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Ribonuclease Structure and Catalysis: Crystal Structure of Sulfate-Free Native Ribonuclease A at 1.5-Å Resolution†

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Received December 29, 1986; Revised Manuscript Received February 25, 1987

ABSTRACT: The structure of native bovine pancreatic ribonuclease A, without the inhibitory sulfate anion normally bound at the active site, has been determined by X-ray diffraction at 1.53-Å resolution. Treatment of a crystal of ribonuclease containing sulfate with an alkaline buffer released most of the sulfate anions. On return to active pH, few of the side chains moved, and the backbone structure remained unchanged. The active site conformation was essentially unchanged except for the replacement of the sulfate anion by a water molecule, which is hydrogen-bonded to histidine-12 and to another water, and for a small movement of the side chain of lysine-41. Histidines-12 and -119, the catalytic basic and acidic residues, have not moved. Thus the distance between them, and the presence of an intervening water, prohibits the possibility of their being hydrogen-bonded together. The structure has been refined by restrained least squares to an *R* factor of 0.17. Analysis of individual atomic temperature factors indicates that the molecule has become less rigid in general but that some regions were particularly affected by loss of the sulfate, while others were relatively unaffected. The active site geometry of native ribonuclease A supports the original in-line mechanism of Rabin and co-workers and is in disagreement with the adjacent mechanism of Witzel and co-workers.

Bovine pancreatic ribonuclease A (RNase A) is a historical landmark in the study of protein structure and function. Because it has long been available in relatively large amounts in good purity, it has provided a model system to study the mechanisms of enzyme catalysis. RNase A was the first enzyme to be sequenced (Smyth et al., 1963; Potts et al., 1962) and one of the first for which X-ray diffraction revealed a three-dimensional structure (Kartha et al., 1967). A considerable amount of work has been done to study the crystal structures of both RNase A and the subtilisin-cleaved form, RNase S (Carlisle et al., 1974; Wlodawer et al., 1982; Wyckoff et al., 1967, 1970), and complexes of these with various inhibitors and substrate analogues (Richards et al., 1971; Richards & Wyckoff, 1973; Borkakoti, 1983). This is the second in a series of nine papers describing a combined low-temperature kinetics and crystallographic study of the structural basis for the catalytic power of RNase A.

Ribonuclease A, a monomeric enzyme of M_r 13 683, is roughly kidney shaped with the active site located in a deep cleft. Figure 1 shows a stereoview of the α-carbon backbone of the native enzyme. The dot in the cleft shows the position of a bound sulfate ion (see below). The enzyme catalyzes the cleavage of RNA, leaving a nucleoside 3′-monophosphate, and is specific for pyrimidines on the 3′-side of the cleavage site.

In addition to the crystallographic work on RNase, many other physical and chemical experiments have been done to elucidate the mechanism of catalysis. The active site residues involved in catalysis have been examined by chemical modification. Two histidines and one lysine were found to be essential to enzymic activity. Carboxymethylation with iodoacetic acid modifies either histidine-12 or histidine-119, but not both, resulting in an inactivated enzyme (Crestfield et al., 1963). The enzyme can also be inactivated by dinitrophenylation of lysine-41 (Hirs et al., 1965). The activity versus pH curve is bell shaped, implicating a base of $pK_a = 5.22$ and an acid of $pK_a = 6.78$ in the free enzyme (Findlay et al., 1962). From kinetic and pH titration data a mechanism was proposed by Findlay et al. (1962) in which two histidines act as base and acid catalysts, while one lysine serves to position the phosphate group of the substrate. This was later elaborated upon by Roberts et al. (1969), who considered NMR and X-ray diffraction data as well as the earlier chemical modification and kinetic data and proposed that the two histidines were His-12 and His-119 and that the lysine was Lys-41.

In this in-line mechanism (Figure 2a), the histidines are on opposite sides of the phosphate moiety with His-12 acting as a base on the 2′-OH of the substrate and His-119 acting as an acid to protonate the 5′-oxygen of the leaving group in the first half of the reaction. The histidines reverse roles in the second half of the reaction: His-12 reprotonates the 2′-oxygen and His-119 deprotonates a water molecule which, again in an in-line manner, attacks the cyclic phosphate intermediate formed as the product of step 1. Lysine-41 appears to be in a position to stabilize the pentacoordinate transition states by either hydrogen-bonding or charge–charge interaction.

