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Where metal ions bind in proteins

(metalloprotein/protein structure/hydrophobicity contrast function)

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ABSTRACT **The environments of metal ions (Li^+, Na^+, K^+, Ag^+, Cs^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}) in proteins and other metal–host molecules have been examined. Regardless of the metal and its precise pattern of ligation to the protein, there is a common qualitative feature to the binding site: the metal is ligated by a shell of hydrophilic atomic groups (containing oxygen, nitrogen, or sulfur atoms) and this hydrophilic shell is embedded within a larger shell of hydrophobic atomic groups (containing carbon atoms). That is, metals bind at centers of high hydrophobicity contrast. This qualitative observation can be described analytically by the hydrophobicity contrast function, *C*, evaluated from the structure. This function is large and positive for a sphere of hydrophilic atomic groups (characterized by atomic solvation parameters, $\Delta\sigma$, having values < 0) at the center of a larger sphere of hydrophobic atomic groups (characterized by $\Delta\sigma > 0$). In the 23 metal-binding molecules we have examined, the maximum values of the contrast function lie near to observed metal binding sites. This suggests that the hydrophobicity contrast function may be useful for locating, characterizing, and designing metal binding sites in proteins.**

Metal binding sites in proteins are varied in their coordination numbers and geometries, their metal preferences, and their ligands (which include backbone carbonyl oxygens; side-chain groups of aspartic, asparagine, cysteine, glutamic, glutamine, histidine, methionine, serine, threonine, and tyrosine residues; and water molecules) (1–5). While the "soft" metal Fe frequently bonds to sulfur ligands from the side chains of cysteine and methionine residues, there are few simple rules that describe the variety of bonding arrangements displayed by harder metals. Despite this variety, we find that many metal sites in proteins share a common feature: they are centered in a shell of hydrophilic ligands, surrounded by a shell of carbon-containing groups.

These centers of high hydrophobicity contrast were first evident to us from visual examination of structures of ionophores and metal–host molecules (6) deposited in the Cambridge Structural Database (7). We then observed that metal sites in proteins (8) share this same qualitative feature.

This visual impression of metals binding to centers of high hydrophobicity contrast can be displayed graphically by a radial distribution plot, giving the mean $\Delta\sigma$ value of protein atoms at distance r from ion binding sites (Fig. 1). The value of $\Delta\sigma$ for a group (14, 15) characterizes its affinity for water ($\Delta\sigma < 0$) or lack of it ($\Delta\sigma > 0$). The distributions of Fig. 1 show that the atomic groups in a sphere immediately around Zn^{2+} and Ca^{2+} ions in several proteins are hydrophilic ($\Delta\sigma < 0$) and in the spherical region farther from the ion are hydrophobic ($\Delta\sigma > 0$). At still greater values of r, $\langle\Delta\sigma\rangle$ approaches 0.007 kcal·mol^{-1}·Å^{-2}, the typical average value for a protein.

