Biochem. J. (1988) 253, 329–336 (Printed in Great Britain)

Co-operativity in seminal ribonuclease function

Kinetic studies

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Kinetic studies with substrates of the hydrolytic rate-limiting reaction step revealed that the non-hyperbolic kinetics of bovine seminal RNAase may not be ascribed to microheterogeneity of the enzyme or to hysteretic effects. The substrate saturation curves with intermediate plateau and the activating and inhibiting effects of the reaction product, respectively at low and high concentrations, are explained in terms of mixed co-operativity, with binding at subsites that is a prerequisite for full activity of the enzyme. A model is proposed that is supported also by the results of binding studies.

INTRODUCTION

Dimeric bovine seminal RNAase shares several enzymic properties with monomeric RNAase A isolated from the pancreas of the same species (for a review on both enzymes see Blackburn & Moore, 1982). The two enzymes have their active-site clefts shaped by identical amino acid residues (Capasso *et al.*, 1983; Suzuki *et al.*, 1987) and have a common mode of action, as BS-RNAase also cleaves RNA at pyrimidine sites with a transphosphorolytic–hydrolytic two-step mechanism (Floridi *et al.*, 1972). However, there are significant differences: BS-RNAase can cleave polyribonucleotides with secondary structures under conditions in which RNAase A cannot (Libonati & Floridi, 1969; Taniguchi & Libonati, 1974), and it is modulated in its catalytic action in the second, rate-limiting, reaction step by increasing substrate concentrations, which generates non-hyperbolic kinetics (Piccoli *et al.*, 1982).

It has been reported that, although the enzyme in its dimeric form displays non-hyperbolic kinetics, a monomeric derivative follows Michaelian kinetics (Piccoli & D'Alessio, 1984). These studies revealed also that artificially dimerized RNAase A shows non-hyperbolic kinetics, just like naturally dimeric BS-RNAase.

Recently, it has been found that an active-site-directed alkylating reagent can discriminate between the two sites on the enzyme (Donadio *et al.*, 1986). Furthermore, the results of binding studies carried out with a series of nucleotides (Di Donato *et al.*, 1987) showed mixed co-operativity for the binding of the reaction product of the hydrolytic step.

The results reported here indicate that the anomalous kinetics of BS-RNAase cannot be ascribed to microheterogeneity of the enzyme, that modulation of the enzyme catalytic activity is not an intrinsic property of BS-RNAase active site, but a property of the dimeric enzyme, and that the reaction products are modulators of the enzymic function, activating at low concentrations and inhibiting at high concentrations. A model is derived, fitting both the kinetic data and also the data on non-hyperbolic nucleotide binding by BS-RNAase (Di Donato *et al.*, 1987).

EXPERIMENTAL

BS-RNAase was prepared from bull seminal plasma (D'Alessio *et al.*, 1972) or bull seminal vesicles (Tamburrini *et al.*, 1986) as described in the references cited. Homogeneous α_2, $\alpha\beta$ and β_2 subforms were separated as described by Di Donato & D'Alessio (1981); α_2 is a stable isoenzyme, selectively deamidated at a single amide group; whereas $\alpha\beta$ and β_2 isoenzymes can transform into α_2 and $\alpha\beta$, respectively. However, under the conditions employed for the assay, their transformation half-time is much higher than 50 h (Di Donato & D'Alessio, 1981).

M(*S*-alkyl)$_2$, i.e. monomeric [*S*-carboxymethyl-Cys^{31},*S*-carboxymethyl-Cys^{32}]BS-RNAase, was prepared as described previously (D'Alessio *et al.*, 1975). The monomeric enzyme, M(SH)$_2$, obtained by selective cleavage of the intersubunit disulphide bridges as described previously (D'Alessio *et al.*, 1975), was used immediately after preparation and stabilized by addition of dithiothreitol to the assay reaction mixture at a final concentration of 20 μM, with the enzyme at a concentration of 0.4 μM. The presence of the two exposed thiol groups and the monomeric state of the protein were monitored as previously described, with dithionitrosobenzoate and by running samples on a Sephadex G-75 column (D'Alessio *et al.*, 1975). We found that the monomeric state and the presence of the exposed thiol groups were conserved for more than 30 h at 25 °C.

A stabilized monomer, with mixed disulphide groups at the intersubunit half-cystine residues, M(*S*-Cys)$_2$, was prepared by the reaction of the native protein (2 mg/ml), in 0.1 M-Tris/HCl buffer, pH 8.6, containing 2 mM-Na_2EDTA, with 25 mM-[^{14}C]cystine (24 mCi/mmol; Amersham International, Amersham, U.K.) for 18 h at 37 °C. The protein derivative with mixed disulphide

Abbreviations used: RNAase A, bovine pancreatic ribonuclease; BS-RNAase, bovine seminal ribonuclease; M(SH)$_2$, monomeric BS-RNAase; M(*S*-alkyl)$_2$, monomeric [*S*-carboxymethyl-Cys^{31},*S*-carboxymethyl-Cys^{32}]BS-RNAase; M(*S*-Cys)$_2$, monomeric [*S*-cysteine-Cys^{31},*S*-cysteine-Cys^{32}]-BS-RNAase; Cyd-3′-*P* etc., cytidine 3′-phosphate etc.; Urd-3′-*P*, uridine 3′-phosphate etc.

