Effect of Antibody Binding on Protein Motions Studied by Hydrogen-Exchange Labeling and Two-Dimensional NMR†

Leland Mayne,*,‡ Yvonne Paterson,§ Douglas Cerasoli,§ and S. Walter Englander‡

The Johnson Research Foundation, Department of Biochemistry and Biophysics, and Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6059

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ABSTRACT: We have used hydrogen-exchange labeling detected by 2D NMR to study antibody–protein interactions for two monoclonal antibodies raised against horse cytochrome *c*. The data show that these antibodies bind mainly to the large 37–59 Ω-loop of the cytochrome *c* molecule. In addition, the results provide some suggestive evidence concerning units of local structural flexibility in cytochrome *c*.

The structural nature of antibody–protein antigen binding sites has been studied by chemical protection, by chemical and genetic modifications of the antigen on binding (Paterson, 1989), and by X-ray crystallographic studies of antibody–antigen complexes (Coleman, 1988; Davies et al., 1988). This paper reports the second application of NMR-detected hydrogen exchange to this problem. In the first (Paterson et al., 1990), hydrogen-exchange results mapped out a region of the protein surface of horse cytochrome *c* that appears to directly reflect the binding site of the monoclonal antibody E8. In the present study, the binding of the E3 and C3 antibodies to horse cytochrome *c* was found to affect the hydrogen-exchange behavior of regions within and beyond the immediate binding site, apparently by restricting local conformational flexibility.

The study of protein internal dynamics has been quite active in recent years, particularly by computational methods (Goodfellow, 1990). Time-resolved fluorescence studies (Axelsen et al., 1991) and other methods including hydrogen exchange have added to our knowledge of protein flexibility. Here we present evidence that antibody–antigen interactions can provide an additional tool for such studies.

MATERIALS AND METHODS

Preparation of Antibody Column. The E3 and C3 monoclonal antibodies are specific for horse cytochrome *c* and bind with high affinity (Carbone & Paterson, 1985). These antibodies were purified by affinity chromatography from ascites tumor fluid by passage over an Affi-Gel 10 (Bio-Rad) column coupled with cytochrome *c* (Sigma, type VI, horse heart) as previously described (Carbone & Paterson, 1985). Antibody was concentrated to 6–9 mg/mL and coupled to Affi-Gel 10 at a ratio of ~8 mg of antibody/mL of gel. Affi-Gel 10 was initially rinsed with 2-propanol and water at 4 °C as recommended by the manufacturer and then allowed to couple with antibody overnight at 4 °C. The coupling was halted, and unbound reactive sites on the Affi-Gel beads were blocked by addition of diethanolamine. The antibody-bound gel was preeluted with 0.1 M acetic acid, then washed with storage buffer (50 mM phosphate, pH 7), and stored at 4 °C. The E3 column had a bed volume of 13 mL containing an estimated 125 mg of E3 and the capacity to bind 13 mg of cytochrome *c*. The C3 column had a bed volume of 21 mL containing an estimated 125 mg of C3 and the capacity to bind 10 mg of cytochrome *c*.

H–D-Exchange Procedure. The H-exchange procedure is outlined in Figure 1. To form the antibody–cytochrome *c* complex, the antibody column was incubated with a 50% excess (~20 mg) of cytochrome *c* (Sigma, type VI, horse heart) in phosphate buffer (pH 7, 50 mM) for 3 h. A small amount of ferricyanide was added to ensure complete conversion of the cytochrome *c* protein to the ferric state. Excess protein and ferricyanide were then washed out with fresh buffer, and H–D exchange was started by washing with D_2O buffer (pD 7, 50 mM phosphate). The column was then sealed and submerged in a 20 °C water bath. Exchange was allowed to continue for times ranging from 0.75 to 213 h. In addition, a zero time point was collected by omitting the exchange buffer wash. Exchange was stopped by washing the column with cold prewash buffer (50 mM citrate, pD 5.5, 4 °C) followed immediately by cold elution buffer (200 mM glycine, 1.0 M KCl, pD 2.5, 4 °C). The 1-mL fractions were eluted, and each was immediately reduced and quenched to a final pD of ~5.3 with a calibrated amount (~0.2 mL) of quench buffer (500 mM phosphate, 40 mM ascorbate, pD 7.9). A rubber bulb air pump was used to provide ~80 Pa (1.5 psi) of air pressure above the column to increase the flow rate. The total time required for elution was ~1 min. For the E3 column approximately 12 mg of cytochrome *c* eluted in 12–16 mL. For C3 approximately 9 mg of cytochrome *c* eluted in the same volume. Protein-containing fractions were pooled and concentrated using Centricon 10 or Centriprep 10 centrifugal ultrafiltration devices (Amicon) at 5 °C. The protein was washed twice with NMR buffer (50 mM phosphate, 12 mM ascorbate, pD 5.3) and brought to a final concentration of ~2 mM in 0.4 mL for NMR measurement. Sodium 3-(trimethylsilyl)tetradeuteriopropionate (TSP) was used as an internal chemical shift standard.

Most peptide NH's in the protein exchange at the same rate as in the free protein (see Table I and Lys 13 in Figure 2), which indicates that the brief acid wash in the elution process has a minimal effect on the measured exchange rates for two reasons. At this temperature and pH (5 °C, pD 2.5) exchange rates even for freely exposed peptides are less than 0.05/min (Englander & Kallenbach, 1984). In addition, the high ionic strength used in the elution buffer has the effect of stabilizing the hydrogen-bonded protein structure against

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* Author to whom correspondence should be addressed.
‡ Department of Biochemistry and Biophysics.
§ Department of Microbiology.

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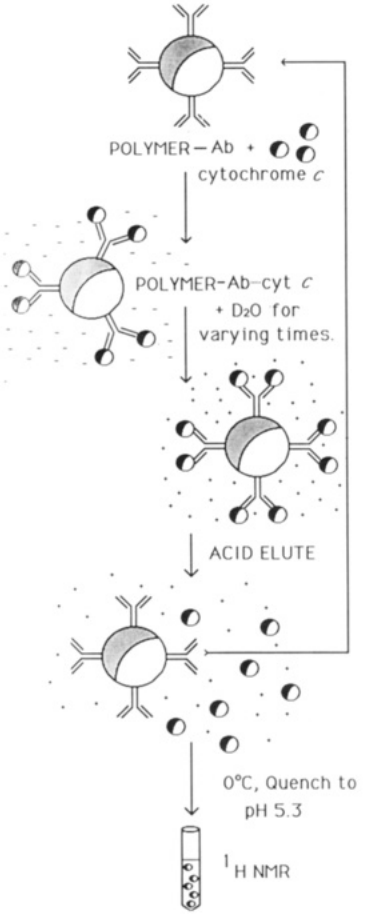

FIGURE 1: Schematic representation of the H–D-exchange experiment showing the binding of cytochrome *c* to the antibody on an affinity column, the exchange period, and elution of the exchange-labeled protein [from Paterson et al. (1990)].

acid denaturation (Jeng et al., 1990; Jeng & Englander, 1991).

