NUCLEAR MAGNETIC RESONANCE STUDIES OF THE STRUCTURE AND BINDING SITES OF ENZYMES, X. PREPARATION OF SELECTIVELY DEUTERATED ANALOGS OF STAPHYLOCOCCAL NUCLEASE

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Abstract.—Five different selectively deuterated analogs of staphylococcal nuclease (Foggi) were prepared having all but a selected few hydrogen atoms in the enzyme molecule replaced by deuterium. The ^{1}H-NMR spectra of these analogs are extremely simplified, since they consist of lines arising only from the remaining protonated sites.

Success in completely analyzing the high resolution NMR spectrum of a protein—and thus deriving detailed information on its structure and function in solution—depends, at least for the present, on the preparation of selectively deuterated analogs of the protein. This procedure[1] allows a simplification of the protein NMR spectrum, so that only the resonance lines from a few selected amino acid residues are observed. We have previously reported[2] the preparation of one such analog (Nase-D1) of the enzyme staphylococcal nuclease and we have been able to completely analyze the aromatic region of its proton NMR spectrum.[3] We have now shown that a variety of similar analogs can be prepared reproducibly and that the protonated amino acid composition can be varied at will.

Materials and Methods.—Isolation and purification of deuterated amino acids from algae: Lyophilized algae (*Scenedesmus obliquus*) grown in 99% D_2O were obtained from Merck Sharp & Dohme of Canada. The cells were disrupted by extracting 250 gm of lyophilized algae with 3.5 l of 10% trichloroacetic acid (TCA) at 70° with agitation for 30 min. After cooling, the mixture was centrifuged for 10 min in a Sorvall RC2-B centrifuge with a GS-3 head at 10,000 rpm at 5 to 10°. The supernatant solution was discarded, and the TCA extraction and centrifugation of the residue was repeated twice.

Chlorophyl and the other pigments were then removed by repeated extraction. The residue was extracted three times with 4 l of a 2:1 ethanol:ethyl ether mixture and five times with 1 l of ethyl ether. Solvent was removed after each extraction by filtration on a large sintered-glass funnel of medium porosity. The weight of the residue after drying *in vacuo* was 130 gm.

Hydrolysis of 126.5 gm of the residue was accomplished by heating 16 hr at 100° with 1.8 l of 6 *N* HCl in a sealed, glass bomb. The hydrolysis mixture was cooled to room temperature and was filtered on a sintered-glass funnel. The insoluble residue (dry weight 20.0 gm). was washed with 750 ml water. Hydrochloric acid was removed by repeated concentration of the diluted filtrate *in vacuo* to a syrup at 35°. Concentration was repeated three times after dilution with 500 ml water. The last traces of hydrochloric acid were removed by drying the syrup in a vacuum oven containing a tray of sodium hydroxide pellets.

The syrup was diluted to 3 l, filtered, and applied to a Dowex-50 column (Biorad; column size, 8.5 cm × 80 cm; resin on hydrogen cycle) at a rate of 60 ml/min. The column was washed with water until a volume of 12 l had been collected. Elution was carried

out with 6 *N* ammonium hydroxide. The first 3.6 l (void volume) was discarded and the following 10 l were collected. The eluate was concentrated *in vacuo* at 35° to 500 ml and then lyophilized (yield: 81.3 gm). The lyophilized product was decolorized by dissolving it in 1.5 l of water and adding 80 gm powdered charcoal (Atlas Chem. Co., Darco G-60). The mixture was stirred for 30 min at room temperature and was then filtered over a thin layer of Hyflo Supercel (Johns Manville). The charcoal was washed with 1 l of water and the washes were collected separately. Colloidal carbon and precipitated amino acids were present in the water wash. This suspension was filtered again; the residue was dissolved in a minimum amount of 0.1 *N* ammonium hydroxide and refiltered. The combined filtrates were concentrated and lyophilized. The final yield was 72 gm of a white powder. Table 1 gives the amino acid composition of this product. The amino acid yield was 70% of the theoretical yield based on the protein content (determined by the method of Lowry) of the deuterated algae.

