Theoretical treatment of tight-binding inhibition of an enzyme

Ribonuclease inhibitor as special case

Jesús M. FOMINAYA, Juan M. GARCÍA-SEGURA, Mercedes FERRERAS and José G. GAVILANES
Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad Complutense, 28040 Madrid, Spain

A general treatment for very tight-binding inhibition is described. It was applied to purified endogenous RNAase inhibitor from rat testis. This treatment discriminates among the different types of inhibition and allows for calculation of the inhibition parameters. When very tight-binding inhibitions are studied at similar molar concentrations of both enzyme and inhibitor, a further approach is required. This is also described and applied to the RNAase inhibitor. A K_i value of 3.2×10^{-12} M was found for this inhibitor protein. On the basis of this result, it was considered inappropriate to classify this type of inhibitor in terms of competitive or non-competitive, as has been done for such inhibitors so far. Functional consequences of this analysis are discussed for the RNAase–RNAase inhibitor system.

INTRODUCTION

The analysis of tight-binding inhibition mechanisms is usually performed according to the graphical methods of Dixon (1972) and Henderson (1972). However, these two procedures present some inconvenience when the K_i value is low (very tight-binding inhibitions). This occurs for ribonuclease inhibitor (RI) proteins. It has long been known that the cytosolic fraction of eukaryotic cells contains a protein able to inhibit neutral and alkaline RNAases (Roth, 1967). Although the function of this protein remains unclear, its molecular interaction with RNAase A, as a model RNAase, has been studied in a number of papers (Blackburn *et al.*, 1977; Blackburn & Jailkhani, 1979; Burton *et al.*, 1980; Blackburn & Gavilanes, 1980, 1982). In accordance with such studies, the inhibition mechanism of these inhibitor proteins was classified as non-competitive. However, further studies revealed the tight-binding character of these inhibitors,

and a competitive mechanism could formally be deduced on this basis (for a discussion see Turner *et al.*, 1983). Nevertheless, it is not biologically meaningful to consider that RI would exert its possible role, as regulator of protein biosynthesis, through competition with cytoplasmic RNA for RNAase binding. This classification of RI as competitive inhibitor may result from an inadequate treatment of the experimental results, owing to the very tight-binding character of this inhibition. In the present paper we propose a general treatment of the very tight-binding inhibition to circumvent these problems, which is applied to the RNAase inhibitor from rat testis.

THEORY

A simple procedure to find both the type of inhibition mechanism and the K_i value for tight-binding systems is

Table 1. Relationships between the parameters of eqn. (1) and classical kinetic parameters for different mechanisms of inhibition

v_0 is the enzyme rate measured in the absence of inhibitor.

Inhibition mechanism	A	B	C
Mixed non-competitive	$\dfrac{V_{max.} \cdot [S]}{\dfrac{[S]}{K_i^{ESI}} + \dfrac{K_m}{K_i^{EI}}}$	$\dfrac{[E]_t}{v_0}$	$[E]_t - \dfrac{[S] + K_m}{\dfrac{[S]}{K_i^{ESI}} + \dfrac{K_m}{K_i^{EI}}}$
Pure non-competitive	$K_i \cdot \left(\dfrac{V_{max.} \cdot [S]}{K_m + [S]} \right)$	$\dfrac{[E]_t}{v_0}$	$[E]_t - K_i$
Competitive	$K_i \cdot \left(\dfrac{V_{max.} \cdot [S]}{K_m} \right)$	$\dfrac{[E]_t}{v_0}$	$[E]_t - K_i \cdot \left(1 + \dfrac{[S]}{K_m} \right)$
Uncompetitive	$K_i \cdot V_{max.}$	$\dfrac{[E]_t}{v_0}$	$[E]_t - K_i \cdot \left(1 + \dfrac{K_m}{[S]} \right)$

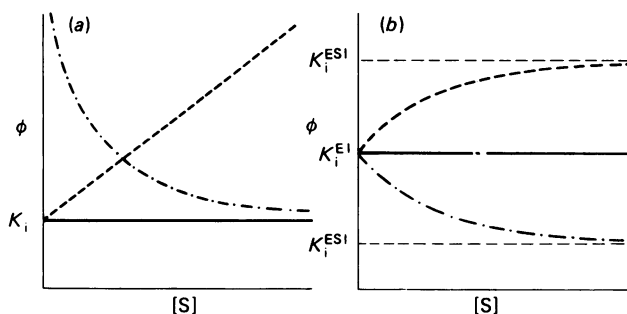Abbreviations used: RI, ribonuclease inhibitor; E, enzyme; S, substrate; P, product.

