REACTION OF PYRYLIUM SALTS WITH AMINO ACID DERIVATIVES.
PART 3.1 RIBONUCLEASE A INACTIVATION BY TRISUBSTITUTED PYRYLIUM SALTS

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Abstract - Treatment of ribonuclease A with 2,4,6-trisubstituted pyrylium salts completely inhibits the activity of this enzyme. The inactivation is probably due to the reaction of the ε-amino group of the active site Lys 41 residue with the pyrylium cation, leading to pyridinium derivatives which hinder the access of substrates to the active site of the enzyme.

Ribonuclease A, a pancreatic enzyme which digests RNA by hydrolyzing its 3',5'-phosphodiester bonds, was extensively studied due to its stability and abundance, and the possibility to reveal novel data related to polypeptide chain folding. The role of disulfide bonds in stabilizing tertiary structures of proteins, as well as enzymatic mechanisms. Classical studies on chemical modifications of ribonuclease by Anfinsen and Moore and Stein as well as X-ray diffraction crystallographic data of Richards and Wyckoff showed that the catalytic groups of the enzyme involve active site His 119, His 12 and Lys 41 residues. Any modification of these residues, such as carboxymethylation of the imidazole moieties (by iodoacetate), or acylation of the ε-amino group of the Lys 41 (by 2,4-dinitrofluorobenzene) led to inactivation of the enzyme. The study of the chemical modification of ribonuclease by means of pyrylium salts (1, 2 and 3) is presented herein.
Although pyrylium salts have been extensively used in chemical synthesis, due to their versatility and accessibility, only a few reports were published regarding their biochemical applications. O'Leary and Samberg investigated the chemical modification of chymotrypsin by 2,4,6-trimethylpyrylium perchlorate (1) and showed that this compound does not modify the catalytic activity of this serine protease, in spite of the reaction of the ε-amino groups of lysine residues from the three polypeptidic chains of chymotrypsin and the pyrylium cations, leading to pyridinium derivatives. The investigation of the reaction of pyrylium salts with a protein possessing lysine residues involved in the catalytic cycle would be of more interest. The ribonuclease A is such a case, the catalytic triad of chymotrypsin being constituted by His 57, Ser 195 and Asp 102 residues which are not able to react with pyrylium salts. Reaction of bovine ribonuclease A (10^{-3} M) with 2,4,6-trimethyl-(1), 2,6-dimethyl-4-phenyl-(2), and 2,4,6-triphenylpyrylium (3) perchlorates (5 x 10^{-2} M), in phosphate buffer (pH 7), for 72 hours at room temperature, followed by the removal of the excess pyrylium salt from the reaction medium, led to modified enzymes which were assayed for their ability to hydrolyze pentauridylate (U₅). In this assay, the modified enzymes were incubated with 10 mM of U₅ at 37°C for 15 min, and the amount of unhydrolyzed U₅ was determined by hplc. The activity of the modified enzyme (compared to that of the native ribonuclease - considered 100% - since it quantitatively hydrolyzed the substrate) vs the reaction time is shown in Figure 1, for the three compounds investigated.
As one can see, compounds (1) and (2) strongly inactivate ribonuclease (for instance 1 reduced at 50% the activity of the enzyme in approx. 8 hours), whereas (3) only slightly influences its activity. Moreover, (1) reacts more rapidly with the enzyme as compared to (2), but at the end of reaction (in the interval of 40-70 h), the two pyrylium salts were similarly effective in inactivating the enzyme. Inactivation is complete in approx. 70 h, for both (1) and (2).

These observation can be explained by considering the reaction of the ε-amino group of Lys 41 with the pyrylium salts (1) and (2). In a previous paper we reported the preparation of some model compounds, from N-α-acetyl-L-lysine and tri-/tetra-substituted pyrylium salts, in an attempt to mimic the reactivity of the ε-amino moiety of proteins. Although ribonuclease possesses eleven Lys residues in its polypeptic chain, not all of these are exposed to the solvent and readily approachable by different reagents. An exception is, the active site residue Lys 41 (which stabilizes the pentacovalent phosphorus in the transition state for RNA hydrolysis) as well as the N-terminal residue, Lys 1. Although the number of lysine residues which were modified by pyrylium salts, could not be determined, our experiments strongly suggest that at least Lys 41 was altered, its ε-amino group being converted to a trisubstituted-pyridinium moiety, as shown schematically in Figure 2.
This modification can also account for the difference observed between the pyrylium salts (1, 2) on one hand, and (3) on the other hand. Triphenylpyrylium perchlorate was the only compound which did not deactivate ribonuclease in this study, preferably because the rather bulky cation could not reach the active site, in order to react with the ε-amino group of Lys 41 (it is known that the active site cavity of ribonuclease is not very large, only one ribonucleotidic moiety being easily accommodated within it). The sterically less hindered pyrylium salts (1) and (2) on the other hand were able to react in this way, affording the substituted pyridinium derivatives. This modified enzyme being much bulkier than the H₂N⁺-group, normally found in the natural one prevents the access of the substrates to its active site. Although less probable, the possibility of the involvement of other amino groups (from lysine residues or other amino acids) in the inactivation of ribonuclease by pyrylium salts, cannot be ruled out.

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REFERENCES AND NOTES


10. A concentrated ammonia solution was added, and the substituted pyridine formed was extracted with Et₂O. The protein was extensively dialyzed against distilled deionized water for removing inorganic ions.

11. Reagents used: U₅ from Serva (Heidelberg: FRG); bovine ribonuclease from Sigma (St. Louis, MO, USA); 1 - 3 were prepared as described in ref 12.


13. Hplc was carried out on an LDC system, consisting of a Rheodyne pump and injector, and a UV-visible variable detector.

14. Activity was calculated with the formula: 100. (mMoles of unhydrolyzed U₅/10 mMoles).


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