Growth on synthetic media: Cultures of *Staphylococcus Aureus* (Foggi) to be grown on media containing deuterated amino acids were first adapted to growth on a synthetic medium. Cells from one agar slant were used to inoculate three, baffled, 250-ml Erlenmeyer flasks containing 50 ml of the synthetic medium. The flasks were incubated at 37° for 16 to 18 hr. Aeration was achieved with a rotary shaker (New Brunswick Model G-53) which described a circle 5 cm in diameter at 200 rpm. The compositions of the synthetic, partially deuterated media used for the production of Nase analogs D1-5 are shown in Table 2. Cotton-plugged, baffled, 2-l Erlenmeyer flasks containing 500 ml of one of the synthetic, partially deuterated media were inoculated with 10 ml of a culture grown on the synthetic medium as described above. The same shaking conditions were used for the 2-l flasks as with the 250-ml flasks. Incubation was at 37° for 36 hr.

After incubation, the broths were pooled, chilled to 5°, centrifuged to remove cells, and saturated with ammonium sulfate. After 1 hr, the broth was filtered on a Lapp filter on filter paper precoated with Hyflo Supercel. The resulting filter cake was sucked dry and stored at −10° to await purification.

TABLE 1. *Composition and yields of deuterated amino acids.*

Composition of Product (percentages)	250 gm algae
Amino acids	
Lysine	4.98
Histidine	1.46
Arginine	3.73
Tryptophan	0.87
Aspartic acid	9.75
Threonine	5.12
Serine	4.40
Glutamic acid	9.99
Proline	5.08
Glycine	5.82
Alanine	8.45
Valine	6.15
Methionine	0.64
Isoleucine	4.17
Leucine	9.97
Tyrosine	1.35
Phenylalanine	3.98
Cysteine	Trace
Ammonia	1.51
Water	12.15
Nitrogen	13.48
Purity	100
Yield	
(gm/100 gm algal cells)	29.0

TABLE 2. *Composition of synthetic media used to prepare the selectively deuterated Nase analogs.*

Components Used for All Analogs	
Deuterated amino acid mixture (Table 1)	148 gm
Glucose	50.0 gm
KH_2PO_4	80.0 gm
Na_2HPO_4	340 gm
$MgSO_4.7H_2O$	1.6 gm
$FeSO_4.7H_2O$	320 mg
Thiamine HCl	40 mg
Niacin	40 mg
Water	40.0 liter
Protonated Amino Acids	
Nase-D1	
L-methionine	7.4 gm
L-tryptophan	7.4 gm
Nase-D2	
L-arginine	20.4 gm
L-methionine	7.4 gm
L-serine	82.9 gm
L-tryptophan	7.4 gm
Nase-D3	
L-tryptophan	7.4 gm
Nase-D4	
L-lysine	86.4 gm
L-tryptophan	7.4 gm
Nase-D5	
L-aspartic acid	169 gm
L-isoleucine	61.7 gm
L-tryptophan	7.4 gm

Purification of Nase samples: The purification procedure used was adapted from that published by Fuchs, Cuatrecasas, and Anfinsen.[4] It consists of two basic steps: (*i*) batchwise adsorption of Nase to phosphorylated cellulose followed by elution; (*ii*) chromatography of Nase on a phosphorylated cellulose column with gradient elution. Our procedure contains several modifications, the details of which are available.[5] Analytical procedures will be described in a subsequent paper.[6]

Results and Discussion.—The distribution of protonated sites in the five selectively deuterated analogs (Nase D1–D5) is indicated in Table 3. The most dramatic simplification of the spectrum is observed in the aromatic region (Fig. 1). It should be noted that the distribution of protonated sites in the aromatic residues is identical for all five analogs. The spectrum of analog D-4 shown in Figure 1 is, therefore, nearly identical to that reported previously for analog D-1 and to that of the other three. This fact, and particularly the finding that the position of the histidine C(2) protons is the same in the fully protonated nuclease

