Co-operativity in seminal ribonuclease function: binding studies

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Binding of nucleotides to bovine seminal RNAase was studied by differential spectrophotometry and equilibrium dialysis. Cytidine 3'-phosphate, the reaction product of the hydrolytic, rate-limiting step of the reaction, was found to be capable, in contrast to related nucleotides, of discriminating between the two structurally identical active sites of the enzyme. Negative co-operativity, with a 'half-of-sites' reactivity, was found at lower concentrations of ligand, whereas at higher concentrations positive co-operativity was detected. These findings exclude that the non-hyperbolic kinetics previously reported for the hydrolytic step of the reaction are due to hysteretic effect. A model of mixed-type co-operativity is proposed for interpreting the binding data.

INTRODUCTION

Studies on structure to function relationships in enzymes have taken great advantage from the comparative analysis of homologous proteins, generated through speciation or differentiation. Bovine seminal RNAase (BS-RNAase) [1] is a dimeric ribonuclease isolated from bovine seminal plasma or seminal vesicles as a set of isoenzymes (α_2 , $\alpha\beta$ and β_2) made up of two types of subunits, α and β , differing from each other by a single amide group [2]. The dimeric structure is maintained by two intersubunit disulphide bonds, as well as by non-covalent interactions [3–5]. Bovine pancreatic RNAase A, one of the most favoured model proteins, is extensively homologous to the subunit of BS-RNAase [1,6].

BS-RNAase cleaves polyribonucleotides, just like RNAase A, through a transphosphorolytic step generating terminal cyclic 2',3'-phosphate nucleotides, followed by a hydrolytic step in which the cyclic phosphates are opened to yield 3'-phosphates [7]. Bond specificity is virtually identical with that of RNAase A [7]. These results are not surprising, as sequence studies have revealed that the amino acid residues interacting with the substrate at the active site of RNAase A [6] are all perfectly conserved in BS-RNAase [8]. It should be added that BS-RNAase is also capable of cleaving double-stranded RNA and the RNA chain in DNA·RNA hybrids under conditions in which these substrates are not cleaved by RNAase A [9,10]. More recently, it has been found that, although the first transphosphorolytic step of the reaction is catalysed by BS-RNAase following classical Michaelis kinetics, in the second, rate-limiting step, non-hyperbolic kinetics are observed [11]. Interestingly, monomerization of dimeric seminal RNAase abolishes these effects, and dimerization of pancreatic RNAase A generates an enzyme with non-hyperbolic kinetics [12]. Recently it has been found, by probing BS-RNAase with active site directed reagents, that 'half-of-sites' reactivity occurs when 2'(3')-O-bromoacetyluridine reacts with His¹² at the active site of the enzyme [13].

In this paper we present the results of binding studies carried out on BS-RNAase by differential spectrophotometry and equilibrium dialysis, with nucleotides as ligands. Our data indicate that only the reaction product of the rate-limiting hydrolytic step, in contrast to related nucleotides, is capable of functionally discriminating between the two structurally identical binding sites on the two subunits of the enzyme. At low concentrations of the reaction product BS-RNAase displays a 'half-of-sites' reactivity whereas at higher concentrations positive co-operativity of binding is detected.

EXPERIMENTAL

BS-RNAase was prepared from bull semen or seminal vesicles as described before [14]. Homogeneous subform α_2 was separated according to [2]. Monomeric bis-S-carboxymethyl-Cys³¹,Cys³²-BS-RNAase was prepared as previously described [3].

[U-¹⁴C]Adenosine 5'-monophosphate (555 mCi/mmol) and [U-¹⁴C]cytidine 5'-monophosphate (517 mCi/mmol) were from The Radiochemical Centre (Amersham, U.K.). [¹⁴C]Cyd-2'-P and Cyd-3'-P isomers were prepared by hydrolysing [U-¹⁴C]polycytidylic acid (Schwarz-Mann, U.S.A.) in 1 M-NaOH for 20 h at 37 °C. The isomers were separated by chromatography on Dowex 1-X8 eluted with 0.025 M-ammonium formate buffer, pH 4.3.

