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The production and X-ray structure determination of perdeuterated *Staphylococcal* nuclease

Theresa R. Gamble [a,*], Karl R. Clauser [b], Anthony A. Kossiakoff [a]

[a] *Graduate Group in Biophysics, University of California, San Francisco, San Francisco, CA 94143-0448, USA*

[b] *Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA 94143-0446, USA*

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Abstract

Staphylococcal Nuclease (SNase) has been chosen as a model protein system to evaluate the improvement in neutron diffraction data quality using fully perdeuterated protein. Large quantities of the protein were expressed in *Escherichia coli* grown in medium containing deuterated amino acids and deuterated water (D_2O) and then purified. The mean perdeuteration level of the non-exchangable sites in the protein was found to be 96% by electrospray ionization mass spectrometry. The perdeuterated enzyme was crystallized and its X-ray structure determined. Crystals of perdeuterated SNase have been grown to 1.5 mm^3. Crystallization conditions, space group and cell parameters were found to be the same for both native and perdeuterated forms of the protein. Comparison of these two forms of SNase revealed no significant structural differences between them at the atomic resolution of 1.9 Å. Data collection using crystals of the perdeuterated protein is scheduled at the Brookhaven High Flux Beam Reactor.

Keywords: Protein perdeuteration; Neutron diffraction; *Staphylococcal* nuclease; Electrospray ionization mass spectrometry

1. Introduction

Neutrons have been successfully employed to study a broad range of biological problems inaccessible by other experimental techniques. When one assesses the literature it is clear that the neutron scattering applications to solution and semi-ordered systems are disproportionately represented when compared to crystallographic studies. This is even more striking considering the fact that neutron diffraction through its ability to locate hydrogen and deuterium atoms can provide a wealth of structural information that far surpasses that of its X-ray counterpart. The paucity of neutron diffraction structures stems from two principal experimental difficulties: (1) the inherently low fluxes that can be generated at the current reactor facilities; and (2) the incoherent scattering effects produced by hydrogens in the sample. The resulting background levels are so high that the dynamic range of the measured intensities is small, which greatly compromises the quality of the data.

To a degree, the flux problems are addressed by the development of new instrumentation and especially the use of sensitive two-dimensional detectors. Traditionally the magnitude of the background due to the hydrogen atoms has been addressed by exchanging all the waters of crystallization for D_2O and using extremely large crystals. However, there are practical limitations to reducing the backgrounds; even if all the waters of crystallization are exchanged, the nonexchangeable

* Corresponding author.

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hydrogens, which make up about 50% of the total atoms in the structure, remain. In addition, because of the buildup of surface deformations inherent in crystal growth, rarely can neutron sized crystals (>3 mm^3) be grown. Limiting neutron diffraction analyses to only those few systems where large crystals can be obtained, and conversely not being able to study systems where the placement of hydrogens could answer fundamental biological issues, presents a situation that is unacceptable.

In the absence of some new advance that can help circumvent these problems neutron diffraction will never reach its full potential. An approach being undertaken in this lab is to use a technique long employed by the neutron low angle scattering community, protein perdeuteration. The rationale is that the incoherent background scatter can be effectively eliminated by replacing biosynthetically all nonlabile hydrogens by deuteriums, which themselves do not have a significant incoherent term. We note, however, that the elimination of the incoherent scattering is very sensitive to the absolute degree to which the protein and the rest of the crystal (mother liquor, inhibitors, etc.) is perdeuterated. For instance, a fully perdeuterated system can offer as much as a 40-fold reduction in sample derived background, however, a 5% contamination of hydrogen into the crystal system reduces the improvement to only 12-fold. Thus the degree to which the backgrounds are actually reduced must wait for experimental verification and will vary between protein crystal systems.

Production of significant amounts of perdeuterated protein is possible because of the availability of robust *E. coli* expression systems that allow for high levels of protein expression in deuterated media. In the following report we describe the production of 96% perdeuterated *Staphylococcal* nuclease (SNase), its crystallization and a comparison to the diffraction data collected from the native material. SNase is a calcium dependent, extracellular enzyme consisting of a single polypeptide chain of 149 residues [1]. It is currently one of the most intensely studied proteins because of the interest in using it as a paradigm for studying structure-function issues in protein folding [2–4]. One particular research area that will be pursued in this study involves a number of unresolved issues concerning its mechanism of action; location of specific hydrogens in the catalytic site could provide crucial insight. Additionally, there is the possibility, given the diffraction quality of the crystals, that we will be able to observe most of the deuterium atoms in the well ordered regions of the protein.

Table 1
Growth media

Rich Media (25 ml) [a]	Algal Media (25 ml)
0.6 g yeast extract	0.25 g algal whole hydrolysate [b] or Celtone powder [c]
0.3 g tryptone	
0.06 g KH_2PO_4	0.06 g KH_2PO_4 [c]
0.3 g K_2HPO_4	0.3 g K_2HPO_4 [e]
0.1 ml glycerol	0.1 ml glycerol [d]
H_2O to 25 ml	D_2O to 25 ml

[a] Scaled down version of SB media (personal communication, Alan Meeker, The Johns Hopkins University School of Medicine, Baltimore, MD).
[b] Can be purchased in two forms: Algal whole hydrolysate-hydrogenated, Algal whole hydrolysate-deuterated (10% H_2O solution, 98 atom % D) (MSD Isotopes, Division of Merck Frost Canada Inc., Montreal, Canada).
[c] Can be purchased in two forms: Celtone-U (hydrogenated) Celtone-D (^{2}H, 97% +) (Martek Corporation, Columbia, MD).
[d] Can be purchased in a deuterated form: Glycerol-d_8 (98.7 atom% D) (MSD Isotopes, Division of Merck Frost Canada Inc., Montreal, Canada).
[e] Made up as stock solution in D_2O when appropriate.