The hydrogen-exchange behavior of free cytochrome *c* is the subject of another paper (J. S. Milne et al., unpublished results). In these experiments exchange was started by passing cytochrome *c* (H_2O, pH 7, 50 mM phosphate, treated with a small amount of ferricyanide as above) through a small gel filtration column (3-mL bed volume, Sephadex G-25 fine, Pharmacia) previously equilibrated with D_2O buffer (pD 7, 50 mM phosphate). This transfer is accomplished in less than 30 s by spinning the column in a bench-top clinical centrifuge. H–D exchange proceeded in sealed tubes in a 20 °C water bath. Exchange was slowed for NMR measurement by reducing the heme with ascorbate and passing the sample through another column equilibrated with NMR buffer (25 mM phosphate, 25 mM acetate-d_3, 12 mM ascorbate, pD 5.3). A total of 17 hydrogen-exchange time points were measured.

NMR Measurements. Two-dimensional COSY spectra were collected on a Bruker AM500 spectrometer. A total of 400 t_1 sets of 1024 complex data points each were collected; 96 or 128 transients were averaged for each t_1 fid. Solvent was suppressed by presaturation during the recovery time. Data processing was done with the programs FTNMR and Felix (Hare Research). Data sets were transformed in the magnitude mode with 2 Hz of line broadening and unshifted sine-bell multiplication in both dimensions. NH–C_αH cross-peak volumes were integrated and normalized against the nonexchanging heme CH–CH_3 bridge 4 cross peak.

For each exchanging peptide NH, the normalized intensities were plotted against exchange time and fit by a first-order rate expression. The measured rate for each NH was compared to its exchange rate in the free protein to determine the degree of HX slowing in the complex. Analysis of protection factors for those residues with factors near 1 indicates that the protection factors are accurate to within a factor of 1.4 for residues with rates in the range of 10^{-3}–0.5 h^{-1}. An analysis of the decay curves was used to confirm the presence or absence of protection.

RESULTS

Table I lists H–D exchange rates measured for the peptide hydrogens observed in the two antibody complexes (k_{bound}) along with the rates in the free protein (k_{free}) (J. S. Milne et al., unpublished results) and protection factors for each position (k_{free}/k_{bound}). Figure 2 illustrates the quality of the H–D-exchange data obtained. Only those hydrogens for which exchange could be followed in both free and complexed cytochrome *c* are listed in Table I. Many peptide hydrogens exchange too quickly to be measured in a COSY experiment, and some (residues 10, 68, 94–99) are too slow ($k_{free} < 10^{-4}$ h^{-1}) to measure under the conditions used here. It is important to remember that the NMR spectra are recorded with uncomplexed cytochrome *c* so any peptide hydrogen that exchanges rapidly in the free protein under the conditions of the NMR measurement will not be seen even though it may be quite slow in the complex.

Most of the measured hydrogens (~23 of 37) exchange at essentially the same rate in the complex as in the free protein (Table I). A number of hydrogens are protected from exchange in each complex. In the E3 complex, residues G29, L32, R38, Q42, N52, W59, K60, L64, K79, and M80 are slowed by factors of 12–60. T19, H33, Y74, and I75 are slowed by factors of 3–7. In the C3 complex, essentially the same residues are protected, but the highly protected set (factors of 11–55) is smaller, containing T19, G29, L32, R38, and Q42, while more residues (H18, H33, N52, W59, K60, L64, Y74, I75, K79, and M80) are protected by factors of 2–6. The factors reported for K79 and M80 are poorly determined as these residues exchange quickly even in the complex. All of the slowed residues are found within three stretches of the primary sequence, namely, residues 18–33, 38–64, and 74–80.

Previous work by other methods has implicated four residues as being in the binding site for each antibody (Paterson, 1989). For the E3 antibody, Thr 47 was implicated by the effect of evolutionarily variant cytochromes on binding (Carbone & Paterson, 1985), Thr 40 was implicated by observing the effect of protein-engineered cytochrome on binding (Collawan, Wallace, and Peterson, unpublished observations), and lysine residues 53 and 79 were implicated by immunoprotection from acetylation (Oertl et al., 1989). For the C3 antibody, Pro 44 was implicated by the effect of evolutionarily variant cytochromes on binding (Carbone & Paterson, 1985), His 26 and Pro 44 were implicated by analysis of tryptic proteolysis (Jemmerson & Paterson, 1986; Cooper et al., 1987)), and lysine residues 53 and 79 were implicated by immunoprotection from acetylation (Oertl et al., 1989). Among these residues, K79 is the only one that is measurable by the H-exchange method; it is found to be slowed in the complex for both antibodies. Residues 29, 38, 42, and 52, which are sequentially close to previously implicated residues 26, 40, 44, 47, and 53, are seen to be protected from H–D exchange in the complex.

