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Properties of Conserved Amino Acid Residues in Tandem Homologous Protein Domains. Hydrogen-1 Nuclear Magnetic Resonance Studies of the Histidines of Chicken Ovomucoid†

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ABSTRACT: Peaks corresponding to the C_ϵ protons of the four histidine residues (positions 58, 111, 123, and 182) of chicken ovomucoid have been assigned in ^1H NMR spectra (360 or 470 MHz) of the native single-chain protein and of fragments of the protein corresponding to its three homologous structural domains. Comparison of the ^1H NMR pH titration behavior of these histidine residues and the deuterium exchange rates of their C_ϵ-H positions show the following: (1) The chemical shift properties of histidine residues 58, 123, and 182 differ despite the fact that the three residues are located in homologous positions in the three tandem domains. (2) The properties of three of the four histidine residues (58, 111, and 123) do not change appreciably when the domains in which they are located are isolated, indicating that their environments are similar in both the fragment and the native protein. (3) The properties of the fourth histidine (182) differ in the isolated domain and in the native protein. (4) The observed properties of the histidine residues stem primarily from intradomain interactions that remain constant in isolated domains rather than from interactions with neighboring domains; an interdomain interaction is required to explain the behavior of only histidine-182. (5) The chemical shift of histidine-111 is affected by the titration of the side chain of aspartate-98 with pH_{mid} 2.6 in native ovomucoid but not in isolated second domain; the chemical shift of histidine-182 is perturbed by the titration of the carboxyl group of the C-terminal cysteine-186 with pH_{mid} 2.4 in native ovomucoid and pH_{mid} 2.6 in isolated third domain.

The avian ovomucoids are glycoproteins whose structure has been shown to be composed of three tandem, homologous, structural domains, each of which is a potential or actual inhibitor of a serine proteinase (Kato et al., 1978). Analysis of the chicken ovomucoid gene has shown that the DNA also is organized into domains. There is one intron within each region of the DNA coding for a structural domain, and one intron is located in DNA that codes for the linkage region between each structural domain (Stein et al., 1980).

As shown in Figure 1, the four histidine residues of chicken egg white ovomucoid are located in positions 58, 111, 123, and 182 (Kato et al., 1978, with revisions). Three of the four histidines occupy homologous positions in the first (His^{58}), second (His^{123}), and third (His^{182}) domains. The second domain contains the additional, nonhomologous, histidine residue (His^{111}). Also indicated in Figure 1 are specific points at which the peptide chain can be cleaved for production of isolated structural domains (Kato et al., 1978; I. Kato et al., unpublished results).

Because of the strong homology among domains, one might predict that the properties of the three conserved histidines would be similar. However, a ^1H NMR study of the histidines of ovomucoid, carried out before the protein was sequenced, demonstrated that this is not the case (Markley, 1973b). The present study was undertaken to determine whether the histidines differ because of intradomain or interdomain interactions. First it was necessary to assign histidine resonances to specific residues in the sequence. Then the properties of each histidine in native ovomucoid were compared with its properties in isolated fragments of the molecule.

Experimental Procedures

Materials. Enzymes and chemicals were from the following sources: CNBr, ^2H_2O (99.7% and 100% isotopic purity), Bio-Gel P-10, and Bio-Gel P-30, Bio-Rad; Sephadex G-25, Pharmacia; Whatman CM-52 carboxymethylcellulose, Reeve

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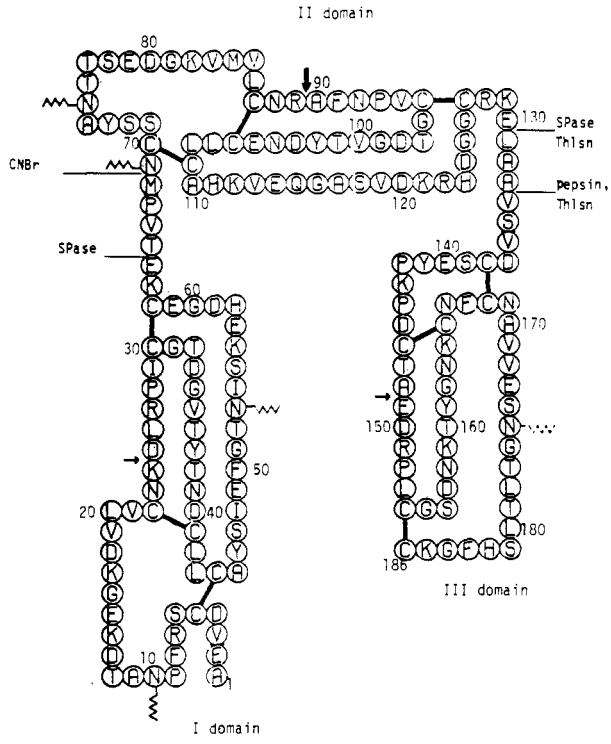

FIGURE 1: Schematic representation of the structure of chicken egg white ovomucoid. The sequence, disulfide bond pairing, sugar attachment sites (saw-toothed lines), and reactive site locations (arrows) are from Kato et al. (1978) with minor revisions. Indicated in the figure are cleavage sites for cyanogen bromode (CNBr) and limited proteolysis sites for staphylococcal proteinase (SPase), thermolysin (Thlsn), and pepsin used to produce isolated structural domains of the molecule.

Angel; thermolysin, Calbiochem-Behring; staphylococcal proteinase (used without further purification), Miles Biochemicals; TPCK–trypsin and porcine pepsin, Worthington Biochemical Corp. Fresh nonfertile chicken eggs were obtained from local sources. All other chemicals were reagent grade or the best commercially available.

Preparation of Chicken Ovomucoid. Chicken ovomucoid was prepared from egg white according to Lineweaver & Murray (1947) as modified by Bogard et al. (1980). Ovomucoid prepared in this fashion is termed crude ovomucoid. Crude ovomucoid was further purified by dissolving 1–2 g of the material in 50 mL of 0.1 M $NH_4C_2H_3O_2$ buffer, pH 4.0, and applying the solution to a 2.5 × 45 cm column of Whatman CM-52 carboxymethylcellulose previously equilibrated with the same buffer. The column was then eluted with 2.5 column volumes of this buffer followed by 5 column volumes of 0.1 M $NH_4C_2H_3O_2$, pH 4.5. Fractions (15 mL) were collected. The absorbance of the eluant was monitored at 280 nm. The major peak of material which eluted at about 3 column volumes was pooled, dialyzed, and lyophilized. Ovomucoid prepared in this manner was pure as judged by electrophoresis on 0.1% sodium dodecyl sulfate ($NaDodSO_4$) gel (Fairbanks et al., 1971) and by amino acid analysis.

Preparation of Chicken Ovomucoid Domains. Chicken ovomucoid was subjected to limited enzymatic cleavage, or CNBr cleavage, as indicated in Figure 1. The identities of isolated domains were verified by amino acid analysis.