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groups, M(*S*-Cys)$_2$, was separated from unmodified dimeric material and from reduced protein on a column (1.5 cm × 60 cm) of Bio-Gel P60 (BioRad Laboratories, Richmond, CA, U.S.A.) topped with 4 ml of thiopropyl-Sepharose 6B (Pharmacia, Uppsala, Sweden). The purified derivative was characterized by amino acid analyses and radioactivity measurements.

Nucleotides (Cyd-2′-*P*, Cyd-3′-*P*, Urd-3′-*P*, Cyd-2′:3′-*P* and Urd-2′:3′-*P*) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and checked for purity by spectrophotometry and paper chromatography as described by Steiner & Beers (1961). Urd-2′:3′-*P* was used after purification on DEAE-cellulose equilibrated in 10 mM-triethylammonium bicarbonate buffer, pH 7.5, and eluted with the same buffer at 50 mM concentration.

Kinetic measurements were performed by a spectrophotometric method at 30 °C in 0.1 M-Tris/HCl buffer, pH 7.3, and by a titrimetric method in 0.05 M-NaCl at pH 7.3, as previously described (Piccoli *et al.*, 1982). Velocity measurements were expressed as $\Delta A/\Delta\epsilon$, where ΔA is the increase of absorbance per min at 290 nm for Cyd-2′:3′-*P* and at 284 nm for Urd-2′:3′-*P*. $\Delta\epsilon$ values are the differences between the molar absorption coefficients of the products (Cyd-3′-*P* and Urd-3′-*P*) and those of the substrates (Cyd-2′:3′-*P* and Urd-2′:3′-*P* respectively) at those wavelengths. The molar absorption coefficients were 1217 $M^{-1}\cdot cm^{-1}$ (at 290 nm) for Cyd-3′-*P*, and 677 $M^{-1}\cdot cm^{-1}$ (at 284 nm) for Urd-3′-*P*, 1068 $M^{-1}\cdot cm^{-1}$ (at 290 nm) for Cyd-2′:3′-*P* and 873 $M^{-1}\cdot cm^{-1}$ (at 284 nm) for Urd-2′:3′-*P*. Measurements were routinely performed in 1 cm-light-path cuvettes. When the absorbance exceeded 1 *A* unit, cuvettes with a light path of 0.2 cm were used.

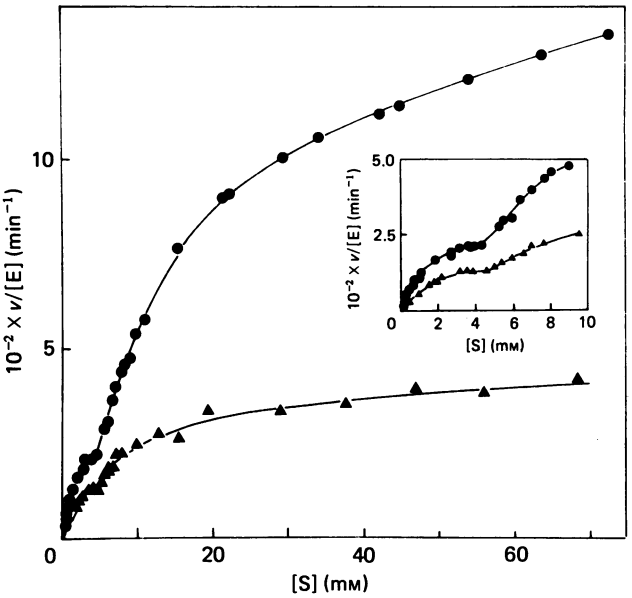


Fig. 1. Saturation curves of BS-RNAase for Cyd-2′:3′-*P* (●) and Urd-2′:3′-*P* (▲) as substrates

In the curve for Cyd-2′:3′-*P* two different experiments are shown, carried out at 30 °C at pH 7.3: in 0.1 M-Tris/HCl buffer by the spectrophotometric assay, and in 0.05 M-NaCl by the titrimetric assay. The curve for Urd-2′:3′-*P* was obtained by the titrimetric assay at 30 °C in 0.05 M-NaCl, pH 7.3. The enzyme concentration ([E]) was 0.2 μM. The inset shows a larger-scale presentation of the experimental data in the 0–10 mM substrate concentration range.

RESULTS

Saturation curve

Fig. 1 shows typical saturation curves at 30 °C at pH 7.3 of BS-RNAase with Cyd-2′:3′-*P*, for a range of substrate concentrations much wider than those previously inspected (Piccoli *et al.*, 1982), and with the other substrate Urd-2′:3′-*P*. The characteristic 'bump' occurs in the same concentration range of 3–5 mM (see Fig. 1 inset) for both substrates. It should be noted that at high substrate concentrations the curves run smoothly and no transitions can be detected. Identical results (not shown) were obtained when the assays were carried out at 23 °C at pH 7.13, i.e. under the conditions in which RNAase A displays abrupt strong transitions in the 30–50 mM concentration range (Walker *et al.*, 1975).