Ado-5'-P, Cyd-2'-P and Cyd-3'-P, Urd-2'-P and Urd-3'-P, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Their purity was checked by spectrophotometry [15] and paper chromatography [16].

Equilibrium dialysis experiments were carried out in home-made Perspex cells made in halves, each containing 250 μ l as total volume and separated by a membrane of Visking tubing 32/32, previous boiled for 30 min in 5% Na₂CO₃, containing 50 mM-Na₂EDTA. The protein and the ligands, dissolved in the appropriate buffers, were introduced in the cells and allowed to equilibrate under rotation (10 rev./min) in a double-walled Perspex cylinder, kept at constant temperature by circulating water between the walls. After a series of preliminary

Abbreviations used: RNAase A, bovine pancreatic ribonuclease; BS-RNAase, bovine seminal ribonuclease; M(S-alkyl)₂, monomeric bis-S-carboxymethyl-Cys³¹,Cys³²-BS-RNAase.

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experiments, equilibrium times were set at 8 h at 25 °C and 3 h at 38 °C. After equilibrium, triplicate 50 μ l portions were withdrawn from each chamber and counted for radioactivity in 3 ml of Lumagel (Lumac, Milano, Italy), with 1 ml of water to avoid microprecipitation. Standard deviation, calculated for each averaged data point, did not exceed 5% of the mean. Protein concentration was determined by amino acid analysis. Controls for non-specific adsorption to the cells or the dialysis membrane, carried out with labelled nucleotides and labelled enzyme prepared as described before [17], were negative.

Difference spectra were determined directly on a Cary 118C spectrophotometer, with a 0.6 mm slit width. Double compartment cells with a 0.438 mm light path for each compartment were used in a thermostatted cell holder.

RESULTS

Spectrophotometric studies

The difference spectra between the binding complexes of BS-RNAase with pyrimidine nucleotides and free nucleotides plus free enzyme were found to be very similar to those recorded for the binding complexes of RNAase A with the same nucleotides under the same conditions [18–21]. Likewise, the pH dependence of the interaction of Cyd-3'-P and Cyd-2'-P with BS-RNAase, measured by plotting as a function of pH the absorbance differences at three different wavelengths with an excess of ligand, indicated great qualitative similarity between the binding interactions of these nucleotides with pancreatic [18,21] and seminal ribonucleases (data not shown).

The stoichiometry of binding was studied with the

Fig. 1. Plot of difference absorbance as a function of the relative concentrations of BS-RNAase and cytidine nucleotides

Experiments were carried out in 0.1 M-sodium acetate buffer, pH 5.0, at 25 °C. The total concentration of BS-RNAase subunits plus nucleotide was 120 μ M in the experiment with Cyd-2'-P (a) and 170 μ M in that with Cyd-3'-P (b).

method of continuous variations [22]. Difference absorbance values at 260 nm for Cyd-2'-P and at 267 nm for Cyd-3'-P were measured at pH 5 for a series of solutions in which the total concentration of BS-RNAase subunits plus nucleotide was held constant, while the relative concentrations of ligand and enzyme were varied. The total concentration of BS-RNAase subunits plus nucleotide was 120 μ M in the experiment with Cyd-2'-P and 170 μ M in that with Cyd-3'-P. The results of the experiments are illustrated in Fig. 1. Binding of Cyd-2'-P produced the maximal difference absorbance with a 1:1 stoichiometry, and the curve shown in Fig. 1(a) is a parabola, i.e. symmetrical within the experimental error. This result indicates that BS-RNAase possesses two binding sites for Cyd-2'-P and that the sites are equivalent and non-interacting. The values of the association constant and of the differential absorption coefficient were determined after a successive optimization procedure, based on the following equation:

$$K = \frac{\Delta A / \Delta \epsilon}{(E_0 - \Delta A / \Delta \epsilon)(L_0 - \Delta A / \Delta \epsilon)} \quad (1)$$

