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Kinetic properties of iodinated ribonuclease A

The use of protein chemical modifications to obtain information about the active sites of enzymes is a well-accepted procedure. Modification of groups not at the active site can potentially provide some assessment of the role of the overall macromolecular conformation in catalysis. The three "exposed" tyrosine phenolic groups of ribo nuclease A (ribonucleate nucleotidotransferase), which are not located at the active site, can be diiodinated; the resultant derivative, I_6-ribonuclease, has been reported to retain full catalytic activity[1]. In this communication we report the results of a kinetic investigation of the transesterification and hydrolytic activities of I_6-ribonuclease with cytidylyl 3′:5′-adenosine (CpA) and cytidine 2′:3′ cyclic phosphate (2′:3′-CMP) as substrates. The results obtained indicate that, although these tyrosines are not at the active site, their iodination alters some of the kinetic properties of the enzyme in a substrate-dependent fashion. In addition the iodinated tyrosines serve as an indicator of an isomerization (or conformational change) of the enzyme which had previously been observed with the temperature-jump technique by measurement of pH changes with a colored indicator following the temperature jump[2].

The procedure of WOODY *et al.*[1] was used to prepare I_6-ribonuclease. The derivative was analyzed by the pH-induced difference spectrum and amino acid analysis. In all respects the analysis of the derivative was the same as previously reported[1] (*i.e.* only the three exposed tyrosines were diiodinated, the remaining tyrosines being unaltered), except that the extinction coefficient at 278 nm, pH 4.5 for I_6-ribonuclease was found to be 10 640 $\pm$ 500 $M^{-1} \cdot cm^{-1}$.

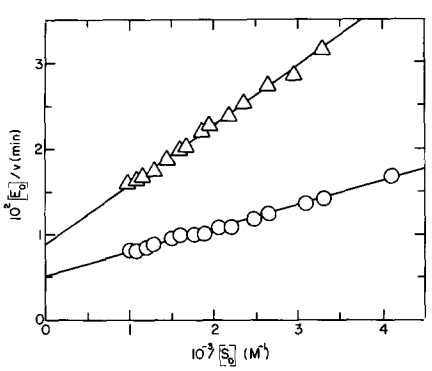
Both ribonuclease and I_6-ribonuclease catalytic activities for transesterification of CpA (Waldhof) and hydrolysis of 2′:3′-CMP (prepared as previously described[3]) were studied at pH 5.5 using a spectrophotometric assay at 290 nm. Plots of the reciprocal initial velocity *versus* reciprocal substrate concentrations are shown in Figs. 1 and 2. The Michaelis constants, K_m, and molecular activities, evaluated from the data in the figures by a weighted least-squares analysis are presented in Table I. (An error of $\pm$ 5% was assumed in the initial velocity.) The evaluation of $(E_0)/v$ for the CpA substrate utilized the same difference extinction coefficient (1150 $M^{-1} \cdot cm^{-1}$)

TABLE I

STEADY-STATE KINETIC CONSTANTS FOR RIBONUCLEASE AND I_6-RIBONUCLEASE

Constants were evaluated by a weighted least-squares analysis of the data in Fig. 1.

Substrate	$K_m \times 10^4$ *(M)*	*Molecular activity* (sec^{-1})
CpA		
I_6-ribonuclease	14.1	2420
Ribonuclease	10.3	2810
2′:3′-CMP		
I_6-ribonuclease	8.01	1.88
Ribonuclease	5.10	3.15



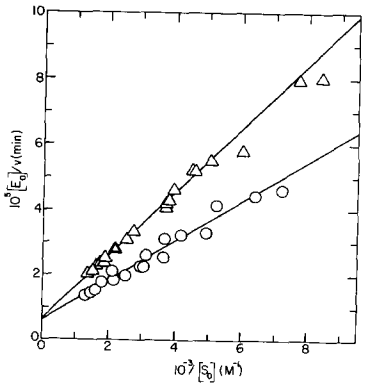


Fig. 1. Double reciprocal plot for I_6-ribonuclease (△) and ribonuclease (○) using 2′:3′-CMP as substrate. Each experimental point is the average of duplicate determinations. The enzyme concentration was $6.36 \cdot 10^{-8}$ M. Initial velocities were determined using a Cary 14 spectrophotometer at 290 nm, 0.2 mm slit width. Solutions at 25° were 0.05 M Tris ,0.05 M sodium acetate and 0.10 M KCl brought to pH 5.5 with acetic acid.