The third domain is only partially glycosylated, and "glycosylated" and "nonglycosylated" third domains can be separated on Bio-Gel P-10 equilibrated with 5% (v/v) formic acid (Kato et al., 1976). Only nonglycosylated third domain was used in the present study. The third domain from staphylococcal proteinase digests of chicken ovomucoid (OMCHI3-SPase)[1] was prepared and isolated by procedures analogous to those published for Japanese quail ovomucoid (Bogard et al., 1980). Limited proteolysis by staphylococcal proteinase at pH 8.2 cleaves bond 130–131 with a high degree of specificity. Pepsin was used to prepare third domain (OMCHI3-pepsin) from differentially deuterium-exchanged ovomucoid since the reaction could be carried out at pH 3 where back-exchange is negligible; pepsin cleaves bond 133–134 (Kato et al., 1976). Third domain was also obtained by limited proteolysis with thermolysin (OMCHI3-Thlsn) which cleaves the connecting peptide between domains 2 and 3 predominantly at position 133–134 and also at position 130–131, yielding a mixture of two species (Kato et al., 1978).

First domain OMCHI1-CNBr was prepared by the procedure of Beeley (1976) except that a 2.5 × 100 cm column of Bio-Gel P-30 in 5% (v/v) formic acid was used to separate the CNBr fragments. The first domain samples were rechromatographed to obtain higher purity. Previous work (Markley & Kato, 1975) demonstrated that back-exchange into histidine C_ϵ-H is negligible under the conditions used for CNBr cleavage at low pH.

A complete procedure for the isolation of second domain (OMCHI2-SPase) will be published separately.[2] A 40-mg sample of OMCHI2-SPase was exchanged in 2 mL of 0.2 M KCL in ^2H_2O at pH* 7.5 and 40 °C. The exchanged protein was passed through a Sephadex G-25 column equilibrated with 1% (v/v) acetic acid in order to stop the exchange and to remove salts. Following lyophilization, the protein was oxidized with performic acid to cleave the three disulfide bonds. After repeated lyophilization, the oxidized protein was digested in 4 mL of 0.1 M NH_4HCO_3 with 1 mg of TPCK–trypsin for 4 h at room temperature.

The tryptic digest was loaded onto a Bio-Gel P-10 column (2.5 × 50 cm) equilibrated with 0.25% (v/v) acetic acid, and the elution of the peptides was monitored by their absorbance at 206 nm. Three distinct peptide fractions (I, II, and III) were obtained; fractions II and III were found to contain histidine by amino acid analysis. Amino-terminal sequence analysis of the histidine-containing fractions showed that fraction II contained a single pure tryptic peptide (Ala^{90}–$CySO_3H$–His^{111}-Lys)[3] and that fraction III was a mixture of peptides. So that the His^{123}-containing peptide could be obtained, fraction III was submitted to high-voltage electrophoresis at pH 6.5 (3 kV, 60 min) and pH 3.5 (3 kV, 40 min) on Whatman 3 MM paper in volatile buffer (pyridine and acetic acid). Histidine-containing peptides were detected by the red color after Pauly reagent treatment of the ninhydrin-positive bands. Two Pauly positive bands (III-a, III-b)

[1] Abbreviations: Hse>, homoserine lactone; $CySO_3H$, cysteic acid; SPase, staphylococcal proteinase; Thlsn, thermolysin; OMCHI, chicken egg white ovomucoid; OMCHI1-CNBr, first structural domain of ovomucoid prepared by CNBr cleavage, composed of residues Ala^1–$Hse>^{68}$; OMCHI2-SPase, second domain of ovomucoid prepared by limited proteolysis by staphylococcal proteinase, composed of residues Thr^{65}–Gly^{130}; OMCHI3-SPase, third domain of ovomucoid prepared by limited proteolysis by staphylococcal proteinase, composed of residues Leu^{131}–Cys^{186}; OMCHI3-pepsin, third domain of ovomucoid prepared by limited proteolysis by pepsin, composed of residues Val^{134}–Cys^{186}; OMCHI3-Thlsn, third domain of ovomucoid prepared by limited proteolysis by thermolysin, a mixture of predominently two species, Val^{134}–Cys^{186} (major) and Leu^{131}–Cys^{186} (minor); RNase A, bovine pancreatic ribonuclease A; TPCK, tosyl-L-phenylalanyl chloromethyl ketone; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt. The notation pH* is used to designate the uncorrected pH meter reading of ^2H_2O solutions measured with electrodes standardized in ^1H_2O buffers.

[2] I. Kato, unpublished results.

[3] The numbering system for native chicken ovomucoid is used to refer to residues of isolated domains.

Table I: pH Titration Parameters for the Histidine Residues of Chicken Ovomucoid and of Isolated Structural Domains of the Molecule from 360-MHz ^{1}H NMR Measurements

assignment[a]	peak designation[b]	protein[c]	pK_a'	histidine protonation: Hill coefficient	histidine protonation: δ_{low} (from DSS)	histidine protonation: $\Delta\delta$	spectral perturbation at low pH: pH_{mid}	spectral perturbation at low pH: δ_{low} (from DSS)	spectral perturbation at low pH: $\Delta\delta$
His^{58}	H3	OMCHI	6.80 ± 0.08	0.84 ± 0.12	7.70	0.91			
	I-H	OMCHI1-CNBr	6.66 ± 0.03	0.92 ± 0.04	7.71	0.90			
His^{111}[d]	H2	OMCHI	6.83 ± 0.04	0.87 ± 0.06	7.70	1.12	2.56 ± 0.17	8.70	0.12
	II-Ha	OMCHI2-SPase	6.76 ± 0.01		7.62	1.14	1.36 ± 0.21	8.67	0.08
His^{123}	H4	OMCHI	5.98 ± 0.04	0.99 ± 0.10	7.77	0.83			
	II-Hb	OMCHI2-SPase	5.87 ± 0.01	0.96 ± 0.02	7.67	0.89			
His^{182}	H1	OMCHI	8.04 ± 0.04	1.26 ± 0.14	7.83	0.98	2.35 ± 0.19	8.70	0.10
	III-H	OMCHI3-Thlsn	7.56 ± 0.02	0.91 ± 0.03	7.91	0.92	2.70 ± 0.15	8.70	0.16
	III-H	OMCHI3-SPase	7.53 ± 0.02	0.96 ± 0.02	7.90	0.90	2.49 ± 0.05	8.65	0.15

[a] Result of this paper. [b] As defined in Figures 2, 3, and 5-7. [c] Notation defined in Figure 1 and footnote 1. [d] Nonhomologous histidine.

were cut out, extracted from the paper with 20% (v/v) acetic acid, and subjected to amino acid analysis and amino-terminal sequence analysis. The main band, III-b, was found to contain a 3:1 mixture of two peptides: His^{123}–CySO$_3$H-Arg^{128} and Val^{113}–Lys^{121}. This mixture could not be separated readily, but since the contaminating peptide does not contain histidine, its presence did not interfere with the measurement by NMR of deuterium exchange at His^{123} C_ϵ-H.

