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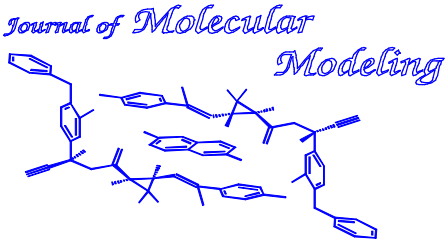

Computational Analysis of cysteine substitutions modelled on the α- and β-domains of Cd_5,Zn_2-Metallothionein 2

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Abstract

Several mutant forms of rat liver Cd_5,Zn_2-metallothionein 2 (Cd_5,Zn_2-MT 2) [1] have been computationally modelled and analysed. All terminal cysteines (5, 13, 19, 21, 26, 29, 33, 36, 41, 48, 57 and 59, Figure 1) have been independently substituted by three other co-ordinating amino-acids (aspartate, glutamate and histidine), and the side-chains of the mutated residues have been modelled to co-ordinate the seven metal ions while minimizing the conformational variations with respect to the wild type protein. We have compared the ability of the putative mutant forms to maintain the MT binding properties. Substitution by aspartate residue best preserves the 3D MT structure. In addition, the mutations C5H plus C21H/E/D show neighbouring impairments that prevent their simultaneous substitution. Although replacement of cysteine by aspartate is feasible in all cases, to our knowledge there is no example of aspartate and cysteine residues co-ordinating to the same zinc atom. Accordingly, the use of histidine or glutamate instead of aspartate cannot be ruled out. The mutant forms in the β-domain of Cd_5,Zn_2-MT 2 have yielded more neighbouring contacts than those in the α-domain, which is corroborated by the accessible surface areas [2] of the sulfur atoms [3] in the native form.

Keywords: Protein modeling, metalloprotein, metallothionein
Abbreviations: MT = metallothionein; Cd_5,Zn_2-MT = Cadmium, Zinc-metallothionein, RMSD = Root Mean Square Deviation; PDB = Protein Data Bank; FEP = Free Energy Perturbation; CnX = mutant form of cysteine n (n = residue number) substituted by X (X = H, E or D, with H = histidine, E = glutamate, D = aspartate); CnX/Y = mutant forms CnX and CnY.

Introduction

Our current interest in metallothioneins (MT) and their α- and β- fragments [3, 4] stimulated us to study the possibility of substituting some cysteine residues to avoid the oxidation problems associated with the apo forms while maintaining the binding abilities of these proteins. Previous studies have either substituted the bridging [5, 6] or terminal cysteines [5, 6, 7] by low co-ordinating residues such as Ser, Tyr or Ala. These mutations altered domain structure by disrupting the normally tight protein clusters.

With the purpose of affecting in a lesser degree the 3D-structure of MT we have only chosen the terminal cysteines

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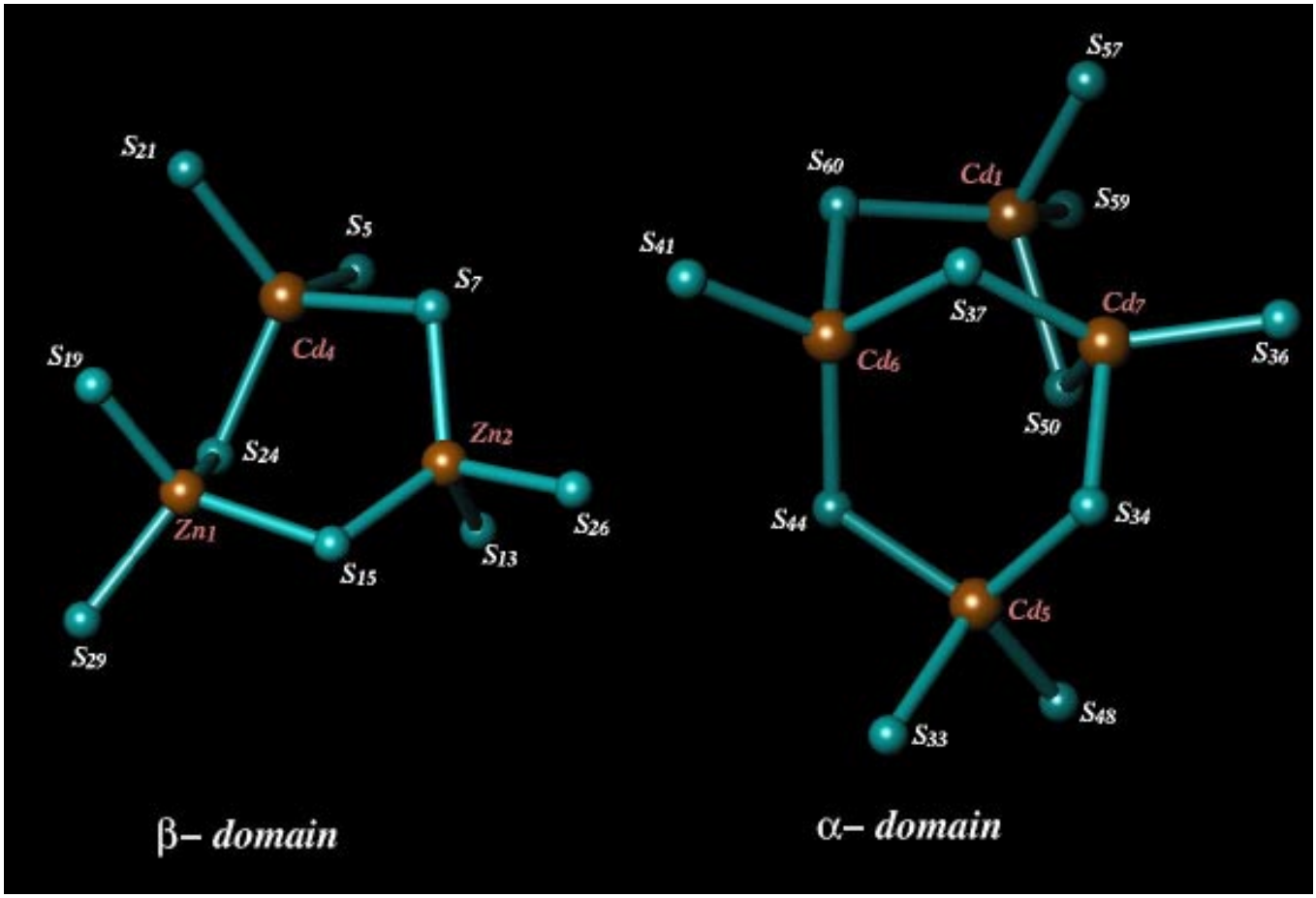

