

Deriving Structures from 2D NMR. A Method for Defining the Conformation of a Protein Adsorbed to Surfaces

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With the development of multi-dimensional NMR methods for the specific assignment of many of the ^1H NMR signals of small proteins, it is now possible to determine three-dimensional solution structures and dynamics for these proteins. Unfortunately these methods fail to provide detailed structural information on proteins bound to macroscopic surfaces because of the very slow overall rotational correlation time of the particle-bound protein. Indeed other spectroscopic and structural methods have provided few details of the structure of proteins when adsorbed to surfaces (1). Commercially, surface-bound, immobilized enzymes provide an important method for efficient utilization of these catalysts in bioreactors. The development of biosensors and novel biomaterials such as self-assembled monolayers (2) requires a better understanding of the structure of proteins bound to surfaces (1). Identification of specific residues of proteins involved in interactions with stationary phase surfaces is critical in understanding their chromatographic behavior (3). Finally, the association of proteins with hydrophobic or glass surfaces has been shown to cause denaturation or partial unfolding of the protein at the surface, with often irreversible loss of the strongly adsorbed protein. It is believed that these irreversibly bound proteins undergo a conformational change to expose a portion of the interior residues to the surface for effective adsorption.

In this report we describe an NMR methodology that for the first time allows us to probe the detailed conformation of a protein bound to a macroscopic surface. This was accomplished by 2D amide hydrogen exchange NMR spectroscopy (4, 5, 6). In a protein, the NH exchange rates for different residues can vary over a factor of greater than 10^8 (4, 5). The rate of amide hydrogen/deuterium exchange depends on the pH (being both acid and base catalyzed), the accessibility of the hydrogen to solvent (stabilization by hydrogen-bonded secondary as well as tertiary structure and finally, local fluctuations of the protein (7). Importantly amide hy-

drogen exchange can be studied under conditions where high resolution resolvable proton NMR signals would otherwise not be observed – this would be true for a protein immobilized at a polymeric or glass surface. However, analysis of the degree of NH exchange is feasible if the protein can be desorbed from the surface and the 2D NMR study of the free protein carried out in solution.

As demonstration of the feasibility of the method, we have used amide hydrogen exchange NMR spectroscopy of lysozyme bound to a hydrophobic chromatographic stationary phase support. Hen egg white lysozyme (E.C.3.2.1.17) was chosen for this initial study because the ^1H -NMR assignments have been made (8), the crystal structure was known to 2 Å resolution (9) and much was known about its adsorption onto hydrophobic substrates (10). Lysozyme is one of the major constituents of protein deposits on contact lenses (11) and is commonly used as a standard in the chromatographic separation of proteins on reverse-phase columns.

In our protocol we first adsorb lysozyme to the hydrophobic surface (solid 5 μ diameter polystyrene divinylbenzene chromatographic stationary phase support; Polymer Labs Inc., Amherst, Mass.). This forms a tightly bound single monolayer on the surface (10). We then expose the surface bound lysozyme to D_2O under fast amide hydrogen exchange conditions (high pH), desorb the protein under slow NH exchange conditions (low pH) using a detergent, and after removal of the detergent, run the high resolution 2D spectra in D_2O solution. The intensities of the 2D NHCH_α TOCSY crosspeaks then reflect the degree of exchange of the specific residue amide hydrogens when adsorbed to the hydrophobic surface.

The stock solution of lysozyme (Sigma) was purified using a home built hollow fiber bundle dialysis device versus 10 mM NaH_2PO_4 pH = 7.4 buffer. An aliquot of the stock solution containing 125 mg of

lysozyme was diluted to 10 mL in buffer and mixed with 10 g of hydrated stationary phase support for 30 min. with stirring. The solution was then removed by the use of a filter funnel with a medium grain glass frit.

Eighty milliliters of buffer were used to wash the stationary phase support in 10 to 20 mL aliquots. For each aliquot a sample of the filtrate was taken for UV determination of protein concentration ($\epsilon_{280} = 2.313$ O.D. mL/mg) to ensure that only an "irreversibly" bound monolayer coverage remained. Typically, out of 125 mg of lysozyme initially added ~25 mg would remain on the support after extensive washing. The stationary phase support has a surface area of ~3 Å/gm and a rough calculation using the molecular dimensions of lysozyme ($4.5 \times 3 \times 3$ nm³) shows that ~50 mg of protein could adsorb to 10 gm of support with monolayer coverage. The 25 mg of lysozyme adsorbed represents 50% coverage which is in good agreement with the 65% coverage by the irreversibly bound layer determined by Schmidt et al. (10) to a similar hydrophobic surface.