where $\Delta A / \Delta \epsilon$ is the ratio between the difference absorbance at a given wavelength and the molar difference absorption coefficient at that wavelength; E_0 and L_0 are the concentrations of total enzyme and total ligand, respectively. The value of $\Delta \epsilon$ at 260 nm, where the difference absorbance was maximal, was found to be 2800 $\text{cm}^{-1} \text{M}^{-1}$. The value of K was $1.3 \times 10^5 \text{M}^{-1}$. This value agrees well with the reciprocal of the inhibition constant calculated from kinetics studies with Cyd-2'-P as an inhibitor (unpublished results). It is also very similar to the values determined by other investigators for the binding of Cyd-2'-P to RNAase A [20,21].

In conclusion, these results indicate that each BS-RNAase subunit possesses an independent binding site for Cyd-2'-P, which is very similar to the binding site of RNAase A for the same nucleotide.

Different results were found when the binding of Cyd-3'-P was analysed with the same experimental approach. Fig. 1(b) shows that the curve which fits the experimental points is not symmetrical as in the case of Cyd-2'-P. The maximal absorbance difference is somewhat lower and is not centred at the 1:1 ratio between the concentrations of sites and ligand, but rather shifted towards lower ratios of nucleotide to enzyme. This suggested non-equivalence among binding sites or, alternatively, interactions among them, such that binding at the second site was negatively influenced by binding at the first site.

These effects excluded [22] application to the case of Cyd-3'-P of the procedure used for the analysis of the binding of Cyd-2'-P to BS-RNAase. Attempts to adapt eqn. (1) met with no success as, without a procedure for the determination of $\Delta \epsilon$, the difference absorbance values measured at various ratios of ligand to enzyme could not be normalized as fractional values of total binding.

Equilibrium dialysis studies

The data collected from studies of the binding of Cyd-2'-P, Ado-5'-P and Cyd-5'-P to BS-RNAase with the equilibrium dialysis method (see Table 1), plotted according to the Scatchard equation, gave linear plots. This indicates that two independent and equivalent binding sites for these nucleotides are present on BS-RNAase. Hill plots of the same data confirmed this

Table 1. Binding parameters for the interaction of BS-RNAase to nucleotides

Enzyme	Ligand	pH	<i>T</i> (°C)	$10^{-4} \times K$ (M ⁻¹)	$10^{-4} \times K_{S1}$ (M ⁻¹)	$10^{-4} \times K_{S2}$ (M ⁻¹)
BS-RNAase*	Cyd-2'- <i>P</i>	5.0	25	13.0		
BS-RNAase	Cyd-2'- <i>P</i>	5.0	25	12.2		
BS-RNAase	Cyd-2'- <i>P</i>	7.3	38	0.6		
BS-RNAase	Cyd-3'- <i>P</i>	5.0	38	—	2.20	0.013
α_2 -BS-RNAase	Cyd-3'- <i>P</i>	5.0	38	—	1.7	0.011
M(S-alkyl) ₂	Cyd-3'- <i>P</i>	5.0	38	0.83		
BS-RNAase	Cyd-5'- <i>P</i>	5.0	38	0.05		
BS-RNAase	Ado-5'- <i>P</i>	5.0	38	0.14		

* Data collected from spectrophotometry experiments. All other data have been obtained by equilibrium dialysis experiments.

indication, as straight lines were obtained with unity values for the Hill coefficients.

The value of the association constant measured for Cyd-2'-*P* at pH 5 agrees favourably with the value determined by difference spectrophotometry (see Table 1). A slightly lower value was obtained for the binding at pH 7.3. Interestingly, both values are comparable with those reported for the binding of Cyd-2'-*P* to RNAase A [20,21], thus confirming the results presented above from the studies of difference spectrophotometry.