The slowed residues in Table I, although widely spread in the primary sequence, are all distributed around the "bottom" of the molecule in the three-dimensional structure viewed in

Table I

residue[a]	H-bond acceptor[b]	free rate[c] (h^{-1})	bound rate (h^{-1})		protection factors	
			E3	C3	E3	C3
Lys 7	Val 3	0.28	0.29	0.32	0.96	0.87
Lys 8	Lys 5	4.9	2.7	3.0	1.8	1.6
Ile 9	Lys 5	0.036	0.039	0.037	0.92	0.97
Phe 10	Gly 6	2×10^{-4}	$\leq 3 \times 10^{-4}$	$\leq 2 \times 10^{-4}$	≥0.7	≥1
Val 11	Lys 7	0.023	0.018	0.015	1.3	1.5
Gln 12	Lys 8	0.16	0.16	0.15	1.0	1.1
Lys 13	Ile 9	0.052	0.042	0.053	1.20	0.97
Cys 14	Phe 10	0.081	0.11	0.086	0.74	0.94
Ala 15	Phe 10	0.51	0.43	0.62	1.2	0.82
His 18	Ala 15	0.47	0.76	0.18	0.62	**2.6**
Thr 19	H_2O	0.37	0.12	0.012	**3.2**	**30**
Gly 29	Cys 17	0.12	0.0018	0.0022	**68**	**55**
Leu 32	Thr 19	0.0022	$\leq 10^{-4}$	1.6×10^{-4}	**≥50**	**14**
His 33	Asn 31 side	0.019	0.0056	0.011	**3.3**	**1.8**
Phe 36	H_2O	0.083	0.082	0.078	1.0	1.1
Gly 37	Trp 59	0.76	0.80	0.89	0.95	0.85
Arg 38	Leu 35	1.4	0.033	0.095	**42**	**15**
Gln 42	H_2O	6.9	0.39	0.0070	**18**	**11**
Asn 52	Thr 49 side	10	0.95	≥5[d]	**11**	≤2[d]
Trp 59	Arg 38	0.15	0.0034	0.053	**45**	**2.8**
Lys 60	Thr 63 side	0.017	0.0012	0.0058	**14**	**2.9**
Leu 64	Lys 60	0.016	5×10^{-4}	0.0085	**32**	**1.9**
Met 65	Glu 61	0.0029	0.0024	0.0036	1.2	0.8
Glu 66	Glu 62	0.62	0.44	0.59	1.4	1.0
Tyr 67	Thr 63	0.033	0.032	0.013[d]	1.0	2.5[d]
Leu 68	Leu 64	0.0004	$\leq 10^{-4}$	$\leq 10^{-4}$	≥.3	≥.3
Glu 69	Met 65	0.010	0.0075	0.0089	1.3	1.1
Asn 70	Tyr 67	0.80	0.79	0.611	1.0	1.3
Tyr 74	Asn 70	0.23	0.072	0.12	**3.2**	**1.9**
Ile 75	Pro 71	0.11	0.018	0.061	**6.2**	**1.8**
Lys 79	Hem prop	22	1.6	7	**14**	**3**
Met 80	Thr 78 side	30	≤1	≥5	**≥30**	**≤6**
Ile 85	Leu 68	1.1	1.1	1.8	1.0	0.61
Arg 91	Lys 87	0.015	0.0093	0.012	1.6	1.2
Glu 92	Lys 88	0.0058	0.0069	0.0074	0.84	0.78
Asp 93	Thr 89	0.016	0.015	0.018	1.1	0.91
Lys 100	Ala 96	0.26	0.30	0.43	0.88	0.60
Ala 101	Tyr 97	0.37	0.51	0.82	0.73	0.45

[a] Boldface indicates those residues that are slowed in the complex. [b] Unless otherwise indicated, acceptors are peptide carbonyl oxygens. Side indicates that the acceptor is part of the side chain of the indicated residue. Hem prop indicates that the acceptor is one of the two propionate groups of the heme. H_2O indicates that the acceptor is a water molecule seen in the crystal structure. [c] J. S. Milne et al., unpublished results. [d] Not a reliable rate due to background noise and a small cross peak.

the standard orientation (Figure 3). However, the surface mapped out by the slowed residues and those otherwise implicated as belonging to the epitope appears to be too large to directly map the epitope. It appears, therefore, that the effect of binding can spread past the boundaries of the epitope itself and slow the hydrogen exchange of residues not directly involved in the binding site.

In prior hydrogen-exchange-labeling experiments with the E8 antibody, the slowed residues and their H-bond acceptors mapped to a compact contiguous region that could be interpreted as directly mapping the epitope (Paterson et al., 1990). Only one slowed residue, Gln 12, mapped outside of that region, at some distance from the other slowed residues. Careful analysis of more recent HX data for free cytochrome *c* indicates a biexponential behavior in the decay of the NMR cross peak associated with Gln 12 (J. S. Milne et al., unpublished results). The fast component of this decay is due to Thr 102, the cross peak of which is nearly degenerate with that of Gln 12 (Wand et al., 1989). It now seems likely that the hydrogen-exchange slowing previously attributed to Gln 12 in fact represents Thr 102, which is adjacent to other residues slowed by the E8 antibody. In the present study it is believed that no such spectral overlap occurs for any of the peaks that are seen to be affected by antibody binding.

The slowing of hydrogen-exchange rates for residues at some distance from the epitope can be expected in the local unfolding model for protein hydrogen exchange, as described below.

DISCUSSION

Hydrogen Exchange. The interpretation of protein hydrogen exchange (HX) data has been the subject of considerable interest (Englander & Kallenbach, 1984; Englander et al., 1988; Englander & Mayne, 1992). For purposes of this discussion we adopt the local unfolding model for protein hydrogen exchange (Englander, 1975; Englander & Kallenbach, 1984; Englander et al., 1992), which takes the view that hydrogens protected from exchange with the solvent (relative to their rates in a free peptide) are involved in hydrogen-bonded structure that must transiently open in order for the HX catalyst (OH^- in these conditions) to gain access and promote exchange. The equilibrium constant, K_{op}, for the transient unfolding reaction that allows the exchange of any particular set of hydrogens can be expressed in terms of the free energy of opening, ΔG_{op}, according to the equation

$$\Delta G_{op} = -RT \ln K_{op} \quad (1)$$

K_{op} is the factor by which a given hydrogen-exchange rate is slowed relative to the rate in an unstructured peptide. Due to the cooperative nature of protein structure, sections of hydrogen-bonded structure involved in the same transient opening motion will tend to have similar NH-exchange rates,

