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Studies of Ribonuclease-A by X-ray and Neutron Diffraction

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Abstract

The structure of monoclinic bovine ribonuclease-A [$P2_1$, $a = 30{\cdot}18$ (12), $b = 38{\cdot}4$ (10), $c = 53{\cdot}32$ (15) Å, $\beta = 105{\cdot}85$ (8)°] has been studied by a combination of X-ray and neutron diffraction techniques. A model based on X-ray data to 2·5 Å resolution has been refined by restrained least squares, with a final $R = 25{\cdot}2\%$ for 951 non-hydrogen atoms and one phosphate molecule. Partially refined coordinates of ribonuclease-S were initially used. A good fit between the model and difference Fourier maps was obtained. Several maps based on neutron diffraction intensities at 2·8 Å resolution and various phasing schemes were calculated and they are discussed in terms of their usefulness in further refinement of the structure.

Introduction

Bovine pancreatic ribonuclease is one of the enzymes whose structure has been extensively studied by a variety of physicochemical techniques. Both the native enzyme and its modification, ribonuclease-S (product of cleavage by subtilisin of a peptide bond between residues 20 and 21), have been studied by X-ray diffraction techniques. The structure of monoclinic ribonuclease-A has been reported at 5·5 Å resolution by Avey, Boles, Carlisle, Evans, Morris, Palmer, Woolhouse & Shall (1967) and later at 2·5 Å by Carlisle, Palmer, Mazumdar, Gorinsky & Yeates (1974). The structure has been independently solved at 2 Å by Kartha, Bello & Harker (1967). The structure of trigonal ribonuclease-S has been reported by

Wyckoff, Tsernoglou, Hanson, Knox, Lee & Richards (1970), followed by detailed studies of the interaction of the enzyme with inhibitors and covalent modifiers (Allewell, Mitsui & Wyckoff, 1973; Wodak, Liu & Wyckoff, 1977). Studies of the monoclinic crystals of ribonuclease-S at 4 Å resolution have been reported by Torii, Urata, Iitaka, Sawada & Mitsui (1978), and recently both ribonuclease-A and ribonuclease-S have been crystallized in an isomorphous (trigonal) form by Martin, Petsko & Tsernoglou (1976). Low-temperature work on monoclinic ribonuclease-A is in progress (D. Tsernoglou, personal communication).

The wealth of structural information available for ribonuclease makes this enzyme a good candidate for neutron diffraction analysis. This technique is complementary to X-ray diffraction and can yield information not readily obtainable by other means (Schoenborn & Nunes, 1972; Bentley, Duee, Mason & Nunes, 1980). This includes the possibility of differentiating N from C or O atoms, thus fixing the orientation of histidines 12 and 119, necessary for understanding the details of the catalytic mechanism. It should also be possible to distinguish exchangeable and nonexchangeable hydrogens, thus complementing the kinetic hydrogen-exchange studies of Schreier & Baldwin (1976). Hydrogen bonds should be observed directly rather than only inferred from the positions of other atoms. Solvent structure should be much more visible than with the X-ray methods.

This communication will describe the steps preparatory to the neutron diffraction analysis of ribonuclease; in particular, the refinement of the model based on X-ray intensities and interpretation of preliminary neutron data. These studies are still under way and the results will be reported fully at a later data.

Experimental

Conditions for the growth of large crystals of ribonuclease-A were found by J. Norvell (1976, unpublished). Lyophilized protein (Sigma type II-A) was dissolved in distilled water at a concentration of 50 mg ml^{-1} and the pH was adjusted to 5·3 by the addition of a 0·3 *M* solution of sodium hydroxide. 5 ml of solution were placed in each scintillation vial and *tert*-butyl alcohol was slowly added to the final concentration of 43%. The vials were left undisturbed on a vibration-free surface for a period of up to six months. Crystals would start growing within several days and reach their maximum size after about a month, but on occasion they would start growing only after several months, yielding the best crystals. The volume of the largest crystal was about 100 mm^3, and a number of crystals between 25 and 35 mm^3 were found. Crystals grew as thick plates, with the shortest dimension along the a^* axis never exceeding 1·5 mm. Unit-cell parameters for deuterated crystals were $a = 30{\cdot}18\ (12)$, $b = 38{\cdot}4\ (10)$, $c = 53{\cdot}32\ (15)$ Å, $\beta = 105{\cdot}85\ (8)°$, similar to parameters reported by other investigators.

