identified by the infrequently observed COSY crosspeaks due to the four-bond coupling between the C2,6H and the CβH resonances. By comparing the chemical shifts of the two methyl resonances of Thr-191 and Thr-196 in the α185-peptide and in the α186{Ala-192}-peptide, we observed that the chemical shift of the methyl doublet at 1.36 ppm remained unchanged in the α185{Ala-192}-peptide, whereas the downfield methyl doublet (1.38 ppm) was shifted by about 0.015 ppm further downfield. Assuming that the Thr residue sequentially adjacent to the amino acid substitution at position 192 would be affected more than the Thr at position 196, we assigned the 1.38 methyl resonance to Thr-191. The remaining sequence-specific resonance assignments could all be obtained from the COSY spectrum. The simplified aliphatic region of the NMR spectrum for the α186-peptide lacking the N-terminal Lys facilitated the assignment and confirmation of many of the remaining aliphatic resonances, especially those from Pro and Asp. The two CαH as well as the two CβH resonances for the two Cys residues (192, 193) were degenerate (Table 1), suggesting that these two side chains are in a similar chemical environment.

Since random coil amino acids do not contain CαH resonances with chemical shifts greater than 4.8 ppm, it has been suggested that the difference in chemical shifts between those in a polypeptide and those in a random coil represents a conformation-dependent chemical shift [6]. Four of the CαH resonances (His, Trp, and both Tyr) have chemical shifts greater than 4.8 ppm, suggesting some amount of ordered structure in the α185-peptide. In addition, the amide resonance of Thr-191 remains a sharp doublet while the other amide resonances are broadened due to exchange. The observation of such a sharp resonance suggests that the Thr-191 amide proton is slowly exchanging due to some feature of an ordered structure within the peptide. For example, such an observation is thought to be consistent with the Thr-191 amide being buried and inaccessible to solvent or with it being involved in H-bonding.

In summary, the total sequence-specific ^{1}H assignment for the α185-peptide was accomplished by analysis of COSY spectra along with spin-decoupling and confirmatory NOE difference experiments. Some ambiguities in the assignments were successfully addressed utilizing additional peptides with selective amino acid substitutions. The chemical shifts of several of the CαH resonances, along with evidence for a slowly exchanging amide at Thr-191 suggest that the α185-peptide may contain a certain amount of non-random coil structure. The role of any such ordered structure in the mechanism of binding to α-bungarotoxin remains to be determined. The assignment of the peptide ^{1}H resonances will facilitate the analysis and identification of chemical shift perturbations observed upon formation of the complex between α-bungarotoxin and the α185-peptide [7].

Acknowledgements—Supported by the NIH (GM32629) and by instrumentation awards from the Instrumentation Program of the Division of Research Resources, NIH (RR03475), the Biological Instrumentation Program of the NSF (DMB-8610557), and the American Cancer Society (RD259). E. H. is an Established Investigator of the American Heart Association.

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Biochemical Pharmacology, Vol. 40, No. 1, pp. 65–68, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

NMR studies of protein surfaces. The interaction of lysozyme with tri-*N*-acetylglucosamine

Methods to identify the surface residues of proteins have several potential applications. Since functional regions of a protein surface may be composed of residues not in continuous sequence, knowledge of surface structure is required for rational design of non-protein mimics of protein function. Also, as illustrated below, mapping exposed amino acid residues may aid in identifying substrate or inhibitor binding sites of enzymes. In addition, it is possible that independent identification of surface residues may supplement the nuclear magnetic resonance data usually employed in determination of a solution structure.

Several nuclear magnetic resonance methods have been used to identify solvent-exposed residues of proteins and peptides. These include measurement of amide proton

exchange rates [1], of relaxation rates [2], of photochemically-induced dynamic nuclear polarization (photo-CIDNP*) [3], and of relaxation induced by paramagnetic cosolutes [4]. The latter method has been applied to cyclic oligopeptides; here we report its application to proteins.

Because of spectral complexity, two-dimensional methods are necessary to correlate surface accessibility with paramagnetic broadening for proteins. The phase-sensitive COSY experiment is well suited for the purpose, since the antiphase nature of the crosspeaks makes their intensity highly sensitive to linewidth [5]. In the experiment to be described, the nitroxyl spin label 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (HyTEMPO) was used as a relaxation probe, and the model protein system examined was hen egg white lysozyme and its interaction with the inhibitor tri-*N*-acetylglucosamine (tri-NAG). The proton NMR spectrum of lysozyme has been almost completely assigned [6], and crystal structures of lysozyme and lysozyme complexed with tri-NAG have been determined [7, 8].

