Use of Fluorine-19 Nuclear Magnetic Resonance to Study Conformation Changes in Selectively Modified Ribonuclease S*

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ABSTRACT: A new technique is described for the study of conformation-related processes in proteins, using nuclear magnetic resonance spectroscopy. The protein is labeled covalently with a small fluorinated molecule at a specific site, and the ^{19}F-nuclear magnetic resonance spectrum of the fluorine probe is used to detect environmental changes. This approach has been used to demonstrate such changes caused by binding of inhibitors to ribonuclease S.

Nuclear magnetic resonance, a physical tool which has yielded extensive information in structural analysis of molecules of moderate size, has been used to a lesser extent in direct structural and conformational studies of macromolecules such as proteins. The large numbers of nonequivalent protons in a native protein produce a ^{1}H-nuclear magnetic resonance spectrum of great complexity with limited resolution of individual resonances even at the highest available magnetic fields. Despite this drawback extensive information on the molecular properties of lysozyme, ribonuclease A, and cytochrome *c* has been obtained from examination of high-field methyl group resonances and low-field aromatic resonances at 220 MHz by McDonald and Phillips (1969). In addition, the properties of myoglobin (Shulman *et al.*, 1969; Kurland *et al.*, 1968) and hemoglobin (Kurland *et al.*, 1968; Wüthrich *et al.*, 1968) have been examined by ^{1}H-magnetic resonance spectroscopy at 220 MHz through studies of high- and low-field resonances which arise from interactions of methyl groups with porphyrin rings. It is therefore obvious that for some proteins whose catalytically or conformationally important residues are unusually shielded or deshielded, useful assignments and analyses can be made, but an examination of the growing catalog of protein spectra serves to show that many interesting proteins are not so fortuitously designed. If nuclear magnetic resonance is to be generally applicable to direct investigation of protein molecules, methods must be devised for simplifying their spectra to permit unequivocal identification of individual resonances and thereby allow interpretation of spectral changes induced by processes of interest.

One approach to this problem has been used by Katz *et al.* (1968), Markley *et al.* (1968), and Putter *et al.* (1969) to study phycocyanin from a blue–green alga and a nuclease from *Staphylococcus aureus*, respectively. Their method employs biosynthetic incorporation of deuterated amino acids into protein with but a few selected protonated amino acids being incorporated. This approach yields vastly simplified protein ^{1}H-nuclear magnetic resonance spectra with the possibility of assigning specific resonances to particular amino acid side chains.

The approach described in this communication is observation of the ^{19}F-nuclear magnetic resonance spectrum of a protein modified by covalent attachment of a small fluorinated probe moiety. Ideally, the probe molecule is introduced into a catalytically or conformationally strategic site on the protein in a way which does not interfere with enzymatic activity. Fluorine is well suited to this purpose, for it is relatively small and easy to introduce as part of small molecules of a variety of reactive specificities. Fluorine nuclear magnetic resonance absorptions occur at lower frequencies than protons, yet they may be observed with drastic modification of proton nuclear magnetic resonance equipment. Fluorine labeling is particularly useful for study of mammalian proteins, since, at the present time, obvious practical considerations limit the biosynthetic selective deuteration method to the proteins of simpler creatures.

In this investigation, the fluorine probes used were trifluoroacetyl groups introduced specifically at lysine residues 1 and 7 of bovine pancreatic ribonuclease S (RNase S). Ribonuclease S is formed from ribonuclease A by subtilisin cleavage of the amide bond between alanine 20 and serine 21 (Richards and Vithayathil, 1959), the full enzymatic activity of the parent enzyme being retained. RNase S is inactivated by removal of the peptide segment containing residues 1–20 (RNase S-peptide), but under appropriate conditions the two sections can reassociate with full restoration of enzymatic activity. When trifluoroacetylated ribonuclease S-peptide is associated with ribonuclease S-protein a fully active enzyme is formed. In addition, the ^{19}F-nuclear magnetic resonance spectrum of the modified S-peptide exhibits significant changes on association with the S-protein, and further changes are observed on binding of various inhibitors to this complex.