Figure 1. *α and β clusters that make up the two-domain structure found for rat liver Cd_5,Zn_2-MT 2. Data taken from [1].*

as possible residues to be substituted. Among the 20 natural amino-acids, the residues with significant binding capacity are cysteine, histidine, aspartic and glutamic [8]. Combinations of these residues constitute the most common co-ordination sites in metallobiomolecules of known crystallographic structure [9] and particularly in Zn metalloproteins [10]. According to these data, we have studied the effect of replacing terminal Cys residues by His, Asp and Glu by means of a molecular modelling approach based on the X-ray structure of rat liver Cd_5,Zn_2-MT 2.

Modelling the positions of side-chains, given that of the backbone, is difficult. Nevertheless, it has been tackled by different authors [11 , 12 , 13] who have succeeded with relatively high accuracy (80%) [14]. To overcome this difficulty, the simplest energy functions, van der Waals and Lennard Jones potentials, have given excellent results for buried residues [15]. However, these simple approaches have failed for totally or partially exposed residues, for which it is necessary to add empirical solvation terms [16 , 17]. Other procedures emphasize an optimal choice of conformation for a particular side-chain by local energy analysis [18], consideration of local backbone conformation [19] or evaluation of neighbouring 3D homology [20]. The weakest point of most side-chain packing methods is the effect of main chain displacements [21].

The ability to co-ordinate metal ions by a wild type protein or any of its putative mutant types requires the study of the conformational space of the apoprotein in a solvated environment in the presence of the metal ions. In accordance with the aim of this study, the co-ordination of the metal ions by MT was assayed by a Molecular Dynamics simulation of the apoprotein solvated with water molecules and Zn^{2+} ions. This did not lead to satisfactory results. The Cd_5,Zn_2-MT 2 structure is due to the co-ordination of the metal ions to the protein, which involves folding. Molecular Dynamics simulation of this system requires too much time to reach the adequate folding. Hence, given the difficulty associated with this method we decided to carry out the replacement of the Cys residues and corresponding structural analysis without including energetic terms. Thus, it was assumed that the mutant types reproduce the binding abilities of native mammalian MTs. The previous assumption entails the local displacement of the side-chain by application of a geometrical refinement with simple energy functions in order to assess the correct geometry. To analyse the ability of the mutant forms to preserve the native structure, individual substitutions of each terminal Cys by Asp, Glu or His were consid-

Table 1. *Statistical analysis of the Zn(II) co-ordination in a set of proteins at high resolution (<2.5Å) from the PDB. Glutamate is found to coordinate to the metal ions either by means of one or both carboxylic oxygen atoms, whilst aspartate coordinates only with one.*

	$Zn\text{-}O_1$ Distance (Å)		$Zn\text{-}O_2$ Distance (Å)		Zn-N Distance (Å)	
	Average	**Range**	**Average**	**Range**	**Average**	**Range**
Glu [u]	2.04	1.91-2.17	2.82	2.60-2.94	—	—
Glu [b]	2.15	2.07-2.18	2.30	2.12-2.48	—	—
Asp [u]	2.00	1.91-2.08	3.30	2.96-3.47	—	—
His	—	—	—	—	2.07	1.91-2.17

[u] unidentate ligand
[b] bidentate ligand

Table 2. *Analysis of the single substitutions of terminal cysteine residues by histidine, glutamate and aspartate in the β-domain of rat liver Cd_5,Zn_2-MT 2.*
*The analysis of the single replacements is based on the conformational variations of the putative mutant form with respect to the native type. The bond distance variation, Δ*d*, corresponds to the difference |ML - MS|, where ML and MS represent the distances between the metal ion and the donor atom (L= N or O). Δτ = angle (LMS), is the bond angle variation.* RMSD*, denotes the root mean square deviation of the backbone atoms of the mutant type with respect to the wild type.* Neighbours*, represents the number of atoms at a distance of less than 3 Å from any of the atoms of the substituted residue in the putative mutant form.*

		Cys 5	Cys 13	Cys 19	Cys 21	Cys 26	Cys 29
	Δd (Å)	0.22	0.22	0.14	0.50	0.47	0.26
	Δτ (deg)	14.9	15.4	26.2	23.7	32.0	7.7
His	RMSD (Å)	[a]	0.06	0.03	0.01	0.00	0.09
	Neighbours	2	1	5	3	4	3
	Δd (Å)	0.13	0.10	0.09-0.19	0.01	0.50	0.01
	Δτ (deg)	16.4	0.6	[b]	5.6	21.0	7.4
Glu	RMSD (Å)	[a]	0.17	0.15	0.17	0.02	0.04
	Neighbours	3	2	4	0	0	1
	Δd(Å)	0.71	0.24	0.00	0.08-0.06	0.15-0.06	0.04
	Δτ (deg)	24.0	1.2	5.8	[b]	[b]	2.4
Asp	RMSD(Å)	[a]	0.16	0.19	0.00	0.00	0.04
	Neighbours	0	0	3	2	1	0

[a] RMSD values for the mutant forms of Cys 5 are not considered because the large flexibility of the N-terminal tail of the protein.

