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DISCUSSION

Session Chairman: Frederic Richards *Scribe*: Lucia Garcia-Iniguez

RICHARDS: We have a written extended comment relevant to this whole fluctuations session from Erik Tüchsen of the Carlsberg Laboratory.

TÜCHSEN: This is a contribution on the dynamic aspect of the atoms in the crystals. We have compared hydrogen exchange rates in crystalline and dissolved hen lysozyme and bovine zinc insulin (4). Crystals were insolubilized by intramolecular crosslinks, introduced by means of glutaraldehyde (1) to permit comparison of dissolved and crystalline protein under identical conditions.

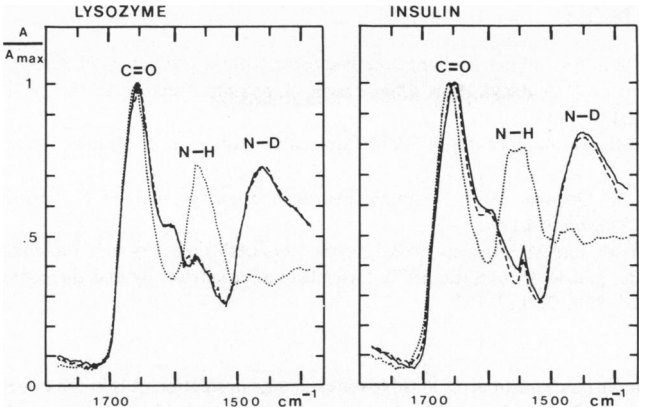

Figure 1 IR spectra of crosslinked crystals of lysozyme and insulin suspended in fluorinated mineral oil. Original transmittance spectra are converted to absorbancy and normalized with respect to the maximum at 1,650 cm^{-1}. Spectra of all-H crystals (....) are shown together with spectra of crystals incubated in D_2O for 2O H at pD 9.7 and 20°C (——) and spectra of crystals heated to 80°C in D_2O (---).

Exchange of peptide hydrogens in the crystals was demonstrated by IR-spectroscopy on suspensions of crystals no larger than a few microns. These crystals were suspended in fluorinated mineral oil after removal of water or deuterium oxide by cryosublimation. In both proteins the amide II band near 1,550 cm^{-1}, due to CONH groups, gradually disappeared when the crystals were incubated with deuterium oxide, indicating replacement of peptide hydrogen for deuterium. In deuterium oxide at pD 9.7, spectra recorded after exchange for 20 h at room temperature and after heating to above unfolding temperature (3) were almost identical. These spectra (Fig. 1) indicate that all peptide hydrogens are accessible in the folded protein molecules.

Kinetics of tritium back-exchange from crosslinked crystals, tritiated under similar conditions, were measured by means of a previously published filtration method (5). In this method, liberation of tritium from a single sample of crystals is followed versus time. Back-exchange from crosslinked lysozyme crystals was indistinguishable from back-exchange of dissolved lysozyme (5) as measured by the gel filtration method of Englander (1) (Fig. 2). For insulin, however, the exchange in crystalline state was considerably slower than in solution, as also observed by Praissman and Rupley (2). These authors concluded that hydroxyl ion catalysis was eliminated upon crystallization or that exchange mechanism was shifted from EX2 to EX1. Our results for insulin are different, since they clearly indicate that the exchange is catalysed by hydroxyl ions, and therefore that the EX2 mechanism is maintained in the crystal state. The insulin data shown in Fig. 2 indicate, in accordance with investigations by Raman spectroscopy (6), that some conformational change of insulin occurs upon crystallization.

