DISCUSSION

Session Chairman: Alan Finkelstein *Scribes:* John Baenziger and Murray Becker

ROGER KOEPPE II: Do the disulfide-cross-linked products retain enzymatic activity, or should they be viewed as "denatured" states that have been "trapped"?

JOSEPH FALKE: In the aspartate transducer there are "trapped" disulfide bond species that retain activity. If the "trapped" species are too far from the native conformation, the protein is inactive. The available evidence argues that the motions occur in the folded state, not a denatured state. However, disulfide formation can trap a nonnative folded state.

KOEPPE: The distance dependence shown in Fig. 3 is very sharp, especially given the log scale. How do you interpret this in molecular terms?

FALKE: The large difference in disulfide formation rates between the 26, 260 and the 26, 267 pairs, despite their nearly equal separation, may result from the fact that residue 267 is two to three times more buried than the other cysteines in the set. If the data for this pair is excluded, there may be a simple exponential decrease in bond formation rate with distance, although many more points are needed to fully characterize this relationship.

KOEPPE: Have you measured the temperature dependence of the disulfide bond formation in these mutants?

FALKE: We have determined that the rates are temperature dependent, but we have not yet examined the temperature dependences in detail. Certainly that is an area worth examining.

KOEPPE: For your first example, the galactose/glucose receptor, the structure was known and the sulfhydryl cross-linking reaction was used to obtain dynamical information. For the second example, the aspartate transducer, however, both structure and dynamics were unknown. In such a case, will not disulfide bond formation be a complicated function of both structure and dynamics, and will not structure prediction be problematic?

FALKE: It is important to measure more than the rates of disulfide bond formation in order to gain information about the proximity of cysteine pairs. The key check is to measure the effect of disulfide bond formation on protein activity. If the rate of disulfide bond formation is fast and the product remains active after disulfide bond formation, a very strong argument can be made that the cross-linked positions are in close proximity in the native structure. If the protein is not active, you may have trapped a species that results from a long range motion.

KOEPPE: If we accept that the mutations do not perturb the native structure of the galactose/glucose receptor, for which you have evidence, could not the newly introduced cysteines nevertheless enhance the local molecular motions in these nonnative structures? The motions of the mutated helices could therefore be more pronounced than in the wild-type helices.

FALKE: The available evidence indicates the surface cysteine substitutions are generally nonperturbing. We have checked the effect of these mutations on a number of properties of the reduced receptors. For each mutant, we determined the dissociation constant for the equilibrium binding of galactose to the receptor, we looked at the dissociation of terbium ion from the Ca^{2+} binding site, and determined the off rate constant for that process, and examined the free energy of unfolding extrapolated to zero urea concentration as a measure of the stability of the protein. In all these tests,the only effect we see is a change in the dissociation of terbium from the metal binding site in the mutant that carries the most buried cysteine 267. In this mutant, the dissociation of metal appears to be a bit slower, although it is less than a 20% effect relative to the wild-type protein. Overall, the lack of large effects is likely due to the fact that we limited our substitutions to residues that are on the surface of the protein.

We have also monitored the effect of substitutions on the fluorine NMR resonances of the five tryptophan residues in the protein. For the most part, the substitutions had no detectable effect on the fluorine chemical shifts and the line widths, which are sensitive to the local environment and rates of motion, respectively. These results indicate that the structural and dynamic perturbations of the substitutions are small or nonexistent. In particular, the 26, 274 mutant in which we detected the 15 Å translation, which is the largest translation of this type that has been detected, is indistinguishable from the native protein in terms of these parameters. Moreover, for this mutant we have compared the T1 relaxation rate of each resonance with the wild-type rate, and we see no differences in this property, which is sensitive to dynamics. Regarding the other receptors, we did see an allosteric effect on the frequencies of the tryptophans near the metal binding site as a result of the 26, 267 substitution, so there is a minor allosteric structural perturbation the receptor containing the most buried cysteines. In addition, in the control substitution, which occurs on the other side of the molecule in the galactose binding cleft, there is a small but detectable effect on both the galactose and metal binding site domains.