TABLE 3. *Protonated sites in the selectively deuterated nuclease analogs.*

Analog	Arg	Asx	Glx	His	Ilu	Lys	Met	Ser	Tyr	Trp
Nase-D1		β-CH_2*	γ-CH_2*	C2-H*			+		2,6-H*	+
Nase-D2	†	β-CH_2*	γ-CH_2*	C2-H*			+	+	2,6-H*	+
Nase-D3		β-CH_2*	γ-CH_2*	C2-H*					2,6-H*	+
Nase-D4		β-CH_2*	γ-CH_2*	C2-H*		+			2,6-H*	+
Nase-D5		†	γ-CH_2*	C2-H*	+				2,6-H*	+

* Indicates protonation by virtue of exchange during HCl hydrolysis.
† Indicates flooding with excess ^1H amino acid.

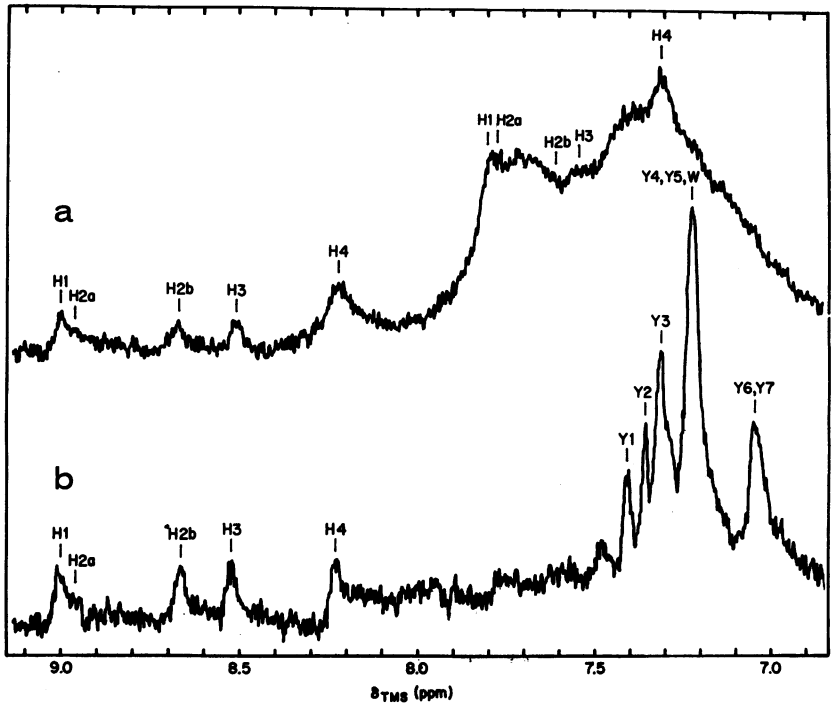

FIG. 1.—Comparison of the ^{1}H-NMR aromatic spectral regions of Nase and Nase-D4 at pH* 6.0. Assignments: His ring C2-H (low field) and C4-H(high field), H1-4; Trp ring C2-H, W; Tyr ring C2, 6-H, Y1-7.
(*a*) Nase, 75 scans.
(*b*) Nase-D4, 228 scans.