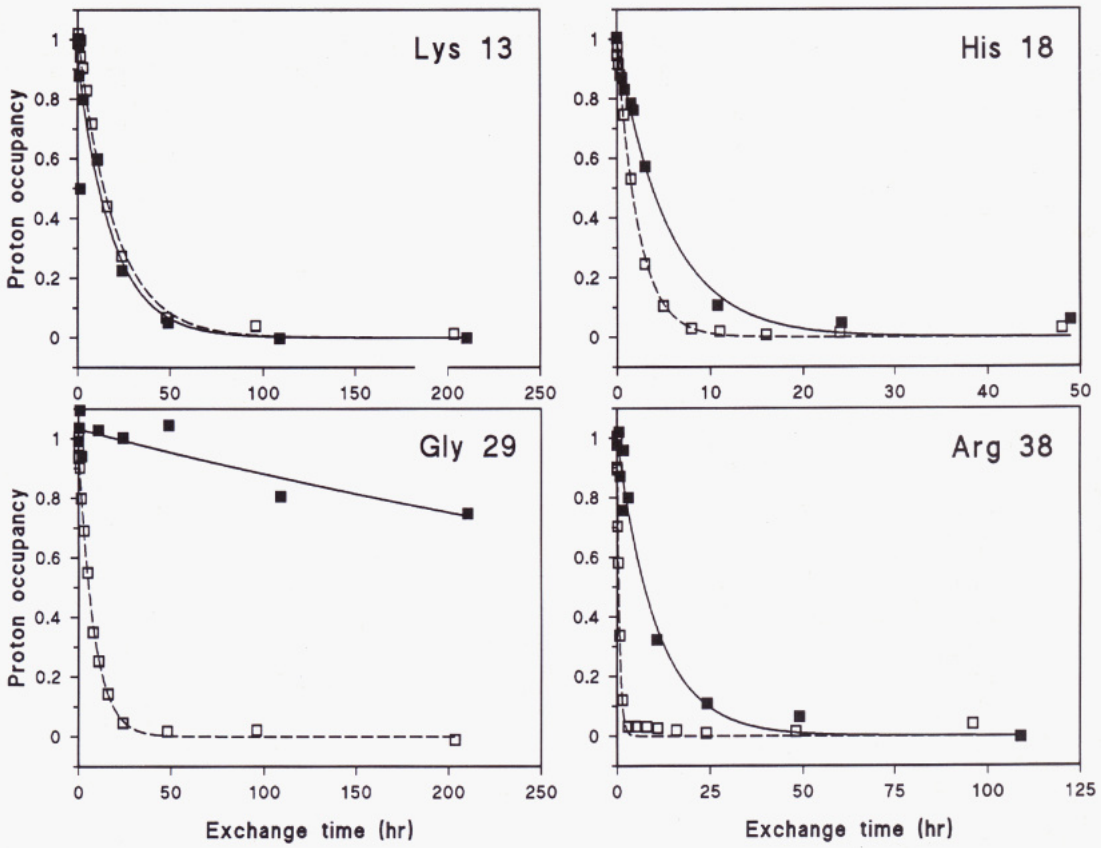

FIGURE 2: Representative hydrogen-exchange curves for some peptide positions showing cross-peak intensity as a function of exchange time for both the free protein (dashed lines, open symbols) and the antibody complex (solid lines, filled symbols).

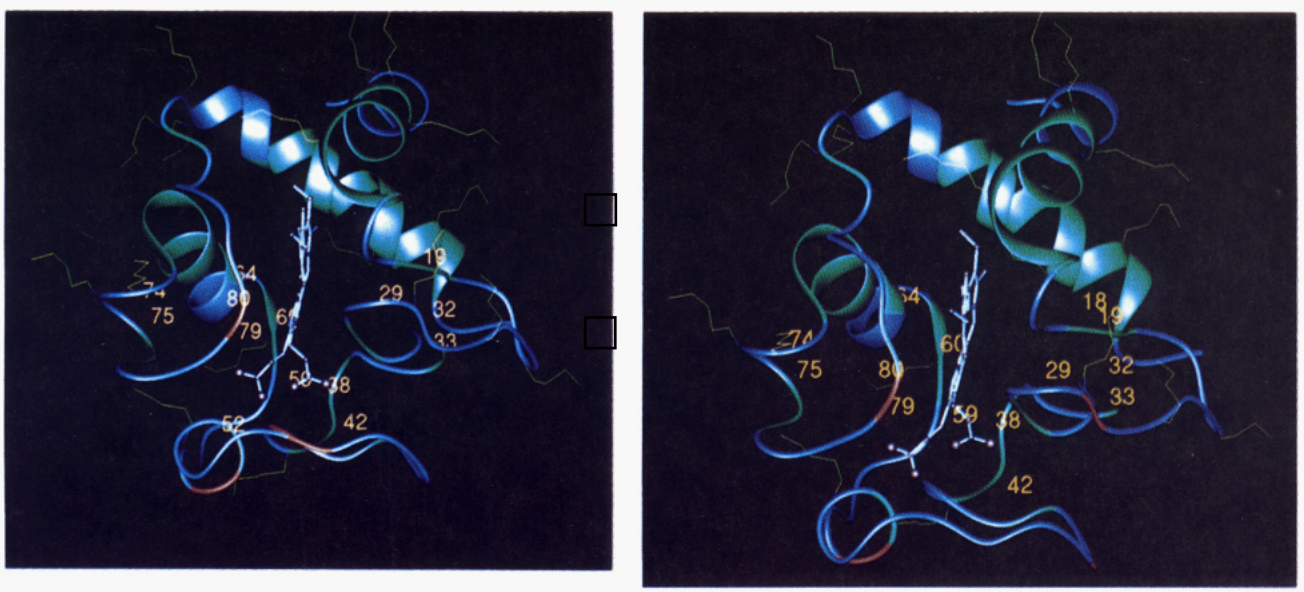

FIGURE 3: Representation of the cytochrome *c* molecule showing results for the E3 complex (left) and the C3 complex (right). Residues that exchange either too fast or too slow to allow measurement of the effect of binding are shown in blue. Measurable residues are shown in green. Peptide positions that are slowed in the complex are labeled with the residue number. Lysine residues that are chemically unprotected in the complex are shown in yellow. The four previously implicated residues are shown in pink, and the heme is shown as a brown ball and stick model. This figure and those that follow make use of coordinates for horse cytochrome *c* kindly provided by G. Brayer (Bushnell et al., 1990).

but this can be obscured by chemical and physical influences (Molday et al., 1972; Englander & Kallenbach, 1984; Robertson & Baldwin, 1991). The term "unfoldon" has been proposed to describe these cooperative units. In this model, the degree of HX slowing is determined by the free energy difference, ΔG_{op}, between the completely folded and locally unfolded forms. There may also be regions of protein structure where H-bond breakage occurs sequentially. For such a "fray", each successive position must be slowed by its own equilibrium ΔG_{op} combined with the summed ΔG_{op} values for those preceding it in the unfolding.

