Lumry, R., and A. Rosenberg. 1975. The mobile-defect hypothesis of protein function. *Colloqs. Int. C.,N.R.S.* **246**:53.
McCammon, J., and M. Karplus. 1980. The internal dynamics of proteins. *C.R.C. Crit. Rev. Biochem.* In press.
Molday, R. S., S. W. Englander, and R. G. Kallen. 1972. Primary structure effects on peptide and group hydrogen exchange. *Biochemistry.* **11**:150–158.
Nakanishi, M., M. Tsuboi, and A. Ikegami. 1973. Fluctuations of the lysozyme structure. II. Effects of temperature and binding of inhibitors. *J. Mol. Biol.* **75**:673–682.
Pohl, F. M. 1969. On the kinetics of structural transition I of some pancreatic proteins. *FEBS Lett.* **3**:60–64.
Richards, F. M. 1979. Packing defects, cavities, volume fluctuations and access to the interior of proteins. Including some general comments on surface area and protein structure. *Carlsberg. Res. Commun.* **44**:47–63.
Richarz, R., P. Sehr, G. Wagner, and K. Wüthrich. 1979. Kinetics of the the exchange of individual amide protons in the basic pancreatic trypsin inhibitor. *J. Mol. Biol.* **130**:19–30.
Rosa, J., and F. M. Richards. 1979. An experimental procedure for increasing the structural resolution of chemical hydrogen exchange measurements on proteins: application to ribonuclease S peptide. *J. Mol. Biol. 133:399–416.*
Schoenborn, B. P., J. C. Hanson, G. D. Darling, and J. C. Norvell. 1978. Real space refinements of neutron diffraction data from carbonmonoxide sperm whale myoglobin. *Acta Cryst.* **34A**(Suppl. 4):65.
Tuchsen, E., and M. Ottesen. 1979. A simple hydrogen exchange method for cross-linked protein crystals. *Carlsberg Res. Commun.* **44**:1–10.
Vincent, J., R. Chicheportiche, and M. Lazdunski. 1971. The conformational properties of the basic pancreatic trypsin-inhibitor. *Eur. J. Biochem.* **23**:401–411.
Wagner, G., and K. Wüthrich. 1979. Correlation between the amide proton exchange rates and the denaturation temperatures in globular proteins related to the basic pancreatic trypsin inhibitor. *J. Mol. Biol.* **130**:31–37.
Wagner, G., and K. Wüthrich. 1979*a*. Structural interpretation of the amide proton exchange in the basic pancreatic trypsin inhibitor and related proteins. *J. Mol. Biol.* **134**:75–94.
Wickett, R., G. Ide, and A. Rosenberg. 1974. A hydrogen exchange study of lysozyme conformation changes induced by inhibitor binding. *Biochemistry.* **13**:3273–3277.
Woodward, C. K. 1977. Dynamic solvent accessibility in the soybean trypsin inhibitor-trypsin complex. *J. Mol. Biol.* **111**:509–515.
Woodward, C . K., L. M. Ellis, and A. Rosenberg. 1975. Solvent accessibility in folded proteins: studies of hydrogen exchange in trypsin. *J. Biol. Chem.* **250**:432–439.
Woodward, C. K., L. M. E llis, and A. Rosenberg. 1975*a*. The solvent dependence of hydrogen exchange kinetics of folded proteins. *J. Biol. Chem.* **250**:440–444.
Woodward, C. K., and B. D. Hilton. 1979. Hydrogen exchange kinetics and internal motions in proteins and nucleic acids. *Ann. Rev. Biophys. Bioeng.* **8**:99–127.
Wüthrich, K., G. Wagner, R. Richarz, and S. Perkins. 1978. Individual assignments of the methyl resonances in the ^{1}H nuclear magnetic resonance spectrum of the basic pancreatic trypsin inhibitor. *Biochemistry.* **17**:2253–2263.
Wüthrich, K., and G. Wagner. 1979. Nuclear Magnetic resonance of labile protons in the basic pancreatic trypsin inhibitor. *J. Mol. Biol.* **130**:1–18.

DISCUSSION

Session Chairman: Hans Frauenfelder *Scribe*: Joseph J. Rosa

WÜTHRICH: Am I correct that for the amide proton of Tyr-23, your mechanism *b* determines the rate at pH > 6? If so, you would predict that the exchange rate at pH > 6 would be unaffected by variation of the denaturation temperature. Is that correct?

WOODWARD: We predict that for any NH proton under conditions where the exchange rate has an activation energy of 20–30 kcal, exchange is by mechanism *b* and there is not a correlation of exchange rate with thermal unfolding temperature.

WÜTHRICH: And that would be the case for the amide proton of Tyr-23?

WOODWARD: I cannot say without looking at the temperature dependence. At pH 6 the activation energy for Tyr-23 varies with temperature; this reflects a change in mechanism.