Preexchange. Native ovomucoid was incubated in ^2H_2O (10 mg/mL) at pH* 8.75 for 5 h at 40 °C to remove intensity from the histidine region of the spectrum which was contributed by slowly exchanging peptide amide protons. Following this treatment, the solution was adjusted to pH* 3 with 1 M ^{2}HCl and lyophilized. This material is termed "preexchanged ovomucoid". For some experiments, samples of the isolated domains were also preexchanged to remove intensity from peptide amide protons. In the case of the first and third domains this was accomplished by adjusting the solution of protein in ^2H_2O (10–30 mg/mL) to pH* 8.0–9.0 at room temperature for 15–30 min. After lowering the pH, the solutions were lyophilized. Preexchanged second domain was prepared by the same procedure utilized for native ovomucoid.

Deuterium Exchange. Preexchanged native ovomucoid (5 mM) was incubated at 40 °C in ^2H_2O containing 0.2 M KCl at pH* 7.50. Aliquots were withdrawn periodically for analysis by ^{1}H NMR or for the preparation of differentially deuterated domains. Exchange was stopped by adjusting the aliquots to pH* 2.5–3.0 with 1 M ^{2}HCl, followed by lyophilization and storage at −20 °C.

Exchange experiments involving the isolated second and third domains were carried out by using the same procedure used for native ovomucoid.

Exchange experiments with the isolated first domain were carried out in a 5-mm NMR tube under the conditions described for native ovomucoid. The exchange reaction was monitored by ^{1}H NMR after the pH* of the solution was adjusted to 5.0 to stop the exchange reaction. The reaction was resumed by adjusting the solution to pH* 7.50, and the incubation was continued at 40 °C.

Titration and pH Measurement. All pH values were determined at room temperature (near 25 °C) by using methods described previously (Markley & Porubcan, 1976). Samples for titration studies were 2 mM by weight of lyophilized protein in 0.2 M KCl in ^2H_2O.

^{1}H NMR Spectroscopy. A Nicolet NT-360 spectrometer formerly at the Purdue University Biochemical Magnetic Resonance Laboratory was used to obtain 360-MHz Fourier-transform spectra. All spectra were obtained at 25 °C; 8K data points were digitized by using quadrature detection. Normally 300–800 transients were averaged by using a 90° pulse (~10 μs). A delay of 2.0–7.0 s following acquisition was used depending on the sample and the need to obtain accurate areas of resonances. The Nicolet NT-470 spectrometer currently at the Purdue University Biochemical Magnetic Resonance Laboratory was used under similar conditions to obtain 470-MHz spectra.

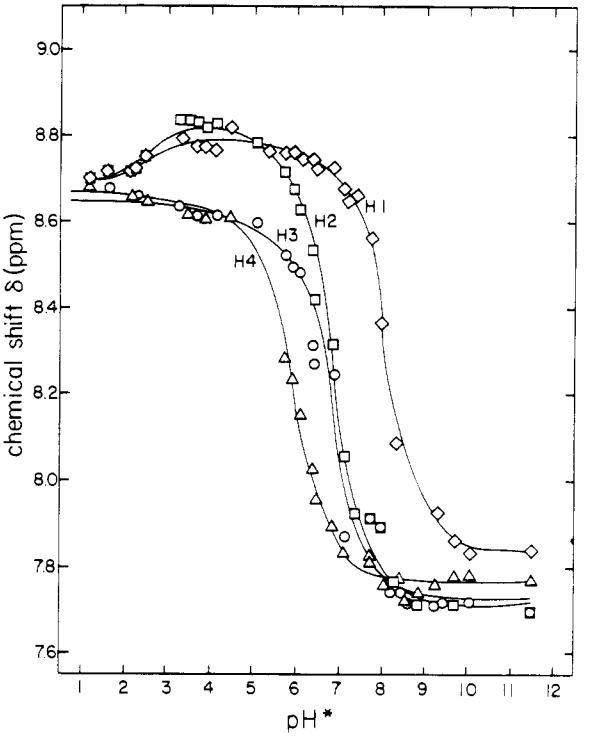

FIGURE 2: pH* dependence of the ^{1}H NMR chemical shifts at 360 MHz of the C_ϵ proton of the four histidine residues of chicken ovomucoid. The titration was carried out at 25 °C in ^2H_2O containing 0.2 M KCl. The histidine peaks (see Figure 3) are assigned as follows (see text): H1, His^{182}; H2, His^{111} (nonhomologous); H3, His^{58}; H4, His^{123}. Analyzed titration parameters are given in Table I. The chemical shifts of His^{111} and His^{182} are affected by protonation of other groups at low pH.

Results

Histidines of Native Ovomucoid. Figure 2 shows the ^{1}H NMR titration curves of the four histidine residues of chicken ovomucoid and identifies the peak labeling scheme used. The curves reported here at 360 MHz are virtually identical with those obtained previously at 100 MHz (Markley, 1973b), indicating that the pH-dependent processes affecting the chemical shifts are rapid on the NMR time scale (Sudmeier et al., 1980). The histidine pK_a' values resulting from these curves (Table I) are identical, within experimental error, with

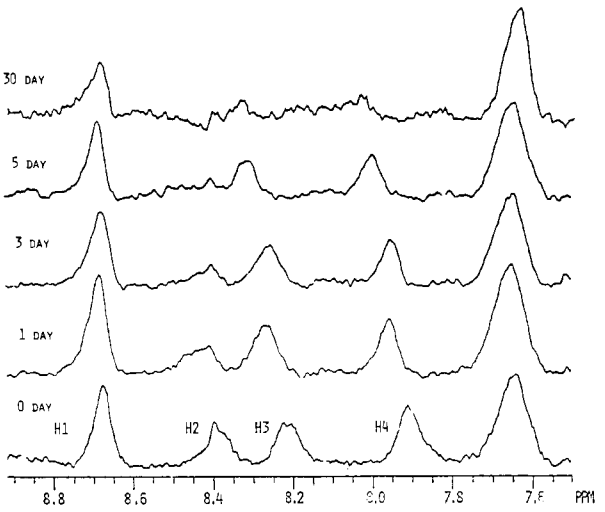

FIGURE 3: Representative 360-MHz ^1H NMR spectra of the histidine C_ϵ-H peaks of chicken ovomucoid (H1–H4) undergoing deuterium exchange. The exchange of 5 mM ovomucoid in ^2H_2O containing 0.2 M KCl, at pH* 7.5, 40 °C, was carried out for the time indicated in the figure. ^1H NMR spectra were recorded at 25 °C by using aliquots that had been titrated to pH* 6.50. The exchange data are summarized in Table II.