2. Methods

2.1. Protein production

Escherichia coli strain AR120 containing a pAS1 plasmid was used for the overproduction of SNase [1]. Medium containing deuterated amino acids, deuterated glycerol, potassium phosphate and deuterated water (D_2O) was developed based on a recipe for rich medium (see Table 1). Deuterated amino acids and their hydrogen containing counterparts were purchased from two sources (see Table 1) and could be directly substituted for one another. *E. coli* was grown in rich medium for 12–18 h at 37°C to produce native SNase (personal communication, A.K. Meeker). The same bacteria were grown in deuterated medium, first for 24

[1] Obtained from A. Meeker and D. Shortle, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD.

h at 30°C, and then for another 24 h at 37°C to produce deuterated SNase. For both culture types, 250 ml of medium was inoculated with 1 ml of *E. coli* grown overnight in rich medium. Cell growth was characterized by cell density at OD_{600} using a Uvikon 860 (Research Instruments International, San Diego, CA).

2.2. Protein purification

The purification method used for both native and deuterated SNase was based on a previous SNase protocol [5] that was further developed by Shortle and Meeker (personal communication). Cells were harvested after the appropriate growth time and a cell pellet was collected by centrifugation ($3000 \times g$, 4°C, 10 min). The pellet was suspended in urea extraction buffer No. 1 (6 M urea/ 25 mM Tris-HCl/5 mM EDTA, pH 8.1) and gently swirled on a rocker box in the cold room for 20 min. The extracted cells were centrifuged at $3000 \times g$, 4°C for 10 min. The supernatant was poured off and discarded; the pellet is loose at this stage. The pellet was resuspended in urea extraction buffer No. 2 (same as above plus 200 mM NaCl) and gently rocked for 30 min, 4°C. Cells were centrifuged at $10\,000 \times g$, 4°C for 15 min and the pellet was discarded. An equal volume of ice-cold ethyl alcohol (200 proof) was added to the supernatant, and incubated undisturbed for 3–5 hours at −20°C. The precipitate that formed at this stage was collected by centrifugation at $10\,000 \times g$, 4°C for 15 min and discarded. An equal volume of ice-cold ethyl alcohol (200 proof) was again added and the entire mixture was incubated, undisturbed, at −20°C for 30 min. The resulting precipitate was collected by centrifugation at 2500 RCF, 4°C for 10 min. The supernatant was discarded and the pellet was drained briefly by inversion and then resuspended in 10 ml column load buffer (6M urea/1 mM EDTA/ 25 mM Tris-HCl/pH 8.1) by gently shaking the solution on a rocker box at 4°C overnight.

The protein, now contained in the suspension, was loaded onto a 1 ml Pharmacia Fast Flow S-Sepharose column equilibrated with column load buffer and then rinsed with 10 bed volumes of the same. Protein was eluted by slowly adding 3 bed volumes of column load buffer plus 70 mM NaCl, followed by 3 bed volumes of column load buffer plus 200 mM NaCl. The protein was precipitated by adding 1/20th volume of 1 M Tris-HCl, pH 7.0, and 3 volumes of ice-cold ethanol (200 proof) and incubated at −20°C for 20 min. The precipitate was collected by centrifugation at 2000 RCF, 4°C for 10 min. At this point the protein is a thick band on an 18% Tris-glycine, SDS PAGE gel (Novex, San Diego, CA). Reversed-phase (Vydac C18 Column, Alltech Associates Inc., Deerfield, IL) HPLC (Waters, division of Millipore, Milford, MA) (buffer A: H_2O, 0.1% trifluoroacetic acid, buffer B: Acetonitrile, 0.08% trifluoroacetic acid) was used to further purify the protein.

2.3. Mass spectrometry

The molecular weights of both native and deuterated SNase were measured by electrospray ionization mass spectrometry [6]. After the protein had been dialysed against the crystallization buffer (10.5 mM potassium phosphate, pH 8.15), the sample was desalted by dialysis, PD-10 Sephadex G-25M (Pharmacia, Piscataway, NJ) or by SEP-PAK® C18 cartridges (Waters, division of Millipore, Milford, MA) and concentrated under vacuum at 37°C (Savant SpeedVac Concentrator, Instruments, Inc. Framingdale, NY). Micromolar solutions (1–10 pmol/μl) of the protein in water/acetonitrile 50:50 (v/v) containing 0.5% acetic acid were directly infused at 1.5 μl/min into a Perkin-Elmer SCIEX API-III triple quadrupole mass spectrometer fitted with an Ionspray articulated nebulizer (SCIEX, Thornhill, Ontario, Canada). The mass spectrometer was operated with the Ionspray needle voltage at 4600 V, the interface plate at 650 V, and the orifice potential at 100 V. The third quadrupole was tuned and calibrated using a 10 pmol/μl solution of horse heart myoglobin (Sigma Chemical Co., St. Louis, MO). Mass spectra were recorded in multiple channel averaging mode, typically combining 5 scans. Data were collected every 0.1 mass units (m/z) with the quadrupole scanning from 600–1600 mass units in 20 s (2 ms dwell time).

