Semi-empirical simulation of Zn/Cd binding site preference in the metal binding domains of mammalian metallothionein

Chia-Ching Chang and P.C.Huang†

Department of Life Sciences, National Tsing Hua University, Hsin-Chu
300 Taiwan, Republic of China

†To whom correspondence should be addressed

Metallothionein, a two-domain protein, naturally binds seven gram atoms of divalent ions such as Zn and Cd. Four of the metals (M1, M5, M6 and M7) are found in the α-domain and three (M2, M3 and M4) in the β-domain. Previous studies have shown that metal ions in the β-domain are more readily exchangeable, and the level of avidity is site specific. By semi-empirical MNDO modified neglect of diatomic overlap calculations, we found the tendency of binding energy for Cd to be M3 > M2 > M4 in the β-cluster and M5 > M7 > M1, M6 in the α-cluster. Thus, the replacement of Zn by Cd can be expected to follow the order M4 → M2 → M3 in the β-domain and M5 → M7 → M1 or M6 in the α-domain. This is reflected by energy differences computed with a series of simulated structures derived from either X-ray crystallography or NMR coordinates.

Keywords: heat of formation/metallothionein/MNDO/quantum/semi-empirical

Introduction

The cysteine-rich mammalian metallothionein (MT) consists of two metal-thiolate clusters, binding avidly seven transition IIB divalent metal ions, such as zinc (Zn) and cadmium (Cd), via mercaptide bonds (Kagi and Schäffer, 1988; Figure 1). The N-terminal half of the molecule forms one cluster, known as the β-domain, which encompasses three metals at sites M2, M3 and M4 based on 111Cd and 113Cd NMR profiles. The carboxyl half of MT constitutes the other cluster, known as the α-domain, which retains four metals (M1, M5, M6 and M7, Nettesheim et al., 1985; Otos et al., 1993). The binding strength varies with the metal (Kagi and Vallee, 1961; Rupp and Weser, 1978; Boulanger et al., 1983; Nielson et al., 1985).

Thus each molecule of native MT would bind seven gram atoms of Zn(II); each of these Zn ions can be replaced by Cd(II) in vitro by simple substitutions (Nettesheim et al., 1985). However, MT purified from Cd-induced cells from various mammalian species generally contains stoichiometrically five Cd(II) and two Zn(II) metal ions (Melis et al., 1983; Nielson and Winge, 1983; Nielson et al., 1985). In addition to stoichiometric measurements, the presence of mixed metals in induced MT has also been demonstrated by X-ray crystallographic (Robbins et al., 1991) and 2-D NMR data (Schultze et al., 1988). In all cases examined, the two Zn(II) ions are found preferentially in the β-domain. As metal–thiolate linkage involves cysteines through tetrahedral coordination, it is unclear why metal affinity is site specific.

Classic simulation of the entire metal-bound metalloprotein has not been feasible because of cumbersome high-level ab initio calculations. However, recent advances in the combinatorial use of quantum (QM) and classic (MM) calculations in molecular simulation have made possible free energy perturbation studies for specific enzymes (Warshel and Levitt, 1976). The QM semi-empirical package MOPAC93 (Stewart, 1993) has been widely used in the management of QM region calculations (Field et al., 1990; Äqvist and Warshel, 1993; Hwang and Pan, 1995; Stanton et al., 1995). This study has been undertaken using the MOPAC93/MNDO calculation (Dewar and Thiel, 1977) in an attempt to provide an explanation as to the basis of the relative energy level for metal preference of binding sites in MT.

