes and, their synthetic biomimetic models.[13,14] In this article, we report upon current progress in the elucidation of the mode of action of a binuclear zinc enzyme, bovine lens leucine aminopeptidase (*bl*LAP).

Bovine lens leucine aminopeptidase

Aminopeptidases are extremely widespread and play a critical role in countless tissue-specific physiological processes in mammals, plants and bacteria. They hydrolyze the amide bond of the terminal amino acid in polypeptide chains thus helping in protein degradation, modification and other vital metabolic processes involving peptides.[10a,15] Of the five types of aminopeptidases isolated to date, leucine aminopeptidase (LAP, EC 3.4.11.1) is one of the best characterized with respect to its sequence, structure and mode of action.



Scheme 1. Solid state structure of the active site of *bl*LAP.

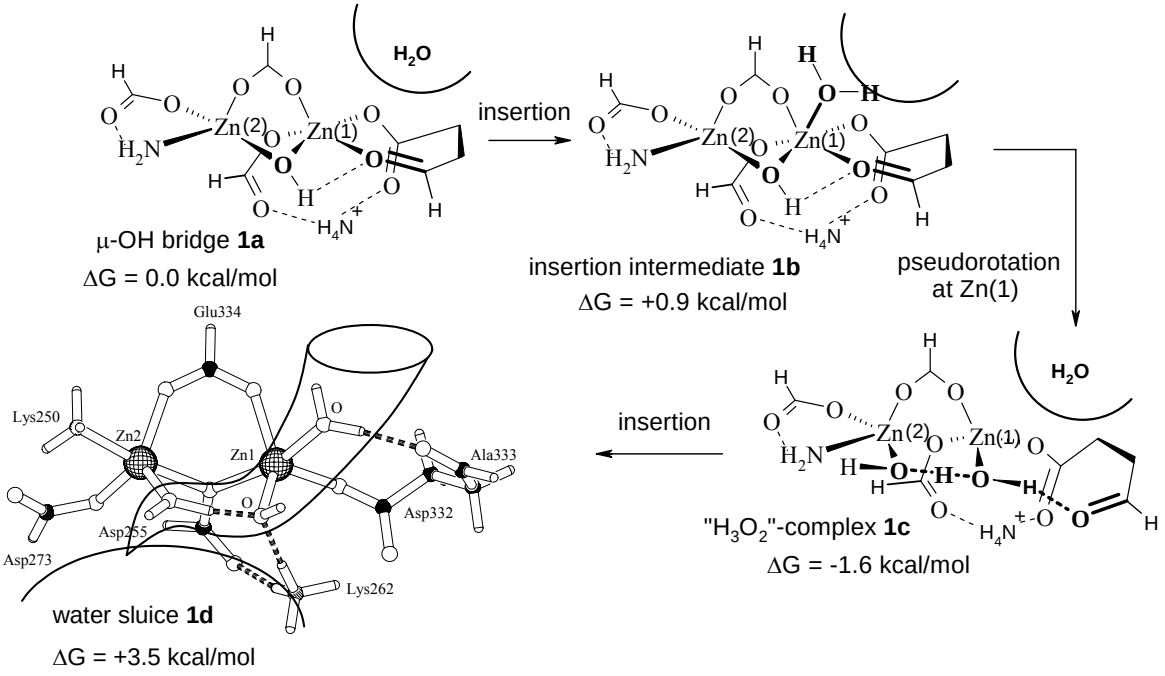
Alterations in LAP activity in humans results in a variety of diseases including cancer, leukemia, cystic fibrosis and eye cataracts and have even been implicated in HIV patho-physiology.[15,16,17] As a consequence, current research efforts are attempting to develop specific inhibitors for LAP.[18] LAP plays a central role in sight processes and, as such, the concentration in cow eyeballs proved to be high enough to be conveniently isolated.[19] Most of the mechanistic work

on this enzyme has thus been performed on the bovine form which has come to be known as *bl*LAP (bovine lens leucine aminopeptidase). Although metalloenzymes are generally characterized by some sort of high substrate specificity, the Zn/Zn-form of *bl*LAP is a notable exception since it exhibits a broad-band activity. It prefers, as its name implicates, to hydrolyze an N-terminal leucine but is also quite capable of hydrolyzing (albeit somewhat slower) all other naturally occurring amino acids with the exception of proline and arginine.[10a]

