DISCUSSION

Session Chairman: David Eisenberg *Scribe*: Lucia Garcia-Iniguez

TORCHIA: I've got two questions. First, you have used the signals of the acetylmethyl of the N-terminal alanine and the signals of the C2 and C4 ring protons of histidine to determine the principal values and orientation of the susceptibility tensor of the metal. In the x-ray structure, the histidine side chain is located on the protein surface and the six N-terminal residues are the least ordered part of the protein. Thus the nuclei used for the tensor determination do not have well-defined positions. Would this not produce significant uncertainties in the determination of tensor elements, especially since disorder would be expected to be larger in solution than in the crystal?

L. LEE: We have used those three assigned resonances as a first approximation to scale the constants in the equation relating the shift to the geometry. We are talking about protons very much removed from the metal. We then use those parameters to test each choice of orientation of the principal axis system based upon the six methyl resonances observed in the study.

SYKES: I think the key thing is that those resonances indeed have some fluctuation in their possible position, but they are quite far removed from the metal (15–20 Å) and they are located at relatively diverse angular orientations. Also, we have added a fourth assigned resonance, a fluoride label on the one SH of parvalbumin, which isn't reported in the manuscript. We used those shifts to make sure that the calculated shifts far away from the metal were not 10 ppm or 0.01 ppm when they were observed to be of the order of 0.5 ppm. Then we moved into the methyl groups and used their shifts to choose amongst the possible orientations of the principal axis system. It may lead to a 10–20% error, but I don't think that it can lead to the kinds of discrepancies that are calculated in the end.

TORCHIA: From your Table II where your observed and calculated methyl values are given, it seems that you have a fair number of discrepancies there too.

SYKES: Yes. By the time we've moved in to nuclei closer to the metal (and by nuclei close to the metal I mean the 6–10 Å), then we are already beginning to see discrepancies of the order of 2–3 ppm. These get out of control as we go even closer to the metal, and become 10–20 ppm.

TORCHIA: Two previous solution studies of the carp parvalbumin (Cave, et al., 1976, *FEBS Letts.* **65**:190 and Nelson, et al., 1976, *Biochemistry* **15**:5552) have resulted in the conclusion that at least 8 of the 10 phenylalanine aromatic rings rotate about the C^{β}-C^{γ} bonds on a time scale of 10^{-3} –10^{-8}s. According to the x-ray structure these residues are within the hydrophobic core of the protein and could not have such motions unless large segments of the protein also moved. In view of these considerations, is it not expected that averaged distances obtained for a dynamic solution structure by the NMR method would disagree with the distances determined by x-ray for the static crystal structure?

SYKES: Certainly there are motions. We would be the first to admit that. A trivial example where the shifts are most certainly wrongly calculated is lys-96 on the surface of the protein, whose position may be different in solution vs crystal because of packing forces. The next example comes from a residue such as phe-57, whose shift is also calculated too large, where in the minimal case there will certainly be flipping of that phenylalanine ring. As for the nuclei about which we think we are going to be able to get the most information, such as those on the amino acid chain which makes the direct loop around the metal ion, there may be some kind of motion here. The x-ray results are an average as well, but are a direct average of position. If the x-ray positions reflect a minimum around which there is some vibrational averaging, then our expectation is that the NMR results, which average less directly because of the r^3 distance dependence, should weight the close contacts more than the far contacts. Thus the observed shifts should be much larger than the calculated shifts, but we observe exactly the opposite. That is, we observe that the calculated shifts for some of these groups are way outside of the range of any of the observed shifts.