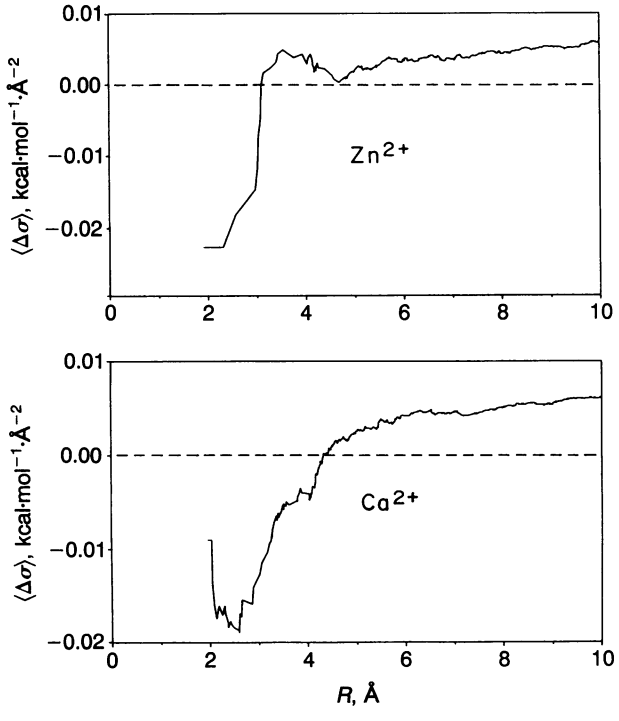

FIG. 1. Radial distribution functions for mean $\Delta\sigma$ values around metal binding sites in proteins. The mean value of $\Delta\sigma$ is plotted against the distance r from the metal site. (*Upper*) For two Zn^{2+}-containing proteins [carboxypeptidase A (9) and superoxide dismutase (10)]. Notice the principal maximum at $r = 3.5$ Å and a possible subsidiary maximum at $r = 6$ Å. (*Lower*) For four Ca^{2+} sites in the proteins [two in parvalbumin A (11), and one each in concanavalin A (12) and staphylococcal nuclease (13)]. For each molecule, coordinates are taken from the Protein Data Bank (8) or the Cambridge Structural Database (7).

The hydrophobicity contrast function, a simple function of the distribution of the atoms within a molecule, gives a quantitative expression to the radial distribution of Fig. 1. The contrast function has the desirable property that is generally a maximum when centered at or near a metal binding site. This function is defined as

$$C(r, \Delta\sigma, R) = \sum_i \Delta\sigma_i r_i^2 - n\langle\Delta\sigma_i\rangle\langle r_i^2\rangle, \qquad [1]$$

where the summation and averages are over all atoms i within a sphere of radius R, n is the number of atoms in the sphere, r_i is the distance from the center of the sphere to the ith atom, and $\Delta\sigma_i$ is its atomic solvation parameter. Hydrogen-atom positions are not usually known for protein structures and are not considered in evaluation of $C(r, \Delta\sigma, R)$. Fig. 2 illustrates

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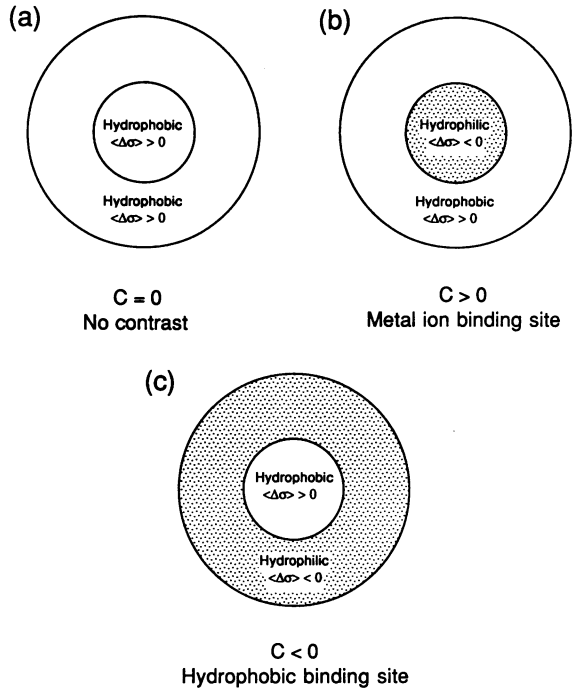

FIG. 2. Schematic illustration of the contrast function C for a uniform hydrophobicity distribution (*a*), a distribution with hydrophobic groups in an inner sphere and hydrophilic groups in the outer sphere (hydrophobic binding site) (*b*), and a distribution with hydrophilic groups in the inner sphere and hydrophobic groups in the outer sphere (metal binding site) (*c*).