The data from two independent experiments with Cyd-2′:3′-*P* at 30 °C at pH 7.3 were plotted according to the Lineweaver & Burk equation and to the Hill equation. Fig. 2 shows that four regions of apparent linearity can be identified on the double-reciprocal plot, for which corresponding regions with different values of the Hill coefficient could be identified on the Hill plot (see Fig. 3). These findings allowed us to calculate a distinct set of kinetic values and of Hill coefficients for each range of substrate concentrations in several independent experiments, with correlation coefficients varying from 0.9 to 0.99.

BS-RNAase is, as isolated, a mixture of three isoenzymes (α_2, $\alpha\beta$ and β_2) made up of two types of subunits differing by a single amide group (Di Donato & D'Alessio, 1981). We thus investigated the possibility that the anomalous kinetics could be due to only one of the three isoenzyme forms, or, alternatively, that they could be generated by different catalytic properties of the active sites of the two subunit types in the dimer, which represents one-third of the enzyme preparations tested. Fig. 4 shows that homologous dimers α_2 and β_2, as well as the heterologous dimer $\alpha\beta$, are all kinetically indistinguishable from the native heterogeneous enzyme.

Effects of monomerization

It has been reported (Piccoli & D'Alessio, 1984) that monomerization of BS-RNAase abolishes the deviations from hyperbolicity in the substrate concentration range 1–10 mM of the saturation curve. Considering that monomeric RNAase A presents non-hyperbolic kinetics at substrate concentrations higher than 30 mM, we investigated the kinetics of monomerized BS-RNAase at higher substrate concentrations. The results, illustrated in Fig. 5, show that up to 80 mM substrate concentration monomerized BS-RNAase follows Michaelian kinetics. The curves obtained with homogeneous preparations of α- and β-subunits were superimposable onto those obtained with preparations of monomers from heterogeneous enzyme.

These experiments were performed with a monomeric derivative of BS-RNAase prepared by selective reduction of the intersubunit disulphide bridges, followed by alkylation of the exposed thiol groups and mild denaturation. As the alkylation and the denaturation steps could have affected the subunit catalytic properties, more native-like BS-RNAase monomers were prepared: (1) by

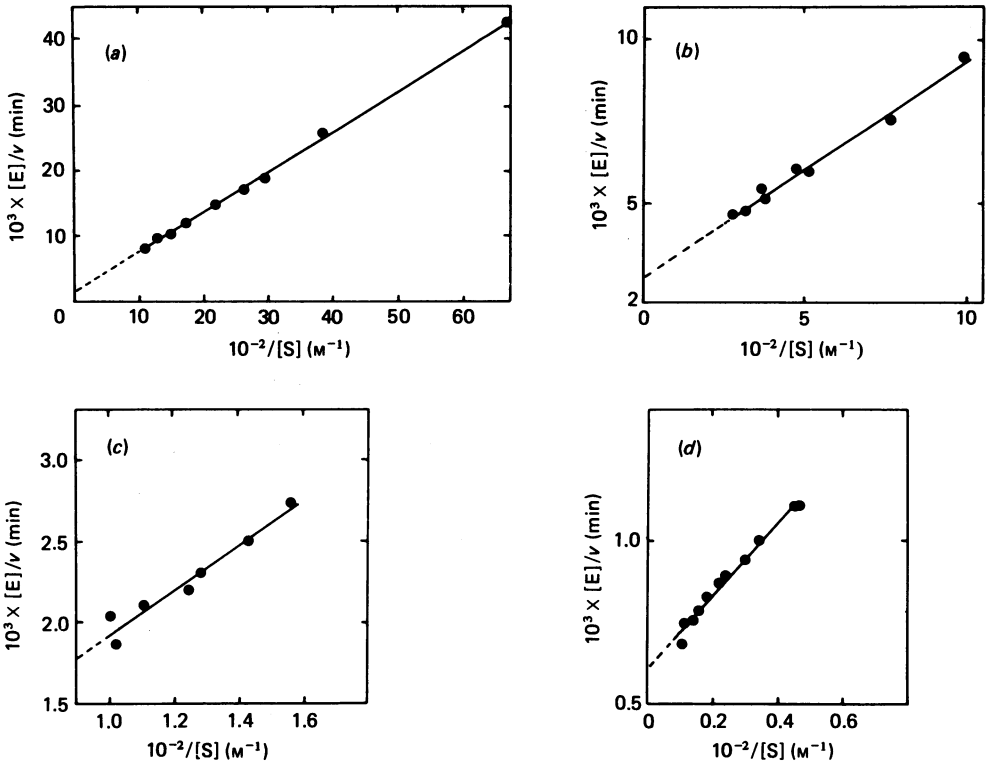


Fig. 2. Lineweaver–Burk plot of the kinetic data shown in Fig. 1.