Experimental Section

Materials. Ribonuclease S-peptide and S-protein (lot 118B-8110, phosphate free) were obtained from Sigma Chemical Company. Ethyl thioltrifluoroacetate was a product of Pierce Chemical Co. Ribonuclease A, cytidine 2′-phosphate, cytidine 3′-phosphate, cytidine 5′-phosphate, yeast ribonucleic acid, and lysine monohydrochloride were products of Sigma Chemical Co. Phenyl isothiocyanate, trifluoroacetic acid, and *N*-ethylmorpholine were obtained from Matheson, Coleman and Bell. Aminopeptidase M (lot 51132) was obtained from Henley and Co., and subtilisin Carlsberg (lot 50624) from Novo Industries, Copenhagen.

ε-Trifluoroacetyllysine and α,ε-bis(trifluoroacetyl)lysine

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TABLE I: Amino Acid Composition of Trifluoroacetylated RNase S-Peptide from Total Enzymatic Hydrolysis.

Amino Acid	nmoles	Ratio[a]	Native Peptide
Asp[b]	1333	1.0	1
Met sulfone	Trace		
Thr	3905	3.0	2
Ser	3930	3.0	3
Glu	2659	2.0	3
Pro			
Trifluoroacetyllysine	2263	1.74	
Ala	5030	3.9	5
Cys			
Val			
Met	1162	0.9	1
Ile	13	0.01	
Leu		0.04	
Tyr			
Phe	1526	1.2	1
His	1191	0.9	1
Lys	316	0.24	2
Arg	1511	1.2	1

[a] Values expressed in residues per molecule. [b] Taken as 1.0 for mole ratio determination.

were synthesized by known methods (Weygand and Geiger, 1956; Schallenberg and Calvin, 1955): ε-trifluoroacetyllysine, reported mp 226–231° dec; found, 222–230° dec; α,ε-bis(trifluoroacetyl)lysine, reported mp 122–123°; found, 121°.

Methods. Ultraviolet absorbances were determined with a Gilford Model 240 spectrophotometer; pH measurements were made using a Radiometer Copenhagen Model 26 or a Sargent Model IR pH meter. ^{19}F-Nuclear magnetic resonance spectra were recorded using a Varian Model HA-100 spectrometer modified to operate at 94.1 MHz, supplemented by a Fabritek Model 1062 computer of average transients. Amino acid analyses were performed on a Beckman-Spinco Model 120B amino acid analyzer.

RNase enzyme activity was measured using the procedure of Kunitz (1946).

Preparation and Purification of Trifluoroacetylated RNase S-Peptide. Reaction conditions were essentially as described by Goldberger and Anifinsen (1962). A solution of 15 mg ($\equiv 1.5 \times 10^{-5}$ mole of lysine residues) of RNase S-peptide in 5 ml of distilled water was adjusted to pH 10 by addition of 5 N NaOH from a micrometer syringe. Ethyl thioltrifluoroacetate (1 ml, 8×10^{-3} mole) was added. The pH was monitored continuously and maintained at 10.00 ± 0.05 by addition of 5 N NaOH from the syringe. The reaction was allowed to continue at room temperature for 90 min with vigorous stirring. After this time, base consumption had effectively ceased. The pH was lowered to 3.85 by addition of glacial acetic acid, and the resultant solution was subjected to gel filtration on a column (3 × 90 cm) of Sephadex G-25 using 0.2 M acetic acid as the eluting solvent. The absorbance of each 5-ml fraction was determined at 230 mμ, and appropriate ultraviolet-absorbing fractions were pooled and lyophilized. This procedure typically yielded 8–10 mg of modified peptide.

Amino Acid Analysis of Modified RNase S-Peptide by Total Enzymatic Hydrolysis. To a solution of 0.2 mg of modified S-peptide in 0.5 ml of phosphate buffer (0.1 M, pH 7.0) was added 0.15 mg of subtilisin in 0.1 ml of the same buffer. After the solution had stood for 3 hr at room temperature, 1.0 mg of aminopeptidase M was added. The solution was allowed to stand for 20 hr at room temperature. The pH of the solution was then adjusted to 2.2 with 6 N HCl, and an aliquot of 0.3 ml of solution was analyzed for amino acid composition.