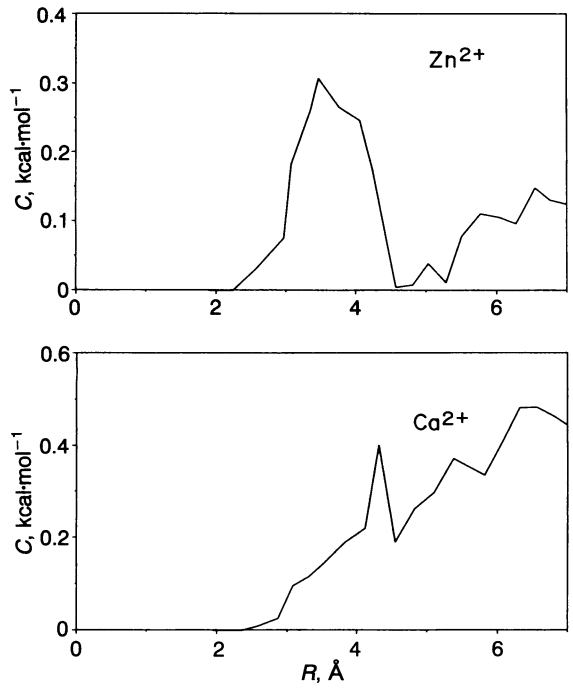

FIG. 3. Hydrophobicity contrast functions (with probe radius R = 7 Å), calculated from Eq. 1. (*Upper*) An average of C for two Zn^{2+} ions [carboxypeptidase A (9) and superoxide dismutase (10)]. (*Lower*) An average of C for four Ca^{2+} ions [two from parvalbumin B (11) and one each from staphylococcal nuclease (13) and concanavalin A (12)].

the meaning of a large value of C and Fig. 3 represents the actual contrast C for the average of two Ca^{2+} sites and four Zn^{2+} sites. The contrast function is defined so that maximal contrast (large C) occurs with the sphere centered at a position having atoms near its center that are hydrophilic ($\Delta\sigma < 0$) and atoms nearer the outer radius R that are hydrophobic ($\Delta\sigma > 0$). The average value of C for all positions within a protein is essentially 0. From the radial distribution plots of $\Delta\sigma$ shown in Fig. 1, it is clear that the most distinctive range of the function is at R values less than 7.0 Å from the metal ion. By trial and error, we found that a scaled average of the contrast functions at 3.5 and 7.0 Å was most effective in detecting metal sites.

METHODS

Algorithm. The hydrophobicity contrast function C is evaluated on a 0.5-Å grid spanning the protein model, with metal atoms deleted. Each grid point is first examined as a potential metal binding site, by ensuring there is no atom within 1.7 Å. If not, C is evaluated for spheres of R = 3.5 Å and R = 7.0 Å. These values of C are averaged to give $\mathcal{C}$ as follows:

$$\mathcal{C}(r, \Delta\sigma) = C(r, \Delta\sigma)_{R=3.5} + SC(r, \Delta\sigma)_{R=7.0}, \quad [2]$$

where S scales the contrast value at 7.0 Å to the value at 3.5 Å. The scale factor is defined as

$$S = \frac{\max[C(r, \Delta\sigma)_{R=3.5}]}{\max[C(r, \Delta\sigma)_{R=7.0}]}. \quad [3]$$

The simplest algorithm is to calculate $\mathcal{C}$ at each grid point by looping over all grid points, finding all atoms within the probe radius R of the grid point, and evaluating their contributions to the contrast function. However, this is extravagantly slow. A revised algorithm loops over the protein atoms, ignoring metal atoms. For each atom, the grid points within the probe radius of the atom are located. For these grid points, the atom's contribution to the terms of the hydrophobicity contrast function is evaluated. For example, if the function of Eq. 1 is used, $\Delta\sigma r^2$ is evaluated for the atom and added to the $\Sigma_i \Delta\sigma_i r_i^2$ for that grid point. Then the program loops over the grid points and calculates the value of C at each grid point. Grid points that are closer than 1.7 Å to an atom are tagged and the contrast function is set to 0 at those points. The grid values are sorted, and the highest 1000 and lowest 1000 grid values are collected and written as "atoms" to a Protein Data Bank format output file. The grid values are also written to an unformatted map file. Using either output file, the contrast function can be viewed on computer graphics. The program also outputs the coordinates of the highest 1000 and lowest 1000 grid points, listing possible ligands within a radius of 4.0 Å. The scaled-average contrast function $\mathcal{C}$ requires that the program be run three times, to find the maximum of the grid at 3.5-Å probe radius and at 7.0-Å radius and to calculate the scaled-average function. Each run of the program requires on the order of 50 min of central processing unit (CPU) time on a VAX 8800. The program can be run on a micro-Vax and is available by request to DAVID%UCLAUE.SPAN@STAR.STANFORD.EDU.