Crystals used for both X-ray and neutron studies were transferred in stages to synthetic mother liquor containing 55% of fully deuterated *tert*-butyl alcohol (d_{10}, Aldrich), adjusted to pH = 5·3 with sodium deuteroxide. This mother liquor was essentially hydrogen-free. The soaking time was about 6 months with several complete changes of solvent.

A new method for mounting crystals for neutron investigation was necessitated by their large size (up to 6 × 5 × 1 mm for crystals actually used) and by the requirement that the shortest unit-cell axis be parallel to the φ axis of the diffractometer. This was accomplished (Fig. 1) by making flat-bottomed quartz tubes of suitable dimensions and mounting the crystals in the mother liquor, immobilized by quartz wool. Tubes were closed by a layer of silicone grease and sealed with dental wax, with all air removed. No crystal movement was ever noticed and the aging process was not detected during two years of experiments.

Crystal fragments used in X-ray studies were usually cut from larger crystals and were mounted in glass capillaries in the usual way. X-ray intensities to 2·5 Å resolution were measured on a Picker FACS-1 diffractometer using the ω step-scan and measuring individual backgrounds for each reflection. Two complete data sets were measured, each on one crystal. Absorption corrections were applied by the method of North, Phillips & Matthews (1968), modified by Santoro & Wlodawer (1980). The data were merged, yielding $R = 2{\cdot}7\%$ ($R = \sum |F_1 - F_2| / \sum \bar{F}$).

Neutron diffraction data were collected using the flat-cone diffractometer at the National Bureau of Standards reactor (Prince, Wlodawer & Santoro,

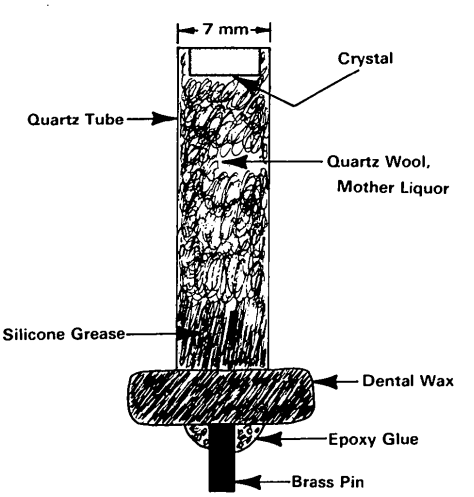

Fig. 1. Diagram of a method used to mount ribonuclease crystals for neutron diffraction studies. The crystal was completely immobilized and immersed in synthetic mother liquor, with the longest reciprocal axis a^* parallel to the axis of the quartz tube.

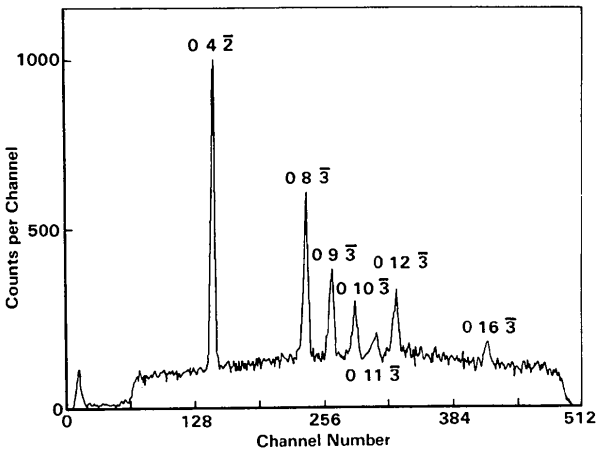

Fig. 2. Example of a frame of output of the linear detector in flat-cone geometry. Counting time 120 s; the beam-stop obscures the lower channels. Seven reflections are clearly visible. The high background is due to scattering by H atoms. Resolution for channel 490 is 2·0 Å.