Nuclear Magnetic Resonance Spectral Measurements. All nuclear magnetic resonance solutions were prepared by dissolving 2×10^{-6} mole of modified peptide in 0.5 ml of ammonium formate buffer (0.1 M, pH 4.5), producing a final concentration of 4×10^{-3} M. Solid RNase S-protein (2×10^{-6} mole) was added to this solution. Inhibitors were added as solids, producing final concentrations of 4×10^{-3} M for cytidine 5′-phosphate, cytidine 3′-phosphate, and cytidine 2′-phosphate, and 10^{-2} M for phosphate. In studying the effect of pH on the spectrum of RNase inhibited with 3′-CMP, an inhibitor concentration of 7×10^{-2} M was used above pH 6.5. Inhibitors were present in saturating concentrations for all experiments, according to binding constants obtained by Cathou and Hammes (1965) from temperature-jump studies on RNase A. Spectra were measured at a probe temperature of 35°, using a capillary of trifluoroacetaldehyde hydrate as an external reference standard. Control solutions were prepared exactly as described above, replacing RNase S protein with RNase A.

Modified RNase S was isolated from the solution used for nuclear magnetic resonance determinations by gel filtration through Sephadex G-25, with 0.1 M ammonium formate, pH 4.5, as eluting buffer. The main body of 280-mμ absorbing material, found at the same elution volume as RNase A in a previous calibration run, was pooled and lyophilized.

Edman Degradation of Modified RNase S-Peptide. An Edman degradation was carried out by the procedure of Konigsberg and Hill (1962). The 19-residue product of the degradation was recovered by gel filtration, eluting from Sephadex G-25 with 0.2 M HOAc as solvent. The phenylthiohydantoin derivative of lysine 1 was not recovered.

Results

The amino acid compositions of modified and native RNase S-peptide are given in Table I, and they indicate that at least 80–90% of the lysine residues of the modified peptide were converted to ε-trifluoroacetyllysine. High and low values shown for threonine and glutamic acid, respectively, are due to one glutamine residue which was not chromatographically separated from threonine under the conditions used.

The ^{19}F-nuclear magnetic resonance spectrum of the modified S-peptide, shown in Figure 1a, consists of a singlet (L) of half-width 2 cps, 9.72 ppm downfield of trifluoroacetaldehyde (external). A second, smaller resonance (K) appears 0.39 ppm downfield of L, and a third (M) appears as a shoulder 0.07 ppm upfield of L. Assignment of the observed resonances was made possible by model studies of ^{19}F-nuclear magnetic resonance spectra of ε-trifluoroacetyllysine and of α,ε-bis(trifluoroacetyl)lysine. The resonances occurred in the same frequency range as those observed for the modified S-peptide. Figure 2a shows the spectrum observed for ε-trifluoroacetyllysine and Figure 2b that observed for α,ε-bis-(trifluoroacetyl)lysine. A mixture of both the singly and doubly acylated amino acids gave the spectrum shown in Figure 2c. In α,ε-bis(trifluoroacetyl)lysine, the ^{19}F-nuclear

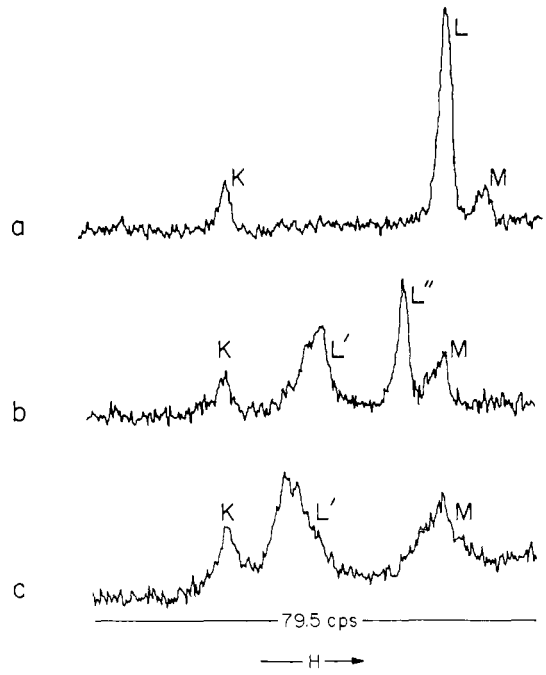

FIGURE 1: ^{19}F-Nuclear magnetic resonance spectra of trifluoroacetylated RNase S: (a) trifluoroacetylated RNase S-peptide; (b) trifluoroacetylated RNase S-peptide associated with RNase S-protein; (c) trifluoroacetyl peptide–protein complex after removal of Lys-1 from the peptide.