Values of Atomic Solvation Parameters. Values are assigned to each atom type, as follows from ref. 15: carbon atoms ($\Delta\sigma$ = 18 cal·mol^{-1}·Å^{-2}; 1 cal = 4.184 J); uncharged oxygen or nitrogen atoms ($\Delta\sigma$ = −9 cal·mol^{-1}·Å^{-2}), charged oxygens ($\Delta\sigma$ = −37 cal·mol^{-1}·Å^{-2}), charged nitrogens ($\Delta\sigma$ = −38 cal·mol^{-1}·Å^{-2}), and sulfurs ($\Delta\sigma$ = −5 cal·mol^{-1}·Å^{-2}). In glutamic and aspartic residues, the two side-chain oxygen atoms are assigned an average ($\Delta\sigma$ = −23 cal·mol^{-1}·Å^{-2}) of the charged and uncharged oxygen values; in arginine and histidine residues the two side-chain nitrogen atoms are assigned the average ($\Delta\sigma$ = −23 cal·mol^{-1}·Å^{-2}) of the charged and uncharged nitrogen values. This use of averaged $\Delta\sigma$ values for charged protein atoms is justified by the fact

that the electron-density maps of proteins do not reveal which atoms are actually charged.

Alternative Ion-Seeking Functions. Several other functions involving atomic properties were tested for their ability to detect metal-ion sites in some of the proteins of Table 1. One of these was the electrostatic potential,

$$V = \sum_i \frac{q_i}{4\pi\varepsilon_0 r_i}, \quad [4]$$

in which q_i is the partial charge on atom i, as given by the library of the CHARMM program, and ε_0 is the dielectric constant of 1. The summation is on all atoms i within a sphere of radius 5 Å, and r_i is the distance of the ith atom from the center of the sphere. Extrema of this function correlated less well with metal sites than the contrast function C. The minima of the electrostatic potential for the proteins tested corresponded to hydrophilic surface features and did not distinguish the ion binding pocket as the minimum value.

Another function tested was the atomic solvation parameter summation function,

$$F = \sum_i \Delta\sigma_i, \quad [5]$$

where $\Delta\sigma_i$ is the atomic solvation parameter of atom i and the summation is on all atoms within a sphere of radius 5 Å. This function correlated better with metal sites than the electrostatic potential. The disadvantage of the atomic solvation parameter summation function was that it could not distinguish between regions with multiple hydrogen bonds and/or salt bridges and the ion binding pocket.

The final function tested was the charge contrast function,

$$C_\pm = \sum_i q_i r_i - n\langle q_i\rangle\langle r_i\rangle, \quad [6]$$

where q_i is the partial charge on atom i, defined in the CHARMM library. The summation is on the atoms within a 7.3-Å sphere. The extrema of this function were similar to those of the contrast function ℭ, though the values corresponding to the ion binding site would often fall well below the highest value (between 100th and 1000th place).