Antibody Binding. Some implications of the local unfolding mechanism for the case of antibody binding can be noted. One expects protection of some residues because the presence of the antibody acts to prevent the breaking of a hydrogen bond by constraining opening motions of either the H-bond donor, its acceptor, or both. In addition, the presence of the antibody itself will act to exclude solvent from contact with

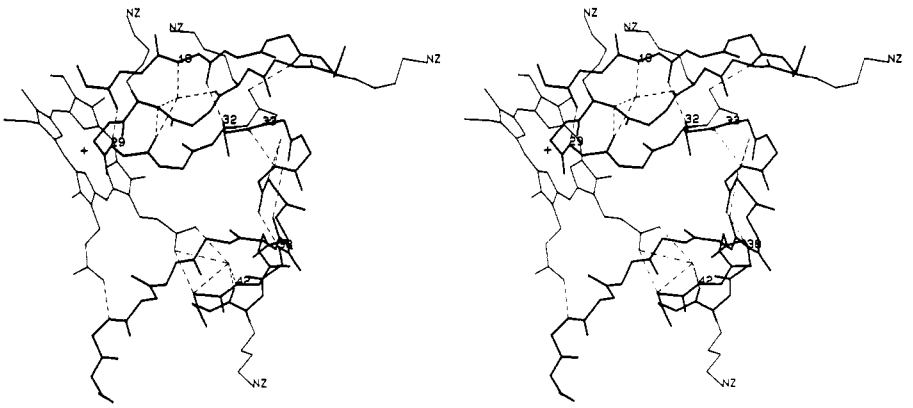

FIGURE 4: Stereoview of a part of cytochrome *c* near the lower right side of the heme cleft showing the hydrogen bonding of residues 19, 29, 32, 33, 38, and 42. Backbone atoms only are shown except for Asn 31 and lysines that are not protected in the complex. The backbone is shown with a thick line; side chains and the heme are shown with a thin line and hydrogen bonds with thin dashed lines. The slowed NH's and the terminal nitrogens of unprotected Lys residues are labeled.

exchangeable hydrogens in the immediate binding surface. This protection may occur even in the absence of hydrogen-bonded structure involving a particular amide, but only those positions that are moderately slow in the free protein, presumably those that are involved in a stable hydrogen bond, will survive to give an NMR signal.

Another important possibility involves the effect of binding on a cooperatively unfolding unit of structure. If a unit of hydrogen-bonded structure, such as a length of helix, tends to open preferentially from one end or another, then any constraint on the residues that initiate the opening will affect the HX rates of residues removed from the actual contact. If the location of a constraint is known, then the remote protection observed can help to map out the structural element that is affected by that constraint.

The exchange of a hydrogen in a complex can be discussed in terms of a reaction diagram:

$$\begin{array}{ccc} \text{cyt}c(\text{H})_{\text{free}} & \xleftrightarrow{1/K_d} & \text{cyt}c(\text{H})_{\text{bound}} \\ \downarrow k_{ex}(\text{free}) & & \downarrow k_{ex}(\text{bound}) \\ \text{cyt}c(\text{D})_{\text{free}} & \xleftrightarrow{1/K_d} & \text{cyt}c(\text{D})_{\text{bound}} \end{array}$$

Here k_{ex}(free) and k_{ex}(bound) are the HX rates of a hydrogen when the protein is free in solution or bound to the antibody, respectively. The measured exchange rate, k_{ex}, is the weighted sum of these two rates, as given by the equation

$$k_{ex} = (1 - F_{free})k_{ex}(\text{bound}) + F_{free}k_{ex}(\text{free}) \quad (2)$$

Here F_{free} is the equilibrium fraction of cytochrome *c* that is not bound to the antibody. For those positions that are unaffected by the binding, k_{ex}(free) = k_{ex}(bound). Positions that are protected by antibody binding, either directly or indirectly due to the inhibition of a mode of local opening, exchange more slowly in the complex. It is conceivable that exchange at some positions may be accelerated in the complex if the binding causes some destabilizing distortion of the protein structure. As implied in eq 1, such destabilization must occur at the expense of antibody binding energy. It is interesting that no such cases have been found in the three complexes so far studied.

The measured HX rate can range from k_{ex}(free) for positions that are unaffected by binding [k_{ex}(bound) = k_{ex}(free)] to a minimum rate of $F_{free}k_{ex}$(free) for the case where exchange is negligible in the complex [k_{ex}(bound) ~0] and can occur only when the complex transiently dissociates. If exchange is faster in the complex, the rate can range upward to $(1 - F_{free})k_{ex}$(bound).

For the present experiments which deal with a tightly bound, stoichiometric complex, F_{free} can be expressed in terms of the dissociation constant, K_d, and the concentration of the complex, [cyt*c*·Ab], as in the equation (derived from a simple binding equilibrium with [cytc_{free}] = [Ab$_{free}$])

$$F_{free} = (K_d/[\text{cyt}c\cdot\text{Ab}])^{1/2} \quad (3)$$

The slowest exchange rate is

$$k_{ex}(\text{min}) = F_{free}k_{ex}(\text{free}) = k_{ex}(\text{free})(K_d/[\text{cyt}c\cdot\text{Ab}])^{1/2} \quad (4)$$

We can then calculate the greatest possible protection factor:

$$p = k_{ex}(\text{free})/k_{ex}(\text{min}) = 1/F_{free} = ([\text{cyt}c\cdot\text{Ab}]/K_d)^{1/2} \quad (5)$$

In the present experiments the concentration of the antibody–antigen complex is (~1–2) × 10^{-4} M in the column solvent space. The maximum protection factors measured are 40–70 for E3 (G29, L32, and R38) and ~30–50 for C3 (T19, G29). If these peptide NH's require equilibrium dissociation in order to exchange, the K_d for these antibodies is ~5 × 10^{-8} M.

Interpretation of Slowing Data. In order to decide, for each protected amide, by what mechanism, direct or indirect, it is protected, we have used the HX data given in Table I and also other published data that implicate particular residues in the binding site and exclude others. This extended data set suggests a consistent picture of the binding site and of the opening motions that are affected upon binding. Figure 3 shows an overall view of the cytochrome *c* molecule and the locations of the slowed residues with the previously implicated residues for the E3 complex (left) and the C3 complex (right). Figures 4–6 provide stereoviews in greater detail of the sections of the protein where slowed residues are located.