At this point a 10 mM NaH₂PO₄ D₂O solution pH* = 7.0 (uncorrected pH meter reading) was prepared and mixed with the lysozyme covered support. The adsorbed protein was then stirred for 45 min. during which time the amide hydrogens that are exposed to solvent can exchange with deuterons. After 45 min. the solution was aspirated away and another 10 mL of the buffered D₂O solution at pH* = 2.5 was added to quench the amide exchange.

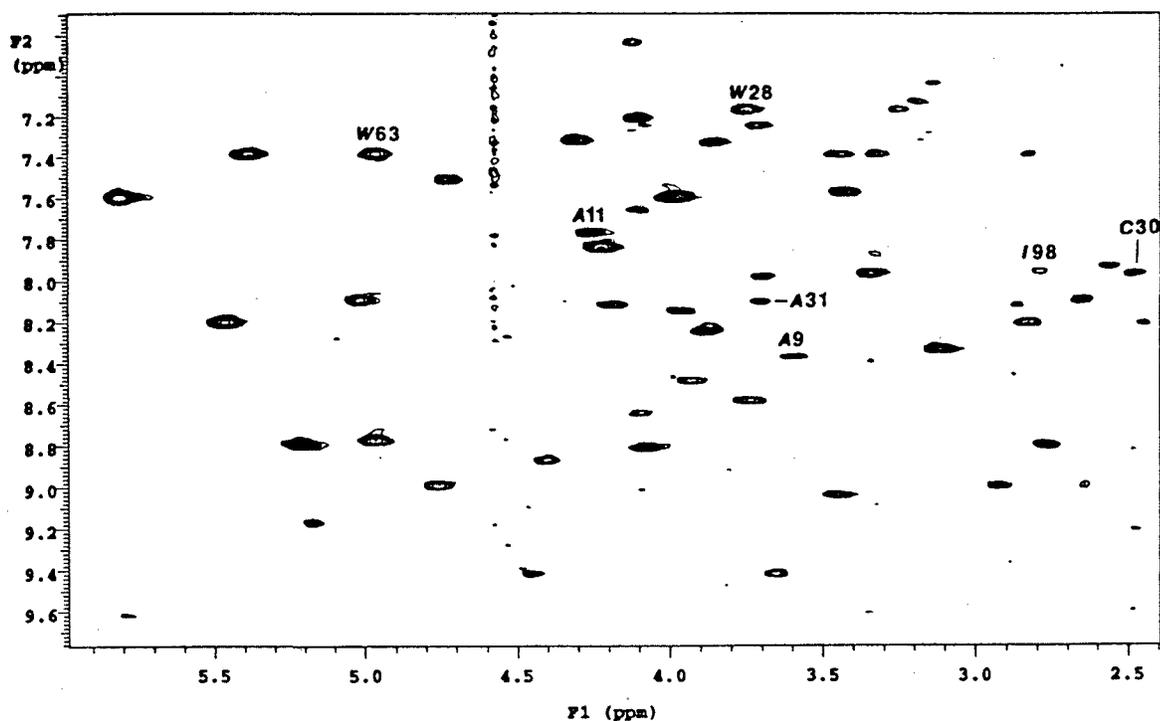
Two aliquots of 0.1% Triton X-114-RS (Sigma) detergent in 10 mL D₂O, pH* = 2.5 were used to remove the tightly adsorbed protein. The reduced form of Triton was used to allow determination of desorbed protein concentration by UV. The ~30 mL of desorbed lysozyme/Triton solution was then concentrated by pressure dialysis (Amicon, Beverly, Mass.) to ~4 mL using a 5000 molecular weight cut-off membrane. The 4 mL was then passed through an Extracti-Gel column (Pierce) equilibrated with pH* = 2.5 D₂O buffer to remove the Triton. The column eluent was further concentrated to ~750 μL and placed in an NMR tube. Approximately 5 mg (0.5 mM) of desorbed lysozyme was obtained by this procedure for the NMR study. Much of the protein loss occurred during the repurification scheme since the adsorbed protein was nearly quantitatively removed from the surface by the detergent treatment.

In the control experiment the identical procedure was used except that only 5 mg of lysozyme was used and no stationary phase support was present. An additional control was run without stationary phase support and without Triton to ensure that any residual Triton not detectable in the 1D NMR spectrum did not interfere with the amide exchange results. This control gave essentially the same integrated area for the TOCSY cross peaks of the NH-C_αH region as the control involving Triton.