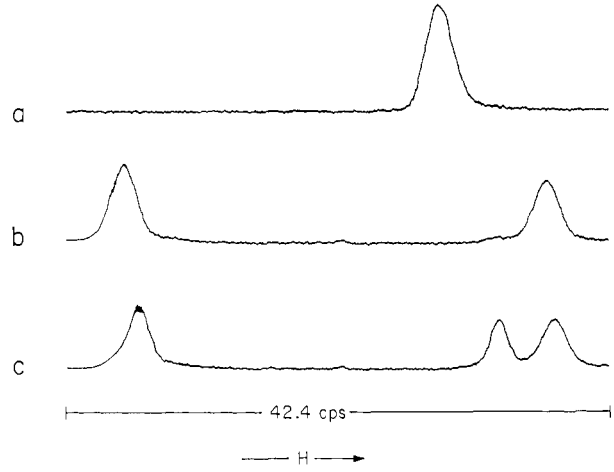

FIGURE 2: ^{19}F-Nuclear magnetic resonance spectra of trifluoroacetylated lysine: (a) ϵ-trifluoroacetyllysine; (b) α,ϵ-bis(trifluoroacetyl)lysine; (c) a mixture of ϵ-mono- and α,ϵ-bis(trifluoroacetyl)-lysine.

magnetic resonance of the ϵ-trifluoroacetyl group occurs 4 cps upfield of that due to ϵ-trifluoroacetyllysine. It was evident from this latter spectrum that the chemical shift of an ϵ-trifluoroacetyl group of lysine is slightly different depending on whether the α-amino group is trifluoroacetylated or free. On the basis of these model studies of trifluoroacetyllysine, peak L in Figure 1a was assigned to the ϵ-*N*-trifluoroacetyl groups of Lys-7 and singly acylated Lys-1 of the modified S-peptide. Peaks K and M in Figure 1a were attributed, respectively, to trifluoroacetyl groups on the α and ϵ amino groups of doubly acylated Lys-1 of the modified S-peptide. Normally the acylating reagent, ethyl thioltrifluoroacetate, is specific for the ϵ-amino group of lysine in proteins (Goldberger, 1967), but a very large excess of the reagent was used to modify the S-peptide, and apparently some reaction with the α-amino group resulted.

When a molar equivalent of RNase S-protein was added to the solution of trifluoroacetylated S-peptide, the nuclear magnetic resonance spectrum changed as shown in Figure 1b. The major peak L appeared to be split into two peaks, L′ and L′′, which moved downfield by 0.22 ppm and 0.07 ppm, respectively. In addition, L′ appeared to be broadened to a half-width of 4 cps from an initial value of 2 cps for L. The minor peak M also was shifted 0.07 ppm downfield but peak K was unchanged by the addition of S-protein. According to the original resonance assignments, these changes indicated that the trifluoroacetyl residue on the ϵ-amino groups experienced significantly different environmental changes. As described in the Experimental Section, the trifluoroacetylated S-peptide–protein complex was subjected to gel filtration and the ^{19}F-nuclear magnetic resonance spectrum of the reisolated material was found to be identical with that shown in Figure 1b, thus demonstrating that the observed spectral changes were due to complex formation. As further proof that the observed changes were due to a specific association of modified S-peptide with S-protein it was shown that addition of a mole equivalent of RNase A to modified S-peptide produced no change in the ^{19}F-nuclear magnetic resonance spectrum of the peptide.

The resonance assignments and interpretation (see Discussion) of these results were tested by examination of the ^{19}F-nuclear magnetic resonance spectra of S-peptide and RNase S after lysine 1 of the peptide had been removed by an Edman degradation. The spectrum of degraded peptide was qualitatively similar to that of trifluoroacetylated S-peptide; peak L was, however, relatively smaller. Addition of the S-protein (Figure 1c) revealed that component L′′ had been removed entirely. Therefore it is reasonable to conclude that in the spectrum of trifluoroacetylated ribonuclease S, L′ corresponded to the ϵ-trifluoroacetyl group on Lys-7, L′′ to the ϵ-trifluoroacetyl on Lys-1, K to α-trifluoroacetylated Lys-1, and M to the ϵ-trifluoroacetyl group on bistrifluoroacetylated Lys-1. This result is consistent with the spectral assignments based on model studies (Figure 2) of ϵ- and α,ϵ-bis trifluoroacetylated lysine. Also consistent with these assignments are the results of integration of the resonances of the RNase S spectrum. The integral of peak L′, due to ϵ-trifluoroacetyl Lys-7, was approximately equal to the sum of L′ (the resonance of ϵ-trifluoroacetyllysine-1) and M (due to the ϵ-trifluoroacetyl group of bisacylated lysine-1). Peaks M and K, which result from bis(trifluoroacetyl)(Lys-1, were equal in area.