Materials and methods

MT is conveniently divided into α- and β-domains, corresponding to α- and β-metal-containing clusters, respectively. Our calculations were carried out using the MNDO method (Dewar and Thiel, 1977) employing the semi-empirical QM chemistry MO package MOPAC93 (Stewart, 1993) originally established with PM3 parameters (Stewart, 1989, 1991). Two sets of coordinate data pertaining to the MT structure were used from X-ray diffraction (Robbins et al., 1991) and NMR [Schultze et al., 1988; Brookhaven Protein Data Bank (PDB)] studies. We reconstructed the metal-binding α- and β-clusters independently by first removing all amino acid residues, except for cysteines (Cys) which, on examination, coordinate specific metals (see Figure 1). The other atoms were restrained to their original positions to preserve the structures of these clusters. The hydrogen and oxygen atoms removed were then returned by QUANTA (version 3.3.1), followed by relaxation with CHARMM (version 2.2; Brooks et al., 1983). If a cysteine did not link with another cysteine in the sequence, it was considered to be an individual cysteine because it must bind metal ions via a mercaptide bond. For example, none of the nine cysteines in the β-domain form a peptide bond with another cysteine in their primary structure; thus the calculated cluster contains nine individual cysteines binding three metal ions. On the other hand, in the α-domain there are six cysteines (Cys33–Cys34, Cys36–Cys37 and Cys59–Cys60) which linked up in tandem pairs as dimers. These dimer peptide bonds are retained in the calculation so as to keep the original structural information. Hence the α-domain contains nine cysteine dimers, five individual cysteines and four metal ions. The MNDO calculations were carried out on the α- and β-clusters where only sulfur and metal atoms were optimized for fixed configuration. As a result, the calculated β-cluster comprises 120 atoms and the α-cluster 139 atoms.

The net charge for α- or β-clusters was −3. As each molecule of MT bound seven divalent cations, a total of 16 possible conformers for the α-cluster and eight for the β-cluster could be configured when all possible combinations of Zn(II) and Cd(II) at different sites were considered. We used dielectric constants of the solvents with the conductor-like screening model (COSMO) method (Klamt and Schurmann, 1993) to
approximate the effect of solvent inside the protein. The values that approximated the protein environment (ε = 1, 2, 3 and 40) were set with a solvent radius limited to 30 Å. It took ~100 min of computer processing time for each fixed configuration and ~300 min of processing time for each relaxed configuration on an RISC/6000 workstation. We obtained the final heat of formation of those structures from the output files, comparing different structures and relative stability with the same atom type in pairs. The heat of formation, ΔHf, obtained from MOPAC by MNDO contains four energy terms as shown in Equation 1:

$$\Delta H_f = E_{\text{elec}} + E_{\text{nuc}} + \sum_A E_{\text{isol}}(A) + \sum_A E_{\text{atom}}(A),$$

where $E_{\text{elec}}$ is the total electron energy, $E_{\text{nuc}}$ is the core-core repulsion energy, $E_{\text{isol}}(A)$ is the energy required to ionize valence electrons of the atoms involved and $E_{\text{atom}}(A)$ is the heat of atomization (Stewart, 1993).

**Results**

**β-Domain: the three-metal cluster**

Cd binding energy in the β-domain. The energy level of Cd at various positions is shown in Figure 2. For conformers in each cluster with an identical atom type, the one having the lowest energy value (obtained by MNDO based on X-ray or NMR data) is set to zero. From Figure 2A, it is apparent that the heat of formation for clusters with Cd at position M4 is lower than those with Cd occupying other positions, e.g. lower
The data shown in Figure 2B also indicate that the energy level of conformers with Zn at position M3 is the lowest and at position M4 the highest compared with conformers containing one Zn and two Cd. The heat of formation of the cluster with Zn at position M3 is lower than at other positions, such as M2, by at least 8.4 and 8.9 kcal/mol at ε = 1 and ε = 40, respectively. The highest energy conformer is the one in which Zn is located at position M4; its relative energy is higher than Zn at M3 by -24.1 kcal/mol at either ε = 1 or ε = 40.

Based on NMR structural data, a lower relative energy difference is observed (Figure 2D). The difference between M3 and M2 Zn conformers is only 1.7 kcal/mol at ε = 1 and 0.9 kcal/mol at ε = 40. The highest relative energy is observed with M4 Cd conformer, which is higher than M3 by 12.9 and 12.7 kcal/mol at ε = 1 and ε = 40, respectively.

**α-Domain: the four-metal cluster — metal binding energy in α-Domain metal binding sites**

A total of 16 conformers are possible when combinations of Cd and Zn at four metal binding sites are considered. The relative values obtained by MNDO for the heat of formation of the α-cluster are shown in Figure 3.