Lower values of association constants, with respect to the values measured for Cyd-2'-*P*, were found for the 5' isomers of cytidine and adenosine monophosphate (see Table 1). Also, in the case of these nucleotides, a comparison of the *K* values with those available in the literature for RNAase A [18,21] indicates that the binding sites on BS-RNAase and on RNAase A have comparable affinities for these nucleotides.

The results of the binding experiments performed with Cyd-3'-*P* appeared more interesting. When the ratio *r* of bound ligand to total enzyme was plotted versus the concentration of total ligand, a biphasic saturation curve was obtained. As shown in Fig. 2(a), a plateau is evident at a ratio of 1 mol of ligand bound/mol of dimeric enzyme; at higher concentrations the curve 'bumps up' again, while other site(s) is (are) titrated. When the data were plotted according to Klotz [23], as illustrated in Fig. 2(b), a slope of 0.58 was calculated at the inflection point of the curve below the plateau, indicating [24] that negative co-operativity occurs at this concentration range.

For the region of higher ligand concentrations, data could not be collected at ratios of bound ligand to enzyme higher than 1.4/1 (mol/mol). This was due to the weak binding affinity of the site(s) titrated at this concentration range, which excluded, for the determination of the concentration of bound ligand, both the use of spectrophotometric techniques (since the ΔA values were too low compared with the experimental error) and radiometric techniques (since radioactive ligand with higher specific activity was not available). However, the data as collected indicate that, beyond the plateau region, a change of trend occurs in the binding process. Although a slope could not be calculated, as an inflection point was not reached, the steepness of the curve strongly suggests (see Figs. 2a and 2b) that beyond the plateau the enzyme binding capacity for the ligand has increased and that in the Klotz plot the slope value for this curve is much higher than 1. This is a clear indication of positive co-operativity in binding.

Fig. 2. Binding of Cyd-3'-P to BS-RNAase

The data from equilibrium dialysis experiments, carried out at 38 °C in 0.1 M-sodium acetate buffer, pH 5.0, are plotted (a) as in a typical binding isotherm, and according to the Klotz equation (b), the Scatchard equation (c) and the Hill equation (d). In each plot two experiments are shown, carried out with total BS-RNAase (○), 51.6 μM final concentration, or α_2 -BS-RNAase (●), 40.4 μM final concentration.

The Scatchard plot constructed with the same binding data is not linear, showing an upward concavity (see Fig. 2c). Hence the analyses of the binding data, with both the Klotz and the Scatchard plots, indicate that Cyd-3'-*P* binds on BS-RNAase to non-identical binding sites or, alternatively, that the sites are identical, but negative co-operativity occurs.

The possibility that the anomalous binding curves could be explained in terms of heterogeneity of sites was considered. The enzyme as isolated, i.e. as it has been used in the experiments reported here, is not homogeneous [2] but is a mixture of three isoenzymes (α_2 , $\alpha\beta$ and β_2) generated by association of two types of subunits (α and β) differing from each other by a single amide group [2]. We thus carried out binding experiments with

homogeneous preparations of the α_2 isoenzyme, the least represented (10%) and the most stable of the three forms [2]. The results fitted the curve obtained with the heterogeneous enzyme (see Fig. 2 and Table 1), indicating that the heterogeneity due to the presence of different isoenzymes in the binding reaction mixture had no effect on binding.

Hence, assuming two initially identical binding sites, the plots of Figs. 2(b) and 2(c) could be interpreted as describing negative co-operativity effects. Two stoichiometric constants were calculated from the Scatchard plot as described by Klotz & Huston [23]: $K_{S1} = (1.98 \pm 0.2) \times 10^4 \text{ M}^{-1}$ and $K_{S2} = (0.012 \pm 0.001) \times 10^4 \text{ M}^{-1}$. It should be noted that the latter value is not as defensible as the former, considering that full saturation by the ligand was not reached (see above). A value for K was calculated also from the Klotz plot [23] and found to be similar, within the experimental error: $K_{S1} = (1.69 \pm 0.18) \times 10^4 \text{ M}^{-1}$.