Addition of an inhibitor, phosphate ion, to the trifluoroacetylated RNase S solution produced the change shown in Figure 3a. Resonance L′ was shifted upfield by 0.03 ppm; K, L′′, and M were unchanged. Addition of 5′-CMP to a fresh sample of modified enzyme produced a similar change as shown in Figure 3b. Addition of 3′-CMP and 2′-CMP induced more marked changes. As shown in Figure 3c, peak L′ was shifted upfield by 0.07 ppm on binding of 3′-CMP. Furthermore, the presence of two component peaks in L′ was fairly clear. Reexamination of the other RNase spectra (1b; 3a,b) raised the possibility that the apparent width of L′ was due partly to the presence of two poorly resolved resonances. The change induced by 2′-CMP binding (Figure 3d) was similar to that of 3′-CMP; the upfield shift of L′ was ~0.02 ppm greater.

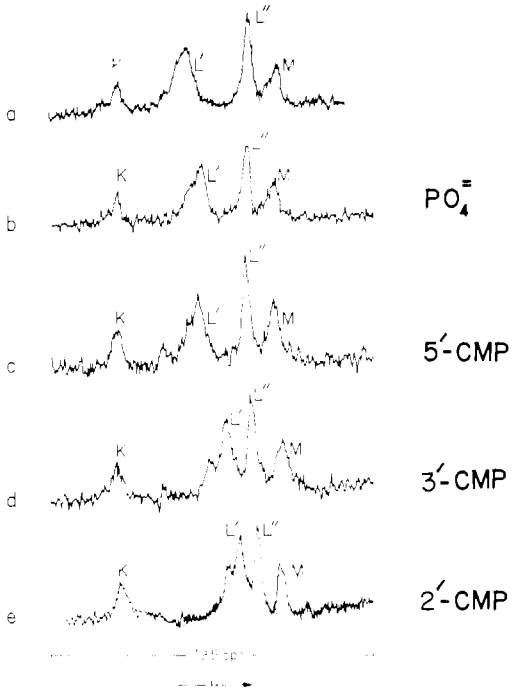

FIGURE 3: ^{19}F-Nuclear magnetic resonance spectra of trifluoroacetylated RNase S (4×10^{-3} M) in the presence of various inhibitors: (a) free RNase S; (b) $+10^{-2}$ M phosphate; (c) $+4 \times 10^{-3}$ M 5′-CMP; (d) $+4 \times 10^{-3}$ M 3′-CMP; (e) $+4 \times 10^{-3}$ M 2′-CMP.

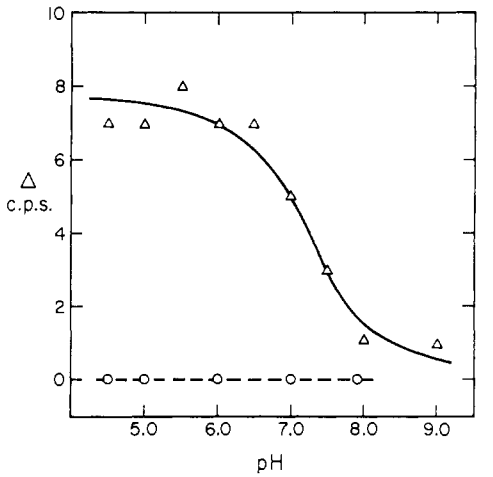

FIGURE 4: Chemical shift of ε-trifluoroacetyl Lys-7 (peak L′) on binding of 3-CMP as a function of pH: (○) position of peak L′ in free RNase S; (△) position of peak L′ in RNase S saturated with 3′-CMP. The solid line is the theoretical titration curve of an ionizable group of pK 7.25.