WÜTHRICH: After we read your paper in *Biochemistry*, we recalled that we once spent 6 months measuring the pH dependence of the exchange in one of the reduced forms of BPTI. This modified protein has a denaturation

temperature of ~65°C, as compared to 95°C for native BPTI. The exchange data, collected for 6 amide protons including Tyr-23 in both the native and reduced proteins, show that the difference in thermal stability is clearly manifested all the way up to pH 10. This clearly shows that for the one proton specifically treated, namely, Tyr-23, your mechanism *b*, is definitely correlated with the thermal denaturation temperature of the protein up to pH 10.

WOODWARD: This experiment is not a test of our model. Process *a* involves thermal unfolding and therefore exchange rates by this mechanism are a function of the thermal stability of the protein. Given a decrease in thermal stability of 30°C it is likely that exchange rates for process *a* are greatly accelerated relative to exchange rates for process *b* (exchange from the folded conformation). If this is so the curves for Fig. 4 *a* will be raised while those for *b* stay the same. Then exchange by unfolding occurs at higher pH's in the derivative as compared to the native BPTI. The critical experiment is to determine whether exchange from the disulfide reduce derivative at high pH is still with low activation energy and if so whether these exchange rates are correlated with thermal unfolding temperature.

I should add that we have looked at the urea dependence of the tritium exchange of the rapidly exchanging protons of BPTI. We find that 8 M urea does not accelerate on exchange rates even though 8 M urea lowers the thermal unfolding temperature of BPTI by 20°C.

Lastly, let me say that there are several protons for which Kurt Wüthrich has reported exchange rates that do not correlate with thermal unfolding around pH 4. Among these are Met 52, Tyr 35, and Ile 18. These are precisely the ones that we would expect to exchange without contribution from the major unfolding process. That is, they exchange with low activation energy around the pH minimum.

VON HIPPEL: The questions I raised in reviewing the papers of both Clare Woodward and Walter Englander had to do with clarifying and identifying the differences between models *a* and *b*, as they are called in this paper. They seem to be approaching one another a bit. We can all imagine model *a* in one form or another. Although it shouldn't necessarily require a global unfolding, it could certainly be an unfolding which involves a small segment and therefore may have different thermodynamic parameters. It remains to define the solvent penetration model specifically in molecular terms. I know that is a hard question. If it were easy it would have been anwered long ago.

Two routes that might be pursued are: 1) the use of the fluctuations discussed earlier in this meeting by the crystallographers and Dr. Karplus in terms of a set of conditional probabilities of channel formation that leads to solvent penetration within the bounds of the observed kinetics and thermodynamics, and 2) the variation in pH dependencies to define local environmental effects on acid and base catalysis which should reflect the specific pathways into the protein.

F. RICHARDS: I would like to make a specific response to Peter von Hippel. We have, in fact, made calculations regarding the fluctuations of cavities of just the kind you are talking about. If you make reasonable estimates of the physical properties of a protein and use its crystal structure, you can "predict" the probabilities of channel formation and from there the hydrogen exchange behavior of BPTI and various other proteins. The theory is not good, but it does show that local fluctuations, without macro-unfolding, are perfectly capable of explaining entry into the protein to the extent necessary to explain exchange behavior.

VON HIPPEL: Can you say anything about the enthalpy of activation of these processes?

F. RICHARDS: No, nothing at all. It's not that kind of a calculation.

KARPLUS: This is to supplement Fred Richards' point in terms of the question of channel formation within BPTI as a function of specific local motions. Although the results have not been analyzed in detail, we've looked at the fluctuations that occur on the picosecond time scale in the N-O distance of all the main chain H-bonds. What one finds is that there is a good correlation between the amide hydrogen exchange kinetics and the very short time fluctuation in their r.m.s. H bond lengths. Obviously, this only gives you some idea of what the local force constant might be and how it varies. Extrapolating to the very large fluctuations that are needed for exchange is difficult. But one can hope to be able to understand why certain protons are slow to exchange and others fast, in various proteins.

MATTHEW: I would like to raise the question of the pH dependence of hydrogen exchange. Unlike model compounds, proteins often fail to obey first-order dependence on hydroxyl ion. We must at least attempt to explain this in terms of the properties of the protein. As is well-known, the transition temperature of proteins is very pH dependent. In terms of solvent penetration, not only must a hydroxide ion get to the amide, but the exchange process *per se* involves a negatively charged intermediate. I would suggest two things: (*a*) the electrostatic field around and within the protein will clearly vary unsymmetrically with pH, such that approach and migration of hydroxyl ions in a given path will either be enhanced or hindered. (*b*) the electrostatic potential generated by the formal charge array can also affect the stability of the charged intermediate required for exchange. The magnitude of these effects can be quite large despite the use of a dielectric constant of 40 within the protein.

These effects would be in addition to the pH-induced conformational fluctuations or electrostatic modulation of substate population.