A different, adjacent mechanism (Figure 2b) was proposed by Witzel and co-workers (Witzel, 1963; Ruterjans & Witzel, 1969; Rubsamen et al., 1974) on the basis of structure–activity relationships of model substrates and NMR and kinetic ex-

† This work was supported by a grant from the National Institutes of Health (GM 26788).

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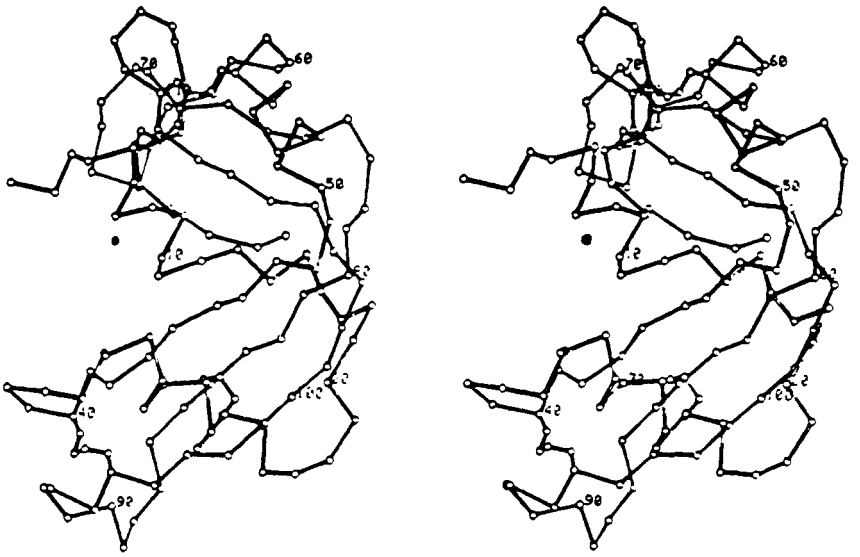

FIGURE 1: Stereo drawing of the α-carbon backbone of RNase A. The dot represents a bound sulfate.

