BOVINE SEMINAL RIBONUCLEASE: NON-HYPERBOLIC KINETICS IN THE SECOND REACTION STEP

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1. Introduction

Bovine seminal ribonuclease is a dimeric enzyme [1] with interchain disulfides linking the 2 subunits [2,3]. It can be isolated from bull seminal plasma [4] or from bull seminal vesicles [5,6], the organ where it is produced. The primary structure of the subunit chain [1,7–9] is strictly homologous to that of pancreatic RNase A from the same species. Especially interesting is the observation that the amino acid residues which were found to interact with the substrate at the active site of RNase A [10] are all conserved at identical sequence positions in BS-RNase [1,7,8]. Hence, it is not surprising that BS-RNase has the same 2-step mode of action (transphosphorolysis followed by hydrolysis of the cyclic phosphate) and the same bond specificity as RNase A [11].

Investigating both the reaction steps catalysed by BS-RNase with appropriate model substrates, we found that the enzyme displays non-hyperbolic saturation curves, but only for the substrate of the second, rate-limiting step of reaction. This phenomenon appeared overlooked in [12] for the limited range of substrate concentrations used. Non-hyperbolic saturation curves have been occasionally reported [13,14] for a small number of oligomeric enzymes, but to our knowledge never for a dimeric enzyme.

Abbreviations: BS-RNase, bovine seminal ribonuclease; RNase A, bovine pancreatic RNase A; Cyd-*P*–Cyd, cytidine-3′-phosphate–5′-cytidine; cyd-2′,3′-*P*(cyclic), cytidine-2′:3′-phosphate (cyclic)

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2. Materials and methods

BS-RNase was prepared from bull seminal plasma [4] and bull seminal vesicles [6] as described. The enzyme preparations used in these experiments were homogeneous by polyacrylamide gel electrophoresis in the presence [1] and absence [4] of SDS and by amino acid analysis [4]. The substrates, cyd-*P*–cyd and cyd-2′,3′-*P*(cyclic), purchased from Sigma (St Louis MO) were checked for their purity by spectrophotometry and paper chromatography.

The enzyme was assayed by:

(i) Spectrophotometric methods [15,16] with a Cary 210 spectrophotometer equipped with cells of various pathlengths and a cell compartment thermostatted at 30°C; the assays were carried out in 0.1 M Tris–HCl (pH 7.3); the reaction with cyd-*P*–cyd was followed at 294 nm, using a molar extinction coefficient $\epsilon = 1930$; that with cyd-2′,3′-*P*(cyclic) at 290 nm, with $\epsilon = 1217$.

(ii) A pH-stat method [17] with a Radiometer pH-M82 pH-meter equipped with a TTT80 titrator, a ABU80 autoburette, a 80 Servograph recorder, a G-2040 C glass electrode and a titration cell thermostatted at 23°C and kept under constant flux of N_2. The reaction mixture contained 0.2 M NaCl.

Linearity of the kinetic progress curves up to ~1 min of reaction was checked for each substrate concentration assayed. The enzyme concentration used in the assay (0.18 μM) was in the range of linearity of catalytic rate with enzyme concentration.

3. Results

Fig.1 shows the kinetic saturation curves of BS-RNase for cyd-*P*–cyd as a model substrate of the