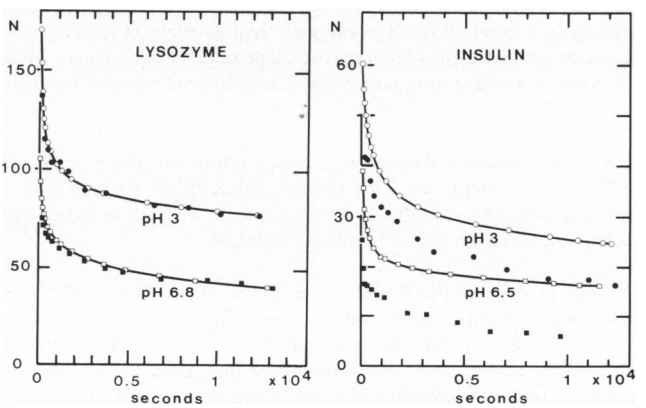

Figure 2 Back-exchange of tritium from crosslinked crystals of lysozyme and insulin at pH values given (-●- and -□-) and from the dissolved proteins in identical buffers (● and ■). N is the number of tritiated hydrogens per protein molecule, disregarding any equilibrium isotope effects.

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To summarize, whichever mechanism is responsible for exchange in solution, it is in the crystal as well. I may extend this into a question to Dr. Petsko. Are the displacements in the crystals big enough to make room for H_2O to get access to the peptide hydrogens?

PETSKO: Yes, provided that in treating the mean square displacements crystallographically you assume that the motion is harmonic, i.e. that the probability of finding an atom a given distance from its average position is Gaussian. If you make that assumption, then a mean square displacement of ~0.3 Å implies a very substantial probability that the atom can be found as far away as 1.5–2.0 Å from its average position, so that there is sufficient leeway in the displacement parameters we see to allow motions of a finite probability large enough to open cavities that could accommodate a water molecule.

BRILL: The analysis presented in the paper under discussion depends upon the assumption that the rotational lattice disorder is negligibly small. Rotational disorder in protein crystals containing high-spin ferric heme can be quantified by single crystal EPR orientation studies (Hampton and Brill, 1979; and see Fiamingo, Thorkildsen, and Brill, this volume). The angles so obtained contain contributions from both molecular and lattice disorder. Results from hemoglobin suggest that most of the disorder is from the lattice, but some arises within the hemoglobin molecule. We observe resonances from the individual hemes, and find the same disorder angle for α and β hemes. There is some difference in the shape of the two distributions.

The rotational disorder in ferric myoglobin crystals, which is much less than that in ferric hemoglobin crystals, is such that half the heme normals lie within a cone of 1.4°. If one assumes all of this to be of lattice origin, and takes the simplest model for the contributions of this angular spread to the mean square atomic displacements, the latter go from zero at an assumed stationary point in the center of the molecule to 0.2 $Å^2$ at an average surface radius of 18 Å. On this basis, the vibrational and conformational contributions to the mean square displacements add to a rotational lattice disorder background which varies between small values and 0.2 $Å^2$ as the positions considered move between points close to the center and the periphery. This upper limit to the effect of rotational lattice disorder can account for a great part of the fluctuations arrived at from the analysis of x-ray diffraction data from ferric myoglobin crystals.

I have a question for those speakers who are going to discuss structural fluctuations in proteins with enzymatic and transport functions. Two problems have been solved in the design of such proteins. One is that of getting a small molecule or part of a big molecule to the active site. The other is that of speeding up some reaction, e.g. the transfer of an electron or the splitting of a bond. While the energetic requirements of making way for the substrate may dominate, it is the reaction itself which is the goal of the overall process. The question is: How might fluctuations play a role in activating reactions? If a role is seen, how do the measurements relate to frequency and activation energy factors?

PETSKO: The question of the rotational disorder in a crystal lattice was taken up to some extent by Sternberg, Grace, and Phillips (1979) (see our references). They tried to analyze this problem in the lysozyme case. We've tried to apply their analysis to the myoglobin results but cannot come up with a convincing axis of rotation that would explain the relative fluctuations we see on the surface of the molecule.