In agreement with the previous work of Paterson and co-workers (Paterson, 1989), the HX results are quite similar for the two antibody complexes. The overall picture that emerges for each antibody is of a binding site that covers much of the large Ω-loop at the bottom of cytochrome *c* (residues 37–59) with some contact at parts of the protein nearby (Figure 3), in particular along the ~25–29 segment and at or near the ~77–80 segment. These two segments run along the front part of the Ω-loop on both sides of the heme cleft. Such a binding site would account for almost all of the amide protons that are seen to be slowed in the complex. There are, however, three areas that appear to be blocked indirectly by the bound

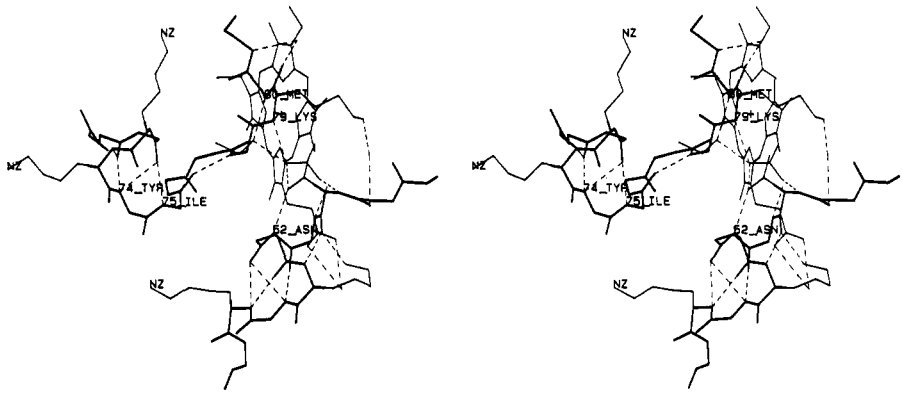

FIGURE 5: Stereoview showing the structure of cytochrome *c* around the lower left side of the heme cleft including slowed residues 52, 74, 75, 79, and 80. Coding as in Figure 4.

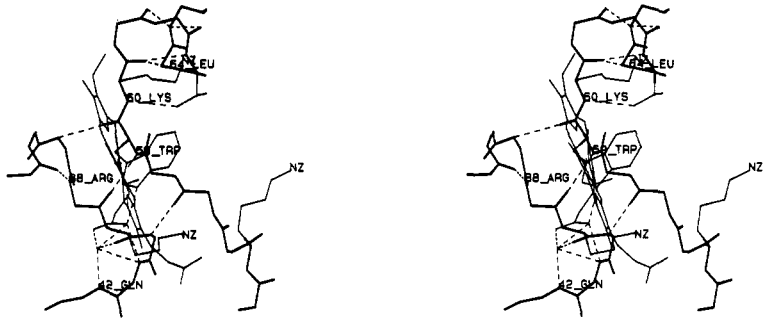

FIGURE 6: Stereoview of the lower back of cytochrome *c* showing the hydrogen bonding of the early part of the 60's helix and slowed residues 38, 42, 59, 60, and 64. Coding as in Figure 4.

antibody. His 18 and Thr 19 (Figure 4) seem to be slowed by an antibody contact in the upper 20's that restricts motion of Thr 19's H-bond acceptor (a water molecular bound between Thr 19) and several residues in the upper 20's. Tyr 74 and Ile 75 (Figure 5) are part of a single turn of helix that might be directly contacted in the complex but is rather far removed from other contact points. It appears that the opening of this short turn of helix from the Ile 75 end is restricted in the complex. A fray from the other end may still be allowed and would explain the relative slowing of 74 ($p = 3.2$) compared to 75 ($p = 6.2$) seen in the E3 complex. Residues 60 and 64 are involved in a rather complicated H-bonded network (Figure 6). It appears that, in the complex, some motion at the beginning of the 60's helix is restricted by contact at or near residues in the upper 50's and/or upper 30's. Residues in the upper 30's are involved with this segment via a network of hydrogen bonds.

Considerations that lead to these conclusions are discussed below in terms of a so-called "lysine perimeter". Previous epitope mapping studies show that many lysine residues are not chemically protected by antibody binding (Oertl et al., 1989) including residues 13, 22, 25, 27, 39, 55, 72, and 73. These unprotected residues define the perimeter of a rather large area (~20 000 Å^2, ~40 Å × ~40 Å) on the front and bottom of the protein. Lys 60 is also on this list but is already behind (separated from previously implicated residues by) lysines 39 and 55. Most of the HX-affected residues are inside of or at the edge of this zone, as are all of the previously implicated residues. Some slowed residues are well outside of the lysine-bounded zone. For this discussion, we divide the HX-affected residues into three groups: those that are within the lysine-bounded area, those that are on its edge, and those that are outside of the zone.

Residues inside the Lysine Perimeter. Residues 38, 42, 52, 79, and 80 are measurable, occur within the lysine perimeter, and exhibit slowed H-exchange in the complex. Residues 36, 37, and 85 are within this area and are not slowed. The unaffected residues help to define the location of the epitope. This is particularly true of residues 36 and 37, since they are very near other residues that are slowed in the complex (residues 38 and 42) or previously implicated (residue 40 for E3 or 44 for C3). Lys 79 is one of the previously implicated residues; the ϵ-amino group of its side chain is protected from acetylation in the complex. Residues 52 and 80 are not directly exposed to solvent, and their HX slowing apparently reflects the protection of overlying structure. Asn 52 is on the inside of a turn of helix. Met 80 is bonded to the side chain of Thr 78, implying that any restriction on the motion of this segment, possibly provided by the contact at the Lys 79 side chain, could protect the Met 80 H-bond. Both residues 79 and 80 have relatively fast exchange rates in the free protein, and thus their protection factors cannot be measured accurately. Given this uncertainty, the fact that Lys 79 is not strongly protected might imply that the backbone of this residue is near an edge of the binding site. This is particularly true in the C3 complex. All other residues within the lysine perimeter exchange too fast in the free protein to be measurable.

Residues on the Edge of the Lysine Perimeter. Residues 29, 32, 33, 59, 74, and 75, at the edge of the lysine perimeter, show HX slowing in the complex. It appears likely that these residues or residues nearby are part of the binding site.