A molecular interpretation of the spectral effects observed upon association of the various inhibitors with the trifluoroacetylated RNase S is difficult due to the complexity of the system but nonetheless desirable. In order to facilitate such an analysis, the effect of pH on the observed spectral changes was undertaken. The inhibitor chosen for this study was cytidine 3′-phosphate since it most closely resembles the product of the enzyme's action on RNA. The results of this study are shown in Figure 4 and they show that resonance L′ (due to the trifluoroacetyl group on Lys-7) is affected by an ionizable group of pK_a 7.2–7.3 on the enzyme–inhibitor complex. In the absence of the inhibitor, no pH effects in the range 4.5–7.8 were observed.

Trifluoroacetylated RNase S was assayed for enzymatic activity simultaneously with a sample of unmodified RNase S, each being prepared by combination of RNase S-peptide and S-protein in a weight ratio of 1:6. Their activities were identical within the accuracy of the technique; both yielded 110 Kunitz units of activity per milligram of enzyme.

Discussion

The objective of the experiments described here was to devise a technique for detecting the small conformation changes often invoked to explain the kinetics and specificities of enzyme reactions. In the model case, RNase S, a fluorine magnetic resonance probe has been employed to seek the occurrence of conformation changes upon enzyme–inhibitor association.

It was possible, by comparison with model compounds, to assign all the observed ^{19}F resonances in the spectrum of trifluoroacetylated S-peptide and to further monitor all such resonances upon association with the S-protein. Thus nuclear magnetic resonance probes were available for the magnetic environments of lysine residues 1 (both α and ε groups) and 7. The chemical environments occupied by these two residues have been delineated for crystalline RNase S (Wyckoff *et al.*, 1967). Lys-1 lies exposed to the solvent, near the surface of the protein, while Lys-7 is inside, close to the active site. In addition to the crystal structure data chemical evidence (Marfey *et al.*, 1965) has suggested that Lys-7 lies very near the active site but does not participate in catalysis or binding. The X-ray data also have shown that in native RNase-S Lys-7 is found in an α-helical segment with His-12, which has been shown by chemical and ^{1}H-nuclear magnetic resonance evidence to be involved in, though perhaps not essential to, inhibitor binding (Crestfield *et al.*, 1963; Meadows *et al.*, 1967). Since the trifluoroacetylated enzyme remains fully active it is likely the trifluoroacetylated peptide is bound to the S-protein in a conformation similar to that adopted by unmodified S-peptide.

If inhibitor binding involves movement of His-12, as might be expected in an induced-fit binding process (Koshland, 1953), it is likely that the rod-like helical segment including modified Lys-7 would be moved also. Any consequent changes in the magnetic environment of the side chain would be reflected in chemical shift or line shape changes of peak L′ in the magnetic resonance spectrum.

Toward this end the binding at pH 4.5 of the inhibitors phosphate, 2′-CMP, 3′-CMP, and 5′-CMP was studied, and the results are summarized in Figure 3. Phosphate and 5′-CMP produced identical changes in the probe spectrum, causing a 0.03 ppm upfield shift of L′ while not affecting K, L″, or M. Binding of 2′- and 3′-CMP produced more marked changes in resonance L′, corresponding to trifluoroacetyl Lys-7, than did the binding of phosphate or 5′-CMP. The upfield shifts obtained upon association of these inhibitors were 0.07 and 0.09 ppm, respectively, and the peaks were resolved into two components. These two components of peak L′ may not be unique to the complex with 3′-CMP. The resolution of L′ in other spectra was not optimal due to the time required to accumulate the necessary signal intensity, and it is possible that the apparent width of L′ in such spectra was due in part to the presence of a shoulder on the low-field side of the peak. In spectrum 3d, the magnitude

of the low-field component was similar to that of peaks K and M. It is possible that the α-modified S-peptide associates with S-protein in a slightly different orientation than does exclusively ε-modified S-peptide, so that the environment of Lys-7 is slightly altered. If that is the case the environmental difference appears to be magnified on small molecular weight inhibitor binding, but does not produce measurable activity loss using RNA as substrate. In all cases, the inhibitor molecule induced a change in the magnetic environment of Lys-7 which appears to place it in an environment more like that of Lys-1. Examination of a crystal structure model indicates that if as a result of inhibitor binding His-12 were drawn closer to His-119 and Thr-45 (other constituent amino acid residues of the active site cluster), Lys-7 would be pulled away from the Arg-39 region of the protein and exposed more completely to the solvent. The fact that resonances K, L″, and M do not shift on inhibitor binding does not necessarily show that Lys-1 is immobile. In the crystal, the amino terminal is surrounded by a large open region, and it could possibly be moved about quite freely without experiencing a detectable change in magnetic environment.