In short, for all but two of the engineered receptors, a variety of sensitive approaches have failed to reveal differences from the wild-type protein. While this evidence is negative, it is likely that any significant perturbation of structure or dynamics would have been detected by at least one of our assays.

ROBERT BLUMENTHAL: If the reaction rates are diffusion limited, one would expect an inverse square distance dependence not a logarithmic dependence.

FALKE: This isn't simple diffusion. There are constraints provided by the structure of the protein. We also need more numbers before we can analyze the distance dependence of disulfide formation rates in too much detail.

RAJINI RAO: You have assumed that the efficiency of disulfide bond formation is the same for all cysteine pairs. The chemical reactivity of any particular cysteine depends on the local environment so that the measured rate of disulfide bond formation may not be proportional to the motion.

FALKE: There will be environmental differences. We measure disulfide formation rates and we can tell you those precisely. It is also useful to try and estimate the time scales of the underlying collisions in order to understand these motions. We have measured an efficiency factor for disulfide formation between free cysteines in solution and have used that efficiency factor to approximate the efficiency factors for cysteine pairs on the protein surface. Although this may provide only a ballpark figure, for residues on the surface of the proteins the reactivities should be similar.

RAO: Could you compare the rates of disulfide bond formation of any pair with the individual rates of modification of a particular cysteine with a sulfhydryl modification reagent?

FALKE: We examined the reactivities of each of the cysteine pairs with iodoacetamide and they were the same within a factor of two, indicating that the net chemical reactivities of the cysteines are quite similar, most likely because they are on the surface of the protein. If we do the experiment with cysteines that are fully buried in the molecule, we find quite a large decrease in the iodoacetamide reaction rates. It is a bit surprising that the cysteine 267 labels at a rate similar to the others, even though it is partially buried. Perhaps the iodoacetamide assay does not detect all environmental and steric differences.

BOB WEIS: How much do the cross-linking rates go up in the absence of glucose?

FALKE: The rates for cysteines in the two adjacent helices go up by a factor as large as 100-fold. We see no disulfide formation for the control pair of cysteines on opposite sides of the molecule in the presence of bound sugar. In the absence of ligand we see rates that are comparable to the rates on the adjacent helices that may be part of some type of global unfolding transition. Alternatively, this bond could result from a large twisting motion that enables these two cysteines on different domains to collide.

ROBERT GUY: I was quite impressed with your work and the potential for using sulfhydryl engineering to analyze protein structure and dynamics. Could you briefly describe how feasible this approach is for other membrane proteins. What experimental conditions are required? Can disulfide bridges be formed in transmembrane and cytoplasmic segments? How difficult is it to determine whether a disulfide bridge forms; especially if formation of the bridge does not alter the functional properties of the protein? Can this approach be used to probe conformational changes such as a movement of the S4 helix in voltage-gated channels or plugging the pore with the inactivation gate?

FALKE: We think that the technology can be applied to other systems. You have to consider each system on an individual basis. One question is how many cysteines the wild-type molecule possesses. In some cases, even proteins containing multiple cysteines can be studied. Ron Kaback's group has engineered cysteines out of a transmembrane protein. That is an approach that can be used if there are not too many cysteines. Another simplification is that cysteines separated by a bilayer generally will not interact. Regarding the chemistry, the disulfide formation reaction uses mild conditions and has been described in our published work.

ALAN FINKELSTEIN: How much material does one need?

FALKE: We detect disulfide bond formation by running the products on a gel. You need enough to detect by Coomassie or silver staining, or whatever antibody staining you are using.