[b] Mutation of Cys by Asp or Glu with both carboxylic oxygen atoms co-ordinating the metal ion.

ered first and then the effect of combined substitutions was taken into account. The best residue is that which introduces fewest steric hindrances and thus causes the least rearrangement of the backbone while maintaining the co-ordination of the metal ions.

A Free Energy Perturbation study (FEP) would be advantageous for evaluating the goodness of the model [22]. However, this is a highly time consuming process and would also involve a previous step of Quantum Mechanics Optimisation of the metal co-ordination in order to obtain the correct forcefield of the system. Finally, the same hypothesis, which assumes that the putative mutant form is able to co-ordinate to the metal ions with the same conformational arrangement as that found in Cd_5,Zn_2-MT 2, should have been taken into account in the FEP calculation. Therefore, we did not consider the calculation of the free energy strictly necessary and

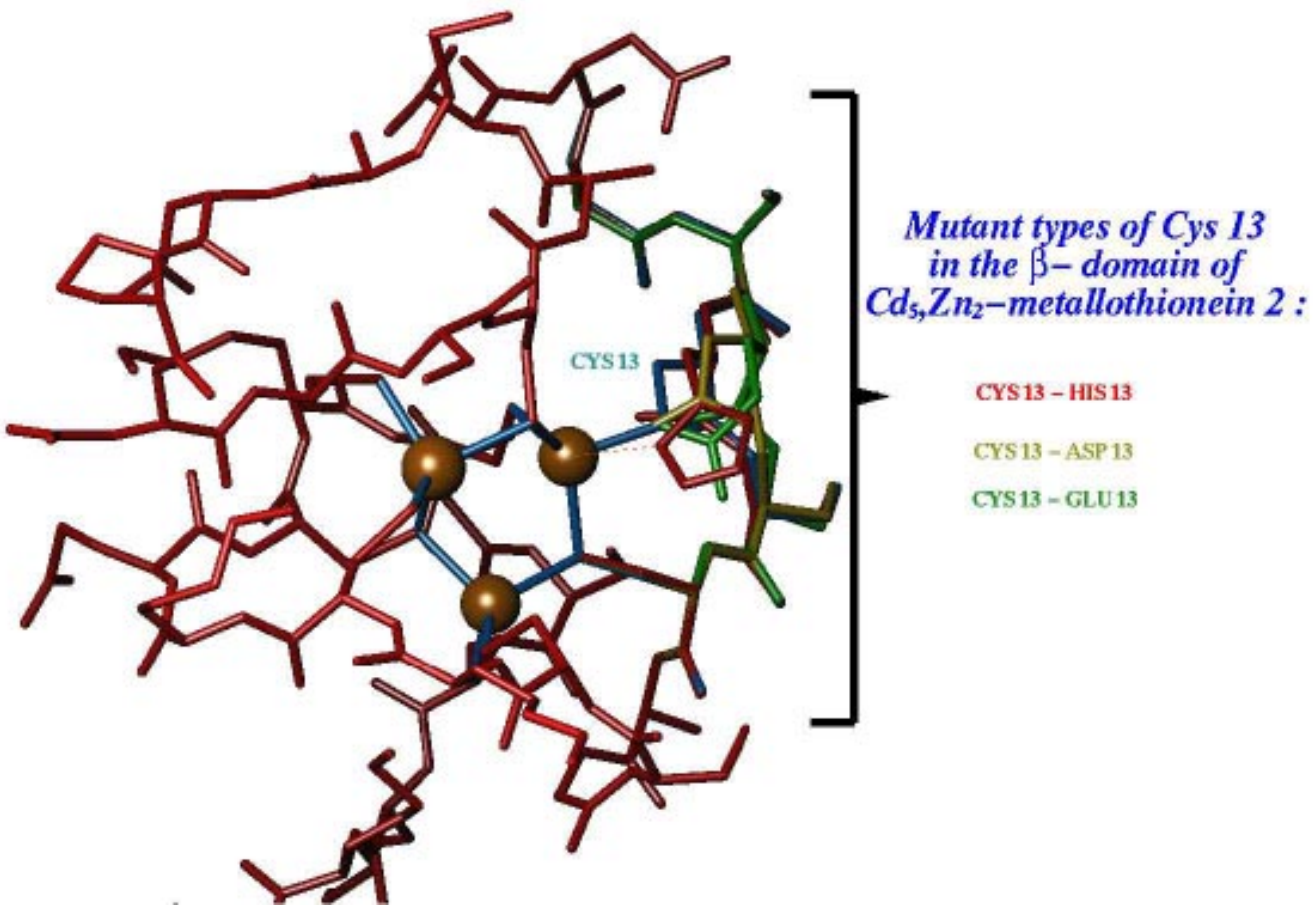

Figure 2. *Mutant types C13H (red), C13D (yellow), and C13E (green) are compared to the wild type form of Cd_5,Zn_2-metallothionein 2. Zn(II) and Cd(II) metal ions are depicted as compact spheres (orange) and their bonds to the protein in blue. The deviation of the imidazol ring of His 13 with respect to the position of the native cysteine initially bound to Zn is identified by a dashed orange line.*

we limited the analysis of the mutant forms to the study of their structural impairments.