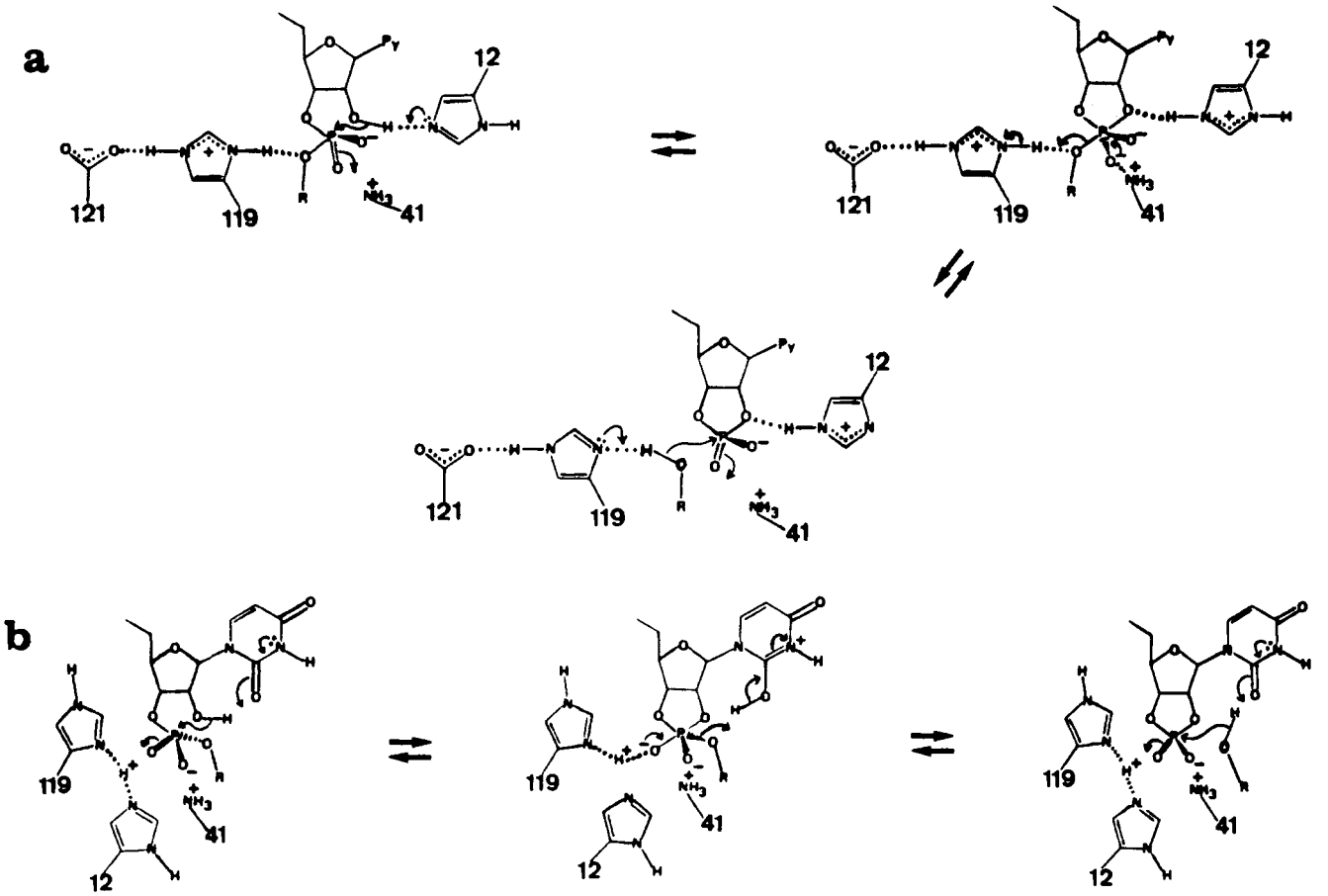

FIGURE 2: Mechanisms proposed for RNase A by Roberts et al. (a) and Witzel et al. (b). Only the first step, formation of the cyclic intermediate, is shown. The second step is the reverse of the first with R = H. The relevant residues are indicated. In mechanism b the necessary pseudorotation to place the leaving group in an apical position is not indicated.

periments. Witzel agreed that lysine-41 was involved in binding but concluded that only His-119 interacts directly with the substrate. His-12 was thought to keep His-119 properly positioned by a histidine-to-histidine hydrogen bond. In this scheme, the 2-oxo group of the pyrimidine ring of the substrate acts as the acid–base catalyst in the reaction while the protonated His-119–His-12 pair stabilizes the negative charge formed in the pentacoordinate transition states.

In the Witzel mechanism the two active site histidines must lie close together. The crystal structures of RNase A and RNase S seen so far suggest that these residues are not close enough for a hydrogen bond to exist between them. Unfortunately, all "native" structures of RNase A and RNase S have a sulfate or phosphate anion bound at the active site, lying between histidine-12 and histidine-119 (Carlisle et al., 1974; Martin, 1978; Richards & Wyckoff, 1971; Wlodawer et al., 1982). The only way to see if the two histidines may hydrogen bond is to examine the active site structure in the absence of any inhibitory anion.

The structures of enzyme–inhibitor and enzyme–substrate–analogue complexes are valuable tools in the study of the mechanism of catalysis, but one must always be aware of

the possibility that the inhibitor or analogue interacts with the enzyme in a manner different from the substrate and transition state. To evaluate properly the effects of binding of these analogues and inhibitors on the active site structure of RNase, it is necessary to have a proper starting point. As Richards and Wyckoff (1971) have pointed out, the effects of the bound anion on the structure of RNase have not been known, and consequently, the changes seen upon binding any substrate or inhibitor relative to the structure with a bound anion may not be the same as the changes that occur in solution. For this reason also, it would be valuable to know the structure of truly native RNase with no anion bound at the active site.

Since ribonuclease has never been crystallized in the absence of either sulfate or phosphate, we decided to try to remove the sulfate from preformed crystals. A Raman spectroscopic study in D_2O by Harada et al. (1982) suggested that in solution the sulfate was released by an increase in the pD to about 8.8. Since crystalline ribonuclease is known to be catalytically active, the active site must be accessible to the mother liquor. Therefore the sulfate should be removable by increasing the pH of the crystals' mother liquor. We report here the first determination of the structure of native, sulfate-free ribonuclease A.