MARK BRAIMAN: Using the efficiency of disulfide formation in air-saturated aqueous solution as an upper limit seems potentially problematic when one considers that your oxidant (oxygen) is much more soluble in hydrophobic solvents than in water. The redox catalyst seems very hydrophobic as well. This would suggest that you might be overestimating collision rates between cysteines located within a transmembrane domain or near one or more hydrophobic residues that could serve as binding sites for oxygen or your redox catalyst. Have you measured the efficiency for disulfide formation in nonaqueous solvents? Could you normalize your data by measuring the formation of the immediate precursor of the disulfide bond (namely the sulfur radical) by a spectroscopic technique such as ESR?

FALKE: The reason that we may get an efficiency decrease is related to the destabilization of the charges involved in the intermediates and the transition state, which are likely to include Cu^{2+}, superoxide anion, and sulfanion. These charges would be present at lower concentrations in a low dielectric environment. We have not used EPR to detect sulfur radical intermediates: that would be a good experiment if it can be done. I can tell you that empirically the disulfide formation rates observed inside the bilayer are slower than those at the protein surface.

BRAIMAN: How do you distinguish between those intrinsic efficiencies and changes in mobilities?

FALKE: One way is to compare the disulfide formation rates between the same two transmembrane helices, starting with the cysteines in the aqueous phase, and moving them down the helices into the lipid phase. Preliminary findings from that type of experiment suggest that the reaction efficiency is lower in the lipid environment. A second approach, as you suggested, would be to compare the efficiencies of model reactions in aqueous and organic solvents. We have not tried that but it is a good idea.

WEIS: In the determination of the aspartate receptor oligomer size, Eq. 5 (the binomial distribution) assumes that cross-linking can take place between all subunits in the oligomer with equal probability. Is this always a valid assumption, especially for tetramers? For example in a tetramer, the formation of one disulfide bond might affect the rate of the second, or if the arrangement of monomers within the tetramer were a dimer of dimers, disulfide bond formation could proceed preferentially among certain pairs of the tetramer and thus appear as a dimeric structure.

FALKE: In that type of experiment, first carried out by Milligan and Koshland, we are not actually measuring rates. We are trying to drive disulfide bond formation to completion within the oligomers. The simple equations in the manuscript will not hold if the formation of the first disulfide bond prevents formation of a second. In that case, the results would deviate from the simple theory and you would have to develop a more complex model to fit the data. For the aspartate transducer, the simple dimer model fits the data quite well.

JUAN BALLESTEROS: With regard to Table 2, when you measure the relative accessibility to PHM, what is the reason why intracellular cysteines show such a difference between the leaky and the intact system?

FALKE: PHM is designed to be a membrane impermeant sulfhydryl modification reagent that can be used to determine which cysteines are in the cytoplasmic and periplasmic compartments. The PHM is added to the external compartment. A periplasmic sulfhydryl should react at similar rates in the two systems, while a cytoplasmic sulfhydryl should react much more rapidly in the leaky system.

BALLESTEROS: Those experiments were done with the unbound receptor. You mentioned that when aspartate was added, the disulfide formation rates changed dramatically. Cysteine 3 and 128 decrease while the other ones increase. Can you relate those differences with the crystal structure?

FALKE: There is only one change in rate that I have a strong opinion about and that is the 128 pair. When the disulfide formation rate was measured for this pair in the absence of aspartate, it was reasonably high. These cysteines are very far apart in the dimer. When the ligand is removed from the protein, the two monomers can transiently separate, because we observe exchange of monomers between different dimers. During such a transient dissociation, it is likely that the monomers can rotate about the bilayer normally. We think that they can rotate independently in such a way that these sulfhydryls can collide and form a disulfide bond.

When aspartate binds to the molecule, the monomers in different dimers no longer exchange, implying that aspartate binding prevents the dimer from separating. The aspartate binding site is at the interface of the two monomers and involves residues from each monomer. That would explain the reason why the disulfide bond formation rate goes down with aspartate. This example also illustrates the importance of measuring the effect of disulfide bond formation on protein activity before interpreting bond formation rates in terms of distance.