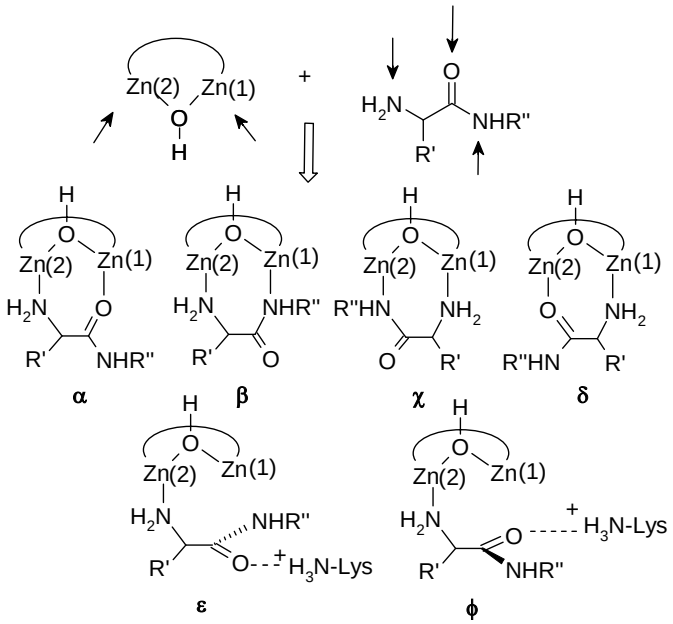
*bl*LAP has a hexameric structure in which each monomer functions independently of the others.[20,21] The active site (one per monomer) is sitting on the edge of two water channels – a large one for the substrate[22] located below the two metal ions (which are unsymmetrically coordinated) and a much smaller one sitting above Zn(1) (Scheme 1).[21,22,23] The metal ions are cocatalytic; activity is only observed when both metal binding sites are occupied.[24] Zn(2) is tightly bound and cannot be replaced by other metal ions except for Co^{2+} whereas Zn(1) can be replaced by Mg^{2+}, Mn^{2+} or Co^{2+} without destroying the activity.[24] Metal substitution in both binding sites affects k_{cat} and K_m values significantly and it is becoming clear that nature sometimes tailors the substrate specificity through metal exchange. For example, a Mn^{2+}-ion in site 1 results in a preference for the CysGly substrate. This modified form of LAP has recently been identified as the main CysGly hydrolyzer in rat liver.[25]

Selected mutagenesis experiments have shown that the Lys262 residue located on the edge of the larger water channel is directly involved in the mechanism of hydrolysis.[26] In addition, Lys262 provides direct hydrogen-bond stabilization in inhibitor complexes.[27] The amino group in the flexible side chain of Lys262 has a radius of allowed motion of ca. 2.5 Å and is positioned directly beneath Zn(1). We have spent quite some time modifying simple models developed by Karplus *et al.*[28] and us[29] to include an NH_4^+ ion (or NH_3 as the situation requires) that can freely move inside of the same radius as the flexible Lys262 side chain.

One of the unusual features in the active site is an trigonal bipyramidal coordination at Zn(1) with one of the ligands being a neutral carbonyl (amide bond to Ala333) and all others "hard", e.g. negatively charged oxygen – a combination which has been shown by *ab initio* calculations to be intrinsically instable.[30] Nature goes to quite some effort to maintain this instable coordination geometry in the active site and we could demonstrate *via* DFT calculations that an extended hydrogen bond network originating from the peptide backbone in the vicinity of Zn(1) is used to "glue" the carbonyl group in place.[29] The second, smaller water channel is located just above and to the left of this loosely coordinated carbonyl group. This recently prompted us to investigate the extent to which single water molecules are capable of interacting with the active site. Our DFT calculations show that a water molecule placed in the general vicinity of the smaller water channel is spontaneously inserted into the Zn(1)-$O_{carbonyl}$ bond (insertion intermediate **1b** in Scheme 2).[31] The Zn(1) in **1b** then undergoes an extremely facile pseudorotation which causes the bridging μ-OH contact to be given up, thus lowering the coordination number on Zn(1) to four. A metal-bound H_3O_2 functionality ("H_3O_2"-complex **1c**) results which is again capable of spontaneously organizing another water molecule originating from the smaller water channel. This process ends with the generation of the water sluice **1d**.[31]



Scheme 2. Formation of a "water sluice" in the active site of *bl*LAP. Relative energies (ΔG) were calculated at the B3LYP/aug-cc-pVTZ//B3LYP/lanl2dz level of theory.[31]



Scheme 3. Possible docking permutations at the active site of *bl*LAP.

These calculations provide a plausible mechanism for continually regenerating a series of species capable of acting as active nucleophiles in the mode of action of *bl*LAP. A key feature is the

role of the weakly bound carbonyl ligand – it functions as a "traffic cop" to direct water molecules coming from the small channel into the heart of the active site where they are activated. In support of our findings, similar H_3O_2 zinc species have been considered in DFT and MD calculations on β-lactamase isolated from *Bacteroides fragilis*.[32] Solid state structures of biomimetic zinc complexes with H_3O_2-functionalities are also available.[13b-f] Kinetic investigations on biomimetic complexes report that zinc bound H_3O_2 species are intrinsically more reactive than μ-OH units.[13b]

Continuing with our investigations, we now consider the process of substrate docking in detail and then discuss our current progress in identifying the active nucleophile(s) as well as the actual mechanism of hydrolysis in the mode of action of *bl*LAP.