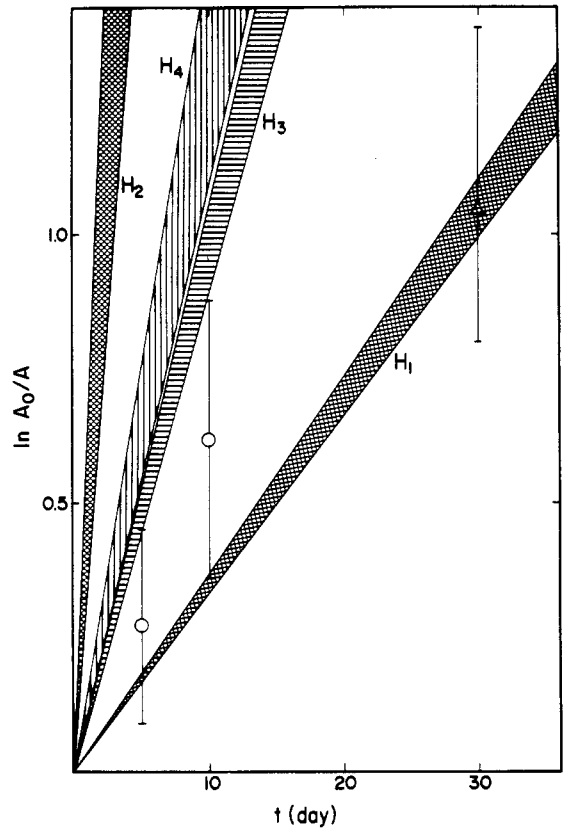

FIGURE 4: First-order plot of differential exchange of histidine C_ϵ-H groups in chicken ovomucoid. A_0 and A are the areas of the ^1H NMR NMR peak of each histidine C_ϵ-H at zero time and time t, respectively. The shaded bars represent the exchange rates determined for the histidine peaks (H1, H2, H3, and H4) of whole ovomucoid. The widths of the bars represent one standard deviation from a nonlinear least-squares analysis of the exchange data (Table II). The point (Δ) is for third domain isolated from ovomucoid exchanged 30 days in ^2H_2O (Figure 5). The points (O) are for first domain isolated from ovomucoid samples exchanged 5 and 10 days in ^2H_2O (Figure 6).

those determined previously (Markley, 1973b). The higher precision of the 360-MHz data permitted a more complete analysis of the transitions that affect histidines H1 and H2 at low pH (Table I).

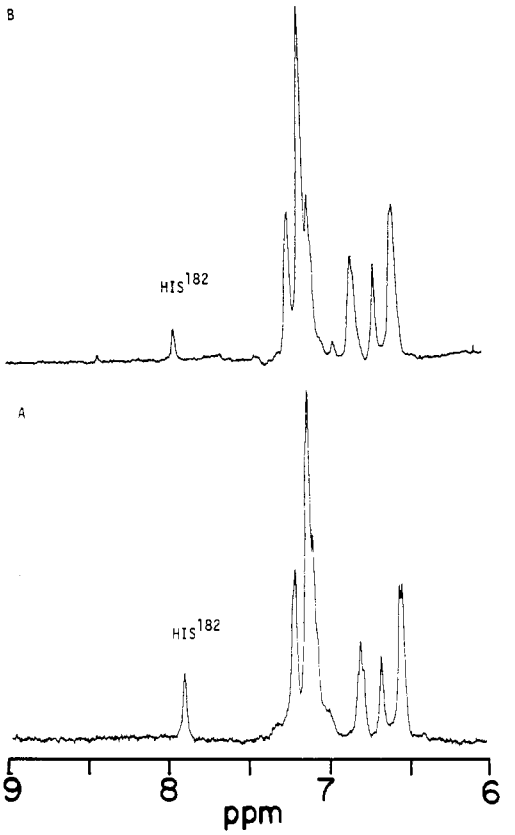

FIGURE 5: 360-MHz ^1H NMR spectrum of the third domain of chicken ovomucoid isolated after limited proteolysis with pepsin (A) from unexchanged protein and (B) from protein that had been exchanged in ^2H_2O at pH* 7.5, 40 °C, for 30 days. The relative area of the C_ϵ-H of His^{182} (III-H) is 0.35 ± 0.10 proton.

Preliminary results (Markley, 1973b) indicated that the C_ϵ-H atoms of the four histidines of ovomucoid exchange at different rates at pH* 7.5 and 40 °C. The present results confirm this (Figures 3 and 4) but show that the earlier exchange half-times were in error. The errors apparently resulted from faulty assumptions made about the base line under the histidine C_ϵ-H peaks (Markley, 1973b, Figure 16) as well as from the limited number of data points used. In the present study, the peaks were resolved cleanly on a flat base line.

Assignments of First and Third Domain Histidines. Third domain and first domain each contain one histidine residue, His^{182} and His^{58}, respectively (Figure 1). These two domains were isolated from ovomucoid samples that had been exchanged previously in ^2H_2O at 40 °C, pH* 7.5. The spectrum of the third domain (OMCHI3-pepsin) made from a sample exchanged for 30 days is shown in Figure 5. The relative intensity of the C_ϵ-H of His^{182} is 0.35 ± 0.10 proton. On comparing this value with those expected for the four histidines after a 30-day exchange, we make a definite assignment of peak H1 of native ovomucoid to His^{182}.

Spectra of the first domain (OMCHI1-CNBr) made from samples exchanged for 5 and 10 days are shown in Figure 6. The relative intensities of the peaks corresponding to the C_ϵ-H of His^{58} are 0.77 ± 0.10 proton after 5 days and 0.55 ± 0.10 proton after 10 days. On comparing these values with the expected intensities of the four histidines of native ovomucoid (Figure 4), one first can rule out peak H1 assigned above to His^{182}. Peak H2, which exchanges much more rapidly, must then correspond to the second domain. The exchange data are not precise enough to decide whether peak H3 or H4 corresponds to the first domain. The assignment of peak H3 to His^{58} was made by difference after the assignments of the

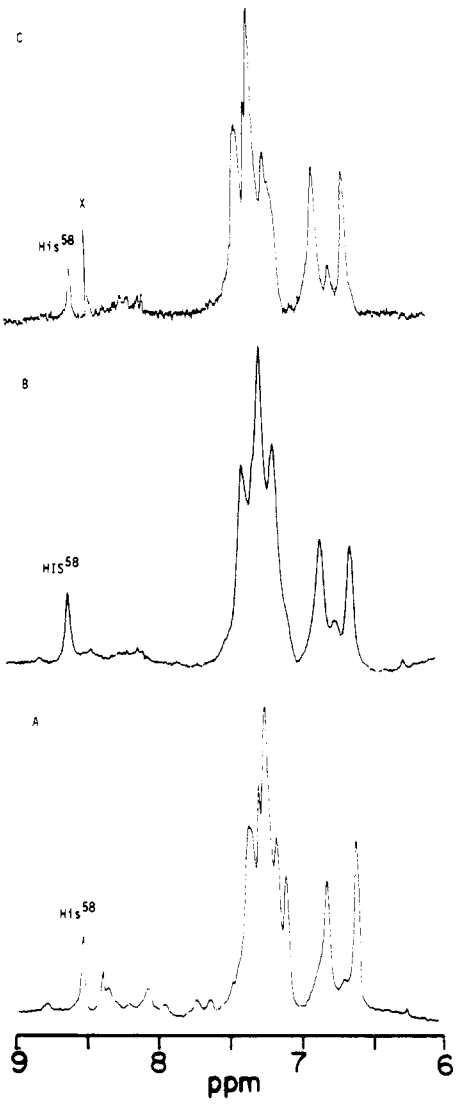

FIGURE 6: 470-MHz ^1H NMR spectra of the first domain of chicken ovomucoid isolated by cyanogen bromide cleavage from protein that had been exchanged in ^2H_2O at pH* 7.5, 40 °C, for (A) 0, (B) 5, and (C) 10 days. The relative area of the C_ϵ-H of His^{58} was 0.77 ± 0.10 proton after 5 days and 0.55 ± 0.10 proton after 10 days. The spectra were obtained at pH* 5.0 at 25 °C. The peak labeled X is formate, an impurity.