Data from four distinct ranges of substrate concentrations, for which linear plots with good correlation coefficients (> 0.99) could be drawn, are shown in the four panels: (*a*) 0.1–0.9 mM; (*b*) 1–3.5 mM; (*c*) 6.5–10 mM

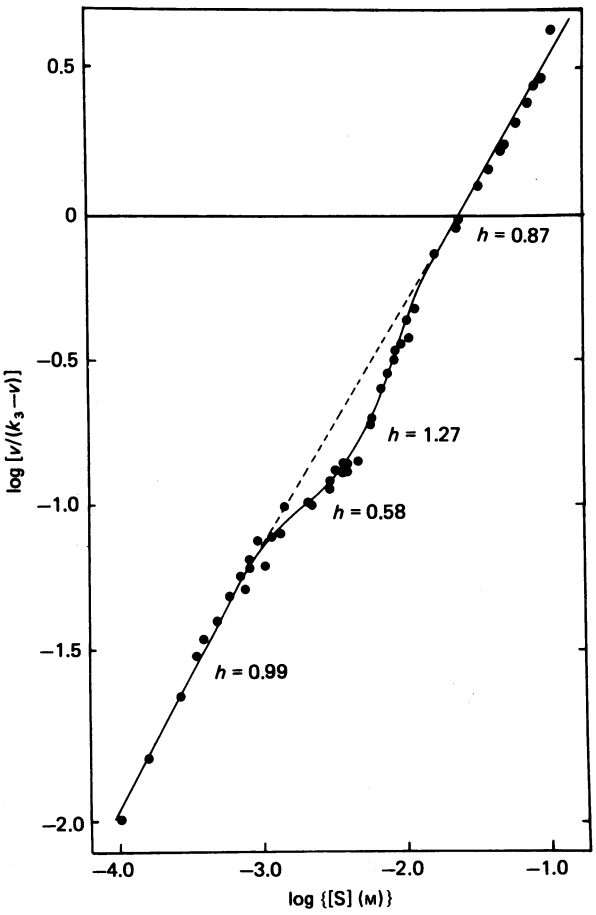


Fig. 3. Hill plot of the kinetic data shown in Fig. 1.

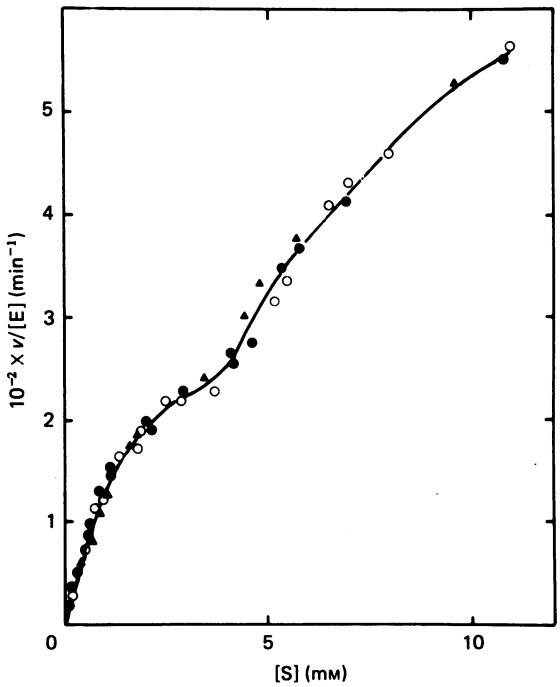


Fig. 4. Comparison of kinetic measurements on the BS-RNAase subforms α_2 (●), $\alpha\beta$ (○) and β_2 (▲) with Cyd-2′:3′-*P* as substrate

The continuous curve fitting the experimental data points is that obtained in a parallel experiment with native heterogeneous BS-RNAase. Data were obtained by the spectrophotometric assay in 0.1 M-Tris/HCl buffer, pH 7.3, at 30 °C with 0.2 μM enzyme concentration.

Table 1. Kinetic parameters for BS-RNAase and its derivatives with cytidine 2′:3′-*P* as substrate at 30 °C at pH 7.3

M(*S*-alkyl)$_2$, M(SH)$_2$ and M(*S*-Cys)$_2$ are monomeric derivatives of BS-RNAase. T_n is the molecular activity per active site: $T_n = k_{cat.}/n$, where n is the number of active sites on the molecule. Thus the values for the monomeric derivatives can be compared with those of the subunit of dimeric BS-RNAase.

Enzyme	Concn. of substrate (mM)	Hill coefficient	T_n (s^{-1})	K_m (mM)
BS-RNAase	0.1–0.9	0.98±0.02	6.31±0.32	4.61±0.12
	1.0–3.5	0.56±0.02	3.00±0.11	1.9±0.3
	6.5–10.0	1.24±0.08	10.80±0.43	14.7±1.2
	20.0–95.0	0.87	13.7	18.2
M(*S*-alkyl)$_2$	1.0–10.0	0.99±0.04	8.2±1.7	5.9±1.7
	0.1–83.0	0.97±0.05	10.6	6.8
M(SH)$_2$	1.0–10.0	1.03±0.06	8.5±0.5	5.5±2.1
M(*S*-Cys)$_2$	1.0–10.0	0.97	12.6	11.3