RONALD KABACK: Why is Cysteine 36 accessible on the surface, but reacts slowly with NEM?

FALKE: What we are measuring in the transmembrane accessibility experiment is the relative rate in the leaky and intact system. When we just measure reaction rates, with PHM or NEM, that residue reacts quite slowly compared with the others, which is consistent with its location in the center of the protein.

BALLESTEROS: I was interested in the residue number 3 with regard to the difference in the rate of disulfide formation in the bound and unbound states. When you have the ligand complexed, the rate of formation of residue 3 increases. When you have the unbound ligand, disulfide formation in residue 3 implies inactivation of the receptor. How do you reconcile these results.

FALKE: The new structure by Kim and Koshland does not extend through the membrane to the cytoplasmic domain where residue 3 is located. Modeling suggests that the transmembrane helices form a 4 helix bundle. In the case of cysteine 3, we found that the disulfide formation rate was fairly rapid and yet inactivates the protein. One way to rationalize that result is to argue that the helix is near the central axis of the molecule. The 3 residue is oriented away from the central axis and when you form the disulfide bond you distort the structure. Basically, it is on the wrong face of the helix.

BALLESTEROS: Wouldn't that imply that there is a rotation about the long axis of the helix that will change the azimuthal orientation of the cysteine?

FALKE: One picture consistent with the result is that there is a rotation of the NH_2-terminal helix about its long axis when the aspartate binds. This could explain the rate, but there are many other possibilities.

OLAF ANDERSON: This is a really neat technique, but I am concerned about the analysis of the oligomer state. To follow up on Bob Weis, in the case of a tetramer, for example, it might be difficult to distinguish a tetramer from a dimer. If the tetramer is formed by lateral association of two dimers, the possibility exists that cysteine 36 can cross-link only with its partner in the original dimer (half-tetramer) and not with either of the residues in the other half-tetramer. If this were the case, the cross-linking pattern for the tetramer would be indistinguishable from that of a dimer.

FALKE: We cannot rule out the very specific case that you mention, namely a dimer of dimers with C2 rather than C4 symmetry. However, I should point out that such a model requires the oligimers to be rigid in contrast to the observed dynamics. Experimentally, one could test your proposal by moving the cysteine to different regions of the monomer surface.

ANDERSON: That you can cross-link at cysteine 128 underlies the need to have a structure. Could it, for example, result from cross-linking of two adjacent dimers?

FALKE: We have observed that the cysteine 128 disulfide formation rate does not dramatically change when the protein is solubilized from the membrane. In the solubilized state, we know the protein is a dimer. I would not rule it out, but it seems unlikely that an intermolecular collision would occur at similar rates in the two systems; thus, we propose that disulfide formation occurs within the dimers. Again, this is reasonable because we know the dimer transiently dissociates to yield exchangable monomers, which could rotate to give the observed collision.

HAREL WEINSTEIN: It was my understanding that the Ca^{2+} binding loop in the galactose-binding protein is very similar in sequence and structure to the classical EF-hands (e.g., calmodulin), but the flanking regions are not like those in an EF-hand. Is this still true?

FALKE: Yes, it is true that there are differences. The COOH-teminal loop has a different structure and the loop bridges a helix and a B strand instead of the two helices that usually flank an EF-hand. However, the number and spatial positions of the coordinating residues are the same as in classical EF-hands. We believe this is the key point for ion binding.

WEINSTEIN: In the dynamics that you measured, you observed a change in the motion of the hinge region upon ligand binding, but no alteration in the Ca^{2+} binding region. Do you have any speculation of the role of the Ca^{2+} binding region?

FALKE: Our published 19F NMR results show very little allosteric coupling between the sugar and Ca^{2+} sites. It is known that metal binding stabilizes the protein, which could be important because the protein exists in an environment rich in bacterial proteases. The site could also function in the docking of the protein to its target transport protein.