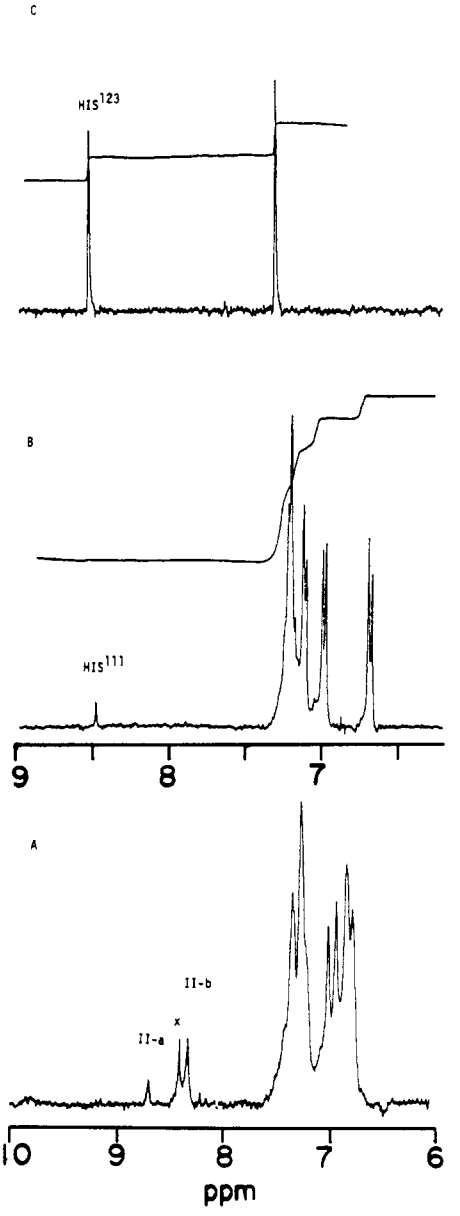

FIGURE 7: 360-MHz ^1H NMR spectrum (A) of the second domain of chicken ovomucoid isolated following limited proteolysis with staphylococcal proteinase after exchange in ^2H_2O at pH* 7.5, 40 °C, for 3 days. The two histidine C_ϵ-H peaks are labeled II-Ha and II-Hb. The sample of second domain exchanged for 3 days in ^2H_2O was digested with trypsin and fractionated. 360-MHz ^1H NMR spectra of the two histidine-containing fractions were obtained: (B) fraction II containing one peptide, Ala-Phe-Asn-Pro-Val-Cys-Gly-Thr-Asp-Gly-Val-Thr-Tyr-Asp-Asn-Glu-$CySO_3H$-Leu-Leu-$CySO_3H$-Ala-His^{111}-Lys, and (C) fraction III-b containing a 3:1 mixture of two peptides, His^{123}-Asp-Gly-Gly-$CySO_3H$-Arg and Val-Glu-Glu-Gly-Ala-Ser-Val-Asp-Lys. The results assign the faster exchanging peak, II-a, to His^{111} and the slower exchanging peak, II-b, to His^{123}. All NMR spectra were obtained at 25 °C with samples at pH* 5.43. The peak in (A) labeled X is from formate, an impurity.

second domain histidines (see below).

Assignment of Second Domain Histidines. Because we do not have a procedure for isolating second domain at low pH, where back-exchange into the histidines can be ignored, it was not possible to use the same assignment strategy for this domain. Instead, we first assigned the histidine C_ϵ-H peaks II-Ha and II-Hb of isolated second domain (OMCHI2-SPase) which exchange at differential rates as shown in Figure 7A. The differentially exchanged domain was digested with trypsin, and peptides containing the two histidines were separated. After a 72-h exchange, the C_ϵ-H of His^{111} retains 20% of its intensity (Figure 7B), whereas the C_ϵ-H of His^{123} retains 73% of its intensity (Figure 7C). With these results we assign peak II-Ha to His^{111} and peak II-Hb to His^{123}.

The assignments are completed by noting that peak H2 of native ovomucoid and peak II-Ha of the second domain have nearly identical pH titration curves, including the inflection at low pH (Figure 8B), and the same deuterium exchange kinetics (Table II). Moreover, the properties of peak H4 and peak II-Hb, while very different from those of the above pair, are similar to one another and quite different from those of peak H3 (Figure 8C; Table II). On this basis we assign peaks H2 and H4 to His^{111} and His^{123}, respectively; peak H3 is assigned to the remaining residue, His^{58}.

Histidines of Isolated Ovomucoid Domains. The pH titration and deuterium-exchange properties of each isolated domain were studied by ^1H NMR. Figure 8 compares the histidine C_ϵ-H titration curves of histidines in individual domains with the curves assigned to the same residues in native ovomucoid. The titration parameters are collected in Table I. The half-times for deuterium exchange at the C_ϵ-H positions are compared in Table II.

Table II: Half-Times for Deuterium Exchange at the Histidine C_ϵ-H Positions in Native Chicken Ovomucoid and in Three Isolated Structural Domains of the Molecule As Determined by ^1H NMR[a]

assignment[b]	peak designation[c]	protein[d]	half-time for exchange (day)[e]	k_2 (M^{-1} s^{-1})[f]
His^{58}	H3	OMCHI	7.1 ± 0.5	140 ± 40
	I-H	OMCHI1-CNBr	12.8 ± 1.7	110 ± 20
His^{111}[g]	H2	OMCHI	1.5 ± 0.5	640 ± 300
	II-Ha	OMCHI2-SPase	1.2 ± 0.3	930 ± 280
His^{123}	H4	OMCHI	5.7 ± 1.1	1100 ± 300
	II-Hb	OMCHI2-SPase	5.9 ± 1.2	1400 ± 300
His^{182}	H1	OMCHI	19.8 ± 1.0	6.5 ± 1.5
	III-H	OMCHI3-Thlsn	6.0 ± 0.6	40.0 ± 9

[a] Exchange was carried out in ^2H_2O at 40 °C, pH* 7.5. [b] From the present work, using the numbering system of native chicken ovomucoid. [c] From NMR data; see Figures 2, 3, and 5–7. [d] As defined in Figure 1. [e] The values represent least-squares analyses of the exchange data fitted to first-order kinetics. Error limits given are one standard deviation. [f] Second-order rate constant for exchange. From eq 1. These values are only approximate since the pK_a' values were determined at 25 °C whereas the exchange was carried out at 40 °C with protein titrated to pH* 7.5 at 25 °C. [g] Nonhomologous histidine residue.