**One Cd/three Zn clusters.** Among four possible α-domain conformers, in which one Cd and three Zn are distributed over
four metal binding sites (M5, M6, M7 and M1) in various combinations, Cd at M5 shows the lowest energy — lower than M7, for instance, by at least 9.7 and 9.1 kcal/mol at $\varepsilon = 1$ and $\varepsilon = 40$, respectively, based on X-ray crystallographic data. The other two higher energy conformers are those with Zn located at either position M1 or M6, higher than M5 by $\sim 14.1$ and $13.3$ kcal/mol, respectively, at $\varepsilon = 1$, and by 12.4 kcal/mol for both at $\varepsilon = 40$ (Figure 3A).

Similar results are obtained in the NMR structures, but the energy level between M1 and M6 is different. The lowest energy is the same as that observed with Cd at position M5. The relative energy difference between M5 Cd and the next lowest energy state M7 Cd conformer is $\sim 4.4$ and 4.5 kcal/mol at $\varepsilon = 1$ and $\varepsilon = 40$, respectively. The other two higher energy conformers, Cd M1 and M6, show a relatively higher energy than M5 by $\sim 18.8$ and 16.2 kcal/mol, respectively, at $\varepsilon = 1$, and by $\sim 18.7$ and 16.6 kcal/mol, respectively, at $\varepsilon = 40$ (Figure 3D).

Figure 3A–F shows the relative energy difference between conformers containing one Cd and three Zn at various positions, with the lowest energy set as the zero point for each group of identical atom-type conformers based on either the X-ray or NMR structure.

**Two Cd/two Zn clusters.** Combinations of two Cd and two Zn located at different sites result in six conformers for the $\alpha$-metal binding cluster. The relative energy for the conformer with Cd at positions M5 and M7 is lower than that for the other five conformers by $\sim 1.5$ and 1.4 kcal/mol at $\varepsilon = 1$ and $\varepsilon = 40$, respectively. The other higher energy conformers are those in which Zn is located at positions M5/M6, M6/M7, M1/M7 and M1/M6. Their relative energy is higher than M5/M7 Zn by 8.9, 18.0, 18.7 and 23.4 kcal/mol at $\varepsilon = 1$ respectively, and by 7.8, 16.4, 17.9 and 21.5 kcal/mol at $\varepsilon = 40$ (Figure 3B).

Similar results are obtained in the NMR structures, but the energy level is different. The lowest energy is the same for M5/M7 Cd, but the relative energy difference between M5/M7 Cd and the next lowest energy state conformer M1/M5 Cd is $\sim 12.0$ and 10.6 kcal/mol at $\varepsilon = 1$ and $\varepsilon = 40$, respectively, in their X-ray structures. For the other four higher energy conformers in which Zn is located at position M5/M6, M6/M7, M1/M7 or M1/M6, their relative energies are higher than Zn at M5/M7 by 12.5, 15.5, 19.1 and 31.0 kcal/mol at $\varepsilon = 1$ respectively and by 11.8, 15.2, 17.4 and 28.5 kcal/mol at $\varepsilon = 40$ (Figure 3E).

Figure 3B and E show the relative energy difference between
conformers containing two Cd and two Zn at various positions, with the relative lowest energy of the conformers set to zero for each group of identical atom-type conformers, in the X-ray and NMR structures.

Three Cd/one Zn clusters. Four conformers of the α-clusters are possible because of different combinations of three Cd and one Zn occupying various sites. In their X-ray structures, the relative energy is lowest when one of the Zn is at position M6 (by at least 3.4 kcal/mol at ε = 1 and by 2.6 kcal/mol at ε = 40), compared with the next lowest energy conformer when Zn is at position M1. The other two higher energy conformers are those in which Zn is located at either position M7 or M5. Their relative energies are higher than those with Zn located at position M6 by ~7.2 and 22.2 kcal/mol for M7 and M5, respectively, at ε = 1 and by ~6.4 and 20.7 kcal/mol for M7 and M5, respectively, at ε = 40 (Figure 3C).