Materials and Methods

Bovine pancreatic ribonuclease (Sigma Chemical Co., type III-A) was crystallized without further purification by addition of methanol to a solution of 3% RNase in distilled water at pH 5.5 until the methanol concentration reached about 40%. The crystallization batch was seeded with 0.5 μL of supernatant from a previously grown batch of microcrystals. Usable crystals (1.0 mm × 0.5 mm × 0.5 mm) were obtained in about 1 week at room temperature.

All of the following crystal manipulations were carried out at 4 °C to minimize the loss of MeOH. A single crystal with some of its mother liquor was removed from the crystallization vial and placed in an empty vial; 70% MeOH, pH 5.5, was slowly added to dilute the mother liquor. Some of this solution was removed from the vial and replaced with fresh 70% MeOH, pH 5.5. This process was repeated until the mother liquor was completely replaced. Then the pH was increased in steps of about 1 pH unit per day to pH 9. The crystal was kept at pH 9 for 8 days, with daily changes of mother liquor. The pH was then returned to pH 6.8 in two steps, allowing 1 day for equilibration at each step.

The crystal was mounted at 4 °C in a 1.0-mm quartz capillary tube with a column of mother liquor on each side to keep the crystal from drying out. The tube was also sealed with machine oil, wax, and Duco cement. About 18000 X-ray reflections were collected on a Nicolet P3 diffractometer at −10 °C, with nickel-filtered copper K_α radiation, to a nominal resolution of 1.53 Å.[1] The data were collected by the full integration method, scanning on ω for a total of 1.0°, counting for 10 s on the background and 20 s on the peak.

The data were processed by the PROTSYS programs (Petsko, unpublished notes) on a PDP 11/60 computer. Radiation damage was determined by monitoring five reflections periodically during data collection. The damage was approximated by a linear decay of intensity with exposure time, total decay being 21%. The intensities of all reflections were corrected to account for the decay rate of 0.13% per hour of exposure time. An empirical method was used to correct for absorption (North et al., 1968). Reflections with intensities of less than one standard deviation were discarded. Between infinity and 1.53-Å resolution, 85% of the theoretical number of reflections were kept, while in the shell of data between 1.62 and 1.53 Å 60% of the data remained. The measured F's were scaled to the corresponding data from a previously refined structure at 1.5-Å resolution of sulfate-containing RNase A at −10 °C by making the sum of the F's equal in the two data sets. The phases from the sulfate structure were then applied to the newly scaled F's. Difference Fourier maps (with coefficients $4|F_{\text{no sulfate}}| - 3|F_{\text{sulfate}}|$ to enhance the differences between the structures) were examined versus coordinates from the original structure on a Vector General graphics system using the program FRODO (Jones, 1978). Alterations in the structure were made to give a better agreement with the electron density.

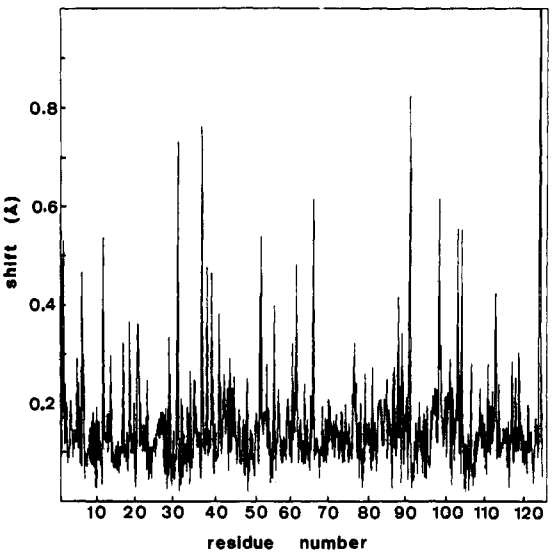

FIGURE 3: Shift in atomic positions due to removal of the sulfate anion from the active site.