Discussion

Assignments. It would have been preferable to have assigned all four histidine peaks by the differential exchange technique (Markley & Kato, 1975). This was possible only for His^{182} of whole ovomucoid and His^{111} and His^{123} of isolated second domain. The exchange results also permitted the assignment of peak H2 of ovomucoid to one of the second domain histidines. We made the remaining assignments after noting that the properties of the two second domain histidine peaks (pK_a', low pH transition, deuterium-exchange kinetics) were nearly identical with those of two histidine peaks in native ovomucoid (Figure 8, Table II). These assignments appear reasonable since duplication of all the properties in a different pair of residues would be highly unlikely. The NMR peak of the one histidine whose properties are greatly different in its isolated domain compared to those in whole ovomucoid (His^{182}) was assigned unambiguously by differential deuterium exchange (Figure 5).

Properties of the Histidines of Ovomucoid and Ovomucoid Domains. Two kinds of information are available for each histidine: its pK_a' value and the rate of exchange of its C_ϵ-H with deuterium from the solvent. At pH values above 5, the exchange rate, k, is given by

$$k = k_2 K_{w,^2H_2O}/(K_a + [^2H]) \quad (1)$$

where K_a is the histidine dissociation constant, $K_{w,^2H_2O}$ is the ionization constant for ^2H_2O (1.35×10^{-15} M^2 at 25 °C), k_2 is the bimolecular rate constant for attack of O^2H^- on the positively charged form of the histidine ring, and $[^2H]$ is the deuterium ion concentration calculated from $p^2H = pH^* + 0.4$ (Vaughn et al., 1970; Bradbury et al., 1980). On the basis of tritium-exchange studies of model compounds, Minamino et al. (1978) determined that the second-order rate constant, k_2, for hydrogen exchange at the histidine C_ϵ-H follows the Brønsted equation. This amounts to a second-order pH dependence of the exchange rate given by eq 1. According to such an analysis, the independent properties of a histidine residue are its pK_a' value and its deviation from the Brønsted plot for exchange of a "normal" imidazole. Minamino et al. (1978) found a linear relationship between log k_2 and the pK_a' of imidazole and histidine derivatives, such as small peptides, that have no macromolecular effects and are completely solvated. They interpreted deviations from this line observed with proteins as indicative of environmental factors around individual histidine residues and found that these deviations correlated well with the degree of solvent accessibility of the histidine residue in the protein.

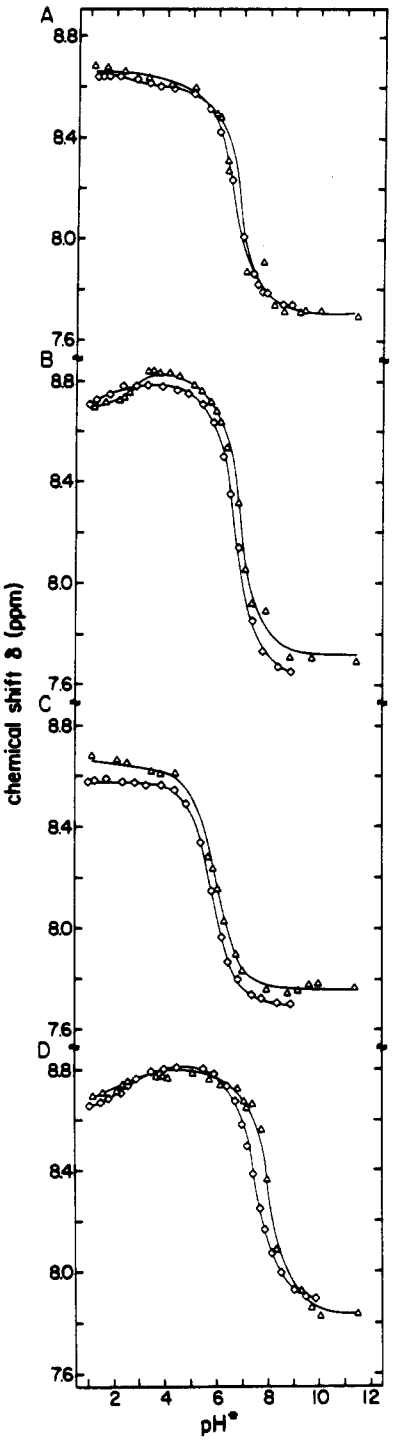

FIGURE 8: ^1H NMR titration curves (chemical shift of the C_ϵ-H vs. pH*) of the histidines of isolated domains one, two, and three of chicken ovomucoid [experimental points (O) and computer-fitted solid lines] at 25 °C in ^2H_2O containing 0.2 M KCl. The titration curves of the same histidines in native ovomucoid under similar conditions are shown for purposes of comparison [experimental points (Δ) and computer-fitted solid lines]. (A) His^{58}, (B) His^{111}, (C) His^{123}, and (D) His^{182}.

The above analysis is probably oversimplified because it does not take into account the effects of neighboring charged groups on exchange (independent of their effects on the pK_a' of the histidine) or the possible participation of neighboring protein

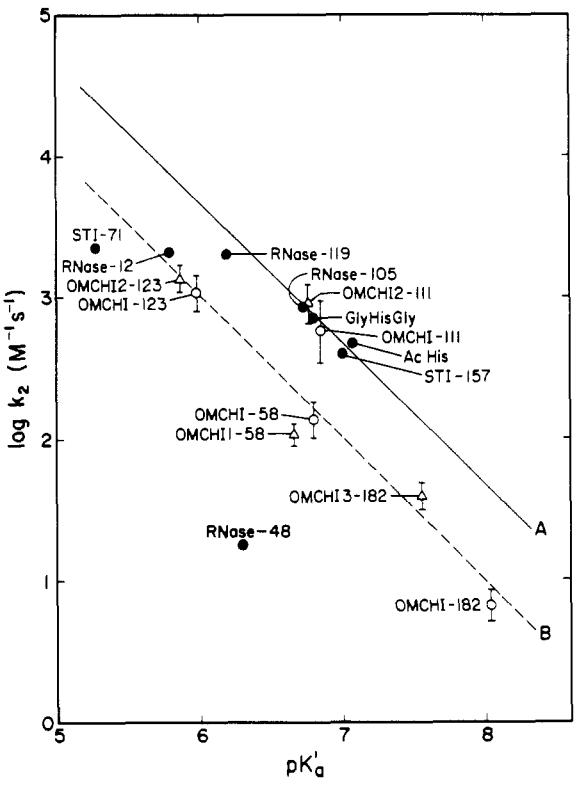

FIGURE 9: Plot of the log of the second-order deuterium exchange rate, k_2, of histidine in model compounds and proteins at 40 °C vs. the pK_a' of the histidine (Brønsted plot). Line A has slope of 1.0 (Brønsted coefficient, α) and shows the curve expected for histidine side chains that are completely solvated. Line B, which is parallel to line A, fits the data for all the conserved histidines of chicken ovomucoid and its isolated domains. Abbreviations and references: (1) OMCHI, whole chicken ovomucoid; OMCHI1, OMCHI2, and OMCHI3, first, second, and third domains of chicken ovomucoid (this work, Table II). (2) Ac-His, N^{α}-acetyl-L-histidine; Gly-His-Gly, glycyl-L-histidylglycine (Markley & Cheung, 1973). (3) RNase, bovine pancreatic ribonuclease A (Markley, 1975a). (4) STI, soybean trypsin inhibitor (Kunitz) (Markley, 1973a; Markley & Kato, 1975). Numbers refer to the residue position in which the histidine is located.