The 600 MHz ¹H-NMR NH-C_αH region of the 30 ms TOCSY spectra of the control and surface adsorbed/desorbed lysozyme is shown in Figure 1. Assignment of the signals was based upon complete analysis of the TOCSY spectra, taking advantage of the reported ¹H assignments of lysozyme at 500 MHz (8). Normally, a series of TOCSY spectra at various time points are taken to measure the rate of NH exchange. Since this has been shown to be first order, comparing the spectra at a fixed time of exchange under conditions where the protein is either surface-bound or not will also provide a measure of the relative degree of NH protection. We define protons as being "exposed" when their crosspeaks present in the control spectrum are decreased in intensity by >40% relative to the surface exposed protein spectrum. Protons are "protected" if the crosspeaks are either absent in the control spectra or increase in intensity by >40% in the surface exposed sample spectra. Three control experiments (without the support present) were used to calculate the percent deviation from the mean of the integrated areas of 39 of the slow exchanging amide proton NH-C_αH TOCSY cross peaks. The mean of the 39 deviations from the means of the integrated areas was 20±13%. Thus, only those changes in integrated area ≥40% were considered exposed or protected after averaging the cross peak areas from two separate adsorbed protein and control experiments. These results are tabulated for the slow exchanging amides [as defined by Redfield & Dobson (8) at pH = 3.8, 35°C, τ_{1/2}, the half life for exchange ≥ 1-5 h] in Table I. Also included in Table I are the integrated intensities of 2 intermediate exchange (1 h < τ_{1/2} < 5 h) and one fast exchange amide hydrogens (τ_{1/2} ≤ 1-5 h) which are protected relative to the control.

All of the amides which are protected (Table I with the exception of the 2 arginines are neutral or hydrophobic residues. A CPK model [Figure