Results and Discussion

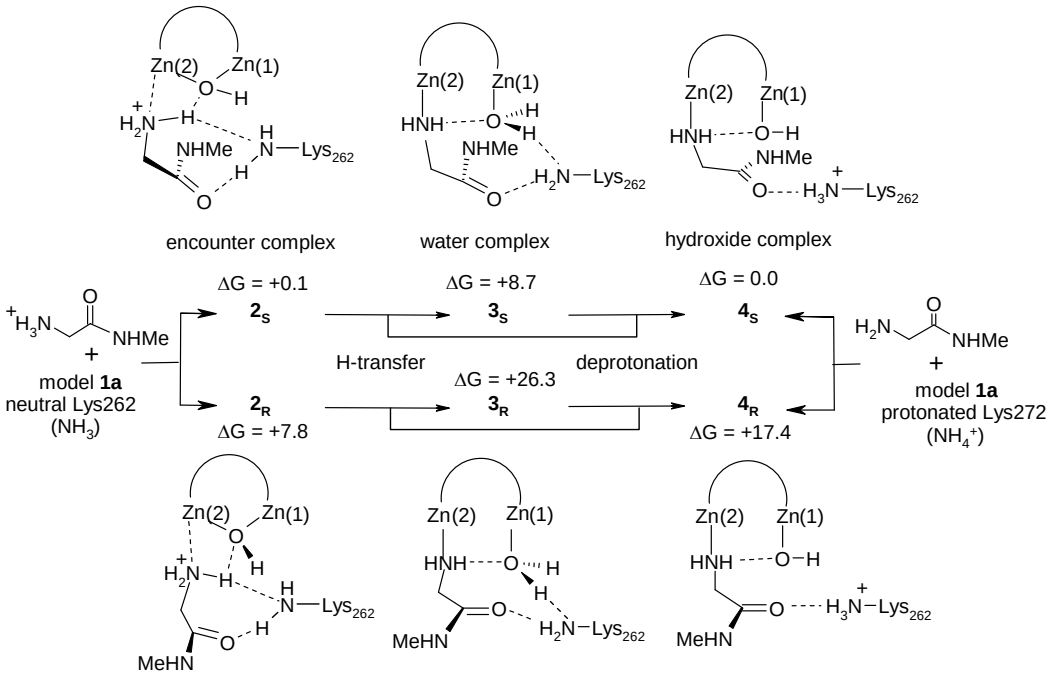
Substrate docking. Several mechanisms for the mode of action of *bl*LAP have been suggested over the past few years with all of the older mechanisms being based on the solid state structures of the available inhibitor complexes.[21-23] In these mechanisms, it is generally assumed that the terminal amino group in the peptide docks onto Zn(2) and the carbonyl group onto Zn(1) (permutation α in Scheme 3). The fact that three additional docking permutations are theoretically possible has not been mentioned in the chemical literature.[8a] The situation is further complicated by a newer AM1/MM[33] study that has provided the first indications that the docking mechanism of a true substrate could deviate from an inhibition mechanism.[34] This study demonstrated that the terminal amino group can indeed dock onto Zn(2); however, neither the carbonyl oxygen nor the NHR group docks onto Zn(1) (permutations ε and ϕ), thus further increasing the number of docking possibilities. In this monodentate situation, the carbonyl group is positioned parallel to the active site *via* a strong hydrogen bond to the catalytic active lysine side chain (Lys262) located on the edge of the larger water channel.[35]

An electrostatic consideration shows that the binuclear active site has a formal negative charge (-1e). Electroneutrality is probably realized through the amino group in the Lys262 side chain (normally protonated under physiological conditions). Due to the broad-band activity of *bl*LAP, practically all amino acids must be able to interact with the active site – and some of the N_{Term} amino groups in these residues have a higher affinity for a proton than Lys262. The docking hypersurface is thus further complicated by the following questions – where is the proton sitting and does it interfere with or modify the docking mechanism?

A detailed analysis of the solid state structure together with simple semiempirical (AM1) calculations revealed that the bidentate permutations χ and δ and all monodentate variations in which N_{Term} docks onto Zn(1) are not possible due to steric interference. A steep pocket wall with a slight bulge allows just enough room beneath Zn(2) for the N_{Term} residue with its smaller side chain R′. In contrast, the region below Zn(1) is freely accessible and the longer peptide chain R′′ can easily be accommodated.[8a] Using our active site model and a small dipeptide, we have explicitly investigated all remaining docking possibilities at the B3LYP/lanl2dz level of theory. In complete accord with the earlier AM1/MM study[35], we could not locate a single bidentate intermediate on the hypersurface – most probably because the substrate backbone must be unfavorably twisted in order

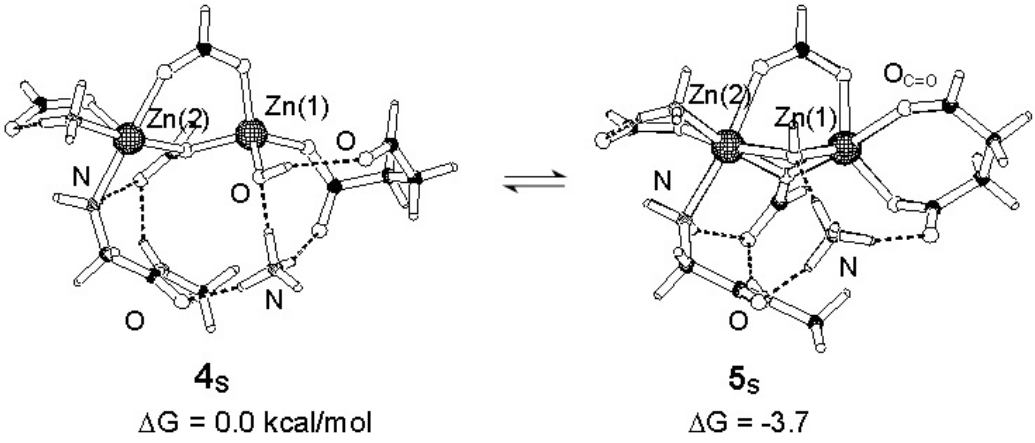
to achieve bidentate docking. These findings strongly indicate that inhibitors (which clearly favor bidentate intermediates[21-23]) are functioning *via* a completely different mechanism than true substrates.