Methods and Results

Hen egg white lysozyme (EC 3.2.1.17) and tri-*N*-acetylglucosamine (*N,N',N''*-triacetylchitotriose) were obtained from Sigma, and 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy from Aldrich. Proton NMR spectra (500 MHz) were measured using 5 mM solutions of lysozyme in D_2O at 35°, pH 3.8, in 25 mM sodium acetate, in the presence and absence of 10 mM tri-NAG, with and without 10 mM HyTEMPO. The line broadening effects of HyTEMPO were monitored by measuring the absolute value volumes of double quantum filtered chemical shift correlated (DQF-COSY) cross peaks. NMR data were processed using FTNMR software (Hare Research Inc., Woodinville, WA) Tri-NAG binds to lysozyme with a dissociation constant of 6.6×10^6 M [9], so that protein–ligand complex formation is complete at the concentrations used for spectroscopy. Titration of lysozyme with tri-NAG showed that on the chemical shift time scale the inhibitor exchanges at an intermediate to slow rate as monitored by the line shapes of the two Trp 62 H2 singlets, free and bound, observable during titration.

Addition of 10 mM HyTEMPO to a 5 mM hen egg white lysozyme solution caused slight overall broadening of resonances as observed in a one-dimensional spectrum (not shown), but it was difficult to detect specific effects because of spectral overlap. Figure 1, which compares the aromatic region of a DQF-COSY spectrum of lysozyme with a spectrum of lysozyme plus HyTEMPO, illustrates the advantage of two-dimensional spectroscopy. Specific effects are obvious; upon addition of HyTEMPO crosspeaks arising from Trp 62, Trp 63 and Trp 123 are broadened beyond detection, while those from the other aromatic resonances are virtually unchanged. That these three Trp side chains are solvent-accessible or partly accessible is supported by the crystal structure of the lysozyme [7]. Two-dimensional detection of photochemically induced dynamic nuclear polarization (photo-CIDNP) has also shown Trp 62 and Trp 123 to be exposed in solution [10]. Direct photo-CIDNP effects were not observed for Trp 63; the clear exposure observed here may arise because the ring of Trp 63, somewhat deeper in the binding cleft than Trp 62, is more readily accessible to the nitroxyl than to the larger flavin photo-CIDNP probe.

The resonances of many residues other than these three tryptophans are affected by HyTEMPO. Attention is focused here on those residues at or near the tri-NAG binding site in the crystal of the lysozyme–tri-NAG complex [8]. These are listed in Table 1, which compares the line broadening effects of 10 mM HyTEMPO on specific crosspeaks in the presence and absence of 10 mM ligand. An example is shown in Fig. 2. Comparing panels (a) and (b) of Fig. 2, it is clear that upon addition of HyTEMPO the C^4H-C^5H crosspeak of Trp 63 and the C^6H-C^7H crosspeak of Trp 123 are completely eliminated by broadening. In the presence of tri-NAG, panels (c) and (d), the Trp 123 crosspeak is still completely eliminated by HyTEMPO, whereas the Trp 63 crosspeak, although attenuated (0.5 its volume in the absence of HyTEMPO, Table 1), is clearly present. This suggests that the Trp 63 side chain is covered by the bound inhibitor, a conclusion supported by the crystal structure. The table indicates similar results for Leu 75, Ile 98 and Ala 107; these are again in agreement with the crystal structures.

Of the other residues in the vicinity of the bound ligand, crosspeaks for Asn 59, Trp 62, Asp 101 and Gly 102 were observed to be either strongly broadened or very strongly shifted when tri-NAG was bound in the absence of

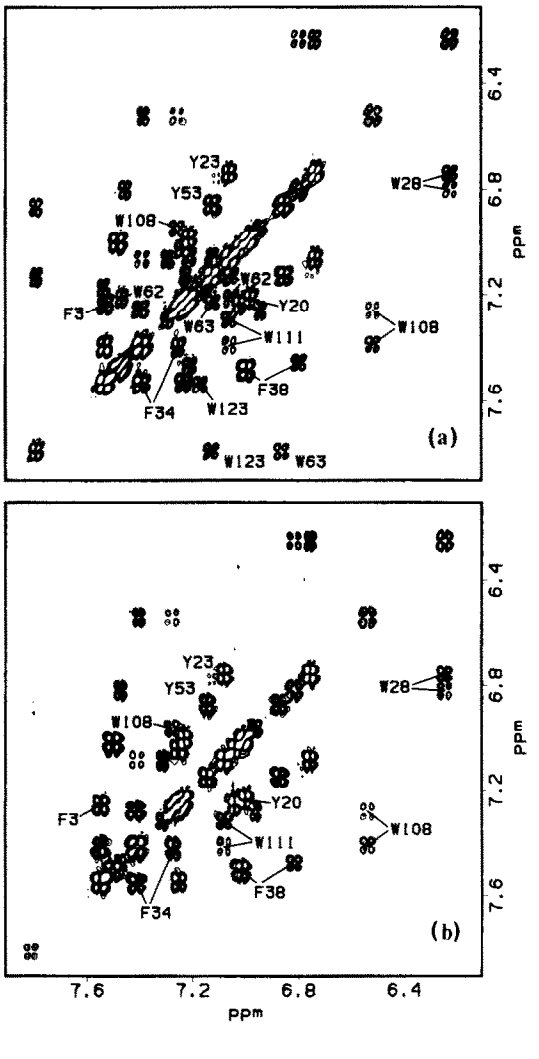

Fig. 1. Effect of HyTEMPO on lysozyme aromatic resonances. (a) 500 MHz DQF-COSY spectrum of hen egg white lysozyme in D_2O, 5 mM, 35°, pH 3.8. (b) Same as (a) with 10 mM HyTEMPO added.