Methods

Several mutations were performed computationally upon the α- and β-domain of the X-ray crystallographic structure of rat liver Cd_5,Zn_2-metallothionein 2 [1]. All terminal cysteines (5, 13, 19, 21, 26, 29, 33, 36, 41, 48, 57 and 59) were replaced by aspartate, glutamate or histidine, independently. The substitution was performed with the program TURBO-FRODO [23] on a Silicon Graphics workstation. The cysteine residues were subsequently replaced by the residues chosen and the backbone conformation was maintained. Meanwhile, the side-chains were rotated to approach the donor atom of the residue to the corresponding metal ion (Zn or Cd). A window of five residues centred on the substituted one was geometrically optimised to avoid conformational deformations. In most substitutions it was considered that aspartate and glutamate behaved as unidentate ligands. However, they can also bind to the metal centre *via* both carboxylic oxygen atoms causing an increase in the co-ordination number of the metal. This was only presumed when the unidentate behaviour led to significant distortions in the structure.

Each mutation was analysed in terms of variations in the native conformation and in the co-ordination geometry about the metal ion, which were estimated by the following differences between the native and the putative mutant forms: 1) metal ion-donor atom bond distance; 2) angle with vertex on the metal ion formed by the donor atom of the mutated residue (O_δ for aspartate, O_ε for glutamate, and N_ε for histidine) and the sulfur atom of the cysteine of the native form; 3) backbone conformation, calculated by the RMSD [24] of the protein backbone; and 4) steric hindrances reckoned by the number of atoms in the neighbourhood of the mutated residue (less than 3 Å distance) impairing the conformational stabilisation.

Table 3. *Variation of the neighbouring contacts for combined substitutions of terminal cysteines in the β-domain of rat liver Cd_5,Zn_2-MT 2.*
Number of new contacts at a distance shorter than 3 Å obtained by the combined replacements of two cysteines with respect to their independent single substitution effect. The total number of contacts is obtained by adding those of Table 2 for the putative mutation considered.

	His 21	Glu 21	Asp 21	His 29	Glu 29	Asp 29	His 26	Glu 26	Asp 26
His 5	16	15	11	0	0	0	0	0	0
Glu 5	-1	0	0	0	0	0	0	0	0
Asp 5	1	1	1	0	0	0	0	0	0
His 19	0	0	0	2	0	0	0	0	0
Glu 19	0	0	0	1	0	0	0	0	0
Asp 19	0	0	0	0	0	0	0	0	0
His 13	0	0	0	0	0	0	-2	2	0
Glu 13	0	0	0	0	0	0	-2	2	0
Asp 13	0	0	0	0	0	0	-1	0	0

Table 4. *Analysis of the single substitutions of terminal cysteine residues by histidine, glutamate and aspartate in the α-domain of rat liver Cd_5,Zn_2-MT 2.*
*The analysis of the single replacement is based on the conformational variations of the putative mutant form with respect to the native type. The bond distance variation, Δ*d*, corresponds to the difference |ML - MS|, where ML and MS represent the distances between the metal ion and the donor atom (L= N or O). Δτ = angle (LMS), is the bond angle variation.* RMSD, *denotes the root mean square deviation of the backbone atoms of the mutant type with respect to the wild type.* Neighbours, *represents the number of atoms at a distance of less than 3 Å from any of the atoms of the substituted residue in the putative mutant form.*

		Cys 33	Cys 36	Cys 41	Cys 48	Cys 57	Cys 59
	Δd (Å)	0.16	0.05	0.22	0.14	0.09	0.07
	Δτ (deg)	6.2	3.2	1.0	4.1	2.5	4.8
His	RMSD (Å)	0.02	0.19	0.22	0.08	0.13	0.04
	Neighbours	0	1	0	0	1	0
	Δd (Å)	0.17	0.03	0.17	0.01	0.05	0.19
	Δτ (deg)	8.2	4.2	1.2	4.9	0.9	3.4
Glu	RMSD (Å)	0.07	0.05	0.16	0.11	0.20	0.07
	Neighbours	0	0	0	3	2	0
	Δd (Å)	0.22	0.25	0.10	0.09	0.23	0.10
	Δτ (deg)	10.5	1.0	0.6	1.5	3.2	1.3
Asp	RMSD (Å)	0.03	0.04	0.15	0.06	0.15	0.04
	Neighbours	0	0	0	0	1	0

Results and Discussion

The zinc-protein structures at high resolution (< 2.5 Å) found in the PDB [25] were used to check the metal co-ordination by histidines, glutamates and aspartates. 24 binding sites were selected and analysed statistically. 67% were constituted by zinc ions tetrahedrally co-ordinated by one glutamate, two histidines and either a water molecule or a carbonyl oxygen belonging to the protein backbone. Consideration of the Zn-O distances less than 2.5 Å (Table 1) allowed division of the previous percentage in two groups. Accordingly, in 21% of the cases the glutamate co-ordinated by means of the two carboxylic oxygen atoms while in the remaining 46% it behaved as an unidentate ligand. Additionally, in 17% of the