BARKSDALE: One of the problems that those of us working with hydrogen exchange have been concerned about is the correlation between the specific behavior of the very highly protected amides as see by Woodward and Wüthrich using n.m.r. and the global exchange properties of proteins observed in the more traditional tritium exchange or IR experiments. Comparison of the overall exchange data by Hvidt and Pedersen (1974) obtained by deuterium infrared measurements with a summation of Wüthrich's specific amide exchange data on BPTI indicates that the very slowly exchanging amides monitored by Woodward and Wüthrich are in fact a microcosm accurately reflecting the overall exchange behavior of BPTI. Interestingly, the distribution of rate constants for BPTI follows a log-normal distribution. By comparison, rate constants for many other proteins, such as lysozyme and myoglobin, are distributed according to a power law. It may be that the distribution function for these large proteins are the sum of several log-normal distributions over the domains of multi-domain proteins.

ENGLANDER: For outsiders this discussion must seem terribly fragmented. I would like to say some things which may give a picture of what is going on. Looking at the structure of BPTI and the location of the very slowly exchanging protons in the β sheet, we can easily see that the small size of BPTI precludes the necessity of penetration by solvent molecules. In fact, two of these very slow protons are on the surface of the molecule, in contact with water. There is no issue of penetration or process *b* for these protons.

With respect to what Jim Matthew said about electrostatic effects on hydrogen exchange, the consequences for hydroxyl ion penetration should be pointed out. The pH dependence for small molecule H-exchange shows acid-base catalysis with a minimum ~pH 3 for peptide hydrogens. In the case of a protein exchanging via solvent penetration, as the pH is lowered the surface positive charge generally increases. The effect, for exchange dominated by a solvent penetration mechanism, should be to promote hydroxyl ion penetration, decrease proton penetration and so move the pH minimum to more acid values. Such behavior has never been observed for any protein I know about. If any changes in the pH minimum are seen, the shift is generally to higher pH, as would be expected for exchange dominated by an unfolding mechanism.

The other issue has to do with the temperature dependence of hydroxyl ion penetration into the protein as required by process *b*. Hydroxyl ion, initially in water, with a rather large hydration sphere, can only be transferred into the protein by stripping off the coordination sphere. This costs on the order of 50 kcal/mol in enthalpy. This means that the temperature dependence of hydrogen exchange via a solvent penetration mechanism should show an apparent activation energy which includes 50 kcal/mol for that step alone plus 20 kcal/mol for the exchange step plus other terms. The sum approaches 100 kcal/mol and is never observed.

MATTHEW: The behavior predicted by Dr. Englander for an electrostatically induced shift of the pH minimum assumes a uniform charge distribution and would apply only if hydroxide migration in the protein's electric field is the only pH dependent property of the protein solvent interactions.

WÜTHRICH: I would like to make one comment and ask one question. The comment regards what Dr. Matthew just said. We have recently published a theoretical interpretation of the exchange data for BPTI which is based largely on considerations of the effect of electrostatic force on the stability. We have measured all the pK values for the ionizable groups in BPTI, so we were able to treat this in a reasonably accurate manner. There was no problem in fitting all the pH dependencies that have been observed using only the equilibria between the variously protonated forms.

I have a question for Fred Richards. Our reference to global fluctuations definitely does not imply a denaturing fluctuation. We do not require the denatured form of the protein, all of the motions are within the conformation space of the globular form. I do not, however, believe that the very high frequency motions of the type encountered in the picosecond range would give rise to sufficiently large fluctuations to allow amide proton exchange. Do you think that what you see as possible channel-forming fluctuations would be fluctuations that can be related to the stability of the protein? That is the crux of the matter. We see internal fluctuations in the native protein which are correlated with stability but which do not promote transitions to the denatured state at low temperature.

F. RICHARDS: Our own calculations are not able to address that question. They are based on the assumption that you can characterize a protein by an isothermal compressibility. We then assume that the cavities in the protein, which already exist in the x-ray structure, fluctuate. We calculate what these fluctuations ought to be, then assume combined probabilities, the way Peter von Hippel suggested, to calculate the probability of forming a channel of the necessary size. The time scale is imposed on this thermodynamic calculation simply by the hydrogen exchange kinetics since this is the only time scale available and everything is scaled on this basis. Doing this, it is perfectly possible to fit the hydrogen exchange data with a single parameter.

The results indicate that small fluctuations are adequate to explain accessibility. With regard to Walter Englander's question, it is not necessary to get hydroxide ion inside the protein, and I don't think anybody believes

that it ever happens. My own prejudice is based entirely on Bill Jencks' suggestion that a cavity is formed within the protein and is filled with a chain of neutral water molecules. Having established that channel, with very low probability in many cases, we now can bring up a hydroxide ion at the protein–solvent interface. With an ice-like proton shift, the hydroxide "appears" in the protein, the charge is transferred, distributed over the peptide, and leaves the way it came. I don't see any problem here. Forming the water channels is a relatively low energy process since we're not desolvating an ion.

ENGLANDER: The process you describe generates, it seems to me, an internal hydroxide ion intermediate that is largely desolvated. As we all know, the equilibrium thermodynamic problem cannot be side-stepped merely by utilizing a clever pathway to achieve this state.