1978). The neutrons were monochromatized by reflection from a 50 × 100 mm graphite crystal of mosaic spread 40′ and the wavelength was 1·68 Å. Neutron flux on the sample, measured with a calibrated fission monitor, was 6×10^4 neutrons mm^{-2} s^{-1}.

Data to 2·8 Å resolution were collected using the diffractometer in equatorial geometry, neglecting intensities falling outside the equatorial plane of the instrument. Each reflection was measured by an ω scan 1·6° wide in 64 steps, with 10 steps on each side used as background. Reflections to 3·2 Å resolution were each counted for 10 min, and to 2·8 Å for 20 min. Two standard reflections were monitored with no change in intensity throughout data collection. A substantial number of Friedel pairs were measured yielding R = 4·4% upon merging. Data were corrected for absorption, but the influence of neutrons with half wavelengths was neglected. The number of observed reflections ($F > 2\sigma$) was 2773, 87% of all reflections at 2·8 Å resolution.

Another data set to 2 Å resolution was collected with the diffractometer operated in flat-cone geometry (Prince *et al.*, 1978). Each layer of reflections was collected by rotation of the crystal around the a axis, in order to limit the number of necessary scans by measuring the most densely populated planes. A complete 360° scan in ψ increments of 0·05° took 60 h. Each general reflection was measured twice and special reflections four times. An example of a frame of the detector output is shown in Fig. 2. These data are still being processed and will be used for future extension of the resolution of the neutron studies.

Refinement of the X-ray structure

The success of neutron diffraction analysis is to a large extent dependent on the quality of the starting model. Since no refined structure of ribonuclease-A was available, it was first necessary to refine a set of atomic coordinates based on X-ray structure factors. Unpublished coordinates and structure factors described by Carlisle *et al.* (1974) were provided by H. Carlisle (personal communication). In addition, unpublished main-chain coordinates and structure factors were provided by G. Kartha, and partially refined coordinates of ribonuclease-S (Powers, 1976) by H. W. Wyckoff.

Although the crystals of ribonuclease-A reported by Carlisle *et al.* (1974), Kartha *et al.* (1967) and in this communication have almost identical unit-cell parameters, comparison of structure amplitudes at 2·5 Å resolution indicates some definite differences. These may be due to the influence of the different solvents used in the crystallizations, namely 50% ethanol, 55% 2-methyl-2,4-pentanediol and 43% *tert*-butyl alcohol respectively, and possibly by changes caused by deuteration of the crystals. Structure-amplitude scaling to Kartha's data yielded R = 12·7%, and to Carlisle's, R = 10·3%. The latter two data sets could be scaled together with R = 12·1%. These numbers are over twice as high as those observed in scaling data collected in this study from two crystals, suggesting that the differences are real. It should be pointed out, though, that the means of data collection differed in all of these investigations, with a linear diffractometer used by Carlisle *et al.* (1974), a manual diffractometer with stationary crystal–stationary counter by Kartha *et al.* (1967) and a four-circle diffractometer in this study. More recent data collected at Birkbeck College by four-circle diffractometry (Daud, personal communication) agreed better with ours (R = 7·8%), but the difference is still much larger than might be expected simply from experimental error.

The restrained least-squares conjugate-gradient procedure of Hendrickson & Konnert (1980), as adapted for a VAX11/780 minicomputer by A. Arnone (personal communication) and this author, was used. One cycle of refinement for 4061 intensities observed between 10 and 2·5 Å and 951 atoms would take an average of 30 min with a total of 24 h of computer time necessary for the complete refinement process. Initially, several cycles of refinement of the coordinates provided by Carlisle caused a drop in the R factor from 45 to only 42% at 3 Å resolution. Since this approach was shown to be unpromising, an alternative one was attempted. Ribonuclease-S coordinates were rotated into the ribonuclease-A unit cell using a program of Hendrickson (1979), by minimizing the distances between C_α positions, using Carlisle's coordinates as guide-posts. Since the C_α coordinates differed by as much as 15 Å for the residues adjacent to the area of proteolytic cleavage in ribonuclease-S, coordinates for residues 17–23 were taken directly from ribonuclease-A. The progress of refinement of a coordinate data set