Similar results are obtained in the NMR structures, but the energy level between M1 and M6 is different. The lowest energy is observed with the conformer having Zn at position M6, which is ~1.1 and ~6.4 kcal/mol, respectively, at ε = 1 and ε = 40. The other two higher energy conformers in which Zn is located at either position M7 or M5 show a higher relative energy than M1 by ~13.2 and 18.4 kcal/mol, respectively, at ε = 1 and by 12.0 and 16.7 kcal/mol, respectively, at ε = 40. The relative energy

Table I. R.m.s. deviation of residue movement

<table>
<thead>
<tr>
<th>Residue</th>
<th>Cd4</th>
<th>Cd3Zn(1)</th>
<th>Cd3Zn(5)</th>
<th>Cd3Zn(6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys33</td>
<td>1.12</td>
<td>1.05</td>
<td>1.30</td>
<td>1.21</td>
</tr>
<tr>
<td>Cys34</td>
<td>0.94</td>
<td>0.81</td>
<td>1.15</td>
<td>0.79</td>
</tr>
<tr>
<td>Cys36</td>
<td>0.98</td>
<td>1.13</td>
<td>1.11</td>
<td>1.25</td>
</tr>
<tr>
<td>Cys37</td>
<td>0.87</td>
<td>0.62</td>
<td>1.19</td>
<td>0.83</td>
</tr>
<tr>
<td>Cys41</td>
<td>1.11</td>
<td>0.86</td>
<td>1.00</td>
<td>0.90</td>
</tr>
<tr>
<td>Cys44</td>
<td>0.92</td>
<td>0.92</td>
<td>0.99</td>
<td>0.77</td>
</tr>
<tr>
<td>Cys48</td>
<td>1.21</td>
<td>1.45</td>
<td>1.32</td>
<td>1.45</td>
</tr>
<tr>
<td>Cys50</td>
<td>1.36</td>
<td>1.14</td>
<td>1.54</td>
<td>1.73</td>
</tr>
<tr>
<td>Cys57</td>
<td>1.48</td>
<td>1.21</td>
<td>1.49</td>
<td>1.52</td>
</tr>
<tr>
<td>Cys59</td>
<td>1.19</td>
<td>1.21</td>
<td>0.86</td>
<td>0.96</td>
</tr>
<tr>
<td>Cys60</td>
<td>0.88</td>
<td>1.03</td>
<td>1.07</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Fig. 3. Comparison of the relative energies of different MT conformers containing the same species and number of metal ions at various sites of the α-domain. (A–C) X-ray structure. (A) The relative energy difference for clusters containing three Zn and one Cd. (B) The relative energy difference for clusters containing two Zn and two Cd. (C) The relative energy difference for clusters containing one Zn and three Cd. (D–F) NMR structure. (D) The relative energy difference for clusters containing three Zn and one Cd. (E) The relative energy difference for clusters containing two Zn and two Cd. (F) The relative energy difference for clusters containing one Zn and three Cd. ( ) ε = 1; ( ) ε = 2; ( ) ε = 3; ( ) ε = 40.
The energy change for conformers with different numbers of Cd was calculated using the COSMO method and approximating the effect of solvent inside the protein. Although the variant dielectric constant must cause different levels of heat of formation for each conformer, the relative energy difference tendency between conformers is observed to be the same. As shown in Figures 2 and 3, the energies for different conformers are comparable when different dielectric constants are followed. For example, based on X-ray crystallographic data, the heats of formation for the β-cluster conformers containing one Cd and two Zn are as follows: -663.4, -770.0, -816.8 and -926.1 kcal/mol at ε = 1, 2, 3 and 40, respectively, with Cd located at position M2; -656.7, -726.9, -809.5 and -918.5 kcal/mol at ε = 1, 2, 3 and 40, respectively, with Cd located at position M3; and -682.3, -788.6, -835.2 and -944.3 kcal/mol at ε = 1, 2, 3 and 40, respectively, with Cd located at position M4. The relative energy differences between conformers M4 and M2 Cd are 18.9, 18.6, 18.4 and 18.2 kcal/mol at ε = 1, 2, 3 and 40, respectively, whereas the relative energy differences between conformers M4 and M3 Cd are 25.6, 25.7, 25.7 and 25.8 kcal/mol at ε = 1, 2, 3 and 40, respectively.