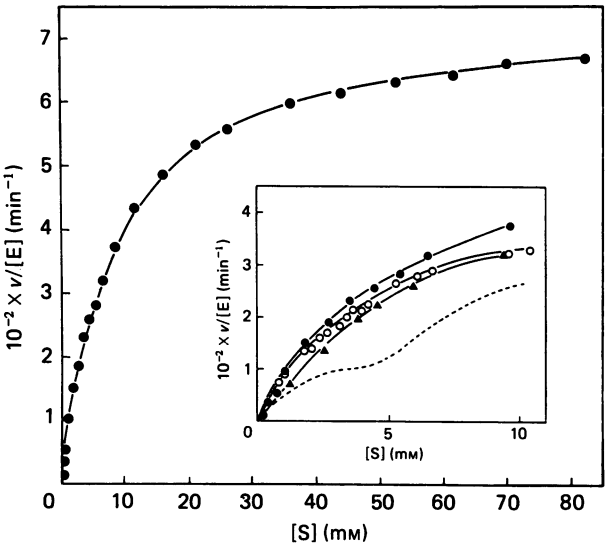


Fig. 5. Saturation curve of a monomeric derivative of BS-RNAase, M(*S*-alkyl)$_2$, with Cyd-2′:3′-*P* as substrate

The inset shows saturation curves in the 0–10 mM substrate concentration range of M(*S*-alkyl)$_2$ (●), M(SH)$_2$ (○) and M(*S*-Cys)$_2$ (▲). The dotted line indicates the saturation curve of native dimeric BS-RNAase. All the experiments were carried out at 30 °C at pH 7.3 in 0.05 M-NaCl by the titrimetric assay for M(*S*-alkyl)$_2$ and in 0.1 M-Tris/HCl buffer by the spectrophotometric assay for M(SH)$_2$ and for M(*S*-Cys)$_2$. The enzyme concentration was 0.4 μM.

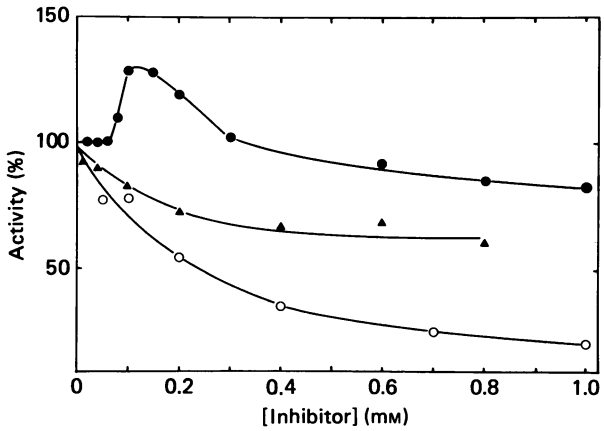


Fig. 6. Effects of Cyd-3′-*P* (●) and Cyd-2′-*P* (○) on BS-RNAase activity with Cyd-2′:3′-*P* as substrate

The effect of Cyd-3′-*P* (▲) on RNAase A activity with the same substrate is also shown. Activity measurements were obtained by the titrimetric assay at 30 °C in 0.05 M-NaCl, pH 7.3. Substrate and enzyme concentrations were 7 mM and 0.2 μM respectively.

reducing the intersubunit disulphide bridges and isolating the monomeric fraction M(SH)$_2$ with free thiol groups at positions 31 and 32, stabilized by low concentrations of dithiothreitol; (2) by making a stabilized monomer M(*S*-Cys)$_2$ with mixed disulphide groups at the intersubunit half-cystine residues. Both monomers gave hyperbolic saturation curves with Cyd-2′:3′-*P* (see Fig. 5 inset). The kinetic data and the values of Hill coefficients determined for these derivatives of BS-RNAase are summarized in Table 1.

Effects of the reaction product

The effects of Cyd-3′-*P* on BS-RNAase activity with Cyd-2′:3′-*P* as a substrate are illustrated in Fig. 6. Low concentrations of Cyd-3′-*P* activate the enzyme up to 30% over the activity value measured in the absence of the nucleotide. Further increases in concentration abolish the activation in an apparently co-operative fashion, and concentrations higher than 0.3 mM are inhibitory. Similar results, i.e. a 20% activation at about 0.1 mM, were obtained with Urd-3′-*P* as an effector.

The results with Cyd-3′-*P* may be compared with those obtained with the 2′-isomer of the nucleotide, which was found to be an inhibitor at all concentrations tested (see Fig. 6).

In a parallel experiment, carried out under identical conditions, the effects of Cyd-3′-*P* were examined on the kinetics of the reaction catalysed by RNAase A (see Fig. 6). As observed by previous investigators (Herries *et al.*,

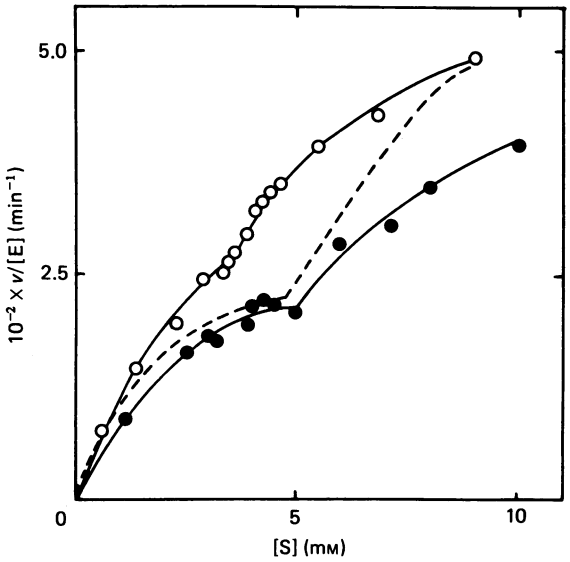


Fig. 7. Effects of Cyd-3′-*P* on the saturation curve of BS-RNAase with Cyd-2′:3′-*P* as substrate at activating (0.1 mM) and inhibiting (1 mM) concentrations

The saturation curve in the absence of Cyd-3′-*P* is shown by the dotted line. Experimental conditions are identical with those described in Fig. 6 legend.