RESULTS

To assess the usefulness of the hydrophobicity contrast function, $\mathfrak{C}(r, \Delta\sigma)$ was evaluated for the 10 metalloproteins and 13 metal–host molecules of Table 1. The 50 highest values for each protein were displayed as dots superimposed on the

Table 1. Hydrophobicity contrast for metalloproteins and metal–host molecules

Protein or other host molecule	Ref.	No. of points examined	Metal ion	Rank of ion site*	Deviation,† Å	Value of ℭ‡	ℭ max/min values§
Proteins							
Calmodulin	16	414,550	Ca^{2+}	1st	0.6	1.0	1.0/−0.7
			Ca^{2+}	2nd	0.2	0.9	
			Ca^{2+}	9th	0.4	0.8	
			Ca^{2+}	29th	0.7	0.8	
Carboxypeptidase A	9	527,067	Zn^{2+}	1st	0.3	0.8	0.8/−0.7
Concanavalin A	12	461,472	Mn^{2+}	1st	0.6	0.7	0.7/−0.5
			Ca^{2+}	22nd	1.6	0.5	
Myoglobin	17	341,412	Fe^{3+}	2nd	0.6	1.0	1.0/−0.8
Parvalbumin B	11	245,384	Ca^{2+}	2nd	1.0	1.2	1.2/−0.8
			Ca^{2+}	4th	1.2	1.0	
Staphylococcal nuclease	13	331,200	Ca^{2+}	2nd	1.3	0.7	0.7/−0.7
Superoxide dismutase	10	314,760	Cu^{2+}	1st	0.4	0.9	0.9/−0.8
			Zn^{2+}	7th	0.2	0.8	
Mn superoxide dismutase	18	392,270	Mn^{2+}	1st	0.5	1.0	1.0/−0.7
Thermolysin	19	547,253	Ca^{2+}	4th	2.8	0.8	0.8/−0.7
			Ca^{2+}	4th	2.2	0.8	
			Ca^{2+}	15th	2.4	0.7	
			Ca^{2+}	60th	0.5	0.6	
			Zn^{2+}	78th	2.0	0.6	
Troponin C	20	23,426	Ca^{2+}	1st	2.4	0.9	0.9/−0.5
			Ca^{2+}	3rd	0.4	0.8	
Host molecules							
Lysocellin	21	29,386	Ag^{+}	1st	1.2	0.5	0.5/−0.4
Monensin	22	31,862	Ag^{+}	1st	0.2	0.7	0.7/−0.4
	23	32,824	K^{+}	1st	1.9	0.6	0.6/−0.4
	22	30,953	Li^{+}	1st	0.5	0.8	0.8/−0.5
	24	31,844	Na^{+}	1st	0.3	0.8	0.8/−0.5
Valinomycin	25	47,230	Cs^{+}	1st	1.4	0.6	0.6/−0.5
	26	47,250	K^{+}	1st	1.0	0.5	0.5/−0.5
	27	48,638	Na^{+}	1st	2.6	0.7	0.7/−0.5
Dibenzo-14-crown-4 thiocyanate	28	23,624	Li^{+}	1st	0.4	0.7	0.7/−0.4
15-Crown-5 bis(isothiocyanate)	29	43,534	Mg^{2+}	1st	0.0	0.7	0.7/−0.7
Benzo-15-crown-5 picrate	30	29,753	Na^{+}	1st	0.5	0.6	0.6/−0.6
Bis(18-crown-6)	31, 32	30,595	Cs^{+}	1st	2.2	0.6	0.6/−0.4
Dichloro-18-crown-6	33	22,154	Mg^{2+}	1st	2.0	0.6	0.6/−0.5

*Range of the highest ℭ value within 3 Å of the metal site.
†Separation of the grid point of this high ℭ value from the metal site.
‡Value of ℭ corresponding to the rank.
§Maximum and minimum values of ℭ for all grid points.

structure of the molecule, as shown for troponin C (20) in Fig. 4. For troponin as well as for each other molecule, high values of the contrast were found to cluster near observed metal binding sites. However, frequently the average deviation between the cluster of high C and the metal site is 2–4 Å. This displacement is diminished if the contrast is computed for spheres of R = 3.5 and 7.0 Å and the two values of C are averaged (as described in *Methods*), giving a composite contrast ℭ. This average contrast ℭ is reported in Table 1. Notice that the value of ℭ near to the metal site is essentially equal to the maximum value of ℭ over the entire molecule. Notice also that the maximum ℭ value usually lies within 1 Å of the actual metal binding site. Deviations greater than 1 Å may be due to metal-bound water molecules that are omitted in the evaluation of ℭ. Omission of these would shift the maximum of ℭ. Deviations of high ℭ values from metal sites are also observed in proteins that bind several metals (see below).