Fig. 2. Double reciprocal plot for I_6-ribonuclease (△) and ribonuclease (○) using CpA as substrate. Each experimental point is the average of duplicate determinations. The enzyme concentration was $1.55 \cdot 10^{-10}$ M. Initial velocities were determined using a Cary 14 spectrophotometer at 290 nM, 0.2 mm slit width. Solutions at 25° were 0.05 M Tris, 0.05 M sodium acetate, and 0.10 M KCl brought to pH 5.5 with acetic acid.

at 290 nm as was experimentally determined for 2′:3′-CMP and 3′-CMP at pH 5.5. The estimated error in molecular activity is ± 10% and that in K_m is ± 15%. The molecular activity values for CpA transesterification are the same within experimental error for ribonuclease and I_6-ribonuclease which corroborates the report of 100% activity for depolymerization of RNA (ref. 1). For hydrolysis of the 2′:3′-CMP substrate, contrary to the results for transesterification, the molecular activity is significantly lower for I_6-ribonuclease than for ribonuclease. For both substrates K_m is larger for I_6-ribonuclease than for ribonuclease. The values of K_m and molecular activity in Table I are in good agreement with those previously reported for the ribonuclease catalysis using CpA (ref. 4) and 2′:3′-CMP (ref. 5) substrates at pH 5.5. The altered kinetic parameters of I_6-ribonuclease could result from perturbations of the active site by introduction of two bulky iodine atoms on tyrosine 115. This residue lies most adjacent to the active site of the "exposed" tyrosine groups[6,7] and is critical in the refolding of oxidized ribonuclease[8]. The values of molecular activity for CpA transesterification are three orders of magnitude larger than those for 2′:3′-CMP hydrolysis, which could imply that the rate-limiting step may be different for CpA and 2′:3′-CMP. This could explain why the molecular activity for I_6-ribonuclease is altered with 2′:3′-CMP as substrate but not with CpA. For example, the molecular activity for CpA is large enough so that a conformational change which is not perturbed by iodination could be rate limiting[2,9], whereas the molecular activity for 2′:3′-CMP could represent the bond-breaking step which is perturbed by iodination. Nevertheless, these interpretations must be accepted with caution since other explanations are certainly possible.

Temperature-jump experiments were performed using an apparatus previously described[9,10]. Solutions containing $6.8 \cdot 10^{-5}$ M I_6-ribonuclease and 0.2 M KCl exhibited

relaxation effects only at 310 ± 10 nm which is the maximum of the pH-induced difference spectra of I_6-ribonuclease. The absorption changes in temperature-jump experiments indicated a rapid release of protons from the diiodinated tyrosine side chains followed by a slower uptake of protons by these groups. The fast process was concomitant with the heating of the solution. The slower process had a relatively small amplitude and could be accurately measured only in the neighborhood of pH 6. The relaxation time characterizing this process was 501 ± 62 μsec at pH 6.0 and 572 ± 74 μsec at pH 6.3. These values are the same within experimental error as the corresponding values previously reported for τ_1 in studies using pH-indicators[2]. This result corroborates the previous interpretation that τ_1 represents an isomerization of the free enzyme. Furthermore, diiodinating the three "exposed" tyrosine side chains has no obvious effect on the dynamics of the isomerization process at the pH values tested.

These results demonstrate that the iodination of tyrosines not at the active site can have subtle effects on the catalytic activity of the enzyme, presumably through alteration of the three-dimensional structure of the enzyme. Moreover, the diiodinated tyrosines serve as an indicator of an isomerization (or conformational change) of the enzyme.

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