The N_{Term} amino group docks onto Zn(2) thus raising its coordination number to six; however, neither the carbonyl nor the amine functionality docks onto Zn(1). Scheme 4 contains the calculated docking mechanism in which differential protonation (N_{Term} or Lys262) was explicitly considered. When a protonated peptide comes within electrostatic contact of the active site (model with neutral Lys262) one of two encounter complexes (**2S** or **2R**) is spontaneously (E_a << 2 kcal/mol) generated. A strong hydrogen bond between the carbonyl and the side chain of Lys262 is generated which positions the peptide linkage parallel to the active site – in an optimal position for nucleophilic attack on the carbonyl function. At this point, a strong differentiation of the two surfaces of the prochiral carbonyl group occurs. The longer peptide chain (simulated by a methyl group) can either project behind (conformation **2s**) or in front (**2R**) the plane of paper. The more stable conformation is **2s** ($\delta\Delta G$ = 7.7 kcal/mol). Due to the fact that hydrogen bonding to Lys262 effectively inhibits rotation about the carbonyl linkage from this point on, a strong preselection of the enantiotopic surface has been reached. Semiempirical AM1 calculations show that longer peptide chains composed of L-amino acids experience an additional steric hinderance by the pocket wall. The enantiotopic differentiation can thus be expected to be considerably larger than calculated for the simple model presented here. These findings are in complete accord with experiment since *bl*LAP consistently differentiates between the D- and L-configurations with the natural L-amino acids being hydrolyzed several orders of magnitude faster than the D-analoga.[35]



Scheme 4. Docking mechanism of a simple dipeptide on the active site of *bl*LAP. Relative energies (ΔG; kcal/mol) were calculated at the B3LYP/lanl2dz level of theory.

After the encounter complex $\mathbf{2_S}/\mathbf{2_R}$ has been formed, there are two different mechanisms for further reaction. A proton can be transferred from N_{Term} to the μ-OH bridge. This breaks the μ-OH bridge and a terminally bound Zn(1)-water complex $\mathbf{3_S}/\mathbf{3_R}$ results. Lys262 then deprotonates the water. Alternatively (and favored since the water complex $\mathbf{3_S}/\mathbf{3_R}$ is clearly less stable), the N_{Term}-proton can be directly transferred to Lys262. Both mechanisms generate the hydroxide complex $\mathbf{4_S}/\mathbf{4_R}$. The docking mechanism is much simpler if Lys262 is protonated instead of the peptide. In this case, the hydroxide intermediate $\mathbf{4_S}/\mathbf{4_R}$ is directly generated. The structure of $\mathbf{4_S}$ (Scheme 5) is quite interesting because the intrinsic instability of the carbonyl ligand (amide bond to Ala333 in the peptide backbone) allows it to move away from Zn(1) in order to stabilize the terminally bound hydroxide ligand. Complex $\mathbf{4_S}$ is not the most stable species on the docking hypersurface. It spontaneously (E_a << 2 kcal/mol) rearranges itself by regenerating a μ-OH bridge $\mathbf{5_s}$ which is more stable than a terminally bound Zn(1)-OH unit (Scheme 5).



Scheme 5. Stabilization of the docking intermediate **4s** through the generation of a μ-OH bridge (species **5s**). Calculated at the B3LYP/aug-cc-pVTZ//B3LYP/lanl2dz level of theory.

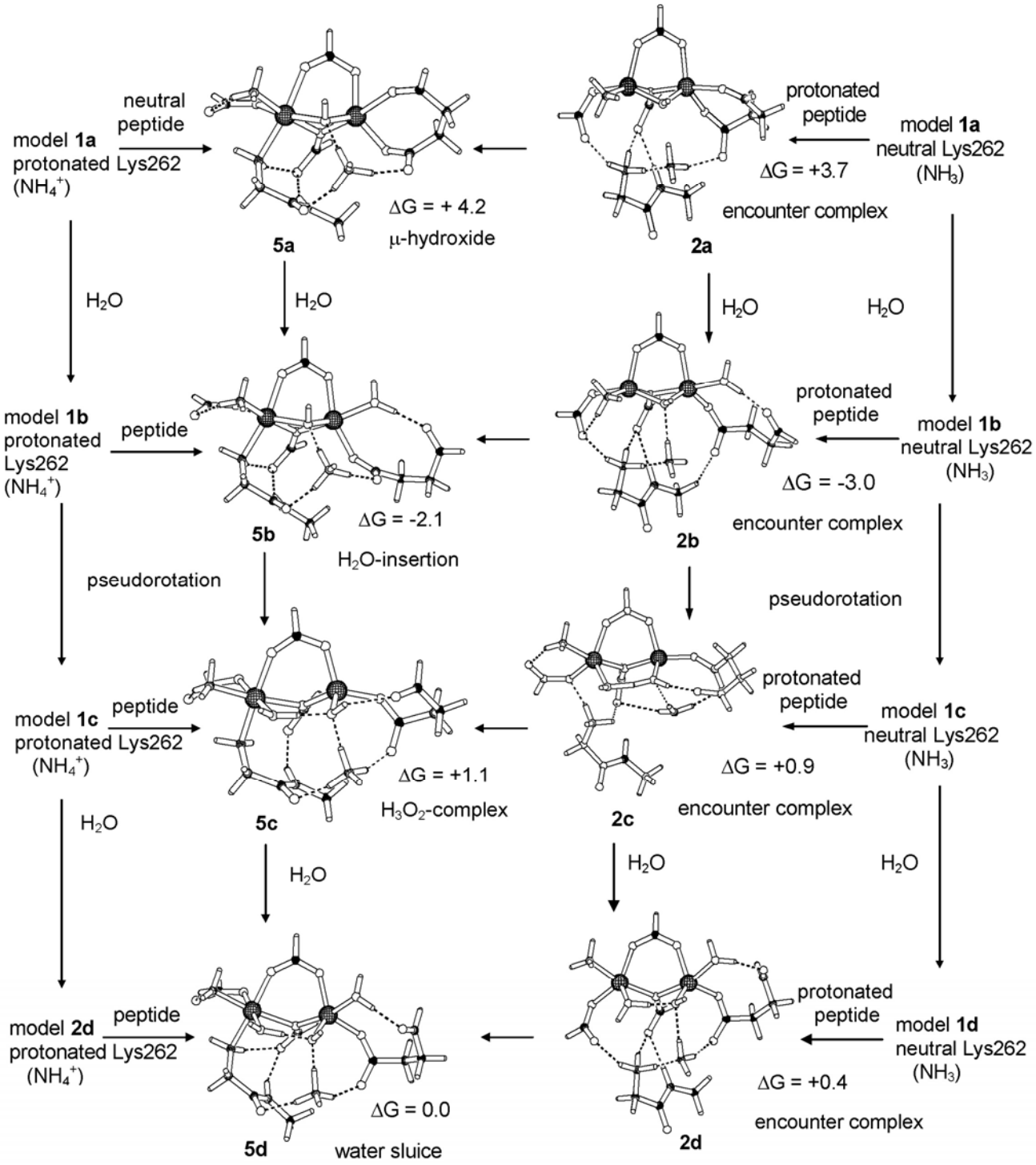
The proton transfer reactions in this docking mechanism are examples for "low barrier" H-transfers which exhibit typical long wavelength IR-frequencies (< 300 cm^{-1}).[36] The hypersurface of such reactions is generally a very flat double potential. Due to the fundamental error in the harmonic oscillator model needed to calculate the frequencies (the longer the wave length, the larger the error) and the "flatness" of the hypersurface, it becomes impossible to accurately pinpoint transition structures for these proton transfers using the density functional B3LYP method. However, it is possible to explicitly follow the "active" frequency in the general area of the transition structure. In this manner, upper limits to the activation barriers can be calculated. In the case of the docking mechanism illustrated in Schemes 4 and 5, all processes are quite facile – even those not explicitly involving hydrogen transfer - and the individual barriers all lie beneath 2-3 kcal/mol. According to these calculations, docking is a thermodynamically controlled process ending with the generation of $\mathbf{5_S}$.