Relaxed cluster

Of the two clusters α and β, only with the former did we obtain convergent results. As mentioned earlier, there are 16 possible conformers in the α-cluster because of various combinations of Cd and Zn binding. Results show that most of the residues move towards the metal sites and some shift more in distance when relaxed (Table I and Figure 4). Based on an r.m.s. deviation analysis, a stronger movement can be seen to occur with Cys50, Cys48 and Cys57 than the other Cys residues, with their r.m.s. deviations being 1.36, 1.21 and 1.48 Å, respectively. When the α-cluster is fully occupied by Cd, the r.m.s. deviations for the residues above are 2.07, 1.40 and 1.21 Å, respectively. The average movement of all-Cd α-clusters is smaller than that of all-Zn α-clusters. The average r.m.s. deviation difference between all-Cd and all-Zn conformers is ~0.06. The average movement for other conformers lies between 1.02 and 1.19 Å.

Discussion

It is apparent that different conformers of MT may exist because of differences in the number of Cd and Zn atoms bound. Each conformer requires a different level of energy for stabilization. It has not been apparent, however, until this study, that different energy levels are required for a given group of conformers that carry the same number of Cd and Zn ions because these metals may occupy different sites.

It is well known that among similar macromolecules those with the lowest energy level will be the most stable by comparison. Conversely, high energy reflects relatively high instability or reactivity. Conformers of the same atom type with a low energy level are shown by a difference in the site of metal binding; some are more stable than others. Our results show that conformers with Cd at position M4 of the β-cluster have the lowest energy level of all conformers, suggesting that Cd at that position is more stable than at other sites (Figure 2A). The preference of Cd for position M4 has been shown by X-ray structural analyses, which have revealed two Zn and one Cd in the β-cluster for Cd-induced MT. It is interesting to note from Figure 2B that, in contrast to Cd, Zn is more stable at position M3 than at position M4. This result can be realized from the energy of various metal clusters, e.g. Cd(M2)–Cd(M3)–Zn(M4) is considerably higher than those of the other two possible conformers. Thus, one would predict that when there are two Zn(II) in the β-cluster they would preferentially occupy positions M3 and M2. This prediction is consistent with the observation made with rat liver MT by X-ray crystallographic diffraction analysis (Robbins et al., 1991). By this calculation, one may also predict that Cd at position M3 is unstable because the energy level of the conformer with M3 Cd is higher than that of the other conformers of the same atom type. This is indeed observed in 111Cd and 113Cd NMR spectral analyses (Kägi and Vallee, 1961; Rupp and Weser, 1978; Boulanger et al., 1982; Nielsen et al., 1985; Otvos et al., 1989; Gan et al., 1995).

Our results are consistent with those obtained with the 111Cd NMR experiments (Otvos et al., 1980) in which the exchange rates of Cd(II) between different metal-binding sites in the β-cluster were measured. The experiments showed that Cd(II)

Fig. 5. A proposed metal exchange reaction path of Cd and Zn in MT. Three of the possible four conformers, namely Zn3, Zn2Cd1 and Cd3, have been shown to exist in MT β-domain. The Zn3 conformer was obvious in Zn7-MT and the Cd3 conformer was deduced by Cd titration (Stillman et al., 1987). The Zn2Cd1 conformer was established by observation with X-ray crystallography (Robbins et al., 1991).
exchange between M2 and M3 sites was quite fast, while the exchange rates between M2 and M4, and M3 and M4, are considerably slower (Otvos et al., 1993). The α-domain of MT has generally been proposed to play a role in metal storage, while the β-domain may be more actively involved in metal exchange.

The relative stability for Zn and Cd in the α-cluster also varies with the sites they bind, as is the case with the β-cluster. Of the four metal binding sites available in the β-cluster, it has been difficult for us to determine which one is more favourable for Cd or Zn. Calculations based on X-ray and NMR structures have shown the same level of energy for positions M5 and M7, which differs from M1 and M6. However, these relative differences are minor, suggesting that binding at these sites would result in almost the same order of stability.