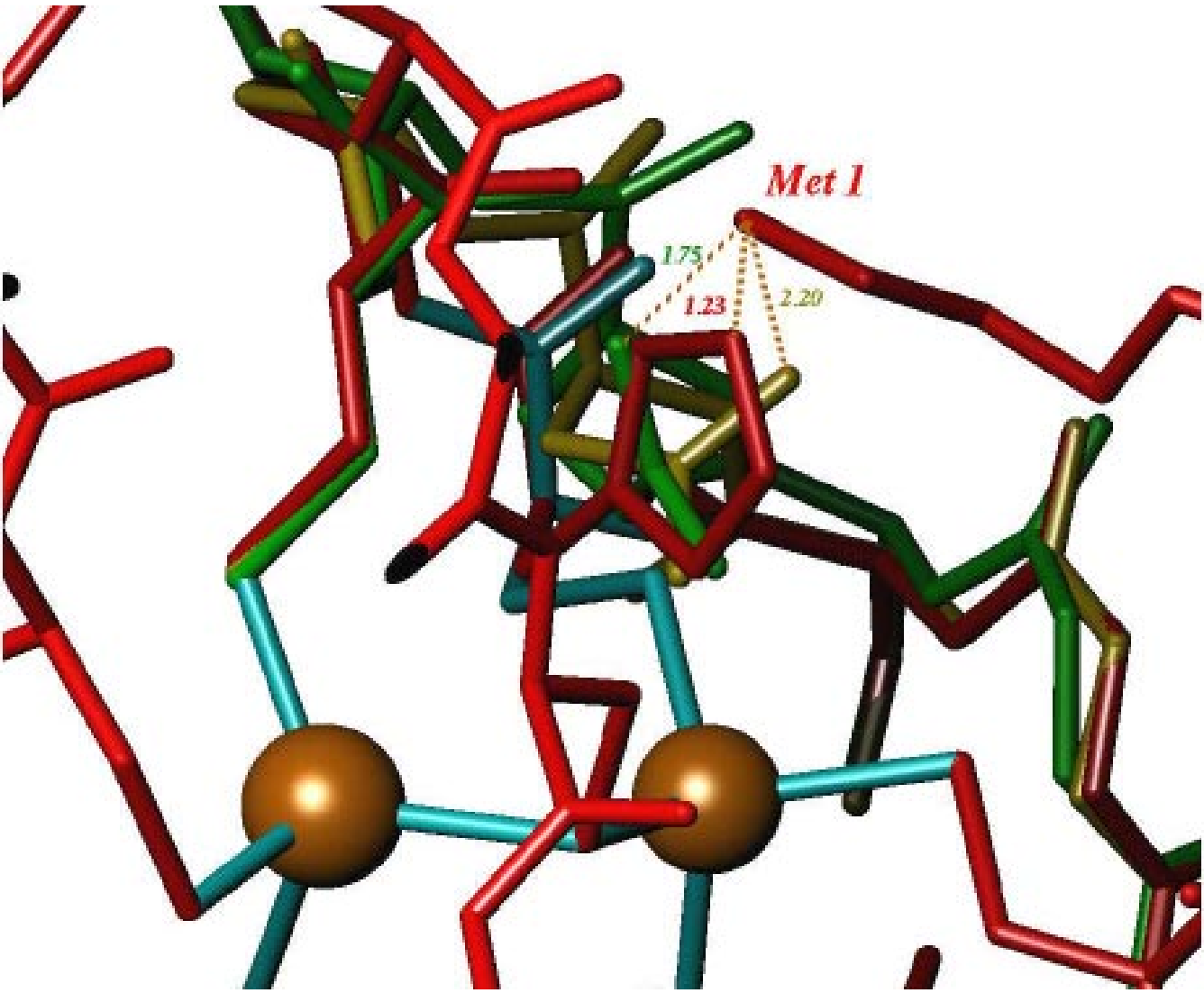

Figure 3. *Mutant types C19H (red), C19D (yellow), and C19E (green) are shown and compared to the wild type form of Cd_5,Zn_2-metallothionein 2. Zn(II) and Cd(II) metal ions are depicted as compact spheres (orange) and their bonds to the protein in blue. Closest distances between neighbouring residues are depicted (His19-Met1: 1.23; Glu19-Met1: 1.75; Asp19-Met1: 2.20).*

total examples found, the binding sites were constituted by three histidines and one aspartate, where the latter co-ordinated only by means of one oxygen. The lower andhigher limits found for the distances between the metal ion and the donor atom (Table 1) were used to evaluate the co-ordination capabilities of the mutant forms.

Analysis of the single substitutions in the β-domain

Results on the single replacements of terminal cysteines in the β-domain of Cd_5,Zn_2-MT 2 are summarized in Table 2. The RMSD [24] of the mutant forms C5D, C5E and C5H were discarded because this residue is in the N-terminal region, where there is a large conformational flexibility of the backbone. However, for the rest of substitutions the maintenance of the backbone conformation with respect to its native form cannot be neglected. Large changes on the conformation would affect the properties of the protein and therefore must be avoided (conformers deviating about 2.4 Å may preserve only 85% of the native contacts occurring between residues located at 8 Å [26]). Nevertheless, none of the studied replacements involved an RMSD value [24] greater than 0.20 Å.

The steric hindrances between the replaced side-chains and the rest of the protein were significant in number for the substitution by glutamate and histidine because of their volume. Consideration of mutants of the β-domain showed that cysteine 13 allows the greater space to accommodate the side-chain of the new residue (Figure 2). This result agrees with the accessible surface area of the sulfur atom of cysteine 13 in the native form (20 $Å^2$), which has the largest area of all