(Nase), lends strong support to the notion that the tertiary structure of all analogs is identical to that of the parent enzyme, since it has been established that differences in chemical shifts between chemically identical residues in a polypeptide chain largely reflect the tertiary structure.[7]

Considerable simplification of the aliphatic region has also been achieved. Spectra of analogs Nase-D3 and Nase-D2 are compared with that of fully protonated Nase in Figure 2 (*a–c*). The chemical shifts of the component protonated amino acids are shown in Figure 2(*d*). It is clear from these spectra that the resonance lines of amino acid side chains in the enzyme are shifted from their positions in the free amino acids. Analog Nase-D3 (Fig. 2*b*) contains only protons introduced through exchange of the deuterated amino acids with HCl during hydrolysis plus those of Trp which was deficient in the deuterated amino acid mixture. The relatively sharp singlet peaks present in the region δ_{TMS} 2.5–4.0 are probably the γ-CH_2 protons of the 18 Glx residues. The β-CH_2 peaks of the 14 Asx residues should have one-fourth the intensity of the Glx peaks and should be barely detectable. The Asx peaks are doublets, and only about 30 per cent of Asx deuterons have been exchanged for protons, as compared with 50 per cent exchange for Glx.

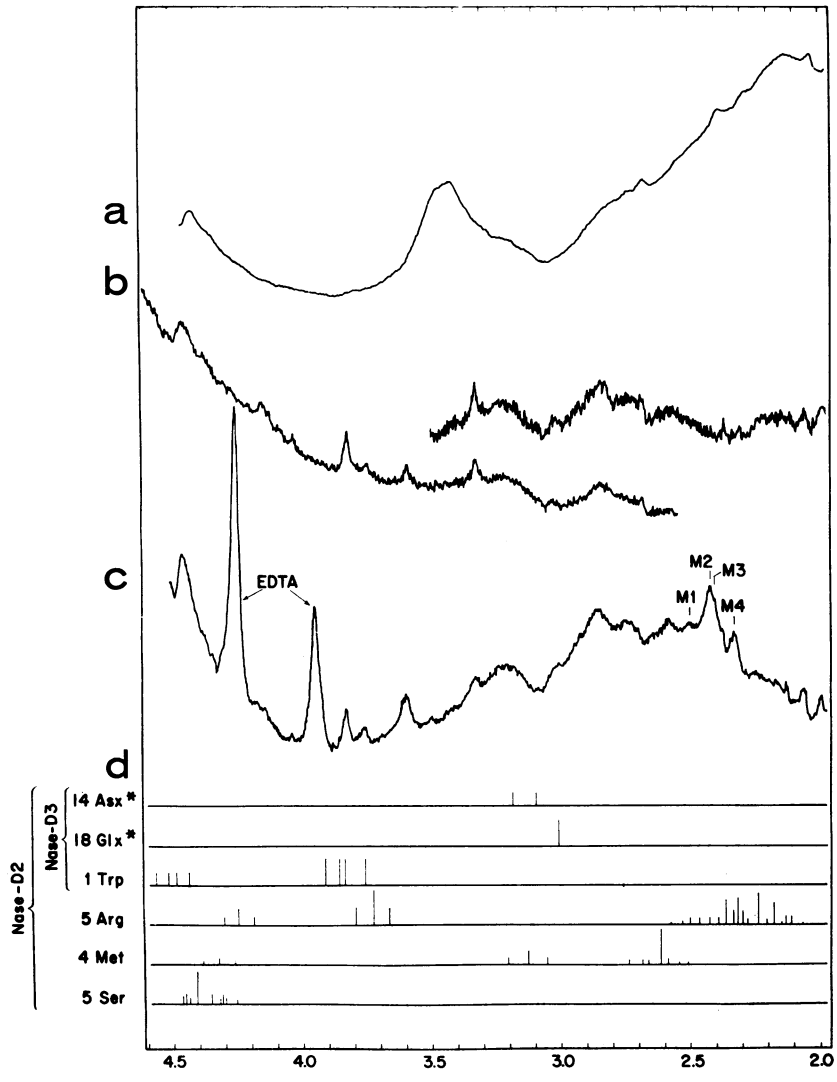

FIG. 2.—Comparison of a portion of the ^{1}H-NMR aliphatic spectral region of Nase, Nase-D3, Nase-D2, and of the aliphatic ^{1}H-amino acids which are present in these selectively-deuterated analogs. All solutions were at pH* 7.25.