A



B

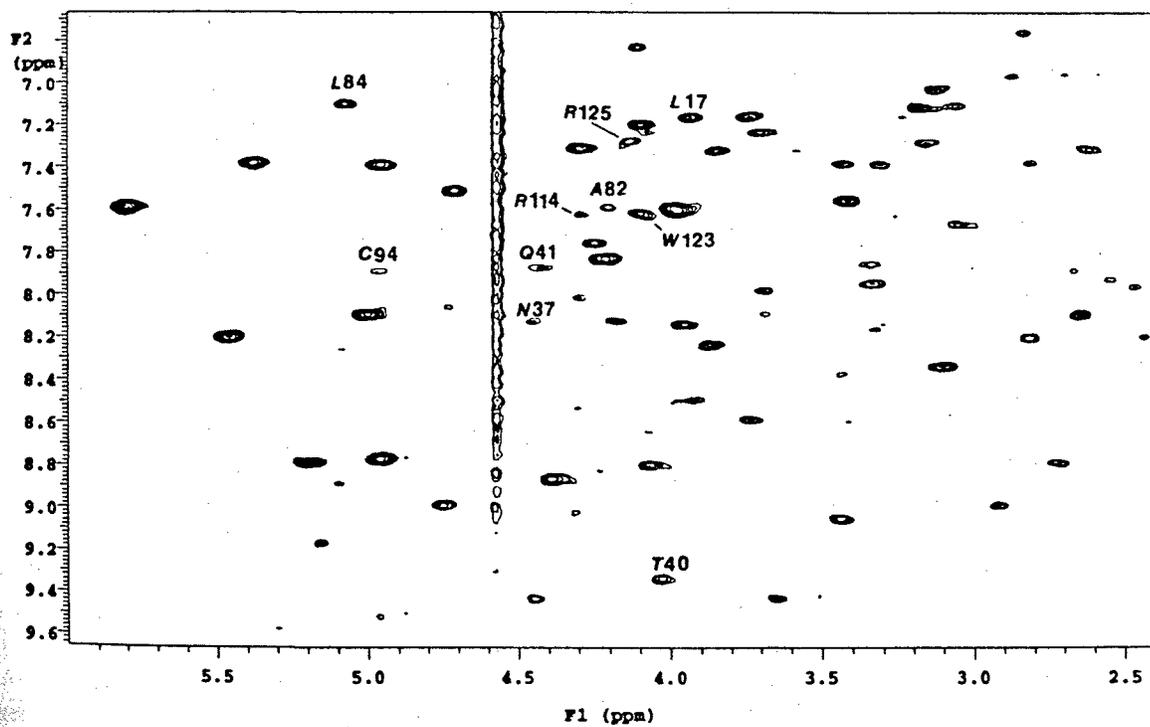


Figure 1: Identical 600 MHz ^1H -NMR TOCSY experiments at 35°C and $\text{pH}^*=2.5$ were run on both the control and adsorbed lysozyme samples. The data was collected in phase sensitive mode with a mixing time of 30 ms using a MLEV17 spin lock and 2 ms trim pulses. 2K points were collected in F2 and 312 in F1 with a sweep width of 7500 Hz and a repetition time of 3 s. 32 transients were coadded at each t_1 increment. The HOD signal was suppressed through low power decoupling at the HOD frequency. The spectra were processed with zero filling to 2K points in the F1 dimension and a 45° phase shifted sine bell apodization in both dimensions.

based upon the crystal structure of lysozyme (9)] shows that all of the protected amide residues (labeled by shading) are found on the surface. Four of the protected amide residues (T40, Q41, A82 and L84) are located close in space at the hinge region between the α -helical and β -sheet domains of lysozyme opposite the active site cleft.

All of the exposed amides – those that show enhanced amide exchange upon binding to the surface – with the exception of W63 are involved in α -helices or β -sheet structures and are mostly buried in the interior of lysozyme (Table I). Of the exposed amides only D52 and W63 show significantly exposed side chains as shown in the CPK model (Figure 2; exposed residues shown by stripes).

The protein amide hydrogens that are observable by 2D amide exchange spectroscopy are mostly the slow exchanging amides (58/129) which are either involved in secondary or tertiary structure hydrogen bonding or buried in the interior of the protein and inaccessible to solvent (12). Surprisingly, one fast exchanging amide hydrogen (R114), and two intermediately exchanging hydrogens (T40 and Q41) $C_{\alpha}H-NH$ cross peaks were observable in the spectrum of lysozyme exposed to the hydrophobic surface which were not present in the control spectrum (Figure 1).

Figure 2A shows that the protected (stippled) amide hydrogen side chains lie on one face of the globular protein in a narrow ridge from R125 to L17. In contrast, in the back-side view (Figure 2B), only a few of the largely buried side chains of the exposed residues (striped) are visible. None of the protected residues are visible in this view. These results suggest that lysozyme is not randomly oriented with respect to the surface, but that it is oriented with a relatively hydrophobic ridge facing towards the hydrophobic surface. This is consistent with the observed retardation of amide hydrogen exchange in binding an amphiphilic helix to a micelle (13), believed to result from burial of the hydrophobic face of an amphiphilic helix into the hydrophobic interior of a detergent micelle.

In addition a number of residues exchange *more* rapidly when the protein is surface bound than when it is in solution. All of these are not exposed to the solvent in the native structure but are located on elements of structure that are likely involved in segmental motion of the two domains that form the

active site cleft (14). Binding of the hydrophobic ridge of the protein to the surface thus appears to induce a conformational change, exposing the active site residues and residues adjacent to the active site to solvent.

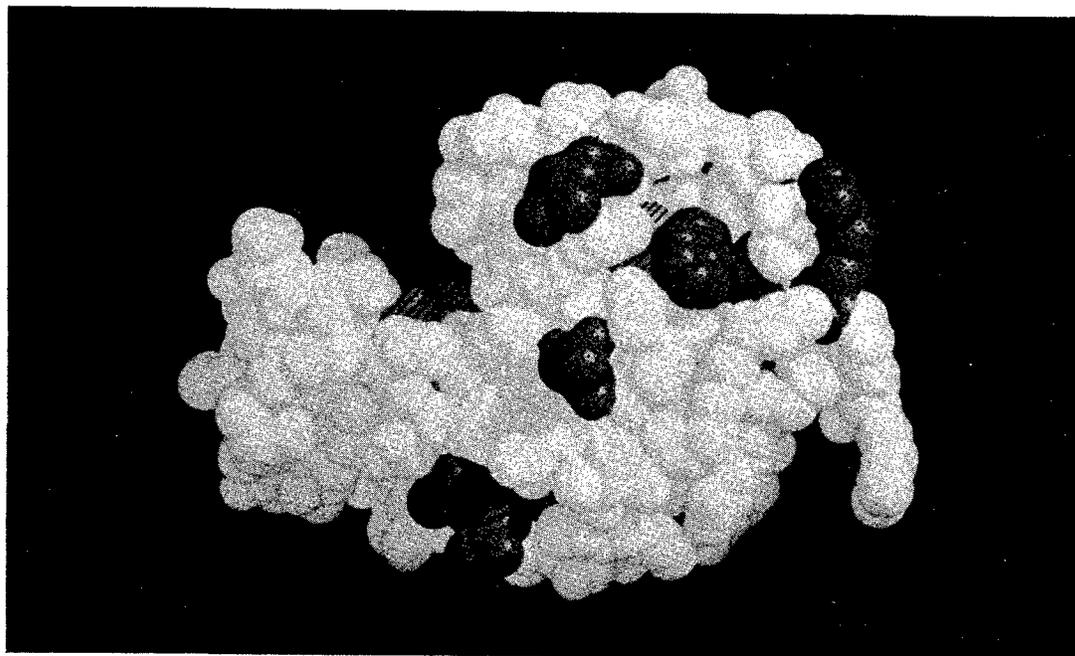
These perturbations in the amide exchange rates do not simply reflect proximity of the protein to the surface because enhancement and protection towards exchange are observed. In addition most of the amide hydrogen exchange rates which are not exposed or protected are the *same* for surface bound or free lysozyme. In contrast only decreases in amide exchange rates are observed in binding an amphiphilic helix to a micelle (13) and in crystalline lysozyme (15).