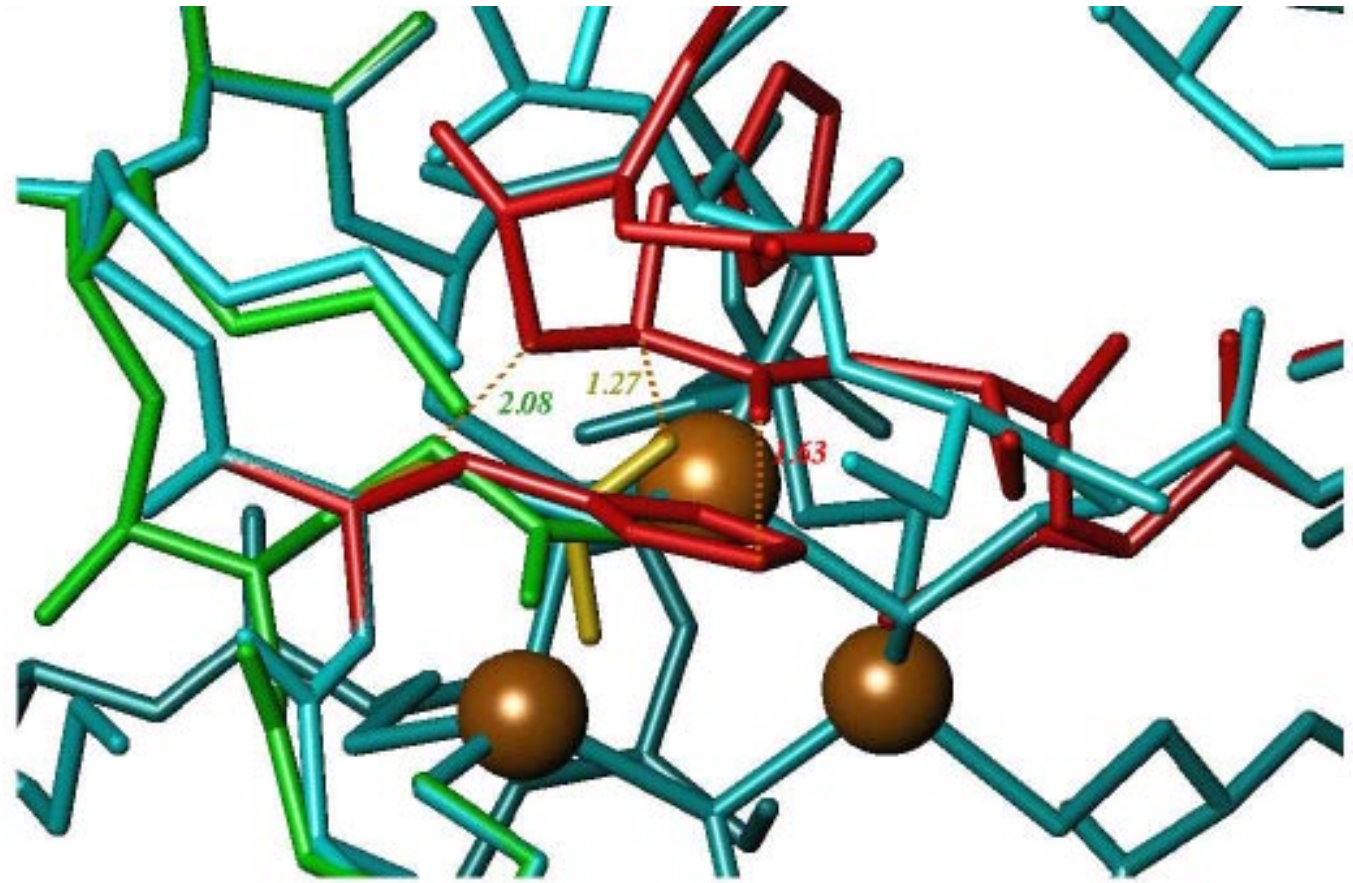

Figure 4. *Interaction between side-chains of the mutant form of C5H and the mutant forms of C21H/E/D (C5H and C21H in red, C21E in green and C21D in yellow). The closest neighbouring contacts are shown. Zn(II) and Cd(II) metal ions are depicted as compact spheres (orange) and their bonds to the protein in blue.*

the S-Cys atoms in the β-domain [3]. The substitution of the remaining cysteines (5, 19, 21, 26 and 29) by His, Asp or Glu produced several neighbouring contacts. In these cases, the percentage of accessible surface areas of the S-Cys atoms in the native form is less than 10%. Replacement of cysteine 19 (Figure 3), which has the lowest sulfur accessible surface area, led to the largest number of neighbouring collisions (a total of 12 contacts) and therefore constitutes the least favoured cysteine to be substituted.

Analysis of combined substitutions in the β-domain

It was assumed that only those residues that co-ordinate the same metal ion (Cys5-Cys21; Cys19-Cys29, and Cys13-Cys26, Figure 1) show a co-operative effect when substituted and thus this effect was disregarded for long range interactions. Table 3 gives the variation of contacts as a consequence of the combined substitutions of these pairs of cysteines, taking as reference the independent single replacement effect. In those cases where two or more Cys residues were co-ordinated to different metal ions, the distances between the residues were long enough in 3D space so that the final result of the substitution could be taken as the addition of their independent effects.

From Table 3 and Figure 4 it can be inferred that the mutations C5H plus C21H/E/D give rise to a high number of contacts and are therefore forbidden. Except for these cases, the rest of combined substitutions could still be accepted as plausible forms for Zn or Cd binding. Note that the combined substitution of cysteines may lead to a decrease in the number of neighbouring contacts. This is the case for mutations C26H plus C13H/E/D (Figure 5).

Experimental studies on the metal binding properties of several β-MT related peptides, where some Cys residues have been substituted by His or Asp, are now in progress [27]. Preliminary results indicate that Co(II), Zn(II) and Cd(II) co-ordinate tetrahedrally to $S_2(Cys)N_2(His)$ binding sites. In contrast, co-ordination is not observed when Cys and Asp ligands are simultaneously present and chemically able to participate in metal binding.