The sulfate-free RNase A structure was refined with the restrained least-squares program of Konnert and Hendrickson (1980) on a VAX 11/780. The refinement was begun with data from 8.0 to 2.0 Å and an overall B factor of 12 Å. After the R factor had dropped from the original 28.3% to 21.4% in six cycles, the data to 1.5 Å were added, and individual B factors were allowed to refine. The refinement was stopped after 11 more cycles when the R factor had stabilized at 17.0%. The root mean square (rms) deviations from ideality for bond distances, planar 1–4 distances, and bond angles were 0.036 Å, 0.069 Å, and 3.9°, respectively.

Results and Discussion

Examination of the $4|F_{\text{no sulfate}}| - 3|F_{\text{sulfate}}|$ map on the graphics system showed that removal of the sulfate from RNase A caused only minor side-chain shifts and no apparent backbone shifts. The refinement confirmed this observation. The average shift in atomic position was 0.15 Å. Figure 3 shows the shift in atomic positions due to removal of the sulfate, most of which are close to the standard deviation of 0.14 Å. Of 951 atoms, only 26 had shifts greater than 0.40 Å, and 15 of these were atoms belonging to lysine residues. All of the lysines in the molecule except Lys-41 are in this group. The ϵ-amino group of Lys-41 has moved 0.38 Å away from its original position near the bound sulfate in the active site.

Most of the lysines had relatively poor density, probably due to a higher degree of mobility than the rest of the protein. One

[1] Although optical theory suggests that the apparent and true resolutions are not always equal, it is the convention in protein crystallography to use the axial spacing corresponding to the highest Bragg angles measured to define the "resolution" of the structure being determined.

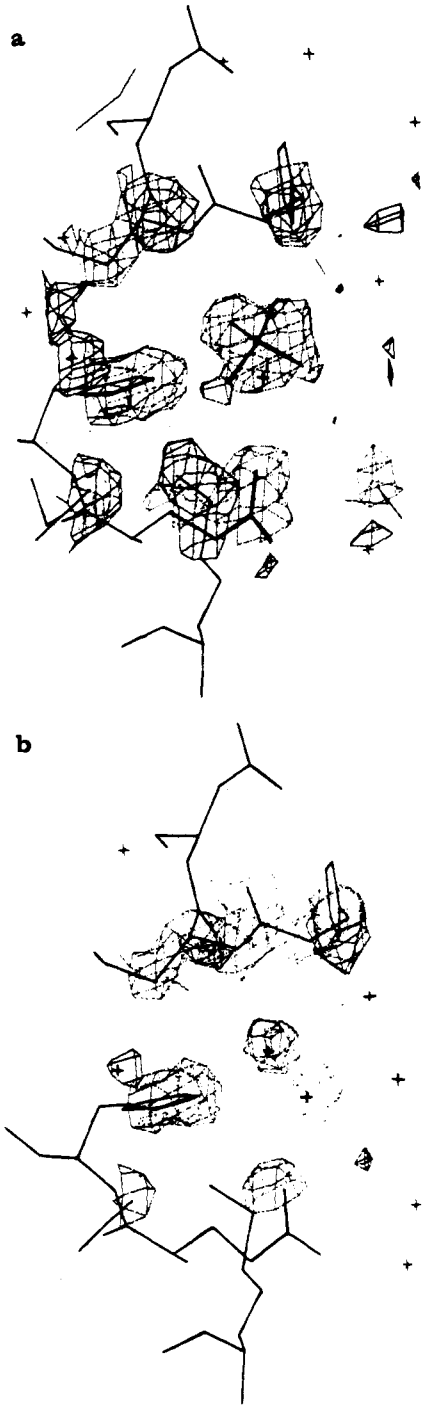

FIGURE 4: Active site structure (a) in the presence of sulfate and (b) in the absence of sulfate. His-119 is at the top, and His-12 is on the left. The difference in the electron density for the backbone atoms is an artifact of the way the picture was displayed with the program FRODO.

would have expected that the most mobile cationic side chains would be most affected by the removal of negative charge from the active site. It is possible that this effect can be felt for some distance, since the α-amino group of lysine-1, which shifted by 0.55 Å, is almost 18 Å away from the position of the bound sulfate. Alternatively, the movement of each of the lysines may be due to the simultaneous loss of several anions. Therefore, the effect would not need to be transmitted over such a large distance. Only one tightly bound anion has been seen in another highly refined (R factor = 0.135) 1.5-Å resolution structure (Burley and Petsko, unpublished results), but it is conceivable that other anions are bound and are indistinguishable from water molecules because of low occupancy.