Discrepancies between high ℭ values and metal sites may suggest other types of binding site or undetected metal sites. For some molecules of Table 1, there are positive discrepancies (points of high contrast not associated with a metal ion). Examples are staphylococcal nuclease (13) (two points) and superoxide dismutase (10) (one point). These discrepancies are located in the active sites and may represent a high tendency of the atomic groups around those points to bind substrates.

Other discrepancies occur in proteins with two or more metal sites. This is not the case in parvalbumin B (11), where there are two Ca^{2+} ions: Of the 50 largest ℭ values, 42 are an average of 1.6 Å from one of these sites and the remaining 8 are an average of 1.9 Å from the other. However, in thermolysin (19) there are four Ca^{2+} sites and one Zn^{2+} site. Of the 50 highest values of the ℭ function, 45 are clustered around two Ca^{2+} sites that are separated by 2 Å, and three others are about 2 Å from the Zn^{2+} site. One other Ca^{2+} site has none of the 50 highest ℭ values nearby. The remaining 3 of the 50 highest ℭ values are not near any known metal binding site. Thus thermolysin exhibits both positive discrepancies, for which a few of the regions of high hydrophobicity contrast are not near known metal sites, and some negative discrepancies, for which one of the observed metal sites is not among the very highest hydrophobicity contrast sites. Thermolysin is the only example in the 23 molecules we examined that displayed negative discrepancies. In proteins where there are positive discrepancies, some may represent metal binding sites, perhaps with lower affinity, that have not yet been identified.

DISCUSSION

Why do metal ions generally bind in regions of high hydrophobicity contrast in proteins? The simplest viewpoint is that the electron distributions in metal ions are highly symmetric, attracting electron-pair donors (Lewis bases) around the ion in a shell. In proteins these electron pair donors are oxygen, nitrogen, and sulfur atoms. The same factors that cause these ligands to bind metal ions also cause them to be strongly solvated by water, and hence they can be described by $\Delta\sigma < 0$. Also because each oxygen, nitrogen, or sulfur atom is covalently bound to a carbon-containing group, atoms with $\Delta\sigma > 0$ are found preferentially in the outer shell. In this explanation, the high hydrophobicity contrast reflects no more than the nature of the metal–ligand interaction and the structure of amino acids.

Other factors may contribute to the tendency of metals to bind at centers of high hydrophobicity contrast. One may be that the hydrophobic sphere restricts flexibility of the site (34), reducing the decrease in entropy associated with binding. This "preorganization" of metal sites would be expected to favor binding (6). Also there is the possibility that the hydrophobic outer sphere provides an interior region of low dielectric that enhances the electrostatic interactions of groups within (38, 39, 43). In this view, high hydrophobicity contrast reflects electrostatic features of the structure and raises the question of whether an electrostatic function might be more effective in detecting metal sites. To investigate this question, we searched for metal sites by direct examination of the distribution of charges on atoms. In doing this we attempted, without success, to find an electrostatic function other than ℭ that correlated well with metal binding. These included the electrostatic potential $\Sigma q_i/r_i$, in which q_i is the partial charge (40) associated with atom i and r_i is its distance from a potential ion site, as well as other functions of q_i and r_i described in *Methods*. Not only did these functions correlate less well with metal binding than did ℭ, but so did other functions of $\Delta\sigma$, such as Eq. **1** with r^2 replaced by r^{-1}, r^0, or r, although the last function was almost as satisfactory as Eq. **1**. Of course, computations of electrostatic functions, such as the electrostatic potential, are impeded with proteins because