Fig. 1. Plots of ϕ (calculated as difference between total enzyme concentration and the fitted C parameter of eqn. 1) versus the substrate concentration for different mechanisms of inhibition

(a) ——, Non-competitive; ------, competitive; —·—·—, uncompetitive. (b) ——, Non-competitive ($K_i^{EI} = K_i^{ESI}$); ------, mixed non-competitive with $K_i^{ESI} > K_i^{EI}$; —·—·—, mixed non-competitive with $K_i^{ESI} < K_i^{EI}$.

Fig. 2. Evaluation of the inhibition constants for different mechanisms of inhibition through plots of ϕ (see the text) (either directly considered or transformed) as functions of either direct or reciprocal substrate concentrations

$$K_1 = K_i^{EI}; \quad K_2 = K_i^{ESI}.$$

Fig. 3. Double-reciprocal plots of the fitted A parameter (eqn. 1) versus substrate concentration for different mechanisms of inhibition

proposed. We have rearranged the general equation of Morrison (1969) (eqn. 11 in his paper) to a more suitable form in order to perform a regression analysis. Hence the initial steady-state rate for any enzyme-catalysed reaction is related to the concentration of a tight-binding reversible inhibitor present in the medium through the following general equation:

$$[I]_t = A \cdot (1/v) - B \cdot v + C \qquad (1)$$

where A, B and C are related to the classical kinetic parameters as shown in Table 1 for the different inhibition mechanisms. Thus the experimental data may be fitted to the theoretical equation through accessible computer programs (Sagnella, 1985).

The B parameter is the same for any type of inhibition, and it allows one to evaluate the enzyme concentration. On the other hand, A and C parameters show different relationships with the substrate concentration, depending on the mechanism of inhibition. A detailed analysis of these relationships should allow calculation of the K_i value as well as discrimination among the different inhibition types. With this goal, it is convenient to plot ($[E]_t - C$), named ϕ, versus [S]. These plots are equivalent to the ones suggested by Henderson (1972) when the slopes of his straight lines are replotted versus the substrate concentration. According to the pattern thus obtained (Fig. 1), it is possible to differentiate the mechanism of inhibition.

It can occur that the experimental data do not allow a discrimination between uncompetitive and mixed non-competitive (with $K_i^{ESI} < K_i^{EI}$) mechanisms. In this case a double reciprocal plot of A versus [S], as mentioned below, will solve the problem.

The inhibition constants can be directly evaluated from plots of ϕ versus [S] for non-competitive and competitive mechanisms. For the uncompetitive mechanism the K_i value can be determined from the intercept of the plot of ϕ versus $1/[S]$ (Fig. 2), and for the mixed non-competitive mechanism the two inhibition constants involved can be determined from the intercept and slope of the plot $(K_m + [S])/\phi$ versus [S].