Extendend docking hypersurface. The generation of a water sluice in the active site of *bl*LAP opens new possibilities for the docking mechanism which is apparently much more complex than we have previously assumed. An important question still open is – at what point in the docking mechanism is a water sluice opened up – before, during or after the substrate has docked? An explicit investigation of this at the B3LYP/lanl2dz level of theory leads to an extended docking hypersurface partially illustrated in Scheme 6. At this point, we limited our calculations to a consideration of the pro-S hypersurface (docking of L-amino acids). Even so, substrate docking is much more complex than Scheme 6 indicates since we have only illustrated the more important intermediates on the hypersurface.

Alternative proton transfer mechanisms leading to one and the same intermediate have been left out as well as all terminally bound hydroxide species such as **4s** (Scheme 5) that spontaneously stabilize themselves *via* regeneration of a μ-OH bridge. The rows in Scheme 6 represent substrate docking; on the left a neutral peptide docks onto various forms (columns) of the active site (protonated Lys262) to yield the μ-hydroxide intermediates **5a-d**. From the right, a protonated peptide (neutral Lys262) leads to the same intermediate. Columns represent the generation of a water sluice. No sluice is present at the top of the column (intermediate **5a**, for example). Successive introduction of water molecules leads to the full-blown generation of the water sluice at the bottom (intermediate **5d**).

The presence of the water sluice does not affect the docking mechanism and *vice versa*. It is significant that every possible combination of both mechanisms represents a stable intermediate on the hypersurface. Explicit calculation of the reaction coordinates connecting each intermediate with the others (indicated by the arrows in Scheme 6) revealed that the entire hypersurface is unusually flat. All intermediates have comparable stabilities (± 2-4 kcal/mol) and all barriers for interconversion between them are also less than 2-4 kcal/mol. Perhaps the best description of the hypersurface is a multi-well potential that is extremely shallow and therefore looks like an egg carton where the "dips" for the eggs are barely deep enough to keep them from rolling out.

For the simple model considered in this study, docking ends with the generation of **5d** which looks to be a perfect candidate for the nucleophilic species.