1962; Walker *et al.*, 1976), the nucleotide was found to be a competitive inhibitor of the reaction at all concentrations tested. The K_i value derived from these measurements (0.64 mM) agrees well with the values reported in the literature (Herries *et al.*, 1962).

Fig. 7 shows the effects of Cyd-3′-*P* at 'activating' and 'inhibiting' concentrations, 0.1 and 1 mM respectively, on the hydrolysis of Cyd-2′:3′-*P* by BS-RNAase. At the 'inhibiting' concentration the reaction is inhibited at all concentrations of substrate, whereas at the 'activating' concentration the nucleotide shifts the whole curve towards higher activity values and tends to abolish the intermediate region of plateau. When the data of the curve with Cyd-3′-*P* at a concentration of 1 mM were plotted in a double-reciprocal plot, the inhibition was found to be competitive, with a K_i of 3.3 mM.

DISCUSSION

The non-hyperbolic saturation curve with an intermediate plateau reported previously (Piccoli *et al.*, 1982) for the reaction kinetics of BS-RNAase with Cyd-2′:3′-*P* could have several explanations. Considering that the curve is obtained for a single reaction step and a single substrate is involved, the range of hypotheses can be restricted to the following: (1) the curve results from mixed co-operativity (Teipel & Koshland, 1969; Cornish-Bowden & Koshland, 1975; Neet, 1980); (2) non-hyperbolicity is produced by interactions of the substrate with distinct conformations of the enzyme, generated by time-dependent hysteretic conformational changes (Neet & Ainslie, 1980); (3) the enzyme is heterogeneous and in the reaction mixture two or more enzyme forms, or two or more types of subunits, are present, one of them with positively co-operative behaviour (Teipel & Koshland, 1969).

The data presented here indicate that hypothesis (3) is not supportable on the basis of the microheterogeneity of the enzyme, as each individual isoenzyme displays a saturation curve identical with that of the heterogeneous enzyme (see Fig. 4). On the other hand, by primary-structure (Suzuki *et al.*, 1987) and X-ray-crystallographic studies (Capasso *et al.*, 1983) and by ^{1}H-n.m.r. analyses (Andini *et al.*, 1983) no other evidence of heterogeneity was ever detected in the protein.

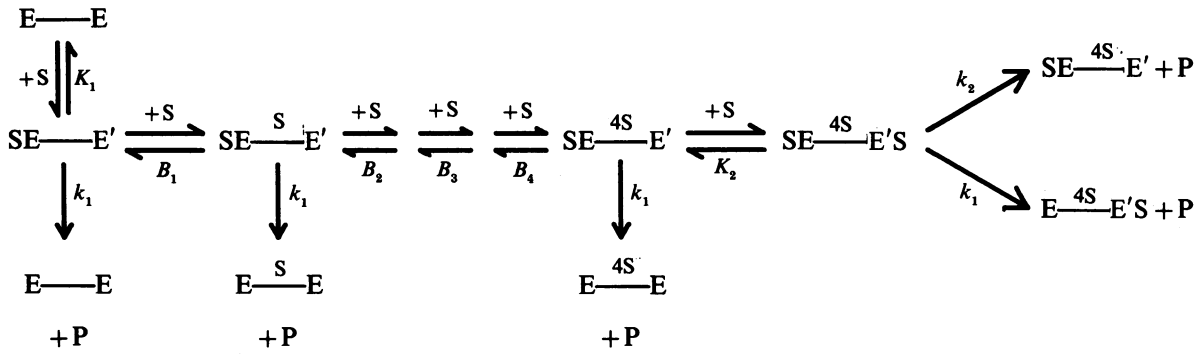
The results of binding studies (Di Donato *et al.*, 1987), showing under equilibrium conditions a non-hyperbolic binding curve with an intermediate plateau, with binding sites not independent or equivalent, lead to the exclusion of hypothesis (2), i.e. that BS-RNAase is a hysteretic enzyme. The results reported here, showing that different monomeric forms of the enzyme follow Michaelis–Menten kinetics, are in line with this conclusion, as they indicate that the non-Michaelian kinetics observed with the dimeric enzyme are not an intrinsic property of the catalytic site of the enzyme, but are exclusive of the enzyme in its dimeric form.

Thus we have to turn our attention to hypothesis (1), which has already been advanced (Piccoli *et al.*, 1982) and has found support from the studies on non-hyperbolic nucleotide binding (Di Donato *et al.*, 1987). However, binding data were not conclusive, as they could not be collected at high ligand concentrations. In the kinetic experiments reported in the present paper, carried out on both substrates of the reaction step under study, we could inspect a 3-log range of substrate concentrations, which allowed a satisfactory analysis of the data with the Hill equation (Cornish-Bowden & Koshland, 1975). This showed that at very low and very high substrate concentrations the Hill coefficient values approach unity, whereas a region of negative co-operativity and one of positive co-operativity were detected at intermediate concentrations. As these four regions of the Hill curve corresponded to apparently linear regions of the double-reciprocal plot of the same data, indicative values of the rate constants and of the K_M values for these four concentration ranges could be calculated (see Table 1). This led to a conclusion of particular interest, namely that at very low substrate concentrations (below 1 mM) the dimeric enzyme behaves like its monomeric derivatives.