RICHARDS: I'd like now to refer to the Konnert-Henrickson axis as the people here who are most directly concerned and experienced with x-ray data refinement problems. There is the assumption in all of these cases of the Gaussian distribution of the fluctuations, but this assumption is clearly not right. It goes back to Debye-Waller to begin at the beginning, and we have a hard time getting over that. Since then—and here's where the Konnert-Hendrickson group have made important contributions —the fluctuations which are normally treated as independent by small molecule people are in fact not independent. You can't have neighboring atoms moving wildly differently from their neighbors; obviously they are coordinated in their movements. This is not explicitly taken into account normally, but it represents a real problem of a fundamental nature.

Dr. Petsko, would you read Dr. Konnert's questions and then answer them?

PETSKO: Sure.

KONNERT (Question read by Petsko): Although the manuscript doesn't say so, I believe that the *B* values were restrained during the refienment to conform to known stereochemistry (Konnert and Hendrickson 1978, *Acta Cryst. A*34,S47). Qualitatively, this means that if an atom undergoes large displacements from the mean positions, then certain other atoms related by approximately known stereochemistry are required to have related large displacements. Were such restraints imposed?

PETSKO: Yes, by the method described in the poster by Hendrickson and Konnert (this volume). That method imposes a kind of common sense geometrical relationship among the temperature factors which seems to hold up reasonably well.

KONNERT: With regard to evaluating the magnitude and extent of anisotropy in the lattice disorder, have you considered looking for diffuse scattering in the vicinity of and in between the Bragg peaks? Very possibly the lattice disorder involves cooperative affects between adjacent unit cells. The result of such cooperative effects on the diffraction pattern would be very similar to those for thermal diffuse scattering, but the temperature dependence would, of course, be different.

PETSKO: This is an excellent point. Diffuse scatter is one of the best ways of looking at coordinated motion and you'll see a spectacular example of that in George Phillips' paper which will follow mine. We have not looked at this in myoglobin in any kind of detail. In long exposures taken with very high intensity x-rays sources there is some quite diffuse scatter in the myoglobin pattern, but we haven't analyzed it. That is clearly something that ought to be done.

KONNERT: Would not a more straightforward method for obtaining the lattice disorder parameters be to carry out a rigid body refinement utilizing the entire molecule and the B-values from the refinement? In this way you could determine both the translational and the rotational parameters of an overall rigid motion.

PETSKO: That is to some extent exactly what Sternberg, Grace, and Phillips did for that paper on lysozyme, and as I said we tried that on the rotational problem of myoglobin and couldn't come up with anything reasonable. Ironically enough, because we had arrived at a measure of the translational lattice disorder from Mossbauer spectroscopy, we did not try a translational rigid body analysis. I think that's something that we should have done, and will go back and do.

RICHARDS: We have another question, this one from anonymous referee: "With regard to long side chains, such as a lysine residue, how can you be sure that the increased motion as one proceeds to the end of the chain is not simply a reflection of the easing of restraints in the refinement?"

PETSKO: That's a good question. There are a number of reasons why we don't believe that's the case. For one thing, values for atoms in the interior of the side chain—which have approximately equal restraints applied to them because they're bonded to the same type of atoms and the same number of atoms—do, none the less, increase as the chain gets farther away from the surface of the protein. But a better way of looking at it is that both the electron density itself and the stereochemical environment make this kind of behavior look perfectly reasonable. When you have a long side chain on the surface of the protein hydrogen bonded to another side chain on the surface, or back to the main chain, the displacements behave exactly as you would expect them to, with an increase as you go around the loop followed by a sharp decrease when you get to the hydrogen-bonded atom. The other point is that for a side chain such as lysine-98, which is free to extend into solution (and presumably wave about), the electron density itself diminishes dramatically as you go along the chain until it is nearly invisible for the terminal atom, in many cases; this is the reflection of a legitimate flexibility of that residue which is being mirrored by the B-parameters.