* Abbreviations: CIDNP, chemically-induced dynamic nuclear polarization; DQF-COSY, double quantum filtered chemical shift correlation spectroscopy; HyTEMPO, 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy; and tri-NAG, tri-*N*-acetylglucosamine, *N,N',N''*-triacetylchitotriose.

Table 1. Effect of HyTEMPO on lysozyme sidechains in the absence and presence of tri-NAG*

Residue	Crosspeak	V/V_o†	
		Lysozyme	Lysozyme + tri-NAG
Asp 52	$C^{\alpha}H$-$C^{\beta}H$	1.0	<0.1
Leu 56	$C^{\gamma}H$-$C^{\delta}H_3$	1.0	0.9
Ile 58	$C^{\beta}H$-$C^{\gamma 2}H_3$	0.9	1.0
Asn 59	$C^{\alpha}H$-$C^{\beta}H$	<0.1	ND‡
Trp 62	$C^{4}H$-$C^{5}H$	<0.1	ND‡
Trp 63	$C^{4}H$-$C^{5}H$	<0.1	0.5
Leu 75	$C^{\gamma}H$-$C^{\delta}H_3$	<0.1	0.8
Ile 98	$C^{\beta}H$-$C^{\gamma 2}H_3$	<0.1	0.6
Val 99	$C^{\beta}H$-$C^{\gamma}H_3$	1.0	0.7
Asp 101	$C^{\alpha}H$-$C^{\beta}H$	<0.1	ND‡
Gly 102	$C^{\alpha}H$-$C^{\alpha}H$	<0.1	ND‡
Asn 103	Not assigned		
Ala 107	$C^{\alpha}H$-$C^{\beta}H_3$	<0.1	0.8
Trp 108	$C^{4}H$-$C^{5}H$	0.9	1.0
Val 109	$C^{\beta}H$-$C^{\gamma}H_3$	0.6	<0.1

* Experimental conditions: 35°, pH 3.8, 5 mM lysozyme, 10 mM HyTEMPO and 10 mM tri-NAG when present.

† V/V_o is the ratio of the crosspeak volume in the presence of HyTEMPO to that in the absence of HyTEMPO.

‡ Not detected in the presence of tri-NAG.

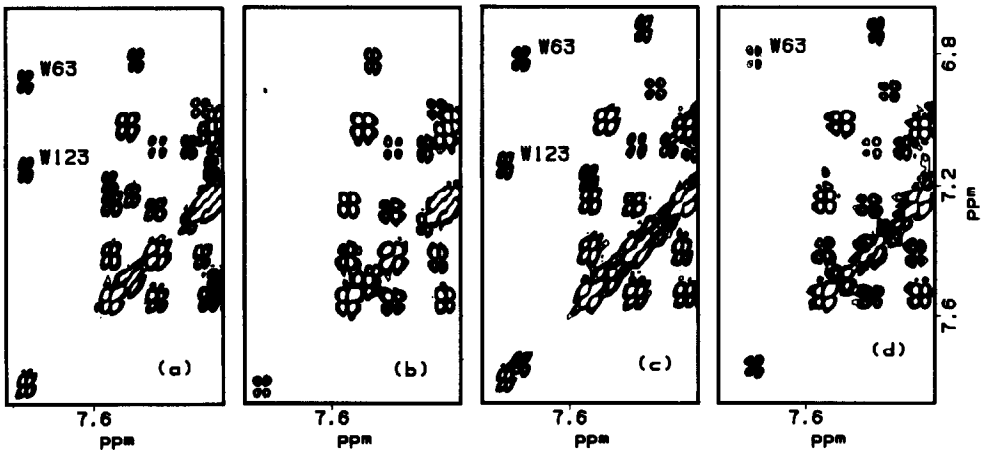

Fig. 2. Effect of HyTEMPO on the Trp 63 sidechain of lysozyme in the absence and presence of tri-NAG. (a) 500 MHz DQF-COSY spectrum of lysozyme in D_2O, 5 mM, 35°, pH 3.8. (b) Same as (a) with 10 mM HyTEMPO added. (c) Same as (a) with 10 mM tri-NAG added. (d) Same as (a) with 10 mM tri-NAG and 10 mM HyTEMPO added.

HyTEMPO. Presumably this indicates that they are involved in the binding region, but their exposure to HyTEMPO cannot be followed. An interesting result was observed for Val 109 and the catalytically important Asp 52. The side chains of these residues and perhaps of Val 99, which are close to but not part of the tri-NAG (A, B, C) binding site, become more rather than less exposed on ligand binding, suggesting a conformation change or concurrent binding of nitroxyl at the D site of the binding pocket.

In summary, this experiment demonstrates that residues involved in a ligand binding site may be delineated by changes in side chain resonance broadening produced by an added free radical.

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