Taken together these data indicate that lysozyme adsorbs to the surface on the side opposite the active site cleft. This protects this narrow ridge of amides from exchange by either blocking solvent access to these residues at the hydrophobic surface or by reducing the rate of local fluctuations in the surface oriented residues. In addition upon binding to the hydrophobic surface a significant disruption of several of the buried α -helices and the active site cleft occurs as the protein partially unfolds at the surface presumably by opening of the "hinge" at the active site (16), exposing a number of buried residues that now show enhanced rate of hydrogen amide exchange. This conformational change alters the amide solvent exposure and/or local fluctuations that allow access of solvent to these interior residues.

This partial unfolding exposes the catalytically important D52 residue and other important residues in the active site cleft. This model is supported by the fact that the enzyme is inactive on adsorption to the hydrophobic surface of alkylated silica (10). These conclusions are also in agreement with the results of Fausnaugh and Regnier (3) based upon analysis of chromatographic behavior of various bird lysozymes that also suggests the protein adsorbs on a side opposite the active site with a contact surface that extends from residues 41 to 102 and 75 to 89. Computer modeling has also revealed a relatively hydrophobic patch identified as a possible binding site in this region and total internal reflection intrinsic fluorescence (TIRIF) shows a decreased quantum yield (and hence altered conformation) of the adsorbed hen lysozyme (17).