The Cyd-3'-P binding data were also analysed with another diagnostic tool [25], the Hill equation. Also in this case an r_{max} value had to be assumed. The plot illustrated in Fig. 2(d), constructed assuming a maximum number of two binding sites, shows a region of negative co-operativity (with an average value for the Hill coefficient lower than 1), followed, after an abrupt transition, by a region with an apparent positive co-operativity, for which an average Hill coefficient higher than 1 can be approximated. No values for the constants could be calculated, as the experimental system did not allow the inspection of binding at very low and very high ligand concentrations.

We also studied the binding of Cyd-3'-P to a stable, fully active monomeric derivative of BS-RNAase, obtained by selective reduction followed by alkylation of the intersubunit disulphide bridges [3]. No co-operativity effects were detected when the binding data from these experiments were plotted according to the Scatchard and the Hill equations (data not shown). An association constant of $0.83 \times 10^4 \text{ M}^{-1}$ was calculated (see Table 1).

DISCUSSION

All spectrophotometric and equilibrium dialysis data on the interactions of BS-RNAase with Cyd-2'-P, Ado-5'-P and Cyd-5'-P lead to the same conclusion: two independent and non-interacting binding sites are available to these nucleotides on dimeric BS-RNAase (Table 1). Moreover, in the case of pyrimidine nucleotides, the values of the constants, the difference spectra and their dependence on pH, reveal striking similarities between the binding processes of pancreatic and seminal RNAases. Hence, they suggest that these nucleotides interact with the two enzyme molecules at very similar binding sites.

However, when the binding of Cyd-3'-P is studied quantitatively, a different picture emerges, as the interaction of the seminal enzyme with this nucleotide has distinctive features, not detectable in the binding pattern of pancreatic RNAase A to the same nucleotide. The interaction of BS-RNAase with Cyd-3'-P is described by anomalous binding curves (Figs. 1 and 2), and a clear stoichiometry of the binding is not easily derived from the available data. The possibility that these results are due to structural differences among the sites binding Cyd-3'-P can be safely neglected. Such differ-

ences would very likely affect also the binding of the Cyd-2'-P isomer and of the other nucleotides. Moreover, in proton n.m.r. studies on the active site histidine residues, no evidence of differences was detected between the two active sites of the two subunits of the enzyme [26]. No heterogeneity was found after structural studies on the enzyme [5,8], with the exception of the presence on the β -type subunit of an amide residue, whereas on the α -type subunit an acidic residue is present [2]. However, this heterogeneity does not affect binding, as the data reported here and obtained with homogeneous preparations of subform α_2 are superimposable upon those collected with enzyme preparations containing all three subforms. These results exclude also another possibility: that the anomalous binding curves are due to the simultaneous presence in the binding mixture of two or more isoenzymes, one of them with positively co-operative behaviour, the other(s) with negatively co-operative or non-co-operative behaviour [27].

'Bumpy' saturation curves, i.e. curves with abrupt transitions, have been found previously in kinetic and binding studies on several enzymes [27-30], including BS-RNAase [11] and dimeric RNAase A [12]. Analyses of these curves have led to the conclusion that they are indicative of mixed types of co-operativity [27,28,31,32]. In the present case, the Klotz and the Hill plots of the binding data are indeed diagnostic of mixed co-operativity. At low concentrations negative co-operativity occurs (in fact, in the saturation curve and in the Klotz plot a 'half-of-sites' reactivity, the extreme case of negative co-operativity, is directly detected) and at higher concentrations there is a clear indication of positive co-operativity.