The changes in magnetic environment of Lys-7, as evidenced by chemical shifts of resonance L′, could be caused most reasonably by (1) electric field effects due to ionizable groups in either the inhibitors or the protein; (2) ring current effects in the inhibitors or the protein; or (3) magnetic anisotropy of groups adjacent to Lys-7, such groups being part of either the inhibitors or the protein. Any one or all such effects could be due to (1) proximity of the inhibitors to the trifluoroacetyl group of Lys-7 or (2) a conformation change in the enzyme. Ring current effects from bound inhibitors are unlikely as the source of the observed spectral changes since phosphate ion caused a chemical shift of resonance L′. The effect of pH on the chemical shift of the major component of resonance L′ helps distinguish between the remaining possibilities. It was observed that an ionizable group of pK_a 7.2–7.3 was responsible for the observed chemical shift in the enzyme–inhibitor complex formed with 3′-CMP. This is most likely due to the charge on histidine-119 which has been shown (Meadows and Jardetzky, 1968) to have a pK_a of 7.4 when the same inhibitor is complexed with ribonuclease A, while histidine-12 has a pK_a of 8.0 in the complex. In the free enzyme, these residues have pK_a values of 5.8 and 6.2, respectively. In addition, the pK_a of the phosphate in 3′-CMP is 6.0 and this would be lowered upon binding to the highly cationic binding site of RNase S (containing histidine-12, lysine-41, and histidine-119). Thus of the three ionizable groups whose pK_a's lie in the range studied, only the His-119 charge produces a measurable effect on the magnetic environment of Lys-7, and then only in the enzyme–inhibitor complex. A calculation from the coordinates of Wyckoff *et al.* (1970) reveals that His-119 imidazole nitrogens would be 10–11 Å away from a trifluoroacetyl group on Lys-7, while those of His-12 would be 12–13 Å distant. While the presence of an inhibitor may decrease the Lys-7–His-119 distance, His-12 (and the phosphate group approximately between the histidines) still should be almost as close to Lys-7 as is His-119. In addition, no change was observed in the chemical shift of the trifluoroacetyl group on Lys-7 in the pH region 8–9 when 3′-CMP was present. This shows that the charge on His-12 has no effect on the observed resonance. It therefore seems unlikely that only His-119 exerts a direct electric field effect on Lys-7. It is also unlikely that the shift of peak L′ is due to hydrogen bonding of some group on the protein to fluorine. Alkyl fluorides hydrogen bond quite poorly as was shown by the proton nuclear magnetic resonance study of Korinek and Schneider (1957). Hydrogen bonding to trifluoroacetate would be expected to be considerably weaker due to electron withdrawal from the fluorines by the carbonyl group.

A more plausible explanation would be that the charged His-119 affects the relative orientations of segments of the protein as they bind the inhibitor. The decrease in the binding constant of 3′-CMP above pH 5.6 has been attributed (Meadows and Jardetzky, 1968) to the titration of histidines 119 and 12. It seems likely that the coulombic attraction between protonated His-119 and the phosphate group of the inhibitor could play an important role in binding of this inhibitor, and that when this bond is broken disruption of the binding site may ensue to some extent. A rearrangement of the peptide segments bearing the active site moieties could well alter the environment of Lys-7; for example, if the distance between His-12 and His-119 were increased as His-119 ceased to be strongly bound to the phosphate, Lys-7 could be carried near the face of the protein cleft near Arg-39. Inspection of RNase S model shows that the trifluoroacetyl group on Lys-7 could be as close as 6 Å to the guanidino group of Arg-39. It would seem then from the ^{19}F-nuclear magnetic resonance data presented here that inhibitor binding causes a small change in conformation of His-12 of the S-peptide which results in its being brought closer to His-119 in the complexed form. When His-119 (pK_a 7.2–7.3) is deprotonated in the complex His-12 (pK_a 8.0 in the complex moves back toward its position in the free enzyme. It should be emphasized that no information is obtainable from the present studies on movements of His-119 since no probe was attached which would monitor such effects.