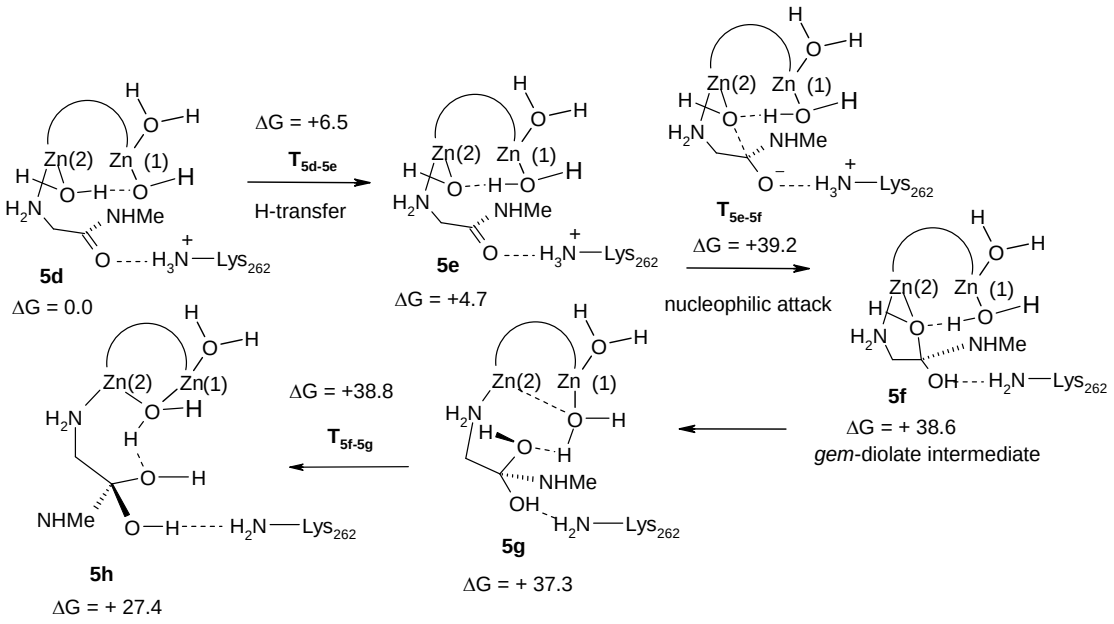


Scheme 6. Partial hypersurface for substrate docking on the active site of *bl*LAP. Calculated at the B3LYP/lanl2dz level of theory.

However, it is obvious that many other species on the hypersurface are also potent nucleophiles (intermediates **4s** or **5c**, for example). It is very clear that the entire mode of action for docking is thermodynamically controlled; all species are in thermodynamic equilibrium with each other.

These results provide the first explanations for the observed "broad-band" activity of *bl*LAP which is quite capable of hydrolyzing N-terminal amino acids except for proline and arginine. With the multitude of possibilities on the hypersurface – not only for docking but also for selection of the nucleophilic species – it is obvious that most steric and electronic constraints in the substrate can easily be overcome simply by selecting different pathways and different "dips" (active intermediates) in the egg carton.

Nucleophilic attack. We are now beginning to investigate the multitude of possibilities for nucleophilic attack on the hypersurface of *bl*LAP. Although this study is far from complete, the water sluice (intermediate **5d**) is the most obvious candidate for the nucleophilic species. All previous mechanisms either proposed (based on the binding pattern observed in solid state structures of inhibitor complexes[21-23]) or calculated (AM1/MM study[35]) for *bl*LAP have assumed that the reaction runs through a classical *gem*-diol/diolate intermediate stabilized by contacts to Zn(1) and Lys262.



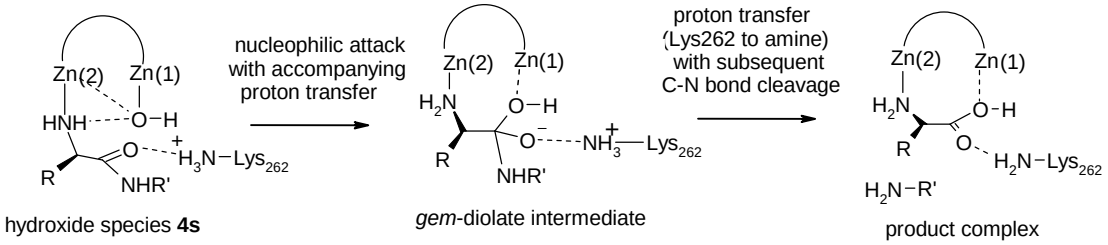
Scheme 7. The first step in a classical two-step mechanism for hydrolysis of the amide bond over a *gem*-diol/diolate intermediate. Energies (ΔG) were calculated at the B3LYP/lanl2dz level of theory and are given in kcal/mol relative to **5d**.

Such a mechanism is indeed possible (Scheme 7) and starts with a relatively low barrier hydrogen transfer in the H_3O_2-functionality in **5d**. The hydroxide ion thus generated on Zn(2) (species **5e**) then swings down and attacks the carbonyl group over the transition structure $\mathbf{T_{5e\text{-}5f}}$ which has an extremely high barrier (ΔG = + 39.2 kcal/mol) to generate an instable *gem*-diol **5f** (ΔG

= 38.6 kcal/mol) in which a hydroxyl-Zn(2) contact is still present. At this point, Lys262 has transferred a proton to the oxygen atom in the former carbonyl group. A spontaneous reorganization (E_a << 2 kcal/mol) of the coordination sphere of Zn(2) releases the Zn(2) bound hydroxyl group. The water left behind is terminally bound to Zn(1) in the intermediate **5g**. Another nearly spontaneous process then reestablishes a μ-OH_2 bridge (species **5h**).

We are now investigating the mechanism of the following C-N bond cleavage step. Similar to nucleophilic attack, this consists of a complex series of proton transfers. Lys262 deprotonates the *gem*-diol and then transfers the proton to the amino group which polarizes the C-N bond sufficiently for subsequent cleavage to occur. It is still unclear if the H_3O_2 bridge is regenerated [pseudorotation at Zn(1) which pulls the second water ligand down between the two zinc ions] before or after C-N bond cleavage occurs. First estimations of the C-N cleavage indicate that this is also a high energy process with a barrier in the same magnitude (≥ 40 kcal/mol) as nucleophilic attack. Which one of the two steps is rate determining in this "classical" mechanism is still unclear.