2.4. Crystallization

Both native and deuterated SNase were crystallized as a complex with Ca^{2+} and the inhibitor thymidine-3′,5′-diphosphate (pdTp) under conditions similar to those previously described by Loll and Lattman [7]. For each batch of protein, hanging drop conditions were optimized by varying the amount of precipitant (methyl pentanediol (MPD)) in the reservoir. The per-

centage of MPD that corresponded closest to single, sharp-edged crystals was used in the reservoir for sitting drop crystallizations. Large crystals were grown by setting up sitting drops ranging from 50 to 200 μl. All crystallization experiments were done at 4°C. Before neutron diffraction data are collected, crystals will be soaked in mother liquor containing 10.5 mM potassium phosphate (pH 8.15), 0.6 mM $CaCl_2$, 1.2 mM citric acid, 0.4 mM pdTp, 60% deuterated MPD (2-methyl-2,4-pentane-d12-diol, 99.0 atom% D, Isotec Inc, Miamisburg, OH), and D_2O.

2.5. *X-ray structure determination*

X-ray diffraction data were collected to 1.9 Å on both native and deuterated SNase crystals using an Enraf Nonius 'FAST' area detector (Delft, The Netherlands) mounted on a Rigaku RU200 X-ray generator (Toyko, Japan) operated at 55 kV, 90 mA. In each case, data were collected on a single crystal at two separate orientations. The crystal-to-detector distance was set at 50 mm and the swing angle (the angle between the direct X-ray beam and the center of the detector) was positioned at 25° corresponding to a maximum resolution of 1.8 Å. Both crystals were oriented with the 4-fold axis along the rotation axis, but no attempt was made to align them precisely. 180° of data were collected at $\chi = 0°$ in increments of 0.1° exposed for 90 s each. An additional 50° of data were collected in the same manner at $\chi = 90°$. The data were processed using MADNES [8] and PROCOR [9]. All refinement was carried out with the program XPLOR [10]. Both R and free R [11] values were monitored during the refinement process. The starting model for refinement was the Loll and Lattman structure for inhibitor-bound SNase [7]. The water molecules were not included. Rigid body refinement was performed using the same model against both native and deuterated data (10 to 2.5 Å). Positional refinement was done incrementally (10 to 2.5 Å, 10 to 2.0 Å, 10 to 1.9 Å) until all data had been included. At this stage, anisotropic scaling factors were calculated and applied to the data. Positional refinement and restrained individual temperature factor refinement was performed in a step wise fashion, alternating with manual corrections using FRODO [12] against Fo–Fc and 2Fo–Fc maps. Fo–Fc maps were inspected for water molecules, which were then added appropriately.

3. Results and discussion

3.1. *Producing perdeuterated snase*

Using the same expression system and purification scheme, but different medium and growth conditions as used to produce native SNase, milligram quantities of highly perdeuterated SNase can be generated.

Three steps were taken to develop optimal conditions for cell growth and protein expression in a deuterated environment. First, cell growth and protein production of native SNase was verified in rich medium. The system's lead time was less than 3 h, lag phase was reached within 10 h, the final cell density (OD_{600}) was between 1 and 3, and protein yield ranged from 80 to 100 mg/ℓ. Second, an alternative source of amino acids, one that could be purchased in both hydrogen containing and deuterated forms, was substituted for yeast extract in the rich medium. All parameters for cell growth and protein yield in this altered, hydrogen containing medium were optimized in a small volume (25 ml medium) and then scaled up (250 ml medium). Cell growth experiments indicated a range for the percentage of the amino acid substitute in the medium which enhanced cell growth (0.2 to 0.4 g/25 ml medium); outside this range cell growth was inhibited (data not shown). Inhibited cell growth at low percentages of amino acid substitute is assumed to be the result of cell starvation. At high percentages of amino acid substitute, cell growth may be hindered by lowered pH (medium pH decreased with increasing amounts of amino acid substitute) or by the presence of small amounts of toxin in the amino acid substitute. Grown at 37°C for 18 h on a large scale, cells in the altered

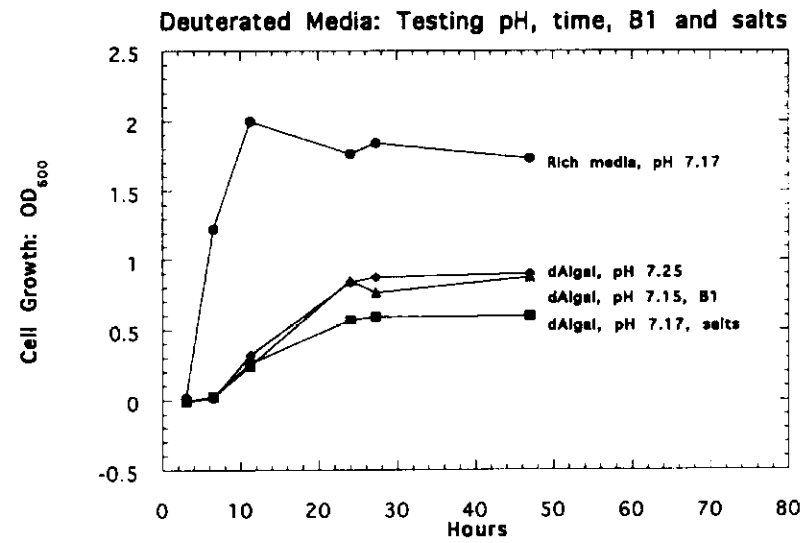


Fig. 1. Cell growth (OD_{600}) versus time. *E. coli* was grown at 37°C in both rich and deuterated media for 47 h.