The negative co-operativity detected in the 1–4 mM range of substrate concentrations can be correlated with the half-site reactivity observed studying (i) the inactivation of the enzyme with an active-site-directed alkylating probe (Donadio *et al.*, 1986) and (ii) the binding of Cyd-3′-*P* to the enzyme (Di Donato *et al.*, 1987).

Furthermore, we report here that Cyd-3′-*P*, in contrast with the 2′-isomer, inhibits the reaction only at high concentrations, whereas it activates at low concentrations. This result is to be expected for a competitive inhibitor of an allosteric enzyme (Segel, 1975).

In conclusion, the data presented here, and those from previous studies (Donadio *et al.*, 1986; Di Donato *et al.*, 1987), favour hypothesis (1), that BS-RNAase is an allosteric enzyme, regulated through site–site interactions generating mixed co-operativity. However, although this appears to be the only hypothesis supported by the experimental data, it must be taken into account that mixed co-operativity can only occur for proteins with a minimum of three sites available to the substrate/ligand, as it can only be described by a third-degree, or higher, equation (Teipel & Koshland, 1969).



Scheme 1. Model for co-operative interactions in BS-RNAase kinetics

E—— and E′—— are respectively enzyme subunits in the substrate-free enzyme and in the enzyme with substrate bound to the other subunit. E^{S}— and E′^{S}— are forms with the substrate bound at the subsites. k_1 and k_2 are the intrinsic catalytic constants of the two catalytic sites.

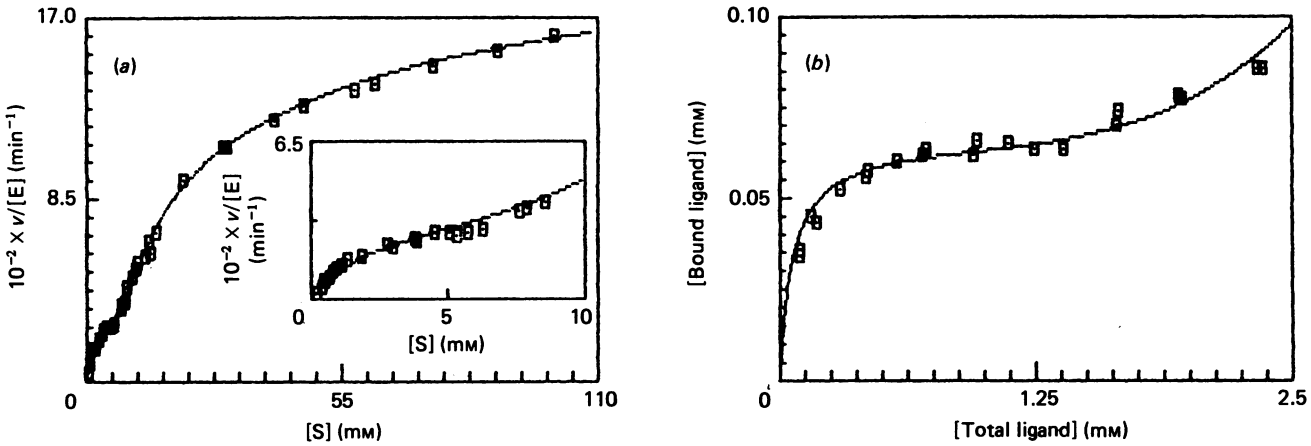


Fig. 8. Comparison between the computer-simulated curves and the experimental data points obtained for the kinetic (*a*) and the binding (*b*) analyses, with Cyd-2′:3′-*P* as substrate and Cyd-3′-*P* as ligand respectively

Table 2. Comparison between the experimentally determined and the optimized parameters used for simulating the BS-RNAase saturation curve

K_1 and K_2 values are reciprocals of K_m at low (< 1 mM) and high (> 30 mM) substrate concentration (see Table 1) respectively. k_1 and k_2 are the kinetic constants per site measured at low (< 1 mM) and high (> 30 mM) substrate concentrations respectively (see Table 1).

	Experimental	Optimized
K_1	216.9 M^{-1}	215 M^{-1}
K_2	54.9 M^{-1}	68 M^{-1}
k_1	378 min^{-1}	330 min^{-1}
k_1+k_2	1644 min^{-1}	1705 min^{-1}

We have already proposed (Di Donato *et al.*, 1987) to reconcile the apparent contrast between the dimeric structure of BS-RNAase possessing only two 'active sites' and its mixed co-operativity behaviour by assuming that subsites are available to the ligand on the enzyme molecule. Although subsites have not been detected in studies of the binding of nucleotides to BS-RNAase (Di Donato *et al.*, 1987), probably because of their low binding constants, chemical (Pares *et al.*, 1980) and crystallographic (Matthew & Richards, 1982) evidence has been obtained for the presence of subsites on RNAase A, an enzyme sharing with BS-RNAase about 80% of its amino acid sequence, including all the active-site residues (Suzuki *et al.*, 1987), and its tertiary structure (Capasso *et al.*, 1983).