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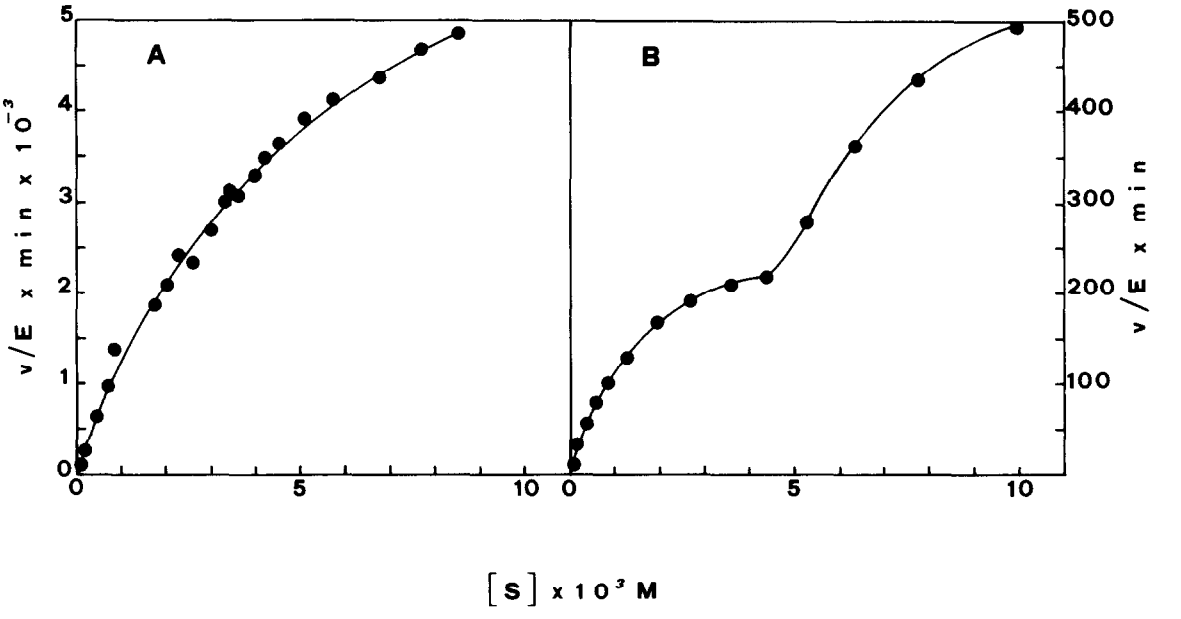

Fig.1. Saturation curves of BS-RNase for cyd-*P*–cyd (A) and for cyd-2′,3′-*P*(cyclic) (B). Initial velocities were determined by spectrophotometric assays in 0.1 M Tris–HCl (pH 7.3) at 30°C. The enzyme concentration E was 1.8×10^{-7} M.

first, transphosphorolytic reaction step (fig.1A), and for cyd-2′,3′-*P*(cyclic) as a model substrate for the second, hydrolytic step (fig.1B). The results indicate that the transphosphorolytic step is catalysed by BS-RNase following classical hyperbolic kinetics up to the substrate concentration tested (10 mM), which is one order of magnitude higher than that tested in [12]. Linear double-reciprocal plots were obtained with the experimental data, from which values of the kinetic parameters were calculated. For an easy comparison of the catalytic properties of the active sites of dimeric BS-RNase and of monomeric RNase A, assayed under identical experimental conditions, catalytic constants are given as turnover numbers: $T_n = k_{cat}/n$, where n is the number of catalytic sites. The values obtained were: for BS-RNase, K_M 8 mM; T_n 83.3 s^{-1}; for RNase A, K_M 5.5 mM, T_n 54.8.

Our investigation of the second step of the reaction gave different results. At low substrate concentrations the saturation curve for cyd-2′,3′-*P*(cyclic) appears to be hyperbolic, although an increasing flattening is evident with increasing substrate concentrations, until a plateau region is reached; at higher levels of substrate the curve rises again in a sigmoidal fashion (fig.1B).

The experiment, carried out with a spectrophotometric assay, was repeated with a different methodology, measuring with a pH-stat the rates of proton release upon hydrolysis of the phosphate cycle by the enzyme. In this experiment we used a preparation of BS-RNase from seminal vesicles and carried out parallel assays with RNase A. The results in fig.2 confirm that the saturation curve of BS-RNase for cyd-2′,3′-*P*(cyclic) is non-hyperbolic and show how RNase A, tested under identical conditions, displays a classical hyperbolic curve for the same substrate. The values of T_n and K_M, obtained from data collected with both assay methods, were computed from double-reciprocal plots, which in the case of BS-RNase were clearly non-linear. An apparent value for V_{max} was taken from the intercept on the velocities axis of the extrapolated extreme portion of the curve. This appeared to be linear over 5–10 mM substrate, and best fits by the least squares method were obtained with excellent correlation coefficients. The values determined for BS-RNase (K_M 19.6 mM, T_n 13.9 s^{-1}) were of the same order as those obtained for RNase A, (K_M 17.6 mM, T_n 22.7 s^{-1}), which were in turn comparable to the values obtained under similar conditions [18].