A similar two-step mechanism over a *gem*-diolate intermediate has been calculated at the AM1/MM level by Clark *et al.* in which they considered species **4s** to be the active nucleophile (Scheme 8).[35] Attack on the carbonyl group with subsequent bond cleavage was also found to be a high energy process; a barrier of 50.5 kcal/mol relative to a μ-OH docking intermediate very similar to **5a** was calculated.



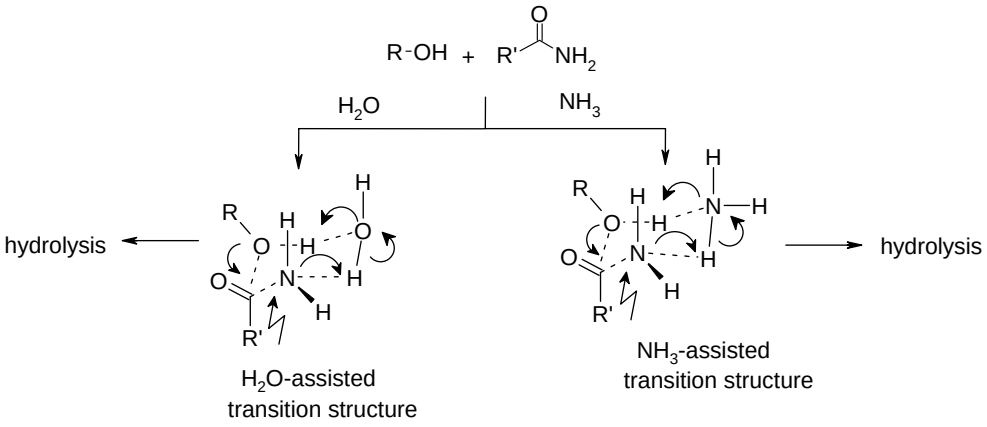
Scheme 8. Classical two-step hydrolysis of the amide bond in the active site of *bl*LAP as calculated at the AM1/MM level of theory.[34]

For comparative purposes, several quantum mechanical studies of simple amide bond hydrolysis in both the gas phase and in water (polarization continuum approximations) report activation energies of 30-50 kcal/mol for the rate determining step [classic text-book base (H_2O) and acid ($H_3O_2^+$) catalyzed mechanisms were considered].[37] In addition, experimental barriers have been determined to lie between 20 and 30 kcal/mol for a series of N-substituted benzamides and toluamides.[38]

Perhaps the largest problem with our study (and the results reported by Clark et al.[35]) is the size of the calculated barrier for nucleophilic attack. This is much too large for an enzymatic process that occurs near the diffusion limit where the maximal barrier *must* be less than 12-15 kcal/mol. Although insufficiencies in the computational methods employed both here and in the independent AM1/MM study[35] could contribute somewhat to uncertainties in the size of the barriers calculated, it is far more likely that fundamental details in the mode of action of *bl*LAP have been missed.

Alternative "non-classical" mechanism. If a "classical" two step mechanism running over a *gem*-diol/diolate intermediate is operative in the active site of *bl*LAP, nature has not gained anything by going to quite some trouble to build a binuclear active site in which a lot of effort has been expended to create and maintain an instable coordination sphere at Zn(1).

First indications of a possible alternative mechanism are provided by a quantum mechanical study of the methanolysis of formamide in water (with reference to the mode of action of serine proteases).[39] With the help of an assisting H_2O or NH_3, a non-classical hypersurface becomes possible in which the nucleophilic attack on the carbonyl bond as well as the C-N bond cleavage occurs simultaneously over a chair-like ring transition structure. This lowers the activation energy needed for methanolysis considerably (ca. 40%) as compared to the uncatalyzed (without H_2O or NH_3) case.[40]



Scheme 9. Alternative, non-classical transition structures for amide bond cleavage.

After completion of substrate docking in the active site of *bl*LAP, a series of potential nucleophilic species (**4**$_s$, **5c** or **5d** for example) are accessed. In each of these species, Lys262 is in an optimal position to catalyze such a concerted, non-classical hydrolysis. In the case of the water sluice intermediate **5d**, the H_3O_2-functionality could also assist in stabilizing a non-classical transition structure – the "hydroxide" attacks the carbonyl group while the "water" stabilizes the transition structure. It is even possible that both "water" assistance from above and "amine" assistance (Lys262) from below the substrate is occurring in **5d** – and both processes work in a synergetic manner to reduce the activation barrier even further. We are currently explicitly exploring these possibilities. First estimations indicate that such a non-classical mechanism is indeed possible in *bl*LAP and it does lead to a reduction in the activation barriers by at least 50% as compared to a classical two-step mechanism.

Conclusions

After having recently investigated the extent to which single water molecules are capable of interacting with the binuclear active site of bovine lens leucine amimopeptidase (*bl*LAP) and

showing that a "water sluice" capable of continually regenerating a series of nucleophilic species is spontaneously generated[31], we have now investigated the process of substrate docking in detail. Due to the presence of several heteroatoms in the substrate as well as two metal ions in the active site, several docking permutations are theoretically possible.[8a] Due to steric constraints in the active site pocket as well as electronic effects – especially the orientation of the carbonyl bond underneath and parallel to the binuclear active site by Lys262 – only two docking permutations are actually generated in which the N-terminal amino group docks onto Zn(2). These differ only in which enantiotopic surface of the carbonyl group is accessible for nucleophilic attack (structures $\mathbf{4_S}$/$\mathbf{4_R}$ in Scheme 4, for example) with the pro-S configuration being clearly preferred. These findings are in complete accord with experiment since *bl*LAP consistently differentiates between the D- and L-configurations with the natural L-amino acids being hydrolyzed several orders of magnitude faster than the D-analoga.[36]

Due to the broad-band activity of *bl*LAP, practically all amino acids must be able to interact with the active site – and some of the N_{Term} amino groups in these residues have a higher natural affinity for a proton than Lys262. We show that the protonation state has no consequence on the docking mechanism as a whole. Both possibilities (Lys262 or N_{term} of the substrate) lead *via* different mechanisms to one and the same docking intermediate ($\mathbf{5_S}$ in Scheme 5).