prepared in this manner is shown in Fig. 3. Thirteen cycles of refinement of 3 Å data, followed by four cycles at 2·5 Å resolution lowered the R factor to 30·9%, with the root-mean-square (r.m.s.) deviation of bond lengths kept at 0·055 Å. Since the refinement would not continue from this point without manual intervention, rebuilding of the model was necessary. This was accomplished using computer graphics at the National Institutes of Health Computer Center. The program *BILDER*, written by R. Diamond and implemented at NIH by G. Cohen, was used to fit the coordinates to maps calculated using as coefficients either $(2F_o - F_c)$ or $(F_o - F_c)$, with about 8% of the atoms removed from phasing in each map in the latter case. It was necessary to make a number of corrections to the main chain, invert several peptide bonds by 180°, and to adjust many side chains. The process of rebuilding was followed by several more cycles of refinement and the procedure was repeated five times, with only the worst fitting areas adjusted in the first three cycles. An overall temperature factor of 6 Å^2 was applied throughout the refinement and no attempt to fit solvent molecules was made, even though a number of peaks due to water and/or butyl alcohol persisted in difference maps. Only a very obvious peak due to phosphate (Kartha *et al.*, 1967) was fitted at the final stages of the refinement.

After 48 cycles of refinement and five of manual chain adjustments the protein model is much improved. The R factor was lowered to 25·2% and the r.m.s. deviation of bond lengths from ideality is 0·042 Å. The range of R factors varies between 20·0% for the shell between 4 and 5 Å to 32·9% for 5–10 Å and 28·0% for 2·65–2·85 Å. Only six amino acid side chains (out of 124) did not have sufficient density in $(F_o - F_c)$ maps calculated after their contribution to phasing was removed. Of the six, four are lysines (residues 37, 41, 91, 98), one is arginine (residue 39) and one asparagine (residue 94). It was not inconceivable that these residues may be disordered in the structure and that the lack of density is not an artifact of refinement. Another 30 side chains have minor breaks in density in maps contoured at 25% of the maximum, but could be unambiguously traced, while 88 side chains fit their respective densities very well. Fig. 4 shows a stereo tracing of C_α positions of the final model. All four proline residues are very well defined in the difference Fourier maps. Residues 42 and 117 are in *trans* configuration and residues 93 and 114 in *cis*, in agreement with Wyckoff *et al.* (1970). It should be pointed out that these configurations were the targets of the refinement procedure, and so some bias is possible, but a complete lack of densities which would permit alternative configurations make this result rather convincing.

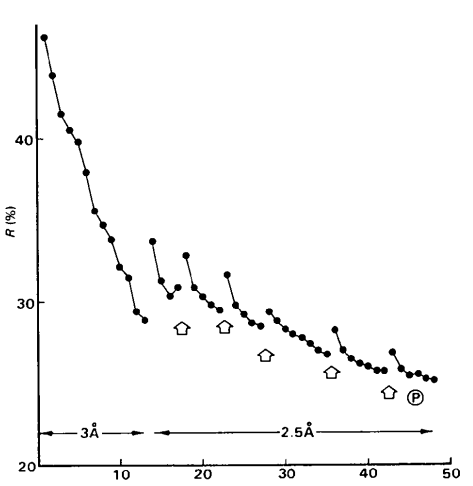

Fig. 3. Progress of the refinement. A model was rebuilt using computer graphics after the refinement cycles marked with open arrows. A phosphate molecule was added as marked by (P).