FINNEY: There are technical queries which bother me as a noncrystallographer. Except for a few honorable cases, all protein structures are experimentally underdetermined. Therefore, you have to feed in additional (chemical) information which in this case is restraints on the refinement. But unless your experiment is perfect, the fact of putting in restraints is likely to force the results of experimental errors into unpredictable regions. The two regions where errors tend to be put, in my experience, are in temperature factors, because they can take up slop, and in the solvent. I wouldn't want to rely on any information which came from a refinement where a considerable amount of chemical information was fed in. We wouldn't know what protein structures were like unless we already knew what protein structures were like. You stress in your paper that a lot of the information that comes out makes chemical sense. If I wanted to be skeptical, I could say it makes chemical sense because you put that chemical sense in to start with. I would like to see refinement work done on a system where no restraints were fed in at all. Then you would know what the extent of your data was, and its reliability, and therefore you could see that the numbers that came out actually came out of the data, and not out of the assumptions that were put in to the refinement. I would like some clarification upon this general point.

PETSKO: The point is very soundly taken, I think, that one has to worry about a refinement in which you assume that you know things about the protein. There are a couple of points about this specific work which I want to mention and then I'll talk in general terms.

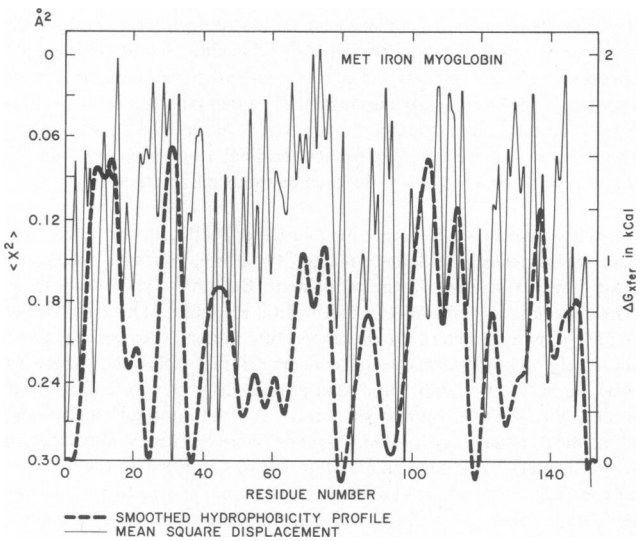
In the original paper in *Nature* in which we describe the calculations in more detail, we talk about some of the attempts we made to determine to what extent the restraints were influencing the temperature factors we obtained. This included, in some regions, eliminating those restraints and refining simply with a normal least-squares procedure that involves no stereochemical connectivity at all, and those numbers agree quite well with the numbers we have from the restrained refinement. We are operating here at a very high resolution, relatively speaking, 1.5 Å, with a large number of measurements. Although we are not dreadfully well overdetermined, we are overdetermined.

In general terms, one can really comment in only a couple of cases. The poster by Hendrickson and Konnert shows results that have actually come out of some refinements in which the restraints were relaxed, i.e. were not applied. There are some unrestrained numbers down there (with respect to B-values), which is what we're talking about. The other analysis done in this way, was rubredoxin, studied by Lyle Jensen and his colleagues at 1.2 Å resolution, where effectively no restraints were applied throughout much of the analysis, and that includes on the temperature factor parameters. Their results are quite similar to ours: the B-values increase as you go from the inside of the protein to the surface of the molecule. They also correlate in a sensible way with structure, being, in general, lower for regions of regular secondary structure or for those atoms used to anchor the iron-sulphur clusters, and higher for atoms on the surface of external loops. Jensen et al. also have given us a very good estimate of the spread in bond lengths and angles that one can expect to find in the course of an unrestrained refinement with very good data at very high resolution. It's that sort of spread that we use in setting the limits to which things can go during the refinements that we do with more limited data. You must understand that these parameters are not rigidly held at all, in the refinement, but rather the atoms are tethered with flexible ropes to positions that maintain more or less expected bond lengths and angles.