A model, based on that already proposed after our binding studies (Di Donato *et al.*, 1987), as well as on the above results and considerations, is illustrated in Scheme 1. The essential features of the model may be summarized as follows: (1) on the free enzyme two sites are available to ligands, including substrates; (2) occupation of either site results in 'switching off' the second site, generating negative co-operativity and making available to ligands and substrates other, weakly binding, non-catalytic subsites; (3) the sequential saturation of the subsites makes available also the second catalytic site, engendering positive co-operativity.

The reported data support this model: negative and positive co-operativity are detectable on analysis of the

kinetic data with the Hill equation; the availability to the substrate of only two sites on the free enzyme is supported by the comparison of the behaviour of the dimeric enzyme at very low substrate concentrations (below 1 mM) with that of the monomeric derivatives of the enzyme.

The formal expression of the model is:

$$v/[E] = \frac{k_1[E'\!-\!\!-ES] + k_1 \cdot \sum_i [E'\overset{S_i}{-\!\!-}ES] + k_1[SE'\overset{S_{ns}}{-\!\!-}ES] + k_2[SE'\overset{S_{ns}}{-\!\!-}ES]}{[E\!-\!\!-E] + [E'\!-\!\!-ES] + \sum_i [E'\overset{S_i}{-\!\!-}ES] + [SE'\overset{S_{ns}}{-\!\!-}ES]} \quad (1)$$

where *ns* represents the number of subsites available to the substrate, and E and E′ denote the catalytic subunit forms binding the substrate to the first and to the second catalytic site respectively. Mathematical treatment of expression (1) leads to the following equation:

$$v/[E] = \frac{2k_1K_1[S] + 2k_1K_1[S] \cdot \sum_i A_i + (k_1 + k_2) \cdot (K_1K_2[S]^2 \cdot \sum_i A_i)}{1 + 2K_1[S] + 2K_1[S] \cdot \sum_i A_i + K_1K_2[S]^2 \cdot \sum_i A_i} \quad (2)$$

where [S] is the substrate concentration, K_1 and K_2 are the intrinsic association constants of the binding sites and k_1 and k_2 their catalytic constants.

We also define:

$$A_i = [(ns + 1 - i)/i] \cdot \prod_i B_i \cdot [S]^i$$

where B_i are the values of the subsite binding constants. The statistical binding factors applied to the first and second catalytic sites are 2 and $\frac{1}{2}$ respectively, whereas $(ns + 1 - i)/i$ are those for the subsites.

It should be emphasized that the treatment used here is based upon the consideration that substrate binding to the subsites is different from binding to the main sites. This implies that we treat the phenomenon as a case of substrate binding to independent classes of sites, one represented by the main sites on the enzyme, the other by the subsites. This is done by using independent sets of statistical factors.

A least-squares procedure was used to fit the curves calculated with eqn. (2) to the experimental data. A computer program was used to obtain the optimized values for the constants, assuming *ns*, the number of subsites, to be equal to 4. A comparison between the optimized and the experimental values is presented in Table 2. The values generated by the iterative procedure for the other parameters (see Scheme 1) were: 6.33 M^{-1} for B_1, 133.55 M^{-1} for B_2, 11.34 M^{-1} for B_3 and 29×10^3 M^{-1} for B_4. The curve obtained by using these values is shown in Fig 8(*a*).

The model was tested also with the experimental data of the non-hyperbolic curve obtained binding Cyd-3′-*P* to the enzyme (Di Donato *et al.*, 1987). From the appropriate equation, solved with $ns = 4$, the curve shown in Fig. 8(*b*) was obtained.

The satisfactory agreement between the calculated curves and the experimental data points indicates that the model is a suitable description of the behaviour of the enzyme, although this does not necessarily imply that the proposed model is the only one that can accommodate the experimental data. Certainly, it is noteworthy that the assumed number of subsites ($ns = 4$) is exactly that experimentally determined for homologous RNAase A (Pares *et al.*, 1980) and that with higher and lower values the fit between the experimental and the simulated model-based curve was completely lost.

As for the molecular basis of the site–site communications, we can speculate that they may easily and effectively occur were the dimeric structure of BS-RNAase just like that of the artificial dimers of RNAase A (Crestfield *et al.*, 1962). The exchange between subunits of the *N*-terminal ends, each including at position 12 a histidine residue that is essential for catalysis, generates composite active sites, made up of residues belonging to both subunits. Any conformational change triggered by the binding of the substrate at one site could thus be easily transmitted to the other site through the same polypeptide chain. Recent X-ray-crystallographic data (Mazzarella *et al.*, 1987) and biochemical information (R. Piccoli, A. Di Donato & G. D'Alessio, unpublished work) appear to support the hypothesis of the exchange of the *N*-terminal ends between subunits of BS-RNAase.

We thank Dr. R. E. Neet and Dr. E. Whitehead for critical reading of the manuscript. This work was financed in part by funds of the Ministero della Pubblica Istruzione and the Consiglio Nazionale delle Ricerche, Italy.

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Received 10 December 1987; accepted 22 March 1988