side chains in catalyzing exchange. The influence of neighboring charged groups on the exchange rate has been documented by Bradbury et al. (1980). Charge effects may explain the fact that exchange data from model imidazole compounds do not all lie on a linear Brønsted plot (Endo et al., 1979). Nevertheless, a Brønsted plot is the most descriptive way to present histidine C_{ϵ}-H exchange data when the histidine pK_a' values are known since it removes the intrinsic dependence of the exchange rate on the pK_a'. Deviation from linearity must result from factors such as solvent accessibility (Kimura et al., 1979), effects of neighboring charge on exchange (in addition to their effect on the histidine pK_a'), or catalysis of the exchange reaction by neighboring groups.

In Figure 9 we plot log k_2 (from Table II) vs. pK_a' (from Table I) for each of the histidines of chicken ovomucoid and its three isolated domains. Other points represent data from this laboratory for model compounds and other proteins. Line A which has a Brønsted exponent, α, of 1.0 includes the data points for the model compounds N^{α}-acetyl-L-histidine (Ac-His) and glycyl-L-histidylglycine (Gly-His-Gly). There is no theoretical basis for the slope of a Brønsted plot. Endo et al. (1979) obtained an experimental slope of 0.75 on the basis of exchange data from three model compounds. Inclusion of all the model compounds they studied gives a slope close to 1.0.

The exchange reaction can be thought of as the specific acid catalyzed addition of a proton to OH^- where imidazole is the acid. The acidity of the C_{ϵ}-H is related to the pK_a' of the imidazole. Presumably all exposed (completely solvated) histidines will fall on line A. Deviations from this line may be illustrated by reference to results with other proteins. The environments of the four histidines of bovine pancreatic ribonuclease A have been characterized by X-ray crystallography [see Richards & Wyckoff (1971)]. The point for His^{105} of RNase A, which is completely exposed to the solvent, falls on line A (Figure 9). The vertical deviations of points for the other three histidines of RNase from line A follow the order of their solvent inaccessibilities: $His^{119} < His^{12} \ll His^{48}$. The environment of His^{48} is so abnormal that its rate of protonation/deprotonation is slow on the NMR time scale (Markley, 1975b). The point for His^{71} of soybean trypsin inhibitor, which gives an abnormally shallow ^1H NMR titration curve (Markley, 1973a), lies well away from curve A, whereas the point for the more normal His^{157} of this protein lies on line A.

Homologous Histidines. Despite the fact that the pK_a' values for the homologous histidines (His^{58}, His^{123}, and His^{182}) of native ovomucoid are dissimilar, their log k_2 values fall on a line (Figure 9, line B) that is parallel to line A but is 0.72 log unit lower. Although, as discussed above, one cannot attribute the abnormal exchange rate of these histidines to a single mechanism (e.g., solvent accessibility), the data are consistent with the three histidines having similar (limited) solvent accessibilities.

The pK_a' values of His^{58} and His^{123} change only slightly when their respective domains are isolated, whereas the pK_a' of His^{182} falls by 0.5 pH unit upon isolation of the third domain. Points for the isolated domains also fall on or near line B. This suggests that the factors that lead to the decreased exchange rates of the homologous histidines of native ovomucoid remain constant when the domains are isolated.

Nonhomologous Histidine. The point for His^{111} of native ovomucoid falls on line A (Figure 9). Neither its pK_a' nor its deviation from line A changes appreciably when the second domain is isolated. From the Brønsted plot one would predict that His^{111} of native ovomucoid or isolated second domain is completely accessible to solvent. None of the histidines of native ovomucoid, however, was polarized in a photo-CIDNP experiment.[4] It is possible that His^{111} is accessible to water but not to the much larger dye molecule used in the photo-CIDNP experiment because of steric hindrance. The only ovomucoid X-ray structure at present is of the third domain from Japanese quail. In this domain, the residue corresponding to His^{111} is an alanine (Ala^{111} of native ovomucoid, or Ala^{40} numbering only the residues of the domain) which is at the surface of the molecule (Weber et al., 1981).

The low pH transition that affects the chemical shift of His^{111} of whole ovomucoid (Table I, Figure 8B) probably arises from the same conformational transition that leads to the huge perturbation of the UV spectrum (Donovan, 1967). This UV transition has been studied in detail for chicken ovomucoid and its isolated domains (March, 1980). March (1980) found that the pH_{mid} of the low pH transition is 2.85 in native chicken ovomucoid and 2.89 in isolated second domain. The transition is thought to result from protonation of an aspartic acid side chain, which causes the breakage of a hydrogen bond between the aspartic acid and the tyrosine whose UV spectrum is perturbed by the transition (March, 1980). The X-ray structure of the third domain of Japanese quail ovomucoid (Weber et al., 1981) shows that Asp^{157} and Tyr^{161} are in

[4] J. L. Markley, D. H. Croll, S. Stob, and R. Kaptein, unpublished data.

position to form a hydrogen bond of the type suggested; in the second domain, the homologous residues are Asp^{98} and Tyr^{102}. ^1H and ^{13}C NMR studies of the Donovan transition in chicken ovomucoid third domain confirm this explanation and further demonstrate that the conformational transition affects only a limited number of residues.[5] Figure 8 indicates that the low pH transition is different for isolated second domain than for native ovomucoid; in the second isolated domain, His^{111} no longer appears to be affected by the Donovan transition which shows a pH_{mid} of 2.9 by UV in OMCH II (March, 1980).

Interdomain vs. Intradomain Interactions. The ability to investigate isolated structural domains has enabled us to determine whether the different properties of the homologous histidines of ovomucoid arise from interdomain or intradomain interactions. This question is of interest because it is not known why proteinase inhibitors like ovomucoid are synthesized as tandem domains, and one would like to know whether the domains behave as isolated beads on a string or interact with one another (Laskowski & Kato, 1980).