Importantly, the surface desorbed lysozyme

A



B

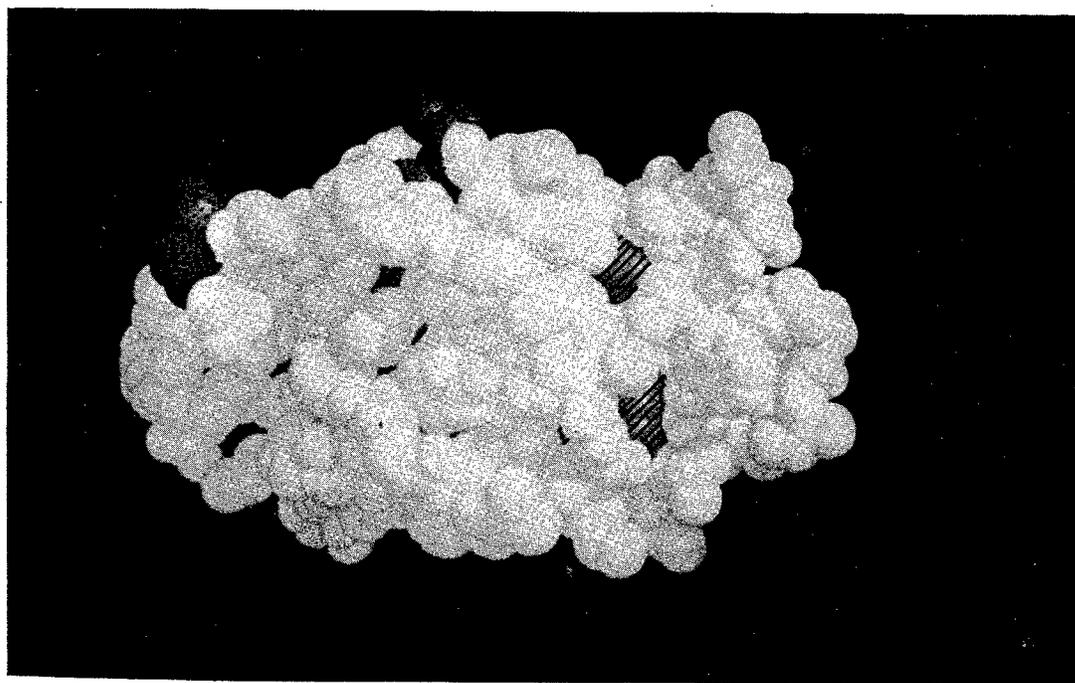


Figure 2: Three dimensional structure of hen lysozyme. A) Front-side view of a CPK model [MIDAS modeling software, UCSF (21)] showing amide NH's which are relatively protected (shaded and stippled) or exposed (shaded and striped) when the protein is bound to the polystyrene surface. The active site cleft is oriented towards the upper left. B) No residues that are protected from exchange (shaded and stippled) can be seen in this backside view of the protein. Several of the side chains of residues that are more exposed (shaded and striped) can be observed in this view.

Table 1: Comparison of Control and Adsorbed Hen Lysozyme Amide Exchange: Integrated Areas of Hen Lysozyme CH-NH TOCSY Cross Peaks

Residue	Control ^a	Surface Adsorbed ^b	Type ^c	Change ^d
A9	1.19		Slow	Exposed
A11	4.26	2.55	Slow	Exposed
L17		2.40	Slow	Protected
W28	4.53	2.73	Slow	Exposed
C30	1.06	0.59	Slow	Exposed
A31	0.68	0.32	Slow	Exposed
N37		1.26	Slow	Protected
T40	0.27	0.71	Inter.	Protected
Q41		1.30	Inter.	Protected
D52	4.71	2.84	Slow	Exposed
W63	4.36	2.47	Slow	Exposed
A82		1.03	Slow	Protected
L84		1.49	Slow	Protected
C94		1.28	Slow	Protected
I98	0.99	0.24	Slow	Exposed
R114		0.87	Fast	Protected
W123	1.15	1.91	Slow	Protected
R125		1.45	Slow	Protected

a.) Blanks indicate no observable cross peak.

b.) The integrated area of the adsorbed protein CH-NH TOCSY cross peaks were normalized via the integrated area of the non-exchangeable Trp 108 H4-H5 cross peak in the control and adsorbed protein spectra. The integrated areas in the table are the average of two control and two adsorbed protein experiments.

c.) Slow, intermediate and fast exchange amide protons are classified as per Redfield and Dobson (4).

d.) Exposed amides are present in the control and diminished by >40% in integrated area or absent in the adsorbed protein spectra. Protected amides are present in the adsorbed protein spectra and diminished in integrated area by >40% or absent in the control spectra.

spectra have identical chemical shifts as the native form spectra. This suggests that while the protein partially unfolds at the surface, it refolds by the same kinetically or thermodynamically favorable pathway upon desorption. Thus, the surface unfolded state may represent a protein folding intermediate of native hen lysozyme. Only a transient folding intermediate has been previously identified in lysozyme (12). By binding the protein to a surface it may be possible to trap a partially unfolded state of lysozyme that bears some resemblance to the transient folding intermediate observed by Miranker et al. (12). This would be complementary to the rapid quench NMR amide hydrogen exchange spectroscopy methods (12, 18, 19, 20) which provides structural details on the initial refolding kinetic intermediates. Our method would presumably provide information on the initial *unfolding* intermediate.

Surface 2D amide exchange spectroscopy offers a new method by which protein adsorption can be monitored at the level of individual residues. This work demonstrates for the first time the feasibility of the method which should be applicable to a number of other enzymes. The method offers a more detailed picture of protein adsorption than provided by currently used techniques (e.g. TIRF, ATR-FTIR and Raman spectroscopy (1)). Future work will include the extension of the methodology to other surfaces and proteins. Indeed at the XVth International Conference on Magnetic Resonance in Biological Systems (Jerusalem, Israel, August 16-21, 1992, abstracts) K. Kawano et al., described similar application of the NH exchange experiment to the binding of lysozyme to hydroxyapatite. They also find that certain residues are protected from exchange when the protein is adsorbed to the surface. However unlike our results no enhancement of exchange is observed.

Acknowledgments

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