The use of ^{19}F probes would seem to be a sensitive method for detecting protein conformation change as evidenced from the results obtained from both (a) association of trifluoroacetylated RNase S-peptide with the S-protein and (b) the association with the protein–peptide complex of various inhibitors. While this method is of course restricted to proteins where suitable chemical modifications are possible, its sensitivity should allow detailed investigations of such systems, hopefully permitting interpretation at the atomic level.

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Characterization of Adenyl Cyclase from the Testis of Chinook Salmon*

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ABSTRACT: Adenyl cyclase activity is found both in the tissue slice preparation and in the homogenate of *Oncorhynchus tschawytscha* testis. The enzyme activity in the homogenate proceeds maximally in the presence of 7 mM F^- and 14 mM Mg^{2+}. The requirement for Mg^{2+} could be replaced by Mn^{2+}. Unlike the mammalian adenyl cyclase, the enzyme from *Oncorhynchus tschawytscha* testis can be easily dissociated from low sedimenting cell fraction by homogenization in 0.01 M Tris-HCl (pH 7.5). Approximately 20% of the testicular enzyme activity appears in the soluble fraction. In the presence or absence of F^- and varying concentrations of Mg^{2+}, the testicular adenyl cyclase was not activated *in vitro* by added salmonid gonadotropin. A slice preparation of the testis, on the other hand, readily responded to added salmonid gonadotropin *in vitro* as evidenced by an increased incorporation of [^{14}C]ATP into cyclic [^{14}C]AMP.

Spermatogenesis in salmonid fish is a cystic, cyclical process where the diploid spermatogonial stem cell is differentiated to haploid spermatids and spermatozoa through a series of successive mitotic and meiotic divisions. A characteristic of salmonid spermatogenesis is the preponderance of one particular cell type at different stages of testis maturation which, in principle, allows biochemical characterization of various cellular processes without cell fractionation. From these considerations, we have chosen salmonid testis as a model system for the study of spermatogenesis (Schmidt *et al.*, 1965; Dixon and Smith, 1968). The endocrine control of spermatogenesis in salmonids and its dramatic response to gonadotropin has been established by the pioneer work of Robertson (1958). Another interesting aspect of this system is the replacement of histones by protamine (Miescher, 1874; Alfert, 1956; Ingles *et al.*, 1966) at a late stage of testes maturation. Further, serine residues of protamine from salmonid testis are phosphorylated both *in vivo* and *in vitro* and the latter phosphorylation is stimulated by adenosine 3′,5′-cyclic phosphate (Ingles and Dixon, 1967; Jergil and Dixon, 1970). Cyclic AMP[1] is now recognized as an intracellular messenger for several hormones in their respective target tissues; this subject has been amply reviewed (Sutherland *et al.*, 1965; Robinson *et al.*, 1968). The formation of cyclic AMP from ATP is catalyzed by membrane-bound adenyl cyclase which has been characterized from fat cells (Birnbaumer *et al.*, 1969), frog erythrocytes (Rosen and Rosen, 1969), and guinea pig ventricle (Drummond and Duncan, 1970). The purpose of the present investigation was to characterize the adenyl cyclase in salmonid (*Oncorhynchus tschawytscha*) testis and to study the nature of its control by salmon pituitary gonadotropin. It is hoped that such a study will be valuable in understanding the hormonal regulation of salmonid spermatogenesis at a molecular level.

Materials and Methods

Collection of Testes. Testes were collected from *O. tschawytscha* during their spawning migration along the Fraser River, British Columbia. Soon after collection, the testes were frozen in solid CO_2 and transported to the laboratory where they were subsequently stored at −80°. In those experiments where fresh tissue was used (*e.g.*, activation of adenyl cyclase by gonadotropin), the testes were transported to the laboratory in ice. The average time lag between collection and experimentation was approximately 4 hr. The ratio of testes weight to the body weight ranged from 0.2 to 1.5%. The predominant germ cells in testes of this size are spermatogonia and spermatocytes.

Chemicals. Uniformly labeled [^{14}C]ATP was purchased from New England Nuclear Corp. Ethanol was removed

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[1] Abbreviations used are: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; cyclic AMP, adenosine 3′,5′-cyclic phosphate.