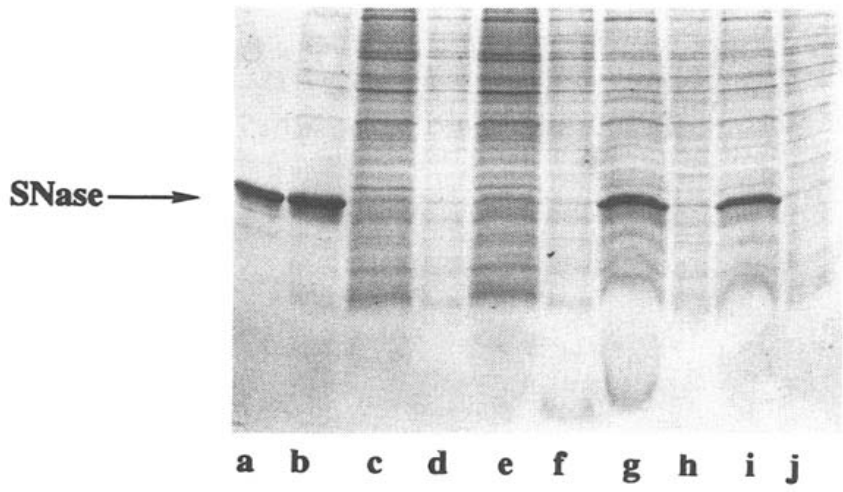


Fig. 2. SNase expression for *E. coli* grown for 24 h at 30°C and 37°C. The lanes shown are as follows: (a) native SNase standard; (b) cells used to inoculate culture, grown at 37°C in rich media; (c) cells grown in rich media, 30°C, 24 h; (d) cells grown in deuterated media, 30°C, 24 h; (g) cells grown in rich media, 37°C, 24 h; (h) cells grown in deuterated media, 37°C, 24 h. Lanes (e), (f), (i), and (j) are identical to (c), (d), (g), and (h) respectively, with the exception that excess ampicillin was added to the medium.

hydrogen containing medium grew to a final cell density which was typically 40% less than cells grown in rich medium; protein yield was decreased by the same amount.

In the final optimization step, all components of the altered, hydrogen containing medium were replaced with deuterated counterparts. Cells grown overnight at 37° in media containing varying amounts of amino acid substitute grew very poorly (final cell density was typically 90% less than cells grown in rich medium); however, the range for optimal growth was comparable to using hydrogen containing amino acids (0.2 to 0.3 g/25 ml medium). In order to improve cell growth, the deuterated medium was adjusted from pH 6 to pH 7 (equivalent to rich medium) and grown at 37°C, for 47 hours; final cell density (OD_{600}) was 50% of cells grown in rich medium (Fig. 1), lead phase was increased by 6 to 11 h, and it took at least 24 h to reach lag phase. The addition of salts ($MgSO_4$ and $FeCl_2$) and vitamins (B1) to the deuterated medium did not alter cell growth significantly (Fig. 1). This improvement in cell growth made by the pH adjustment, however, did not correlate with protein production. Cells grown under these conditions did not express SNase (Fig. 2). In order to address this problem we took advantage of the fact that the pAS1 plasmid used in this expression system contains a temperature sensitive lambda repressor (14); protein production can be suppressed at 30°C and induced at 37°C. Using this inherent characteristic of the expression system, cells were grown up in deuterated medium to lag phase (24 h) at 30°C and then incubated at 37°C for another 24 h. Final cell density (1 to 3 OD) and protein yield (80 mg/ℓ) were comparable to cells grown in rich medium. The effect of deuterium and deuterated nutrients in slowing down cell growth and altering expression protocol is not unique to this project; deuterium isotope effects have been shown to affect both cell lag and log phase, as well as cell division itself [13,14]

3.2. *Protein purification and characterization*

Purification of perdeuterated and native SNase was always done in tandem, the native preparation acting as an internal control for the purification process. As documented in Fig. 3, the perdeuterated protein behaved identically to the native protein at each step in the purification process. Reversed-phase HPLC, phenyl

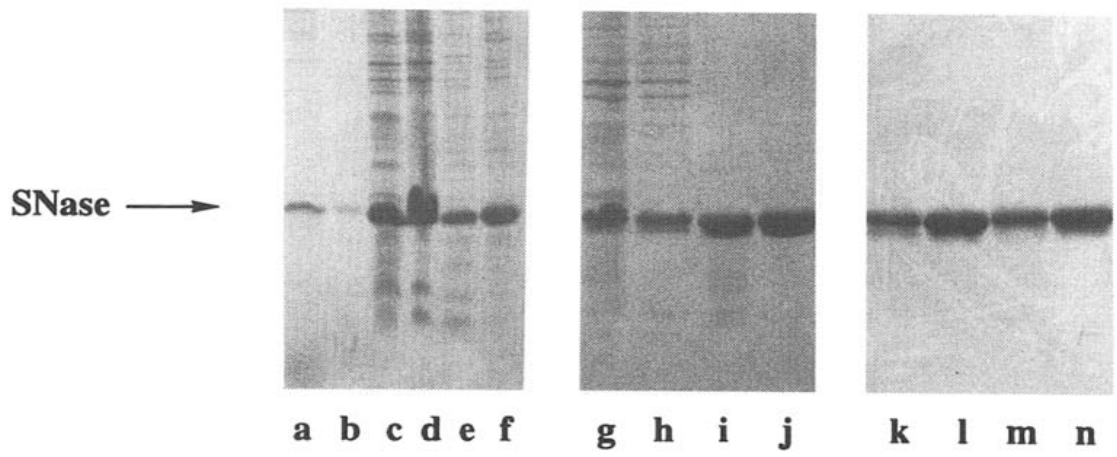


Fig. 3. Parallel purification of native and deuterated SNase. The lanes shown are as follows: (a) native SNase, cell supernatant after harvest, (c) native SNase, Urea extraction No. 1, pellet, (e) native SNase, Urea extraction No. 1, supernatant, (g) native SNase, Urea extraction No. 2, (i) native SNase, loaded onto S-sepharose column, (k) native SNase, eluted off S-sepherose with column load buffer plus 70 mM NaCl, (m) native SNase, eluted off S-sepherose with column load buffer plus 140 mM NaCl. Lanes (b), (d), (f), (h), (j), (l) and (n) are deuterated SNase under the identical conditions described above.