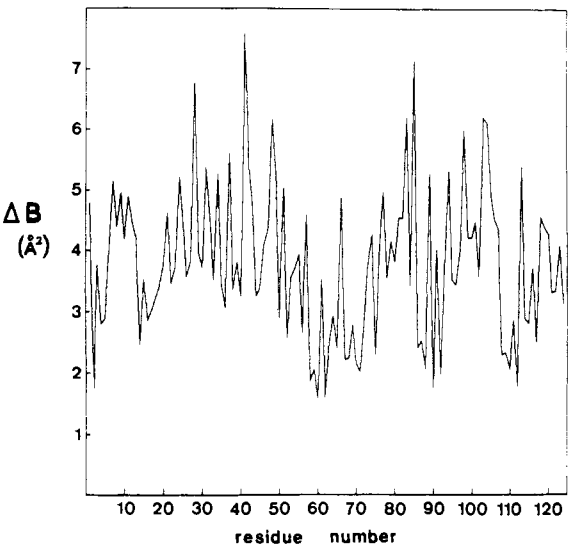

FIGURE 5: Change in B factor of all atoms averaged over each residue.

This possibility might be distinguished by performing an electrostatic field calculation on the sulfate-free structure and comparing it with the calculation for RNase with sulfate in the active site (Matthew & Richards, 1982). If the effect is felt over a great distance, then one would expect the calculation to show that the loss of sulfate has changed the electrostatic environment of remote regions of the protein.

The active site as a whole has retained its conformation. The sulfate has been replaced by one water, located within hydrogen-bonding distance of His-12. The water is in a position previously occupied by one of the sulfate oxygens. There is no electron density at the sulfur position. This water is close to another water in the active site and appears to be involved in a hydrogen-bonded network. Thus the active site has not needed to distort to compensate for the loss of the sulfate.

The two histidines of the active site (12 and 119) have moved very little: the distance between the ϵ-nitrogen of His-12 and the δ-nitrogen of His-119 increased from 6.05 to 6.26 Å. Histidine-12 appears to be hydrogen-bonded to the new water through its ϵ-nitrogen and to the carbonyl of threonine-54 through its (protonated) δ-nitrogen. Histidine-119 is hydrogen-bonded via its ϵ-nitrogen to the carboxyl group of aspartate-121. Contrary to results reported by other groups (Wyckoff et al., 1970; Borkakoti et al., 1982; Borkakoti, 1983), but in agreement with Wlodawer et al. (1982), we see only one well-defined position for His-119. The positions of some of the active site residues with and without the sulfate are shown in Figure 4.

Glutamine-11 is the only other active site residue that appears to have moved. The side-chain amide group has rotated slightly, so that the oxygen is closer to the original location of the sulfate. Another major movement that was detected in the protein is the rotation of the isopropyl side chain of the C-terminal valine by about 180° and a translation of the β-carbon. This movement shows up prominently in Figure 3 as a relatively large shift in position of the two γ-methyl groups of the valine side chain. The actual shift was about 2.6 Å (the maximum value on the graph was set to 1.0 Å to increase the detail in the rest of the graph). The other prominent shifts seen on the graph correspond to the terminal groups of the side chains of Asp-38, Arg-39, Leu-51, Thr-87, Asn-103, and Asn-113.

A plot of the change in B factor averaged over each residue versus residue number (Figure 5) shows that the overall B factor has increased by 3.9 Å^2 after the loss of sulfate, indi-

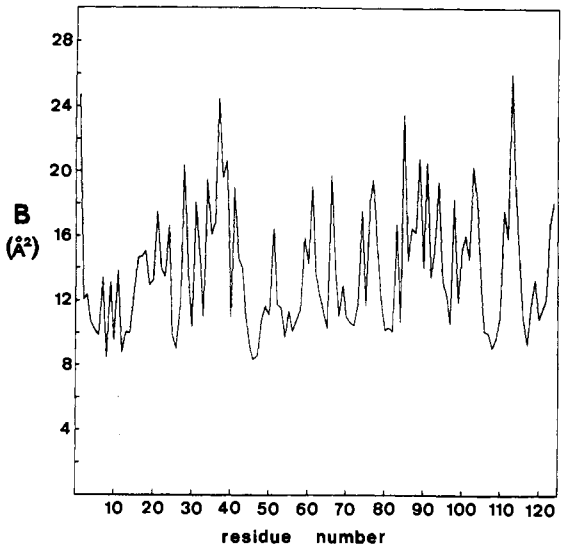

FIGURE 6: *B* factors of all atoms of the sulfate-free structure averaged over each residue.