One other piece of information is that the x-ray structure is unfortunately not one of the highly refined modern structures and when you "grow" protons on this structure you find, just by visual inspection, that for many protons the structure just doesn't look good. There are protons on glycine residues involved in the loop around the metal that point right at the metal ion and that doesn't seem reasonable. Consequently, we think a cause of error bigger than motional averaging is in the x-ray based proton coordinates. Changes in the proton coordinates required to bring the NMR and x-ray into agreement do not necessarily involve big differences in the heavy atom positions, and we hope to generate a more accurate starting structure before we go into these subtle details of motion. I want to move the distance of closest approach of several protons farther away than is indicated by the x-ray position. The catastrophe situation would be if, as others believe, proteins in solution don't move around a local minimum described by the x-ray

structure, but exhibit huge fluctuations. Then there would the possibility of nuclei spending much more time far away from the metal ion.

TORCHIA: One final point. You also have very strong angular dependencies in your shifts. Have you considered that fluctuations in the orientations of these groups might explain the kind of discrepancies you are seeing?

SYKES: The shifts are always going to involve the averaging over instantaneous values of $(3\cos^2\theta - 1)/r^3$ for each possible conformation. But I still think that moving the atoms in space to positions that bring the calculated shifts within the range of the observed shifts will, as the first iteration, bring us to a better structure than exists at the present moment.

DOBSON: I have two points. First of all, I wonder about your attribution of the fundamental reason for the lack of high correlation between the x-ray and NMR data to errors in the x-ray structure. As well as the consequences of fluctuations of protein groups themselves, I am thinking about the effects of fluctuations in the groups binding the metal ion. These could give rise to a time dependence of the susceptibility tensor, causing the shifts to be averaged in a more complicated way than occurs because of the protein motions themselves. I don't think that one can eliminate this type of averaging by looking at the expected distance dependence. I think there is much evidence for this type of averaging in small molecule studies and wonder if you can comment on this for parvalbumin.

Secondly, if you don't take the fluctuations into specific consideration, I worry about the method you are suggesting for the refinement of the structure. NMR has very high potential for accurate structural measurements because of the high distance dependence of the shifts. However, one has also to consider that averaging effects come in very strongly when there is such a strong distance dependence. I think one of the powerful applications of NMR is to use this fact to look at these fluctuations. I wonder if you have further evidence to eliminate these types of fluctuations and if not, do you think that they are so small that one can neglect them in this type of refinement procedure?

SYKES: On your first question, there are certainly very rapid vibrational-like fluctuations of the ligand field which are involved in the spin lattice relaxation time of the electron, for example. For any conformation, an average tensor will result which is a reasonable thing to work with. If this protein is undergoing, for example, a two-state conformation exchange on a slower time scale, one will have two separate tensors for each conformation and will have to average not only changes in the geometric factors but changes in the susceptibility. That's a possibility, but I know of no evidence in the protein to suggest that we must worry about that kind of a process going on. The residues in the immediate vicinity of the metal ion are involved in a tight turn around the metal ion, and the metal ion is held extremely tightly with a binding constant of $\sim 10^{-9}$ M. Thus we rather favor that the motion is reasonably constrained.

As for as your second point, we are proceeding on two fronts. In addition to the NMR data collection we are collecting new x-ray data on parvalbumin and, in conjunction with crystallographers, hope to refine the structure crystallographically to a higher level.

BRILL: Our poster deals with fluctuations in the *g*-tensor of a paramagnetic ion in a protein crystal. The *g*-tensor is related to the susceptibility tensor, and we are able to comment on the fluctuations in principle values and orientation.

DOBSON: I would like to ask whether you looked at the shifts with lanthanides other than ytterbium. One of the advantages of looking at the effects of different lanthanides is that you might get some idea about the factors controlling the susceptibility tensor. Related to this, can you look at the way in which the lanthanide ion is coordinated in the protein and understand the orientation of the susceptibility tensor? I think that one of the problems in other studies is that one has not been able to rationalize clearly the nature of the susceptibility tensor. This leads to uncertainty in the interpretation. Can you comment further on the susceptibility tensor in parvalbumin?

SYKES: The answer to both of those questions is yes. In a separate paper (see reference 25) we have looked at a series of other lanthanides. However, because of the "susceptibility line-broadening mechanism" the choice of ytterbium gives us the best resolution of the shifted resonance, and we have not compared the spectra with the other lanthanides in detail.