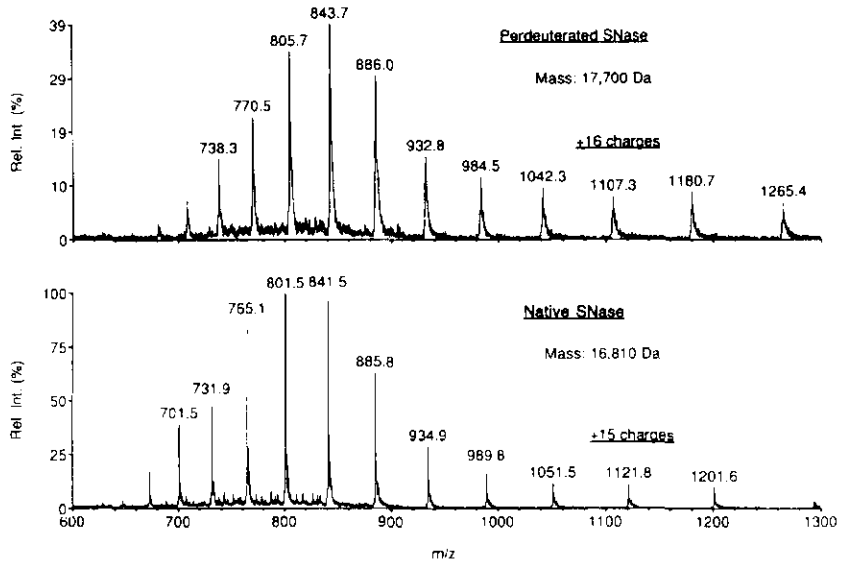
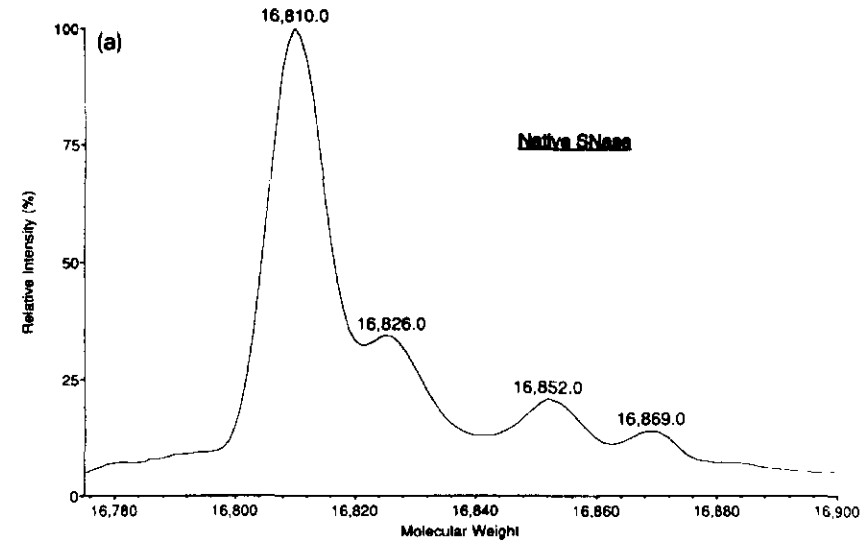


Fig. 4. Electrospray ionization mass spectra of perdeuterated and native SNase. Since the mass spectrometer measures mass-to-charge ratio (m/z), the labeled series of peaks in each spectrum represent a single intact protein species, but with a range of charged states ($+14$ to $+26$). The different charged states arise from multiple protons binding to the protein from the acidic sample solution infused into the ion source of the mass spectrometer. Since members of a charged state series must increment in an integral fashion, the intact mass is easily deduced by a mathematical algorithm relating the series members.

superose, and hydroxyapatite [7] were all tried for further protein purification. In terms of final protein yield, reversed-phase HPLC was the most successful final purification step.

In the purified protein, not all hydrogen atoms will be substituted by deuterium. SNase contains a total of 1205 hydrogen sites. These sites can be classified depending on their relative exchangeability in solvent: 921 (76.4%), are non-exchangeable, bound to carbon in amino-acid side chains or the alpha-carbon of the peptide backbone; 284 (23.6%) are exchangeable, bound to amino, carboxyl, hydroxyl groups and to amide groups in the side chains of asparagine and glutamine or the peptide backbone [15]. Since both perdeuterated and native SNase were purified in aqueous hydrogen containing solvents, some of which were denaturing, only non-exhangeable sites of perdeuterated SNase can be expected to remain deuterated.