(*a*) Nase; 135 scans. (*b*) Nase-D3; 198 scans. (*c*) Nase-D2; 118 scans; peaks assigned to the methyl groups of the four Met residues are labeled "M1-4." (*d*) Peak positions of aliphatic ^{1}H-amino acids present in Nase-D3 and Nase-D2.

Analog Nase-D2 (Fig. 2*c*) should contain the protons present in Nase-D3 plus protons from 5 Arg, 4 Met, and 5 Ser residues. As expected, there are no peaks in the spectra of Nase-D3 that are not also present in spectra of Nase-D2. The difference between the two spectra (Fig. 2*b* and *c*) should give the peaks corresponding to Arg, Met, and Ser. The only additional *single* resonances that are resolved, however, are in the region around δ_{TMS} 2.6. These peaks are marked

M1–M4 in Figure 2*c* and probably correspond to the methyl peaks of the four Met residues. They have intensities of three protons each and lie 0.1–0.3 ppm upfield from the methyl peak of free Met.

The Lys region of the spectrum of Nase-D4 at 100 and 220 MHz is shown in Figure 3. All EDTA has been removed from this sample of Nase-D4. At 220 MHz the γ-CH_2 peaks of Lys are resolved as a separate peak. However, even at 220 MHz no peaks corresponding to single Lys residues may be resolved. The difference between the spectra of analogs Nase-D4 and Nase-D3 should give resonances due only to Lys. Figure 4 shows the results of an attempt to decompose the envelope of the Lys-ϵ-CH_2 region of Nase-D2 at 100 MHz. The background spectrum for non-Lys resonances (Fig. 4*e*) is the corresponding region of the spectrum of Nase-D3. The gains of the two spectra were normalized by reference to an adjacent Glx peak common to both. The envelope of the

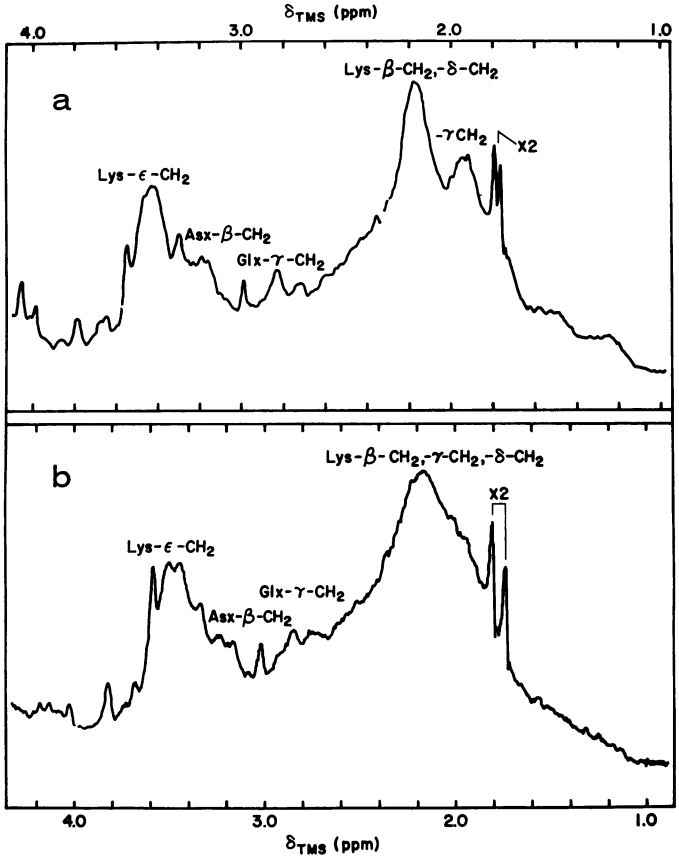

FIG. 3.—Comparison of a portion of the ^{1}H-NMR aliphatic spectral region of Nase-D4 (pH* 7.25) at 220 and 100 MHz.