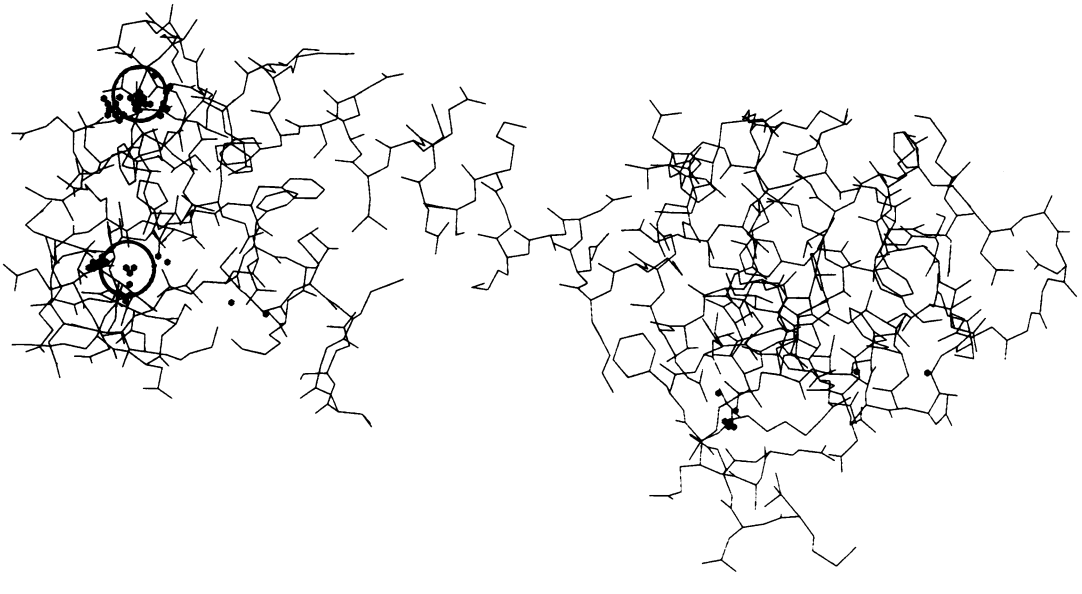

FIG. 4. Scaled-average hydrophobicity contrast function ℭ in chicken skeletal muscle troponin C (20). Two bound Ca^{2+} ions are shown as circles in the left (C-terminal) domain. Shown as dots are the highest 50 values of the hydrophobicity contrast function. Notice that some 40 of these 50 are clustered near the two bound metals.

we do not generally know the positions of protons or the precise partial charge on most atoms.

We conclude that the hydrophobicity contrast function ℭ detects metal binding sites in proteins because it directly reflects structural characteristics of metal sites and that it may also indirectly represent some electrostatic and hydration components of the free energy of metal binding. ℭ of course contains no information about the covalent contributions to metal binding or about the organization of the binding site through conformational change; thus ℭ would not be useful for detecting binding in cases where these effects predominate. The shapes of *d* orbitals would be expected to be more important for the binding of transition metals than for the alkali and alkaline earth metals. More elaborate, nonspherical contrast functions might be able to discriminate between binding sites for different metals.

To the extent that the hydrophobicity contrast function describes the environment required for metal binding, it may find use in searching for subsidiary binding sites and in the design of metal binding sites. Subsidiary metal sites may be missed during initial inspection of electron-density maps in cases where the metal is at low occupancy or is of low atomic number (e.g., Li^+, K^+, Mg^{2+}) but might be revealed by the contrast function. To aid in the design of metal sites, the contrast function provides a quantitative characterization. It could be used both in *de novo* design or in redesign to increase metal ion affinity for a binding site, perhaps creating a more stable protein (41, 42).

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