As for the stoichiometry of binding, the first supposition may be that of two binding sites per dimer. Assuming that this is the case, the values of the microscopic constants for the two initially identical binding sites with strong negative co-operativity can be derived [23,24] from the values of the high affinity (K_{S1}) and low affinity (K_{S2}) stoichiometric constants (see Table 1). These site constants K_1 and K_2 would be respectively equal to $(0.99 \pm 0.1) \times 10^4 \text{ M}^{-1}$ and to $(0.024 \pm 0.005) \times 10^4 \text{ M}^{-1}$. A similar value for K_1 , equal to $(0.85 \pm 0.1) \times 10^4 \text{ M}^{-1}$, can be calculated from the Klotz plot of the same data. Interestingly, the affinity constant for Cyd-3'-P of monomerized BS-RNAase is very close ($0.83 \times 10^4 \text{ M}^{-1}$; see Table 1) to the value of K_1 . Considering that the binding of Cyd-3'-P to the single sites on free monomers mimicks the binding of low concentrations of ligand to either site on the monomers linked in the native dimer, these data support the assumption that two sites are available to Cyd-3'-P on the dimeric enzyme at zero ligand concentration.

On the other hand, as it has been previously noted [27], 'bumpy' curves due to mixed types of co-operativity may be generated only when at least three sites are involved in the binding process. BS-RNAase is a dimer, with one active site per subunit. However, the enzyme is a ribonuclease, or, more generally, a 'catenase' [33], i.e. an enzyme which, interacting with a polymeric chain-like substrate, has to have a multiplicity of sites interacting with the several linked monomers making up the substrate. In fact, Matthew & Richards [34], studying pH-dependent electrostatic effects in pancreatic ribonuclease, identified five anion binding sites on the enzyme. Interestingly, Walker *et al.* [35] considered

Scheme 1. Model for the binding process of Cyd-3'-P to BS-RNAase

E- is a BS-RNAase subunit in the ligand free enzyme; E'- and F- are subunits which have undergone conformational transitions upon ligand binding by the enzyme. P is the ligand (Cyd-3'-P), bound at the main sites (PE-) or at the subsites (P).

binding of the substrate at extra sites as a basis for interpreting the anomalous saturation curves of RNAase A at high concentrations of substrate. Direct evidence of subsites on RNAase A was obtained by Pares *et al.* [36], probing the enzyme with affinity reagents. The inability in our studies on the binding complex of BS-RNAase with Cyd-3'-P to detect any signs of interactions at these putative subsites may simply mean that these interactions are very weak.

Based on the data and the considerations presented above, we propose for the binding process of cyd-3'-P to BS-RNAase the model which is outlined in Scheme 1. The main features of the model can be summarized as follows: (1) ligand-free BS-RNAase possesses two identical binding sites for ligands (Cyd-3'-P or other nucleotides); (2) interaction of cyd-3'-P at one of the sites of the ligand-free enzyme results in 'switching off' the other potentially available binding site, thus generating half-of-sites reactivity; (3) subsites with very weak binding constants are available on the BS-RNAase molecule only to Cyd-3'-P and only after one main site is occupied by this nucleotide; (4) full occupation of the subsites results in a conformational transition, which leads to the formation of an activated form of BS-RNAase with both the main binding sites actually available to the ligand. This generates positive co-operativity.

It should be underlined that although the available data are easily interpreted by the model, this may not be considered more than a working hypothesis for further studies on the complex binding and kinetic processes of BS-RNAase.

The special effects produced by binding of Cyd-3'-P, compared with the other nucleotides, are likely to be related to its nature of reaction product. We have recently found (unpublished work) that, in contrast with its 2'-P isomer, Cyd-3'-P acts at very low concentration as an activator of the regulatable, rate-limiting step of the ribonuclease reaction.

The results of these binding studies, indicating co-operativity in binding of Cyd-3'-P to BS-RNAase, shed light on the anomalous kinetics observed when the

hydrolytic rate-limiting step of the reaction of BS-RNAase [11] is studied. They exclude the possibility that the saturation curves with intermediary plateau may be due to hysteretic effects occurring on the enzyme active site(s). They have also to be related to recent results obtained from probing with alkylating reagents the active site of the enzyme [13]. We found that the reaction of an affinity reagent, such as 2'(3')-O-bromoacetyluridine, at His¹², occurs with 'half-of-sites' reactivity, whereas bromoacetate does not discriminate between the two active sites of the enzyme.

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