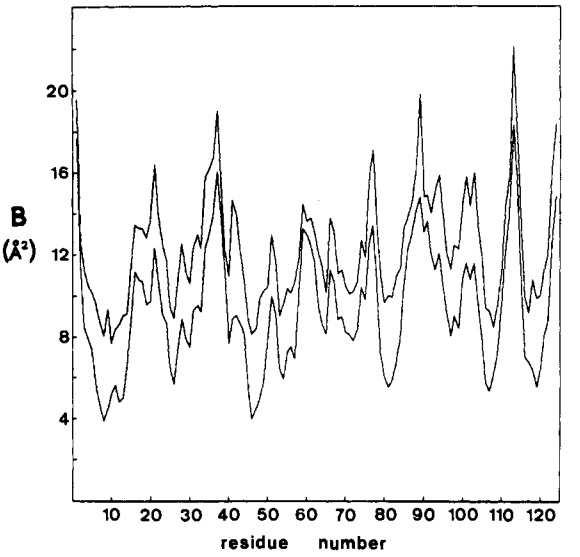

FIGURE 7: *B* factors of only the backbone atoms averaged over each residue. The upper curve is for the sulfate-free structure while the lower is for RNase with sulfate bound.

cating an overall loosening of the protein. This effect has been observed before in studies of the affects of electrolytes on the thermal denaturation temperature of RNase A (von Hippel & Wong, 1965; Ginsburg & Carroll, 1965). In these studies the presence of sulfate increased the transition temperature significantly above that observed in the presence of chloride. Although the overall difference in *B* factor between sulfate-free and sulfate-bound RNase is consistent with these data, comparison of *B* factors between two different crystals is always dangerous. A difference in lattice disorder may be misinterpreted as a change in mobility. However, this is unlikely in the present case, since despite the general increase in *B* factor there are still some residues for which ΔB is relatively small. Comparison of Figure 5 with the absolute *B* factors for each residue in the sulfate-free structure (Figure 6) shows that there is little correspondence between the magnitude of the change in *B* factor and the magnitude of the *B* factor. This is reasonable since it is possible for a remote, mobile side chain to be less affected by a change in electrostatic environment than a less mobile side chain that is close to the site of the change. An example of this is Lys-41, which had the largest increase in averaged *B* factor but did not have the largest *B* factor even after this increase. One might have predicted that Lys-41 would have a large change in *B* factor due to its proximity to the sulfate.

There are three important minima in Figure 5 covering residues 58–65, 86–93, and 108–112. These minima are reflected in Figure 7, a plot of the *B* factors (backbone atoms only) of RNase with and without sulfate bound at the active site. The two curves have nearly the same shape, but they are much closer together in these same regions, indicating that this trend is not biased by the *B* factors of the side chains. All of these regions contain turns and disulfide bridges. Because they were relatively unaffected by the removal of the sulfate, while the molecule in general became considerably looser, these may be regions that are stable to denaturation in general. The regions encompassing residues 15–35, 75–85, and 95–105 have relatively large increases in *B* factor. These all correspond to extended sections of mostly α-helix or β-sheet which are relatively close to the surface of the protein. Further studies of structural changes at the approach to denaturation are currently being pursued in our laboratory.

The maintenance of the active site geometry upon removal of the inhibitory sulfate anion appears to exclude the possibility of a hydrogen bond between His-12 and His-119 as proposed by Witzel and thus lends more support for the mechanism proposed by Rabin et al.

ACKNOWLEDGMENTS

We are grateful to Dr. David R. Rose for helpful discussions.

Registry No. RNase, 9001-99-4; His, 71-00-1; Lys, 56-87-1; Gln, 56-85-9; sulfate, 14808-79-8.

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