In answer to your second question, we have looked at the carboxyl, carbonyl, and water ligands to the metal in the principal axis system. They form an almost perfect trigonal anti-prism of the kind that you see in the x-ray structure of small molecule lanthanide complexes. The z-axis points along the three-fold axis. It's the first system where we've been able to rationalize the principal axis system.

LLINÁS: You say that you can follow the step-wise addition of the first metal and then the second. After the first addition of metal I presume you observe some shifts, right?

L. LEE: Yes.

LLINÁS: Then on filling the second site you will observe a different chemical shift pattern on the spectrum. Can you distinguish the linear superposition of paramagnetic shifts caused by both metal ions from conformational changes at the first site induced by the binding second metal? It seems to me you should have both effects going on at the same time.

L. LEE: There is a sequential binding of the two metals to the calcium saturated protein. In this study we are working at particular metal to protein ratios, and therefore are only focusing on the first site filled by ytterbium, which is the EF-binding site of parvalbumin.

SYKES: Confusion arises because one is used to thinking of the fast exchange limit. In the slow exchange case, appropriate here, the resonances appear at their shifted position as the first site is filled. If the second metal is also near the nucleus, that resonance will disappear and reappear in a new position as the second site is filled. Thus you see separately all the species that are possible: ytterbium in the first site, calcium in the second site; ytterbium in the second site, calcium in the first site, and ytterbium in both sites. Consequently there are none of the problems possible for metal binding, which is in the fast exchange, such as estimating the influence of a second site.

LLINÁS: Have you tried to fill the first site with calcium and the second with ytterbium and vice-versa?

L. LEE: Because of the relative affinities of ytterbrium and calcium for the two sides, the experiment you describe is not straightforward. We have always looked at the EF-site only. We have not tried to look at the structure of the CD-site.

LLINÁS: The spectrum looks quite impressive from the point of view of spreading the resonances over a wide chemical shift range. I wonder if you couldn't use that effect to attempt selective Overhauser experiments combined with the paramagnetic shifts. That would provide a second triangulation quite independent of the first. That way you could refine the conformational interpretation.

SYKES: The difficulty with these experiments is that the T_1's of the shifted resonances are very short.

WÜTHRICH: You conclude that the x-ray structure might not be accurate because your calculated pseudo-contact shifts overestimate what you see in the experiments. I would just like to comment that this is what we consistently found when working with hemeproteins. In all attempts to compare calculated with observed pseudo-contact shifts in hemeproteins, we always had to introduce a "fitting factor" of the order 0.7–0.85 in order to make the calculated shifts comparable to those actually observed. We have usually attributed the need for this reducing factor to the electron delocalization in the heme group.

KARPLUS: I am curious whether the substitution of the 3^+ for the 2^+ ion would make a significant difference, because the 3^+ ion structure has not been well determined. Obviously that substitution would pull everything in and that could affect your results.

SYKES: If you pull the nuclei in, it should affect the results in the wrong direction; that is, the observed shifts should be bigger than the calculated ones. Relating to the substitution, the x-ray structure of paravalbumin used terbium as one of the heavy metal derivatives and showed no difference in structure at the level of the x-ray resolution between the trivalent and the divalent ion. In addition, Matthews has substituted each of the lanthanides for the calcium ions in thermolysin and seen essentially no difference in the x-ray structures. There seems to be no strong evidence that there is any change in structure due to the trivalent lanthanide substitution, although I think if we are going to argue that we are more sensitive than the x-ray structure we can't argue that if the x-ray structure doesn't appear to change, there is no change. I think the most subtle measurement is that of Horrocks using the laser luminescence of the lanthanides. The lanthanides generally prefer a higher coordination number in free solution, but when Horrocks studied the luminescence of terbium or europium bound to the EF-site of parvalbumin as a measure of the number of water molecules bound to the metal ion, he saw one. This is the same as for calcium in the EF-site in the crystal structure. This would seem to imply that the structure is the same around the lanthanide.