Analysis of the substitutions in the a-domain

Analysis of the single replacements in the α-domain is summarized in Table 4. According to the RMSD values [24], the substitutions of cysteines 36 and 41 by histidine and cysteine

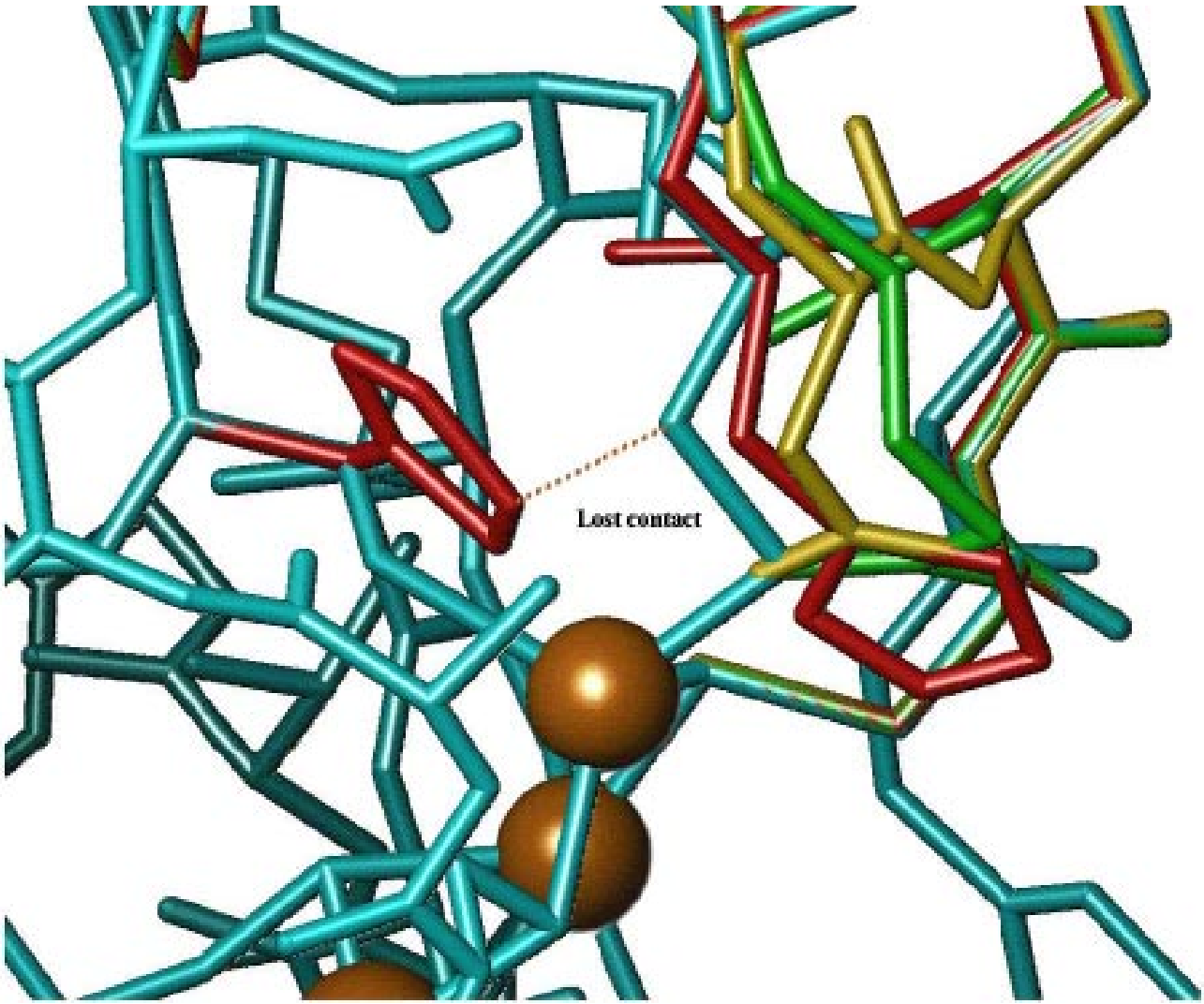

Figure 5. *Interaction between side-chains of the mutant form of C26H and the mutant forms of C13H/E/D (C26H and C13H in red, C13E in green and C13D in yellow). The closest neighbouring contacts are shown. Zn(II) and Cd(II) metal ions are depicted as compact spheres (orange) and their bonds to the protein in blue.*

57 by glutamate gave rise to the greatest changes in the backbone. However, they are smaller than 0.22 Å, and thus any considered substitution does not cause significant changes in the backbone conformation. In addition, the geometrical restraints imposed by the co-ordination of the metal ions are fully accomplished by all single substitutions.

The neighbouring contacts found for each mutant type agrees with calculations for the sulfur accessible surface areas for cysteines 33, 36, 41, 57 and 59 [3]. Cysteine 57 shows the smallest sulfur accessible surface area on the α-domain (3.45 $Å^2$), while cysteines 33, 41 and 59 present larger accessible surface areas than those found in the β-domain (about 20 $Å^2$). Consequently, the substitution of cysteine 57 by histidine, glutamate or aspartate gives rise to steric hindrances to accommodate the replaced side chain in the protein (Figure 6).

In order to assess whether the steric effects could preclude Zn_4-αMT cluster formation, the C57H mutant form of mouse α-MT fragment was cloned in *E. coli* and the peptide was recovered from cell cultures. Preliminary circular dichroism studies on metal binding indicate that Zn_4 and Cd_4 cluster formation occurs, probably with a similar architecture to that found for the native domain [28].

If considering simultaneous substitutions, only two pairs of terminal cysteines in the α-domain are linked to the same metal ion (Figure 1). The only mutant type with co-operative effect, unless long distance interactions were considered, was found for the substitution of cysteine 57 by aspartate and cysteine 59 by glutamate appearing an additional contact in the neighbourhood of aspartate 57.