The calculated K_i values can be confirmed through a double-reciprocal plot of $1/A$ versus $1/[S]$, as shown in Fig. 3. These plots are also useful to discriminate among
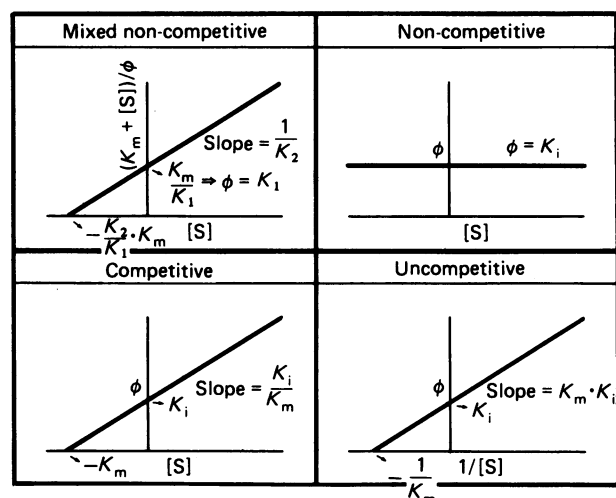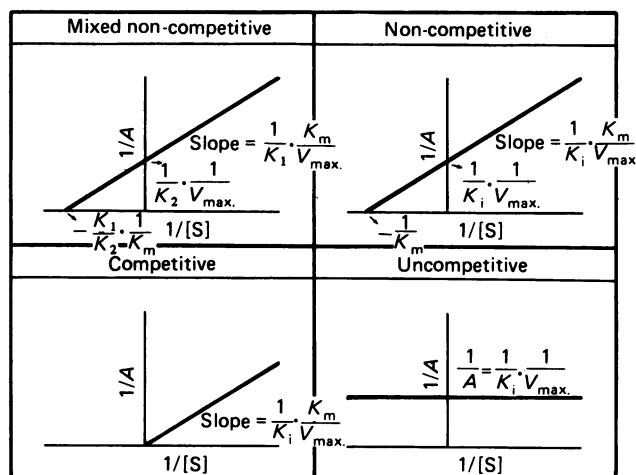
different inhibition types, and only a doubt about the pure or the mixed inhibition type may result if non-competitive mechanism is found. This doubt will be resolved by using the above-mentioned direct plot, ϕ versus [S]. It is instructive to consider that when K_i is very small all the types of inhibition will appear in the direct plot of ϕ versus [S] as non-competitive mechanisms. This fact has a functional meaning because in such a situation no competition between inhibitor and substrate will be possible.

MATERIALS AND METHODS

The ribonuclease inhibitor employed throughout this work was isolated from rat testis by using a procedure similar to that of Blackburn *et al.* (1977). The inhibitor protein was purified to homogeneity as demonstrated by
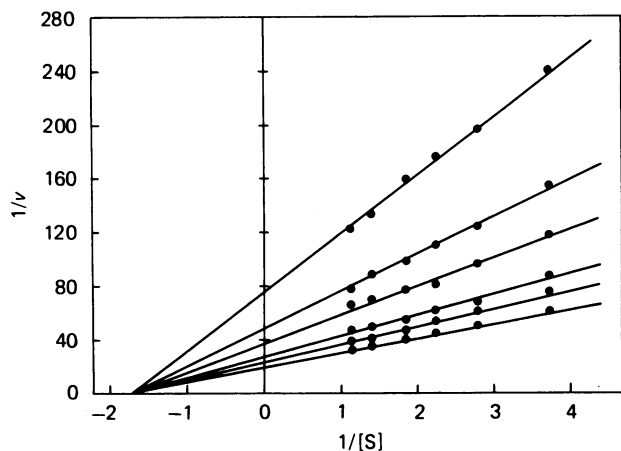
Fig. 4. Lineweaver–Burk plot of the inhibition of RNAase A by rat testis RI

All the assays were performed at 0.5 nM enzyme concentration. Inhibitor concentrations were: 0, 0.070, 0.131, 0.240, 0.298 and 0.375 nM. The substrate concentration is expressed as mmol of nucleotide/l. The enzyme rate is expressed as ΔA_{260}/min in the assay conditions.
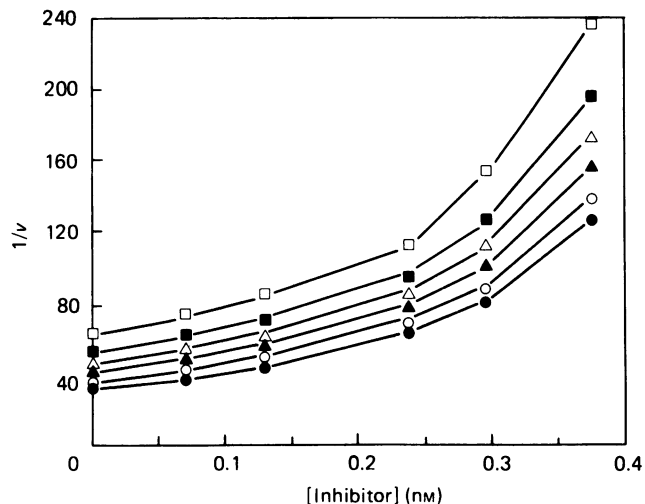