Electrospray ionization mass spectrometry was used to determine the level of perdeuteration in the protein (see Fig. 4). As shown in Fig. 5a, the measured mass of native SNase is 16 810 daltons, matching the theoretical value. The measured mass of perdeuterated SNase is 17 700 dalton (Fig. 5b), an increase of 890 dalton. If this mass increase is assumed to be a result of deuterium incorporated at non-exchangeable sites only, then an average of 96% of the non-exchangeable



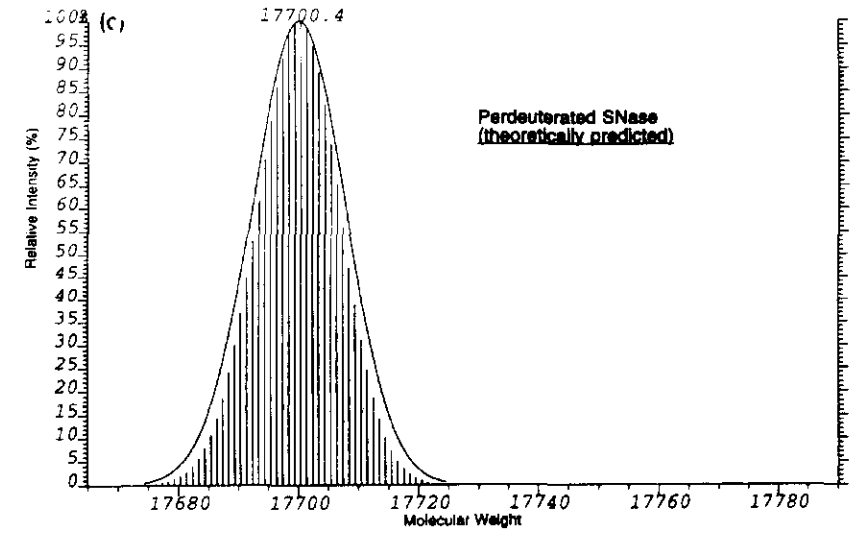


Fig. 5. Electrospray ionization mass spectra. Deconvolutions of the original mass spectra, containing multiply charged states, demonstrating intact species typically present in (a) native SNase and (b) perdeuterated SNase. Peaks corresponding to mass increases of 16 and 42 dalton are consistent with partial methionine oxidation and N-terminal acetylation respectively. A theoretically predicted spectrum for perdeuterated SNase, (c) constructed by presupposing a uniform deuterium relative abundance of 96% at all non-exchangeable sites, and the same instrument resolving power as that used for the experimentally obtained spectra.

sites in perdeuterated SNase have been replaced with deuterium. Typical preparations of perdeuterated SNase yielded >90% deuterium replacement. The upper limit to the level of perdeuteration obtainable is set by the amino acid substitutes, which are generally rated at 97–98% deuterium incorporation (based on initial isotope sources not amino acid products).

The differences in peak width seen between the actual mass spectrum of the perdeuterated SNase and its theoretically predicted spectrum (see Fig. 5c) suggest that the distribution of deuterium among non-exchangeable hydrogen sites is non-uniform. Mass spectral peak width is dependent upon both the resolving power of the instrument and the isotopic distribution of masses present in a sample. The theoretical prediction of the peak width, using the OPUS software package (VG Instruments, Manchester, UK), for perdeuterated SNase is subject to two assumptions: a uniform deuterium relative abundance of 96% at all non-exchangeable sites, and the same instrument resolving power as that used for the experimentally obtained spectra. The peak width of a theoretically predicted spectrum of the native SNase matches the experimentally obtained spectrum (data not shown), confirming the actual instrument resolving power and accounting for naturally occurring isotopic abundances. The actual peak width obtained for perdeuterated SNase is clearly wider than theoretically predicted. In order to create a wider peak without altering the mass, the deuterium distribution must be such that some non-exchangeable sites contain less than 96% deuterium while others contain more. LeMaster and Richards found, when examining isotopically labelled amino acids from protein hydrolysates, that certain non-exchangeable proton sites had reduced isotopic enrichment [16].

3.3. Crystallization

The two main goals in terms of crystallization were to: 1) determine the crystallization conditions for perdeuterated SNase, and 2) to optimize conditions so that large neutron sized crystals could be grown. The same basic protocol resulted in both native and deuterated SNase crystals, although the precise crystallization conditions needed to be optimized independently for each protein preparation. The optimal amount of precipitant (MPD) in the reservoir varied between 20 and 30%. For hanging drops (5–10 μl protein/calcium/inhibitor/precipitant solution), crystals appeared after 4–5 days and grew for up to two weeks. The morphology of the crystals is identical between native and deuterated SNase; the crystals are elongated along one axis and grow rapidly in this direction, but not the other two. (see Fig. 6a and 6b). Crystal shape varied with increasing reservoir precipitant concentration; the edges of the crystals become less angular and more rounded, the overall shape less symmetric and more irregular. Using native SNase, the optimized hanging drop conditions were applied to 50 μl sitting drops in order to grow large crystals. Crystals appeared within two weeks and continued to grow for two weeks, ultimately reaching a size of 0.5 to 1.0 mm^3. Seeding and pulsing techniques were tried to increase the size of these crystals, but both of these methods were unsuccessful because they caused excessive nucleation both in the drop and on the existing crystals. This multitude of nucleation sites is a function of the high concentration of protein necessary to grow these crystals (20 mg/ml); any disturbance to the equilibrium of the drop causes crystal formation. The best results were obtained by using large sitting drops (200 μl); crystals took at least 3 weeks to appear and continued to grow for another month. The largest native crystal was 9.5 mm^3, the typical range of the larger crystals being 1 to 3 mm^3. For the deuterated protein, the largest crystal grown from a 200 μl sitting drop was 1.5 mm^3, the range for the larger crystals being 0.7 to 1.0 mm^3. We do not believe that the observed difference in crystal size between native and deuterated SNase is caused by any property inherent to the protein itself. Crystal size appears to be linked to the number of crystals that form in any given sitting drop which is itself a function of the percentage of MPD in the reservoir and the number of nucleation sites in the drop. Because these parameters vary with each batch of protein and are impossible to control precisely, it is a matter of chance that singular, and thus larger, crystals did not grow from the deuterated protein.