Gly 29 (Figure 4) is not exposed to solvent and its H-bond acceptor, Cys 17, is well outside of the lysine-bounded area. It appears that there may be contact along the segment including residues 25–28. (His 26 is one of the previously implicated residues in the C3 case). Gly 29 is the acceptor for a β-turn H-bond from Lys 27. The terminal amines of lysines 25 and 27 are unprotected in the complex, indicating that this may be at an edge of the binding site.

His 33 is slightly slowed ($p = 3.3$ for E3 and 1.8 for C3; Figure 4) while its neighbor Leu 32 is among the most slowed ($p > 50$ for E3 and 14 for C3). This difference seems to

reflect the fact that His 33 is exposed and bonded to an exposed side chain (Asn 31) while Leu 32 is not exposed to solvent and is involved in an internal H-bond to Thr 19. A transient change in conformation involving a large number of residues would be required to break the 32–19 H-bond while the movement of a single side chain could break the 33–31 bond.

Trp 59 is H-bonded to Arg 38, which is believed to be in the binding site (see above). It is also involved in a hydrogen-bonded network which includes residues 60 and 64, discussed below.

Tyr 74 (Figure 5) and Ile 75 are the last two residues in a single turn helix. There seem to be two possible mechanisms for their protection. There could be contact at or near Pro 76 (residue 75 is not exposed and its NH acceptor, Pro 71, is hidden behind lysine perimeter residues 72 and 73). Alternatively, there could be extended effects on the contact that protects Lys 79 and Met 80 that are transmitted via the backbone and a β-turn H-bond between Ile 75 and Thr 78. If the greater protection of residue 75 over 74 apparent in the E3 data is significant, it may imply that residue 74 has other opening modes available to it such as an opening of the helix from the other end (Asn 70).

Residues outside the Lysine Perimeter. Residues 18, 19, 64, and to some extent 60 are outside of the lysine-bound area and appear to be slowed by indirect, cooperative effects.

His 18 (Figure 4) is slowed only in the C3 complex and only by a factor of 2.6. It appears that it is protected by the same constraint that slows Thr 19 in the C3 complex. Thr 19 is slowed (Figure 4), but it lies behind unprotected lysine residues 25 and 27. Its acceptor is a water molecular that is also H-bonded to several residues in the 25–31 segment. The most likely cause of slowing is that this water is less labile in the complex due to constraints on the motions of the 25–31 segment. Evidence for such constraints is also seen in the case of Gly 29. That constraints on the motion of this segment in the E3 complex still allow some freedom for the water–Thr 19 bond to break is evident from the low level of protection.

Since Lys 60 (Figure 6) is not chemically protected, any direct contact cannot, in either the E3 or C3 complex, include its terminal amino group. Antibody blocking studies (Carbone & Paterson, 1985) also indicate that Lys 60 is not central to the E3 epitope since both E8 and E3 can simultaneously bind horse cytochrome *c* and Lys 60 is protected in the E8 complex (Paterson et al., 1990). In addition, Lys 60 is partially hidden behind the unprotected lysines 39 and 55 and the unslowed residues Phe 36 and Gly 37 (see Figure 6). Its acceptor, and side chain of Thr 63, is even farther away from the major part of the epitope. It seems apparent then that any motion that is restricted by the antibody must include a motion of the backbone of residue 60 away from its acceptor. As previously mentioned, some contact at residue 60 seems possible but unlikely. It seems more likely that the motion of the ~56–60 segment is restricted. This segment includes the short section of β-sheet hydrogen bonding that forms the neck of the large Ω-loop (37–59) at the bottom of the molecule (Figure 3) and which appears to form the major part of the binding site. Such restriction of motion could be through contacts at or near residues 56, 57, and the side chain of 58. In addition, because of the rather large number of H-bonds involving this segment its motion could be restricted by the stabilization of hydrogen bonds from these residues to residues 37–40. As already seen, residue 38 is slowed, as is the bond from residues 59 to 38 which is part of this β-structure. Thr 40 has been previously implicated in the epitope; substitutions at this site markedly reduce antibody binding (Collawn, Wallace, and Paterson, unpublished observations). One other linkage exists which might also be relevant with regard to the slowing of Trp 59. The indole NH is H-bonded to one of the heme propionates which is also bonded, via the same oxygen and its partner, to several residues in the middle of the Ω-loop. This connection is further supported by the observation that breaking the indole NH to propionate bond by chemical modification of the indole destroys antibody binding (Cooper et al., 1987).

The restriction of motion that slows Lys 60 exchange also seems to affect Leu 64. The two H-bonds (Lys 60 to Thr 63's side chain and Leu 64 to Lys 60) may form a cooperative unit which requires the concerted breaking of both bonds. In the E3 case the lower protection factor seen for Lys 60 ($p = 14$) relative to Leu 64 ($p = 32$) is presumably due to some residual opening possible due to local motions involving the side chain of Thr 63. This situation appears to be similar to the case of residues 32 and 33, discussed above.

It is particularly interesting to note that the H-bonded hydrogen between the NH of Gly 37 and the C=O of Trp 59 is not slowed. Given the evidence that the segment containing residues 59 and 60 is restricted, this indicates that residue 37 is able to move as part of some other independent opening motion.

CONCLUSIONS

The HX results together with previous data provide evidence that, for both the E3 and C3 antibodies, the binding site on cytochrome *c* consists mostly of residues in the 40's and 50's (the 37–59 Ω-loop) with some contact with residues in the mid-20's, lower and upper 30's, and upper 70's. Some effects of this binding also appear to spread beyond the area of direct contact, apparently by restricting local unfolding motions. The precise extent of the binding site and the details of the local opening motions perturbed by binding are not completely determined by these results. Additional structural information such as an X-ray structure of the complex would be quite helpful in removing these ambiguities. The size of the proposed epitope is ~1200 Å^2 (~20 Å × ~30 Å), still slightly larger than crystallographically measured epitopes for protein antigens of similar size (Davies et al., 1988). This may indicate that cooperative effects play an even larger role than indicated above.