In previous studies, Otvos et al. (1989) proposed that intramolecular metal exchange could take place between two β-domain clusters. Based on our results, we predict that the order of metal replacement from Zn(II) to Cd(II) between β-domain clusters is in the order M4 > M2 > M3. This reaction flows along a path with several intermediate states, as if metal exchange follows an avenue towards the lowest energy state (Figure 5). Although the effect of solvation energy was large when the calculation was made under different dielectric constants with the COSMO method, the energy level tendency at different dielectric constants was the same under a given specific condition. Hence, the solvation effect does not perturb the relative reaction path. Our calculations also indicate that Cd at position M5 is more stable with a low relative energy at ~10 kcal/mol, with the next most stable position being M7. Thus it can be deduced that the order of stability for Cd binding is M5 > M7 > M1 or M6 in the α-cluster. In contrast, selective stability for Zn binding at various sites within the α-domain is arbitrary.

We find that the relative energies obtained from NMR are similar to those from X-ray structural analysis for the α-cluster but are lower for the β-cluster. The order of metal change is, however, similar. It is possible that the β-domain is more flexible in the NMR structure. Studies with 113Cd NMR have revealed that the chemical shift signal of Cd at position M3 is broad, and at times undetectable under perturbed conditions, implying that Cd at position M3 is relatively unstable (Otvos et al., 1989).

The net energy difference among the conformers with different dielectric constants was not apparent. One reason may be that the conformers exposed to the solvent are similar in that the metals are deeply bound inside the peptide cluster and thus little affected by their solvent environment.

In our MNDO calculation, the MT structure was fixed for its amino acid backbone so as to preserve the microenvironmental constraints for metal binding. Each of the atoms was also relaxed to monitor if the fixed structures were necessary. The results showed that when relaxed, thus removing all of the constraints for specific metal stability, residue Cys50 shifted the most in distance relative to the original metal positions (Table I and Figure 4). This implies that residues adjacent to the metal-connecting residues may provide the constraint on which selectivity and stability is based. The residue moving the furthest distance may play an important structural role during the metal binding process.

X-ray crystallographic and NMR observations suggest that the β-domain is more flexible than the α-domain, as supported by our computation in this study. Our previous studies with site-directed mutagenesis have shown that Cys50 in MT is not among the nonconsequential Cys residues, such as Cys7, that can be readily substituted with an apparent effect on detoxification function. Replacement of Cys50 by Tyr50 or Ser50 resulted in collapse of the β-domain, as demonstrated by an NMR spectral analysis (Chemaik and Huang, 1991; Cismowski and Huang, 1991). The difference between averaged r.m.s. deviations for α-clusters carrying either all-Cd or all-Zn also offers a mechanistic explanation for an earlier observation by Kagi and Vallee (1961) that the binding of Cd was stronger than Zn in the native MT.

The relative heat of formation between different conformers has provided hints with which to interpret results from our calculations using the QM/MM hybrid is representative of the calculation of the total region. A hybridized computational simulation contains both the QM and MM considerations and has been used extensively in the simulation of enzyme mechanisms (Warshel and Levitt, 1976; Field et al., 1990; Åqvist and Warshel, 1993; Stanton et al., 1995; Hwang and Pan, 1995). However, most of the regional QM analyses involved only a few atoms so as to afford the calculation time. The MM regime is therefore valuable because it provides additional structural information. In our study, we have applied the QM semi-empirical calculation and designed regions that suitably mimic the whole MT structure. We have been able to show that by fixing the amino acid backbone, such a calculation can yield information consistent with that predicted of the domains.

Acknowledgements
We thank Professor J.-K. Hwang and Dr Charles Brooks for valuable comments, and the National Center of High-performance Computing for the use of their facilities. P.C.H. is on leave from the Johns Hopkins University. This work was supported in part by grants NSC 84-2311-B007-021 and 85-2113-M007-032 from the National Research Council, Republic of China.

References

1171
C.-C. Chang and P. C. Huang


Received March 8, 1996; revised July 29, 1996; accepted August 16, 1996