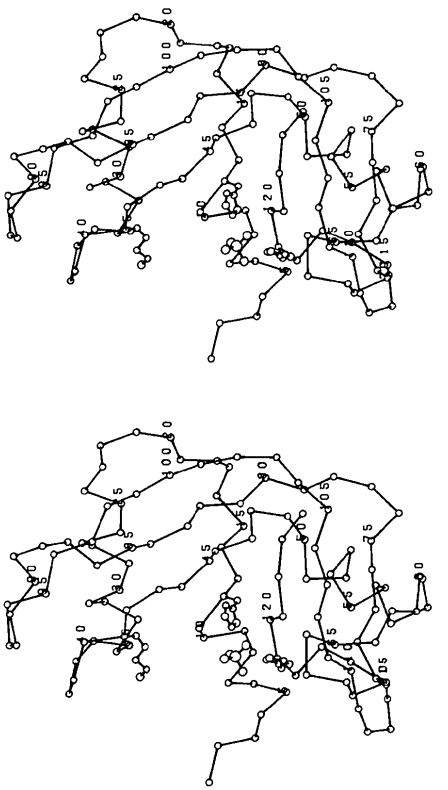

Fig. 4. Stereotracing of C_α positions and the phosphate near the active site in the final model. Side chains of His-12, His-119, and Lys-41 are also marked. The orientation of the molecule is similar to that presented by Richards & Wyckoff (1971). This figure was prepared using a program of R. Feldmann at the National Institutes of Health.

No detailed analysis of the secondary structure and of hydrogen bonding was done at this time since this is one of the aims of the neutron diffraction studies. However, it appears that residues 3 through 12 form a partially distorted α helix, as noted by Wyckoff *et al.* for ribonuclease-S, but its extent appears to be larger than previously reported for ribonuclease-A. Another stretch of α-helix can be found for residues 24–34, in agreement with both Wyckoff *et al.* (1970) and Carlisle *et al.* (1974), and between 51 and 58, as reported by

Kartha *et al.* (1967). All of these helices are considerably distorted.

This set of atomic parameters has been deposited in the Protein Data Bank. Copies can be obtained from Brookhaven National Laboratory, Upton, NY 11973, USA, or from the Crystallographic Data Centre, University Chemical Laboratory, Cambridge, England.

Preliminary results of the neutron study

Structure amplitudes obtained from deuterated ribonuclease crystals are quite different for the X-ray and neutron cases. This is especially obvious at low resolution; for example, the extremely strong X-ray reflection 003 is weak for neutrons, with 002 being very strong in the latter case. Scaling of 2559 reflections observed in the two data sets for the resolution range of 10–2·8 Å yields $R = 32{\cdot}7\%$, with the discrepancies as large as 50% below 6 Å and quite uniform around 31% above that value.

Several ways of phasing neutron diffraction data have been proposed in the past. Schoenborn (1969) calculated Fourier maps of myoglobin at 2·8 Å resolution by applying X-ray phases directly to the neutron structure amplitudes. It was estimated that the deviation from the 'true' phases was 40° (Schoenborn, Nunes & Nathans, 1970). An alternative approach was to calculate phases based on the positions of all non-hydrogen atoms, with the estimated deviation from the 'true' phases being the same as in the former case (Schoenborn *et al.*, 1970). Both of these approaches were tried with the ribonuclease data. The average phase difference between phases calculated using X-ray and neutron scattering factors was only 16° and the maps calculated using either of the phase sets and the observed neutron structure amplitude were virtually identical. These maps were also quite similar to a map calculated using X-ray amplitudes and phases, which is not surprising in view of the predominant importance of phases in map calculations. Proper estimate of the $F(000)$ term was quite important in map calculation, since the shift in contouring level can yield spurious differences in otherwise very similar maps. This is of particular importance in neutron maps, in which negative features are expected and should not be arbitrarily omitted. Unfortunately this parameter is very dependent on the H/D ratio in the protein and is subject to substantial errors in the early calculations.

The agreement between observed neutron structure amplitudes and those calculated on the basis of non-hydrogen atom positions was fair, with $R = 35{\cdot}4\%$. This value falls in between the values for the initial and final X-ray models, indicating a good probability of successful refinement, even though almost half of all the atoms in the structure were H and were not used in structure factor calculations.

While both $F_o.\alpha_c$ and $(2F_o - F_c).\alpha_c$ neutron maps looked quite similar to the respective X-ray maps, this was not true for $(F_o - F_c').\alpha_c'$ maps, in which F_c' and α_c' were calculated after about 8% of the chain was removed. These maps based on X-ray structure amplitudes were successfully used to adjust the model, but they were very difficult to interpret in the neutron case. Lowering the number of removed atoms did, however, improve the difference maps, and this technique appears to be the least susceptible to the bias in the initial phase set. It appears that the most promising approach to the refinement of the neutron structure of ribonuclease is a procedure similar to that used in the X-ray refinement, namely alternation of automated refinement and corrections based on partial maps. A similar approach (but using a different refinement scheme) was also chosen for triclinic lysozyme by Bentley *et al.* (1980). Initial positions of most of the H atoms can be predicted on the basis of the coordinates of the refined non-hydrogen atoms, and the decision between H and D can be made by checking the difference Fourier map. Ultimately this can be treated as an occupancy problem and the probability that a given atom is either H or D can be incorporated as a refinable parameter. The refinement of the structure of ribonuclease-A based on neutron diffraction data is still in progress and will be reported in detail in the future.