We then went on to demonstrate that the water sluice mechanism is clearly coupled with substrate docking – and both possibilities lead to a complicated hypersurface in which a multitude of stable intermediates and possible nucleophilic species are generated which are in thermodynamic equilibrium with each other. The hypersurface is characterized by very low barriers for interconversion – all of which possess similar stabilities. This is also in accord with the observed broad-band activity of *bl*LAP. It is clear that most steric and electronic constraints in the substrate can be overcome by simply selecting a different pathway on the hypersurface.

Our calculations illustrate the fact that considerable care should be exercised in interpreting experimental information gained *via* inhibition studies of enzymes. All available solid state structures of inhibitor complexes of *bl*LAP exhibit a bidentate binding mode reminiscent of permutation α in Scheme 3.[21-23] True substrates, however, dock in a monodentate manner with the carbonyl group being directly activated by Lys262. In the inhibitor complexes, Lys262 provides secondary hydrogen-bond stabilization.

Although the textbook concept of "*transition state analogues*" has been and still is quite popular, it is our opinion that most inhibitors do not provide an adequate "model" for the transition structure involved in the actual catalysis.[11] Inhibitors interact strongly with the active site – often forming covalently bound enzyme-inhibitor complexes - and thus slow down or completely stop the catalytic turnover simply because the active site is effectively "blocked". It is quite feasible (and very likely, as exemplified by this study) that true substrates react *via* a completely different mode of action.

Current work in progress as well as an independent AM1/MM study[35] at a considerably lower level of theory indicates that a "classical" two-step hydrolysis over a *gem*-diol/diolate intermediate has a prohibitively high barrier ($\geq$ 40 kcal/mol). This is directly comparable with calculations on the *uncatalyzed* gas phase hydrolysis of an amide bond[38] as well as experimentally determined barriers

(between 20 and 30 kcal/mol) for the hydrolysis of a series of N-substituted benzamides and toluamides.[39] The calculated barrier for hydrolysis of a peptide bond *via* a "classical" mechanism in the heart of *bl*LAP is thus much too large for this nearly diffusion controlled enzymatic process, a fact which we attribute to an oversight of fundamental details in the mode of action and *not* to insufficiencies in the computational method employed. First indications of an "non-classical" alternative in which an assisting H_2O or NH_3 (both of which are present in the active site of *bl*LAP as a Zn-bound solvent molecule and the side chain amino group in Lys262) can play a critical role in lowering the barrier *via* stabilization of a concerted 6-membered ring transition structure have been reported in a quantum mechanical study of the methanolysis of formamide.[40] We are currently beginning to investigate this possibility and hope to be able to provide a complete theoretical description (catalytic circle) of the mode of action of this interesting broad-band hydrolase in the near future.

Computational Details

The calculations reported in this article were performed with the gradient-corrected hybrid B3LYP[40] density functional using the Gaussian03[41] program package. The lanl2dz basis set (D95[42] basis for the first row atoms and the Los Alamos effective core potential for the zinc ions[43]) was employed for geometry optimizations throughout since extensive calculations on similar binuclear zinc complexes have clearly demonstrated that this medium-sized basis set delivers qualitatively and quantatively similar results as the much larger aug-cc-pVTZ[44] basis.[13a,14,31] Somewhat better energies were obtained for selected reactions (Scheme 5 for example) by single point calculations at the B3LYP/aug-cc-pVTZ//B3LYP/lanl2dz level of theory. These results did not differ significantly from the results obtained at the B3LYP/lanl2dz level. All species found on the hypersurface were characterized as energetical minima or transition structures *via* vibrational analyses at the B3LYP/lanl2dz level of theory. Default convergence criteria were used and no symmetry was employed in any of the calculations. All relative stabilities reported are gas phase Gibb's free energies that contain standard thermochemical (298 K) and vibrational corrections. For the sake of readability, chemical drawings were employed to illustrate the calculated structures in many of the schemes. Calculated coordinates (pdf-files) and tables of absolute energies/thermodynamical corrections for all stationary points found on the hypersurface can be obtained from the authors upon request.

References and Notes

1. A "*mechanism*" is generally understood as being a series of individual molecular reactions, one coupled right after another, which finally convert an educt into a product in a linear fashion. The active site of enzymes is, however, not always limited to a single mechanism. Often several alternatives coexist and the terminology "*mode of action*" is used to describe the complete manifold of mechanistic possibilities.

2. *Chemical Reviews* has devoted three special issues to this topic with the general themes „*principles of enzymatic catalysis*“ (*Chem. Rev.* **2006**, *106*, 3029), „*biomimetic inorganic chemistry*“ (*Chem. Rev.* **2004**, *104*, 347-1200) and „*bioinorganic enzymology*“ (*Chem. Rev.* **1996**, *96*, 2237).
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4. Both *Accounts of Chemical Research* (*Acc. Chem. Res.* **2006**, *39*, 71) and *Chemical Reviews* (*Chem. Rev.* **2000**, *100*, 351) have devoted special issues to this topic.
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