All the cysteines are conserved in each domain, and the three isolated domains are expected to have similar folding geometries. Their structures probably resemble that of the third domain of Japanese quail ovomucoid (Weber et al., 1981). The X-ray structure suggests an explanation for the unusually high pK_a' value of His^{182} in the third domain of chicken ovomucoid. (The pK_a' value of His^{182} of this residue also is high in Japanese quail ovomucoid third domain.[5]) The C_o-carboxyl group of the C-terminal Cys of Japanese quail ovomucoid third domain is approximately 5 Å distant from the N_{ϵ_2} atom of His^{182} (Weber et al., 1981). The presence of the negatively charged carboxyl group would elevate the pK_a' of His^{182} via electrostatic interaction above its normal value of 6.5. Similarly, the pK_a' value of the carboxyl group of Cys^{186} should be depressed from its normal value of 3.9. The chemical shift of His^{182} is perturbed by a transition at low pH (Table I) which probably corresponds to the pK_a' of the C_o carboxylate of Cys^{186}.[6] Similar folding geometries for the three domains would lead to similar solvent accessibilities for the three conserved histidines. The exchange data for these histidines are consistent with their having similar solvent accessibilities. The difference in pK_a' between His^{58} and His^{123} probably results from amino acid differences that create differing electrostatic environments at the histidines.

One must postulate an interdomain interaction to explain the large difference between the pK_a' of His^{182} in whole ovomucoid and in the isolated third domain. The pK_a' difference apparently does not result simply from the formation of a new amino terminus, because the titration behavior of His^{182} is nearly identical in third domains that have very different amino-terminal arms (OMCHI3-Thlsn and OMCHI3-SPase; see Figure 1).

Conclusions

These results support the idea that the structural domains of ovomucoid have conformations that are determined primarily by intradomain interactions; but side chains of residues in different domains within the molecule also interact. These studies bear on the common use of homology to extend NMR assignments from one protein to another related protein. The present results indicate that such arguments are valid only for very closely related proteins. The most closely related domains of chicken ovomucoid by sequence homology are the first and second domains. Recent CD experiments indicate that the secondary structures of domains I and II are more closely related to one another than to that of domain III (Watanabe et al., 1981). The properties of their conserved histidines also are quite similar. The third domain histidine (His^{182}) differs from the other homologous histidines as the result of its interaction with the C_o carboxylate of Cys^{186}. Several pairs of ovomucoid domains from various birds have been found to differ by replacement of only a single amino acid residue;[2] it will be interesting to use NMR spectroscopy to determine how far such minor differences are propagated through the protein structure.

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[6] This perturbation earlier was attributed (Markley, 1973b) to the low pH transition detected by UV spectroscopy (Donovan, 1967). Since Asp^{157} and Tyr^{161} are too far apart in quail ovomucoid third domain (Weber et al., 1981) to provide a direct mechanism for perturbing the chemical shift of His^{182}, and in light of the fact that the conformational changes associated with the Donovan transition are limited,[4] it is unlikely that the low pH transition affecting His^{182} and the Donovan transition are related.

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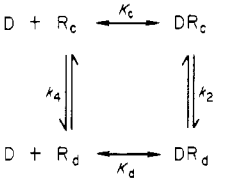
Conformations of *Torpedo* Acetylcholine Receptor Associated with Ion Transport and Desensitization†

Richard R. Neubig, Norman D. Boyd, and Jonathan B. Cohen*

ABSTRACT: Rapid-mixing techniques have been used to measure under identical conditions both the kinetics of binding of [^{3}H]acetylcholine (AcCh) to nicotinic receptors in postsynaptic membranes isolated from *Torpedo* electric tissue and the kinetics of AcCh-induced efflux of $^{22}Na^+$ from the vesicles. The binding studies define the rate constant, k_s, of the slow conformational transition resulting in the receptor conformation binding AcCh with high affinity [K_d = 2 nM, k_{dis} = 0.04 s^{-1} at 4 °C [Boyd, N. D., & Cohen, J. B. (1980) *Biochemistry 19*, 5344–5353, 5353–5358]]. For AcCh concentrations between 0.1 and 15 μM, k_s was characterized by a Hill coefficient of 1, $(k_s)_{max} \sim 0.8$ s^{-1}, and 10 μM AcCh produced a half-maximal rate. The observed $^{22}Na^+$ efflux, measured at times from 24 ms to 1000 s, defined the initial rate of $^{22}Na^+$ efflux (rate constant, k_o, a parameter proportional to the number of open channels) and a rate constant (k_d) for desensitization. Values of k_d were determined for AcCh concentrations between 0.1 and 1 μM where desensitization occurred before full release of $^{22}Na^+$ from the vesicles; for higher AcCh concentrations only k_o was determined. The observed concentration dependence of flux desensitization was the same as that of the conformational transition (k_s) defined by ligand binding, a result indicating that the high-affinity receptor is functionally desensitized. For AcCh concentrations to 300 μM, k_o was characterized by a Hill coefficient of 1.9 ± 0.1, $(k_o)_{max} \sim 420$ s^{-1}, and 150 μM AcCh produced a half-maximal response. These results establish that in the absence of agonist less than 1 channel in 10^7 is open and that the binding of two AcCh is necessary for channel activation. Comparison of the parameters characterizing [^{3}H]AcCh binding and channel activation indicates that the transient low-affinity conformation detected in binding studies reflects a desensitized receptor conformation that limits ion transport at subsecond times.

The binding of acetylcholine (AcCh)[1] by the nicotinic cholinergic receptor results within a fraction of a millisecond in a permeability response, the opening of a transmembrane ion channel. Binding of AcCh at equilibrium does not produce the response, since exposure to a constant concentration of AcCh for seconds or longer results in a reversible decline (desensitization) of the permeability response. Channel activation and desensitization have been interpreted by models in which the receptor (the AcCh binding protein and its ion channel) exists in distinct conformations differing both in ligand affinities and in the functional state of the ion channel [for reviews, see Colquhoun (1979) and Adams (1981)]. The simultaneous determination of the kinetics of binding of AcCh and of the functional state of the ion channel would provide direct evidence for the postulated receptor conformations.

Nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue provide a unique preparation for the measurement of both the kinetics of ligand binding and the associated permeability response [for a review, see Karlin (1980)]. The effects of cholinergic agonists on the kinetics of binding of radiolabeled α-neurotoxins provided the first evidence of slow conformational transitions associated with the binding of agonists (Weber et al., 1975; Weiland et al., 1977). The results were generally compatible with a reaction model (eq 1) in



(1)

which the receptor contains a single class of AcCh binding sites that exists in the absence of ligand in two interconvertible conformations, R_c and R_d, binding agonist with low (K_c) and high (K_d) affinity, respectively. This model is similar to that proposed by Katz & Thesleff (1957) and Rang & Ritter (1970) to account for desensitization at the vertebrate neuromuscular junction, and it was reasonable to assume that R_d represented a functionally desensitized conformation, while binding to R_c was related to channel activation.

Further quantitative definition of receptor conformational equilibria has resulted from the introduction of rapid-mixing and ultrafiltration techniques to measure the kinetics of binding of [^{3}H]AcCh and [^{3}H]carbamylcholine (Boyd & Cohen, 1980a,b) and the use of stopped-flow fluorescence to analyze the binding of a fluorescent agonist (Heidmann & Changeux, 1979a,b, 1980). The general conclusions from both studies were similar and provided additional data compatible with the

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[1] Abbreviations: AcCh, acetylcholine; α-BgTx, α-bungarotoxin; Carb, carbamylcholine; TPS, *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7).

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