The author thanks G. Kartha, H. Carlisle, and H. W. Wyckoff for their unpublished diffraction data on ribonuclease, W. Hendrickson and G. Cohen for computer programs, and D. Davies, A. Santoro, E. Prince, M. Navia, and R. Bott for helpful discussions.

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Crystal Structures of 2,3,4aβ,6,7,8aβ-Hexamethyl-4a,5,8,8a-tetrahydro-1-naphthoquin-4α(and 4β)-ol*

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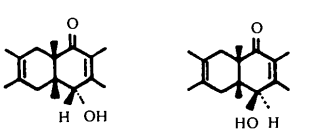
Abstract

Crystals of the 4α-ol are triclinic, $P\bar{1}$, $a = 7{\cdot}660\ (1)$, $b = 8{\cdot}194\ (1)$, $c = 12{\cdot}981\ (2)$ Å, $\alpha = 75{\cdot}17\ (1)$, $\beta = 79{\cdot}57\ (1)$, $\gamma = 65{\cdot}19\ (1)°$, $Z = 2$, $R = 0{\cdot}043$ for 2438 reflexions. The 4β-ol is monoclinic, $P2_1/n$, $a = 7{\cdot}671\ (1)$, $b = 16{\cdot}877\ (2)$, $c = 10{\cdot}994\ (1)$ Å, $\beta = 92{\cdot}27\ (2)°$, $Z = 4$, $R = 0{\cdot}041$ for 1640 reflexions. The molecules have different, ring-flipped conformations, so that in each molecule the hydroxyl group is pseudo-equatorial to the cyclohexenone ring. The two conformations produce differing intramolecular nonbonded contacts, which account for the varying photochemical reactivities in the solid state, and suggest that the photorearrangements in solution proceed from different conformations from those observed in the crystal lattice. Both structures contain intermolecular O(4)—H···O(1) hydrogen bonds.

Introduction

A study of the photochemistry of various substituted 4aβ,5,8,8aβ-tetrahydro-1-naphthoquin-4-ol systems [*e.g.* (I), (II)] has revealed reactivity differences, with either no solid-state reaction or formation of different photoproducts in solution and in the solid state (Appel, Greenhough, Scheffer, Trotter & Walsh, 1980). To establish the reasons for these different results and to provide structural data which might indicate the factors influencing the reaction pathways, a structural study of the starting substrates has been undertaken. The present paper describes the structures of 2,3,4aβ,6,7,8aβ-hexamethyl-4a,5,8,8a-tetrahydro-1-naphthoquin-4α-ol (I) and -4β-ol (II).



(I) (4α-OH) (II) (4β-OH)

Experimental

Recrystallization from petroleum ether/ethanol gave well formed colourless chunks of the 4α-ol and large colourless plates of the 4β-ol; fragments were cut, of dimensions *ca* $0{\cdot}3 \times 0{\cdot}3 \times 0{\cdot}3$ mm and $0{\cdot}6 \times 0{\cdot}2 \times 0{\cdot}2$ mm, respectively. Preliminary unit-cell data were obtained from precession photographs, and accurate unit-cell parameters (Table 1) were determined by a least-squares analysis of the setting angles of 25 reflexions automatically located and centred on an Enraf–Nonius CAD-4 diffractometer ($\theta = 12$–$21°$ for the 4α-ol, 8–12° for the 4β-ol; graphite-monochromatized Mo $K\alpha$ radiation). Space groups were

* IUPAC names: 4α(and 4β)-hydroxy-2,3,4aβ,6,7,8aβ-hexamethyl-4a,5,8,8a-tetrahydro-1(4*H*)-naphthalenone.

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