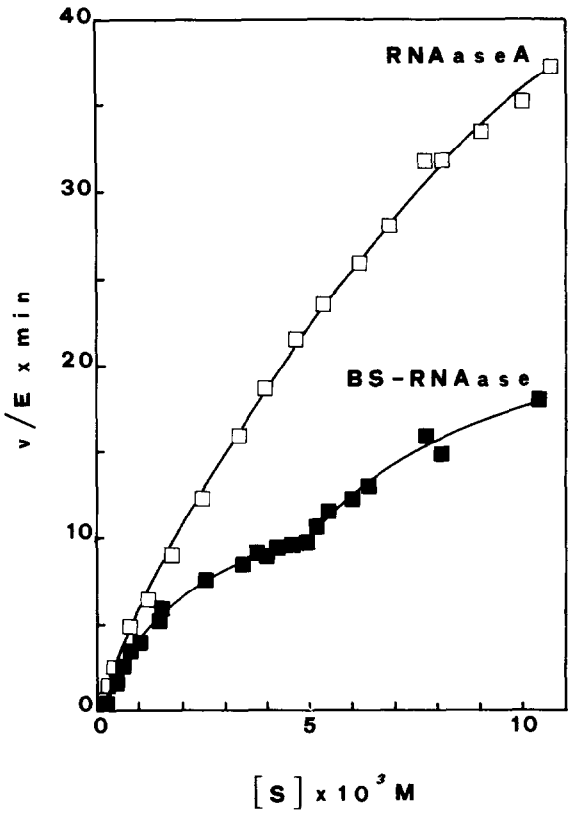

Fig.2. Saturation curves of BS-RNase and RNase A for cyd-2′,3′-*P*(cyclic). Initial velocities were determined with a pH-stat method at pH 7.13, ionic strength 0.2, at 23°C. BS-RNase was 5 μg/ml and RNase A 3.5 μg/ml. Velocity values are plotted as μmol substrate consumed . min^{-1} . mg enzyme $protein^{-1}$.

4. Discussion

The kinetic data presented here indicate that the transphosphorolytic step of the reaction catalysed by BS-RNase follows typical Michaelian kinetics. The hydrolytic step, which is found to be rate-limiting, is catalysed with non-hyperbolic saturation curves for the substrate. This phenomenon was probably not detected in [12], as a rather limited range of substrate concentrations was investigated: 0.04–0.3 mM, compared with 0.1–10 mM here.

Non-hyperbolic saturation curves were first discussed in [13] and have been found for another tetrameric enzyme [14]. They may be produced by the presence in the reaction mixture of isoenzymes catalysing the same reaction with different rates [13,19]. BS-RNase, as isolated [20], is constituted by a set of isoenzymes (α_2, $\alpha\beta$, β_2), generated through the association of 2 types of subunits: α and β. The α-subunit differs from the β-subunit for the absence of a single amide group [20]. Recent results (unpublished) indicate that the amide group in question is that of Asn_{67}. This residue is far away from the subunit active site and also from the intersubunit region, as shown by the tri-dimensional structure of the protein [21]. Thus it would seem very unlikely that the side chain of this residue could affect the catalytic act of the reaction, without significantly affecting the K_M, i.e., the binding of substrate.

Another possible basis for non-hyperbolic saturation curves could be the slow isomerization of the enzyme between conformers of different catalytic activities. These hysteretic effects [22,23] would be induced by increasing concentrations of substrate, binding at the subunit active site. However, we did not observe any deviations from linearity in the kinetic progress curves (from a few seconds to over a minute) obtained with BS-RNase at all substrate concentrations tested.

Another, more likely hypothesis can be advanced, based on the available data. BS-RNase may represent an enzyme subjected, in the rate-limiting step of the reaction, to regulation of its activity through allosteric interactions. These would involve the substrate and/or the product, generating at low concentrations negatively co-operative effects, while at higher concentrations positively co-operative effects would be promoted. This hypothesis requires [13] that for a dimeric enzyme, extra site(s) for the effectors be present on the enzyme molecule, besides the two active sites. Extra sites may not be unlikely on an enzyme molecule which interacts with a polymeric substrate, such as RNA. They have been identified on RNase A [17,24] and considered as the basis for interpreting some unusual kinetic properties of the enzyme [17].

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