LIPPARD: To comment on your remark about protons, a number of x-ray crystallographic studies of transitional metal complexes recently have shown that in certain cases the aliphatic CH groups will orient to bind or pseudo-bind the hydrogen atom to the transition metal. This may conceivably be happening in changing from 2^+ charged species to a 3^+. I was curious to know what sort of difference in distance you would require in order to get shifts that would match the experimental ones.

SYKES: For the nuclei very close to the metal ion for which there are the biggest discrepancies, we are talking about movements in the order of ~ 0.5–1 Å.

LIPPARD: The 0.5 Å you can probably explain by C-C bond rotations.

SYKES: However, you're bringing the protons towards the metal.

LIPPARD: That's right. There would be a difference between the 2^+ and 3^+ ions. I don't know which way it would go because these particular cases haven't been looked at, and the cases that have been looked at tend to be low valent transition metals such as molybdenum and tungsten compounds. The lanthanides have not been looked at in any detail, but there is a sort of an interaction that one is beginning to recognize in accurate crystal structures of transition metal complexes where aliphatic hydrogens do turn in towards the metal, and that's the thing one should be aware of.

DOBSON: I'd like to comment on Professor Wüthrich's remark. Is the point that in hemeproteins one is comparing a calculated tensor with experimental shifts? In the parvalbumin work, one is obtaining a tensor experimentally by using shifts of protons from distant parts of the molecule and then predicting shifts of groups which are close to the metal ion. The relative effects in the parvalbumin case couldn't be corrected by a simple weighting factor in the calculations.

SYKES: I think that's exactly right, Chris. We deduce a principal axis system and susceptibility tensor elements from the elements shifts of the distant protons. The question is why does this tensor not predict the correct shifts for nearby protons.

WÜTHRICH: This is exactly what we have seen in our studies with hemeproteins, and that's why I mentioned it. We determined the *g*-tensor from the hyperfine shifts of protons which are relatively far from the heme iron, and compared the calculated shifts with the experimental shifts for protons at closer distances. The calculated shifts were always too big. What is the possibility that electron delocalization might play a role in these lanthanide complexes? Can this be properly excluded?

L. LEE: We considered the possibility of contact contributions in our analysis. We have tried to minimize the possibility of such contributions. First of all, we are considering the lanthanides, for which the *f*-electrons are inner core electrons when compared to the transition ions in which outer valence electrons are involved. Secondly, of the lanthanides, ytterbium is the best because it has the smallest contact contribution. In addition, we are looking at protons which are several bonds removed from the metal; it might be more important for carbons or oxygens directly bonded to the metal. From these considerations we have deemed the contact contribution to be negligible.

WÜTHRICH: Yes. I understand what you have done, but I'm not talking about the contact shifts on the observed protons, but about the reduced electron density at the metal ion. What is the effective electron localization at the metal ion? Are you certain you can use a point-dipole approximation in calculating your pseudo-contact shifts? If you have appreciable delocalization of the unpaired electrons from the metal ion, this could effectively reduce the resulting pseudo-contact shifts without necessarily causing contact shifts on the observed protons.

SYKES: I think that the question of electron delocalization isn't anywhere near as severe for these metals as for a heme group.

HENDRICKSON: A question of clarification: I wonder why you seem to be waiting for the crystal structure to be better. Why can't you just tell us what the structure should be?

SYKES: I think it's that Lana has only two hands and so much time. I think that next thing is to start the refinement that you suggest.

HENDRICKSON: I'm curious whether you think you are close enough to be within the realm of that refinement. What is the sensitivity of the method?

SYKES: If we take all the nuclei that are calculated to be outside of the range of observed shifts and move them so that their calculated shifts are within the bounds of the most upfield and the most downfield observed shifts, we will get a much better constraint on the structure.