3.4. X-ray structure determination

Native and deuterated SNase crystals grow in the same space group and have identical unit cell dimensions (see Table 2); this is consistent with the parameters reported by of Loll and Lattman for native,

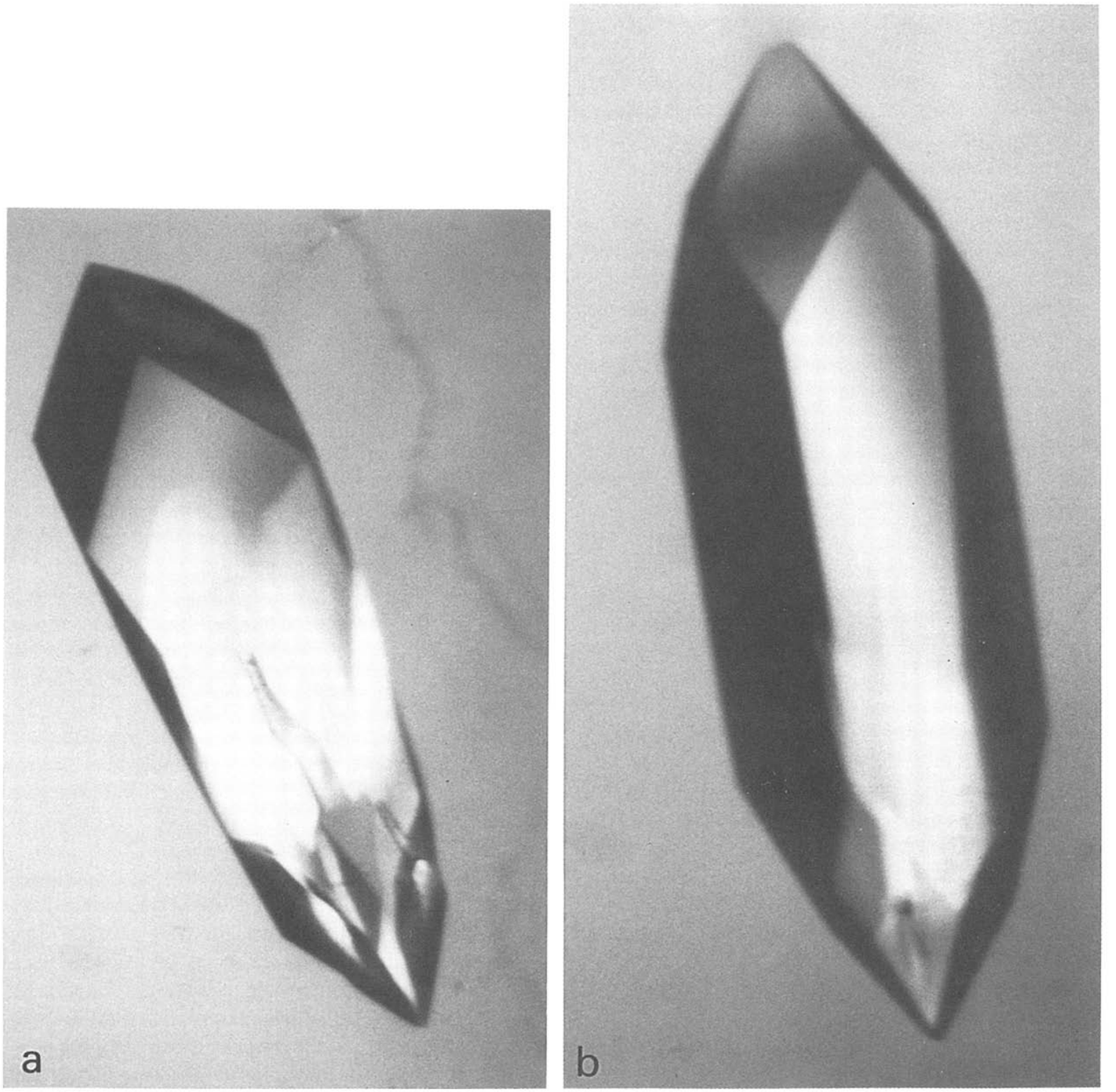


Fig. 6. SNase crystals. Typical morphology of protein crystals grown using (a) native and (b) deuterated SNase. At the time of the photo, the native crystal was 6.25 mm^3, and the deuterated crystal was 1.27 mm^3.

inhibited SNase [4]. The R_{merge} for the two orientations of each crystal, total number of observations, and total number of reflections indicate that each crystal type behaved similarly during data collection and processing. The completeness to 1.9 Å resolution of each data set is better than 95%, with completeness greater than 90% in the highest resolution shell. The two data sets are very similar as evidenced by an R_{scale} between them of 4.05%. No sigma cutoff was applied to either set of data, thus all reflections were used in refinement. Rigid body refinement yielded similar R values using the same starting model for both data sets. Anisotropic scale factors were comparable for the two data sets and lowered the R factor by 2% in each case. From Table