ROSE: I'd like to comment that in addition to the detailed atomic information it's also possible to back off and take a bird's eye view of the molecule. If you look at the chain sequential hydrophobicity as it lies along the polypeptide chain, that correlates very well with the mean-squared displacements shown in Figs. 3 and 4 of your paper. If you plot the free energy of transfer from aqueous to organic solvent against the residue number, you find for myoglobin that there are parts of the chain where the hydrophobicity is at a local maximum and other parts of the chain where it is at a local minimum. The parts of the chain at a local minimum are presumably the parts that are sitting at the solvent-accessible exterior of the molecule, and the local maxima in hydrophobicity are then the parts that ought to be nailed down. You can see the correlation from my figure (below).

LUMRY: I get the impression that the diffuse scattering is an important factor, that it contains a lot of information and that sometimes it jeopardizes the results of looking at the temperature ellipsoids themselves. Would you comment?

PETSKO: We don't have any calculations that would give us any experience on it, but my feeling agrees with yours. I think that the neglect of the diffuse scatter is probably a relatively serious thing and that in some cases—particularly proteins which are not nearly so well packed as they are in these particular crystals—that that's going to get us into a



lot of trouble unless we at least look at it very carefully. It does have extra information of great importance, and it's related to these correlated motions that we spoke about earlier.

LESK: I'd like to ask a little bit more about your citation of rubredoxin in answer to John Finney's question. If we were naive we would all believe that the surface regions of the protein are both more flexible and also more disordered. The strength of your results is that by doing things as a function of temperature you can sort one effect out from the other. I'm not aware that rubredoxin was ever studied as a function of temperature. If not, how you can sort out disorder effects from motions within a single molecule?

PETSKO: O.K. I haven't talked at all about the temperature-dependent work that we did, and in the case of rubredoxin you are correct that no temperature-dependent work has been done. There the strongest evidence for the quality of the results comes again from the comparison of two related rubredoxins in two different crystal forms, rather like our comparison of the lysozyme structures.

KUNTZ: You said earlier that these pictures are done from a harmonic analysis and would tie in with the high frequency motions we saw in Richard Feldmann's film "Molecular Dynamics of Pancreatic Trypsin Inhibitor" and in my films on "Protein Surfaces" (Kuntz and Connolly). Your channel for getting the oxygen in started hot at the top but didn't stay hot all the way through. Musn't you have low frequency motions that allow the oxygen to actually penetrate there?. What is your feeling about what the high frequency motions tell us about the low frequency motions, which we know must be the important ones?

PETSKO: Obviously, low frequency motions are extremely important both for the exchange processes and for certain types of biological function. I'm not sure that we can have much to say about those unless they represent states that occur with a fairly high probability.

HARVEY: Can you say something about the errors that are introduced by the harmonic assumption? Do you think the true motions are probably larger or smaller than you are coming up with as a consequence of anharmonicity?

PETSKO: I think maybe Martin Karplus could make a comment about that but I don't know how to answer.

RICHARDS: We'll conclude with a comment by Hans Frauenfelder on his own paper.

FRAUENFELDER: I would like to add two things. One is the factor three. We have had some difficulties about whether X^2 means x-squared or 3 X^2 or 1/3 X^2. In any detailed comparison for instance between Brill's and our data we should be careful, because I think there it may be a factor of 3 off—in the right direction, I hope. When you take a motion like that, the X^2 that you measure is really one- or two-dimensional depending on whether it's a point you turn or an axis. We have to check that.

The second point is about the large scale motions. I think it's difficult to get this information at present from x-ray. We have indirect evidence of large scale motions of the protein from measuring the binding in solvents of various viscosities, where we can explain all the features by not assuming static barriers inside the protein but by assuming opening and closing of channels. From the data, the information we find is that large-scale motions could certainly exist. They would explain all the data with fewer parameters than one needs if one has just a standard set of barriers, and one then gets information about the time scale which checks remarkably well with Mossbauer effect data. There one can get the X^2 and the time dependence because a natural time scale is built-in. So I think one will have to look for the large scale motion at the beginning, and get it from other tools, not the x-ray.