Fig. 5. Dixon plot of the inhibition of RNAase A by rat testis RI

Experimental conditions were those indicated in Fig. 4 legend. Substrate concentrations, expressed as mmol of nucleotide/l, were 0.271 ($\square$), 0.362 ($\blacksquare$), 0.450 ($\triangle$), 0.546 ($\blacktriangle$), 0.720 ($\bigcirc$) and 0.894 ($\bullet$).

the single protein band obtained in SDS/polyacrylamide-gel electrophoresis after silver staining, and its amino acid composition (not given in the present paper) shows a considerable similarity to the ones previously reported (Blackburn et al., 1977; Burton et al., 1980; Burton & Fucci, 1982). Ribonuclease A, phosphate-free (Worthington), has been used in this study, as model RNAase. The enzyme activity was determined by measuring the absorbance at 260 nm due to acid-soluble products released by RNAase from the yeast RNA substrate, after the required incubation time at 37 °C, as described previously (García-Segura et al., 1985, 1986). The yeast RNA employed as substrate was previously purified through saline precipitation, further ethanol precipitation and exhaustive dialysis. The enzyme reaction was started by the substrate addition; previously, the mixture containing both rat testis inhibitor and RNAase A was incubated for 10 min at 37 °C. The substrate concentrations were in the 0.5–1.5 K_m range (non-saturating conditions).

The experimental results were fitted to the corresponding kinetic equation [eqn. (8) in the Results and discussion section] by the non-linear-regression facility of the statistical software package BMDP (Dixon, 1981). The mentioned equation is linear in the parameters to be fitted. However, a non-linear-regression analysis was found more useful. It allows one to fit the parameters of that equation as well as p and $[E]_t$ as constants (see below).

RESULTS AND DISCUSSION

The Lineweaver–Burk plot of the RNAase inhibition by rat testis RI is shown in Fig. 4. This plot suggests a non-competitive mechanism of inhibition. However, a standard Dixon (1953) plot of these data does not allow graphical calculation of the K_i (Fig. 5). This fact should be interpreted as a consequence of tight-binding inhibition (Morrison, 1969, 1982). A similar result was found by Turner et al. (1983) in their studies on pig thyroid and
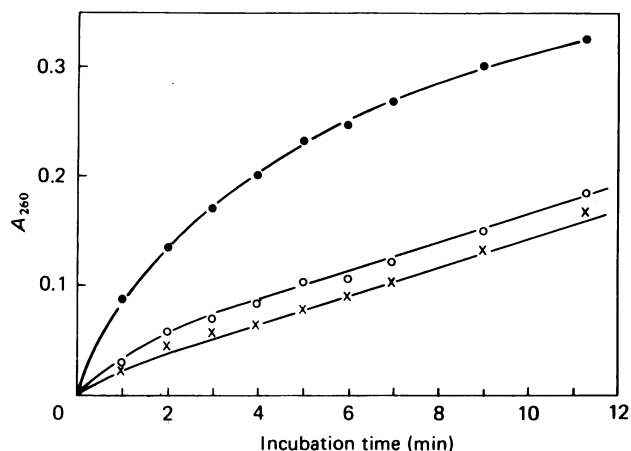

Fig. 6. Kinetics of the degradation of RNA by RNAase A in the presence of rat testis RI

The A_{260} of acid-soluble nucleotides released in the assay medium is plotted against the incubation time. The enzyme reaction was carried out without RI ($\bullet$), with RI and preincubation with RNAase A before the addition of RNA as starting reagent ($\times$) and with RI and no preincubation with RNAase A, which was added as starting reagent ($\bigcirc$). The concentrations employed were: 0.5 nM-RNAase A, 0.282 nM-RI and 2 mM-RNA (expressed as mmol of nucleotide/l).

liver RI. In addition, these authors found a slow binding character for such a RNAase inhibition. However, our results (Fig. 6) seem to indicate that the rat testis RI does not exhibit a slow binding character, or at least not so pronounced as that found by Turner et al. (1983). The small kinetic differences between the preincubated and non-preincubated mixtures could be explained by considering simple diffusional constraints due to the macromolecular nature of the inhibitor.
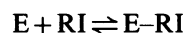
Anyway, a non-classical treatment of the kinetic results must be carried out owing to the tight-binding type of this inhibition. In fact, it is not correct to assume, as in the classical methods, that free and total inhibitor concentrations are roughly the same. In order to avoid such difficulties we applied the procedure described in the Theory section. This is based on the reasonings of Morrison (1969, 1982), as occurred for the graphical methods of Dixon (1953) or Henderson (1972). But, in these graphical methods, if the K_i values are too low in comparison with the enzyme concentration employed, the determination of both inhibition parameters and mechanism might become unfeasible. In the Dixon (1972) method this is due to the difficulty of drawing a very smooth curve, and in the Henderson (1972) method to the strong similarity among the very small slopes of the straight lines obtained at different substrate concentrations.