The observed long-range effects are unlikely to represent electrostatic or solvent-mediated effects since in some cases, Phe 36 and Gly 37 for example, the apparent remote effect can skip over some residues that lie between the apparent epitope and the remotely affected residues. The fact that two antibodies, previously known to bind in nearly the same area, slow the HX rates of the same set of residues, both those that appear to be slowed directly and those that appear to be slowed indirectly, gives further confidence that these apparent cooperative effects are a real reflection of the dynamics of cytochrome *c*. No such effects were seen in a previously study using a different antibody (Paterson et al., 1990). The differences seen between the E3 and C3 antibodies lie mostly in the magnitude of protection factors rather than in the residues protected. In the C3 antibody complex, the highly slowed residues (19, 29, 32, and 42) are grouped in the lower right front part of the cytochrome *c* molecule (in the standard orientation; see Figure 3), while the distribution of slowed residues is more generally around the bottom of the molecule. The highly slowed residues in the E3 complex are more evenly distributed among the affected residues. These differences may indicate that the C3 antibody either has more contact in the lower right area of the molecule or has interactions that

are "stronger", at least in the sense that they are more effective at restricting motions that allow hydrogen exchange.

In this regard it should be noted that different residues on the surface of the antigen buried by complexation with the antibody may contribute unequally to the affinity of the complex. Novotny et al. (1989) have used crystallographically defined antibody–antigen interfaces to calculate the free energy of the interaction. On this basis they define the *functional epitope* recognized by an antibody to consist of the relatively small number of residues within the buried interface that make the largest contributions to the stability of the complex. Thus the antibodies E3 and C3 may bury similar surfaces on cytochrome *c* but have different *functional epitopes*. It is possible that residues that interact strongly with the antibody will be less likely to separate in the complex and therefore will be more effective at inhibiting opening motions. If so, these residues may induce higher HX protection factors than those that are simply buried by the intersurface contacts and held less rigidity.

Our results with E3 and C3 contrast with the case of the E8 antibody (Paterson et al., 1990). In the E8 case, all of the slowed residues appear to be part of the epitope. In the complexes discussed here, many residues appear to be slowed by indirect cooperative effects. We suggest this difference lies in the relative rigidity of the epitopic regions recognized by these antibodies. HX data for free cytochrome *c* show that the large 38–59 Ω-loop of the molecule, the central part of the E3/C3 epitope, is much less stable than the 60's and 90's helices which are central to the epitope of the E8 antibody. One expects that local unfolding may begin from an unstable part of the structure and spread into more stable parts rather than the other way around. Thus the effect of antibody binding at the bottom of the molecule where many peptide hydrogens are fast exchanging may be propagated to more stable parts of the molecule, while E8 binding at the relatively rigid 60's and 90's helices does not affect exchange rates in more labile regions.

Comparisons of the free and complexed form of protein antigens have been made for several crystallographic structures of lysozyme and neuraminidase in antibody–antigen complexes with different conclusions. Lysozyme shows no significant conformational change on binding to antibody D1.3 (Amit et al., 1986), but backbone movements of 1.0–2.0 Å occur on binding of the antibody HyHEL 5 (Sheriff et al., 1987). Such motions are also seen in the anti-neuraminidase structure (Coleman et al., 1988). Rotations about side-chain bonds in residues both in and remote from the epitope also occur in antibody–lysozyme complexes (Davies et al., 1988). These findings indicate changes can take place in the three-dimensional fold of the antigen that improve the complementarity between the interaction sites. Our results extend this notion to indicate that cooperative changes on binding may involve the dynamics of the antigen as well as its three-dimensional structure.

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REFERENCES

Amit, A. G., Mariuzza, R. A., Phillips, S. E. V., & Poljak, R. J. (1986) *Science 233*, 747.

Axelsen, P. H., Gratton, E., & Prendergast, F. G. (1991) *Biochemistry 30*, 1173–1179.

Bushnell, G. W., Louie, G. V., & Brayer, G. D. (1990) *J. Mol. Biol. 213*, 585–595.

Carbone, F. R., & Paterson, Y. (1985) *J. Immunol. 135*, 2609–2616.

Colman, P. M. (1988) *Adv. Immunol. 43*, 99.

Cooper, H. M., Jemmerson, R., Hunt, D. F., Griffin, P. R., Yates, J. R., Shabanowitz, J., Zhu, N. Z., & Paterson, Y. (1987) *J. Biol. Chem. 262*, 11591–11597.

Davies, D. R., Sheriff, S., & Padlan, E. A. (1988) *J. Biol. Chem. 263*, 10541.

Englander, S. W. (1975) *Ann. N.Y. Acad. Sci. 244*, 10.

Englander, S. W., & Kallenbach, N. R. (1984) *Q. Rev. Biophys. 16*, 521.

Englander, S. W., & Mayne, L. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 243–265.

Englander, J. J., Englander, S. W., Louie, G., Roder, H., Tran, T., & Wand, A. J. (1988) in *Structure & Expression, Volume 1: From Proteins to Ribosomes* (Sarma, R. H., & Sarma, M. H., Eds.) pp 107–117, Adenine Press, Schenectady, NY.

Englander, S. W., Englander, J. J., McKinnie, R. E., Ackers, G. K., Turner, G. J., Westrick, J. A., & Gill, S. J. (1992) *Science 256*, 1684–1687.

Goodfellow, J. M., Ed. (1990) *Molecular Dynamics Applications in Molecular Biology*, CRC Press, Boca Raton, FL.

Jemmerson, R., & Paterson, Y. (1986) *Science 232*, 1001.

Jeng, M.-F., & Englander, S. W. (1991) *J. Mol. Biol. 221*, 1045–1061.

Jeng, M.-F., Englander, S. W., Elove, G. A., Wand, A. J., & Roder, H. (1990) *Biochemistry 29*, 10433–10437.

Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11*, 150–158.

Novotny, J., Bruccoleri, R. E., & Saul, F. A. (1989) *Biochemistry 28*, 4735–4749.

Oertl, M., Immergluck, K., Paterson, Y., & Bosshard, H. R. (1989) *Eur. J. Biochem. 182*, 699.

Paterson, Y. (1989) in *The Immune Response to Structurally Defined Proteins: The Lysozyme Model* (Smith-Gill, S., & Sercarz, E., Eds.) pp 177–189, Adenine Press, Schenectady, NY.

Paterson, Y., Englander, S. W., & Roder, H. (1990) *Science 249*, 755–759.

Robertson, A. D., & Baldwin, R. L. (1991) *Biochemistry 30*, 9907–9914.

Sheriff, S., Silverton, E. W., Padlan, E. A., Cohen, G. H., Smith-Gill, S. J., Finzel, B. C., & Davies, D. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8075–8079.

Wand, A. J., DiStefano, D. L., Feng, Y., Roder, H., & Englander, S. W. (1989) *Biochemistry 28*, 186–194.

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