Table 2
Data collection and refinement statistics

Parameter	native SNase	deuterated SNase
space group	$P4_1$	$P4_1$
cell parameters (Å)	$a=b=48.39$, $c=63.45$	$a=b=48.38$, $c=63.41$
R_{merge} (%) [a]	5.06	5.55
total No. of observations	36686	44428
total number of reflections	11071	12976
completeness (%)	95.4	96.6
resolution	15–1.9 Å	15–1.9 Å
R_{merge} between 1.97 and 1.9 Å (%)	18.7	16.2
completeness at highest resolution (%) (1.97–1.9 Å)	90.2	93.8
$<I/\mathrm{sig}I>$ between 1.97 and 1.9 Å	3.22	3.45
rigid body R value (%)	24.4	24.9
anisotropic scale factors		
native SNase	$B11=-2.53$, $B22=-2.53$, $B33=5.05$	
deuterated SNase	$B11=-2.96$, $B22=-2.96$, $B33=5.91$	
both cases	$B12=B13=B23=0.00$	
final R value (%)	19.0	19.3
total No. of water molecules	88	89

[a] $R_{merge}=\Sigma_i\Sigma_j|<I_i>-I_{ij}|/\Sigma_i\Sigma_j I_{ij}$, R_{merge} and R_{scale} (see text) are calculated in the same manner.

2 it can be seen that the crystals diffracted better in the direction of the 4-fold rather than perpendicular to it. After refinement with 10 to 1.9 Å data, the model converged at an R value of 24.6% for the native data and 24.3% for the deuterated data, water molecules were added (57 to the native structure, 65 to the deuterated structure). This first round of water addition, together with positional and individual temperature factor refinement improved the R values by 4.3% in each case. In two more rounds, waters were added to both structures giving a total of 88 water molecules for the native structure and 89 water molecules for the deuterated structure. The final R value for the native structure was 19.0%, and for the deuterated structure, 19.3% (see Table 2). The most significant peaks found in a difference map comparing native and deuterated data and using the final native coordinates for the phase information were only 0.11 $e/Å^3$. This difference map is another indicator that perdeuteration did not significantly alter the structure or diffraction characteristics of the protein.

Comparison of the final native and deuterated backbone atoms (Cα,C,N) along with the calcium atom and the inhibitor yields an RMS deviation of 0.08 Å. The largest concerted differences between backbone atoms are found at residues 45 to 51 (0.13 to 0.21 Å). These residues constitute a flexible loop near the active site which previous investigators have found to be partially disordered [4,14]. This flexibility is reflected in temperature factors which are higher than average. The only other differences between the two structures are observed for several of the lysine residues and for Ser141. The largest differences between side chain atoms are also found at these lysine sites, which are located at the surface of the molecule, are exposed to solvent, and are therefore inherently flexible. Ser141 is the last residue of the C terminus to be seen in the electron density; residues 142–149 are presumed to be disordered. All the water molecules except 5 have corresponding mates in the native and deuterated structures; no water with a temperature factor less than 30 $Å^2$ differs from its mate by more than 0.3 Å. Comparison of the two final structures of native and deuterated SNase reveals no conformational alterations caused by the perdeuteration method itself.

4. Conclusion

The purpose of this work has been twofold: (1) to demonstrate that highly perdeuterated protein can be readily produced using established cell lines and protocols and (2) to show that perdeuteration itself does not conformationally alter the protein under study.

Because deuterium isotope effects are known to alter cell division and enzyme function, cell growth and protein expression in a deuterated environment are not expected to be directly comparable to native conditions. The *E. coli* system used in this study was particularly sensitive to the parameters of time and pH; cells in deuterated media took longer to grow than the same cells in hydrogen containing medium and a lowered pH shown to be tolerable to cells in hydrogen containing medium, hindered growth and expression significantly in deuterated medium. Inherent features of the cell line were utilized to overcome observed isotope affects such that cell growth and protein expression were comparable to cells grown in rich medium. Taking into account the ways that deuterium can affect cell behavior, we feel that other cell lines could be optimized to express highly perdeuterated protein.

The expected decrease in background in neutron diffraction data depends directly on the level of protein perdeuteration. It was therefore essential to find a technique that could quantitatively assess the incorporation of deuterium at the hydrogen atom sites. Electrospray ionization mass spectrometry proved to be an excellent candidate for this analysis – the technique requires no special treatment to the protein other than the removal of salts, is accurate to within one or two daltons for proteins of this size, and is becoming more accessible to the scientific community at large. The mass spectrometry data demonstrated that incorporation of deuterium at the non-exchangable hydrogen atom sites can consistently reach 96%, a percentage which approaches the theoretical limit imposed by the deuterated nutrients contained in the medium.

Most importantly, we have shown that perdeuteration itself does not alter the structural features of the protein. The native and deuterated versions of SNase behaved similarly throughout the purification procedure and crystallized under the same experimental conditions. X-ray diffraction was used to compare the diffraction characteristics and structure of the native and deuterated protein, first by direct comparison of the diffraction data, then by analysis of difference Fourier maps made between the two data sets, and lastly by independent refinement and comparison of the two final structures. All of these comparisons confirm that there are no significant structural differences between native and perdeuterated SNase.

Until neutron diffraction data have been collected and analyzed, we cannot quantitatively assess the extent to which protein perdeuteration can enhance the technique. However, there is good reason for optimism given the level of deuterium incorporation, crystal size and quality, and the unaltered state of the perdeuterated protein demonstrated by this work. We hope that protein perdeuteration will enable a much broader range of structural problems to be studied by neutron diffraction than has been possible in the past.

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