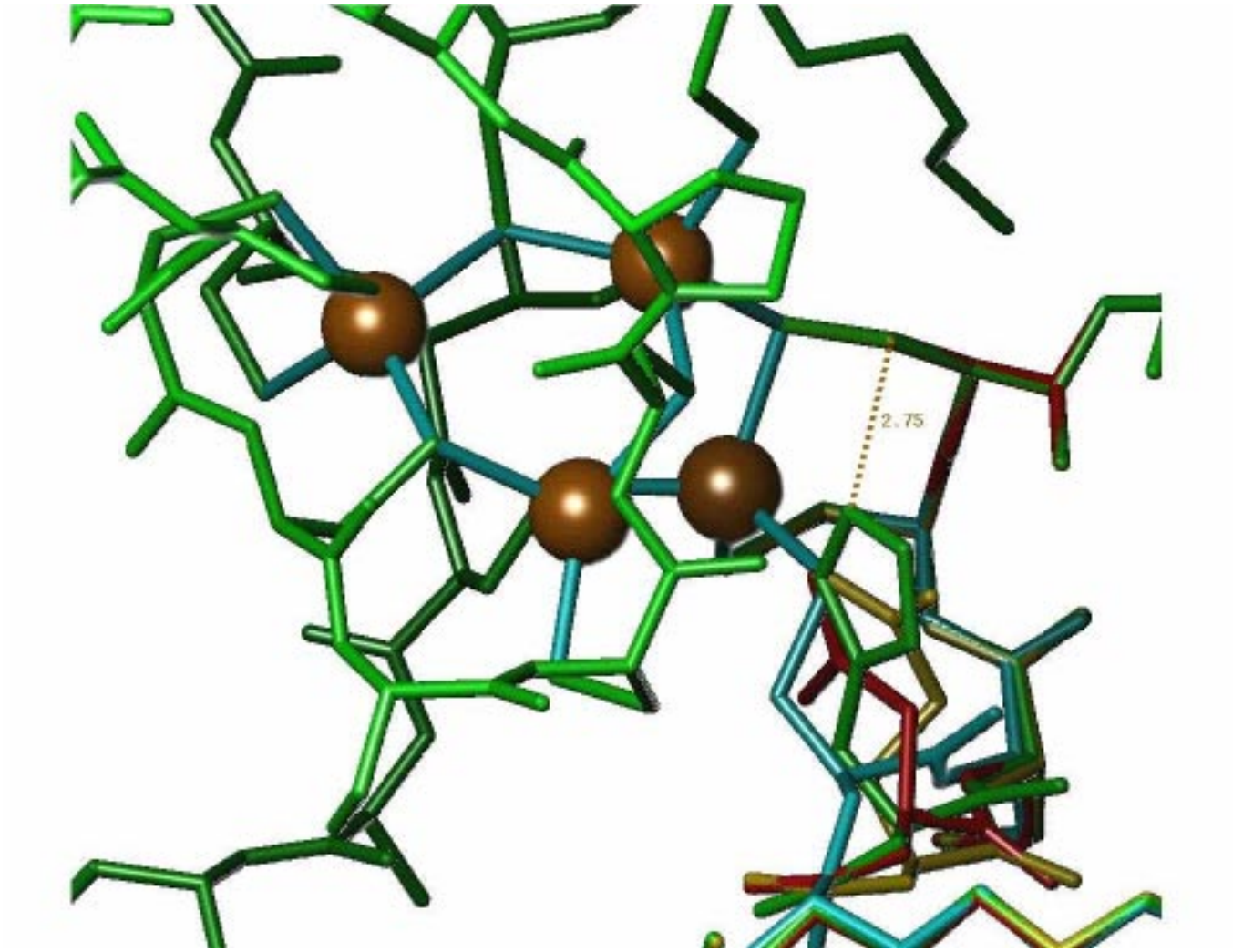

Figure 6. *Representation of main contacts found for the replaced side-chains of the mutant forms C57D (red), C57E (yellow), and C57H (green) within its neighbourhood. Zn(II) and Cd(II) metal ions are depicted as compact spheres (orange) and their bonds to the protein in blue.*

Conclusions

The conformational parameters analysed (RMSD, bond distance and bond angle variations and neighbouring contacts) have indicated that all single substitutions of terminal cysteines in rat liver Cd_5,Zn_2-metallothionein 2 by His, Asp or Glu are feasible. However, substitution of those cysteines highly buried has led to difficult accommodation of the replaced side-chain, especially with glutamate and histidine due to their large side-chain volume. The possible hindrances have been evaluated by the number of neighbouring contacts. In general, the putative mutant forms of the β-domain have shown a greater number of neighbouring side-chain contacts if compared with the α-domain and therefore the latter appears to be preferable for terminal Cys substitutions. Replacement of cysteine 19 is the most unfavourable case because it gives rise to the largest number of neighbouring collisions. The remaining mutations are viable with small perturbations in the native protein structure.

Aspartate has shown to be the best substitute of terminal cysteines of Cd_5,Zn_2-metallothionein 2 if compared with histidine and glutamate, the two latter showing similar conformational impairments. It should be noted that aspartate is not found in combination with cysteine co-ordinating to the same metal ion in the database of proteins [25]. More detailed conclusions in respect to a particular position in the mutated MT sequence are summarized below.

Single substitutions in the β-domain can be categorized in three main groups according to the side-chain neighbouring collisions:

Cys 5 and Cys 13 : Asp > His > Glu
Cys 19 and Cys 29 : Asp > Glu > His
Cys 21 and Cys 26 : Glu > Asp > His

Analogously, single substitutions in the α-domain have led to:

Cys 33, Cys 41 and Cys 59 :	Asp ≈ His ≈ Glu
Cys 48 and Cys 57 :	Asp ≈ His > Glu
Cys 36 :	Asp ≈ Glu > His

The simultaneous substitution of more than one terminal cysteine co-ordinating the same metal ion has afforded good results for all possible combinations except for the substitution of cysteine 5 by histidine, which precludes substitution of cysteine 21 by histidine, aspartate or glutamate residues, owing to significant neighbouring collisions.

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