(*a*) Nase-D4; 82, 50-sec scans at 220 MHz. (*b*) Nase-D4; 225, 250-sec scans at 100 MHz. A doublet originating from an unknown contaminant is marked $\times 2$.

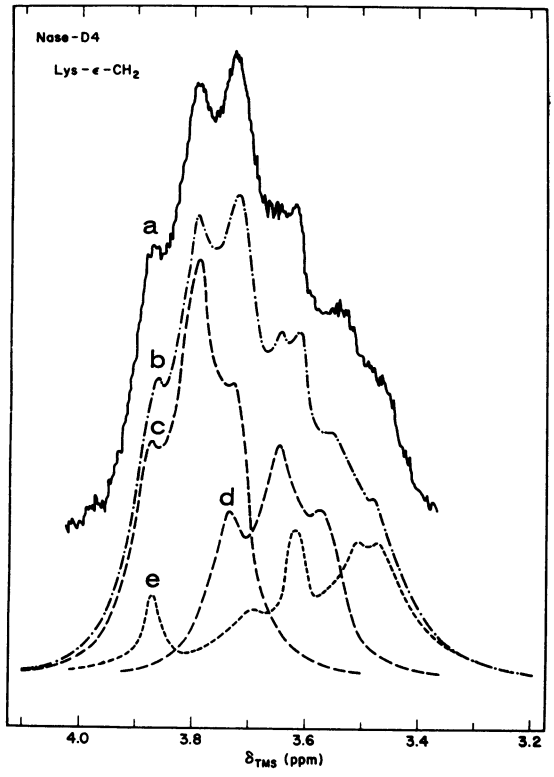

FIG. 4.—Decomposition of Lys-ε-CH_2 region of the ^{1}H-NMR spectrum of Nase-D4. This region may be represented by two classes of Lys: 14 unshifted Lys, and 9 Lys shifted 0.15 ppm upfield.

(*a*) Nase-D4, pH* 7.25; 225 scans at 100 MHz.

(*b*) Fitted spectrum: sum of curves (*c*), (*d*), and (*e*).

(*c*) Unshifted component of Lys-ε-CH_2 spectrum; area corresponds to 14 Lys residues.

(*d*) Shifted component of Lys-ε-CH_2 spectrum; area corresponds to 9 Lys residues.

(*e*) Background of non-Lys resonances; spectrum of Nase-D3 with gain normalized with that of curve (*a*).

experimental spectrum (Fig. 4*a*) may be approximated (Fig. 4*b*) by adding two assymetric triplets (Figs. 4*c* and *d*) to the baseline. The chemical shifts of the two triplets are 3.38 and 3.53 ppm, whereas, the chemical shift of the ε-CH_2 resonances of free Lys is 3.52 ppm at this pH (7.25). The splitting constants of the triplet at 3.53 ppm are equivalent to those of free Lys. The apparent increase in splitting of 1 Hz seen in the peak at 3.38 ppm may be a result of partial nonequivalence of the shifted peaks. The line widths of the two triplets (Fig. 4*c* and *d*) are both 8 Hz. The results of the curve fitting indicate that the Lys-ε-CH_2 region of Nase may be represented by two classes of Lys: 14 unshifted Lys, and 9 Lys shifted 0.15 ppm upfield. The spectra shown in Figures 3 and 4 do illustrate, however, an inherent limitation of the microbiological selective deuteration procedure. When a large number of chemically identical residues (23 Lys in this case) are present in the polypeptide chain, resolution remains incomplete. There are two possible procedures for further simplification of the spectra: (1) selective deuteration by chemical synthesis, or (2) preparation of analogs cleaved at one point in the polypeptide chain, separation of the N-terminal and C-terminal fragments and recombination of the fragments derived from different selectively deuterated analogs; this procedure has been used for the assignment of histidine 12 in pancreatic ribonuclease (8).

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