We have found such difficulties in the study of the RNAase inhibition by rat testis RI, and neither mechanism of inhibition nor K_i values could suitably be determined. When the experimental data were fitted by regression analysis to eqn. (1) (see the Theory section), a null K_i value was obtained. This occurs for inhibitors, such as the RNAase inhibitors, that require to be assayed at molar concentrations lower than those of enzyme. If this condition is not established in our case (assay of similar concentrations of both RI and enzyme), inhibition values over 90 % are obtained, and thus the enzyme rates determined are greatly inaccurate. When this situation is found, a value of $[E]_t/K_i = 110$ is obtained from eqn. (1). At such a ratio, virtually all of the inhibitor molecules are bound to the enzyme (Henderson, 1972) and eqn. (1) becomes:

$$[I]_t = [E]_t \cdot (1 - v/v_0)$$

which is equivalent to considering that the K_i value is zero. Then, no information can be obtained by an analysis such as the ones proposed by Dixon (1972), Henderson (1972) or that described in the present paper.

Thus, when these situations are found, it can be assumed that the overall K_i value is so small that no reversion of the enzyme–inhibitor complex is produced by the substrate added. Then, a new kinetic treatment must be carried out by considering that independent reactions are established:

$$E + RI \rightleftharpoons E-RI$$

with $K_i = [E][RI]/[E-RI]$

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E-S \overset{k_3}{\rightarrow} E + P$$
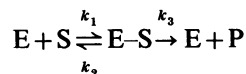
From the conservation equations for both enzyme and inhibitor:

$$[E]_t = [E] + [E-S] + [E-RI] \tag{2}$$

$$[RI]_t = [RI] + [E-RI] \tag{3}$$

and considering that $K_m = (k_2 + k_3)/k_1$, the steady-state equation:

$$d[E-S]/dt = 0 = k_1 \cdot [E] \cdot [S] - (k_2 + k_3) \cdot [E-S] \tag{4}$$

can be transformed into:

$$[E-S] \cdot (1 + K_m/[S]) = [E]_t - [RI]_t + [RI] \tag{5}$$
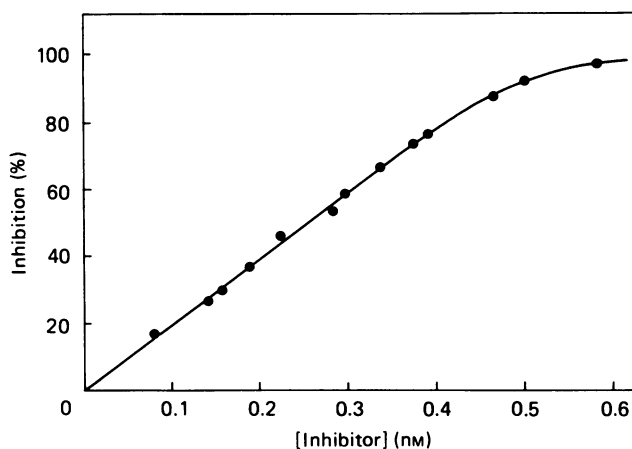

Fig. 7. Degree of inhibition of 0.5 nM-RNAase A by increasing amounts of rat testis RI

The substrate concentration in the assay medium was 7.4 mM.

Considering the rate equation $v = k_3 \cdot [E-S]$, where $k_3 = V_{max.}/[E]_t$, it can be deduced:

$$v = (V_{max.}/[E]_t) \cdot ([E]_t - [RI]_t + [RI]) \cdot \{[S]/([S] + K_m)\} \tag{6}$$

and then:

$$v/v_0 = v' = 1 - [RI]_t/[E]_t + [RI]/[E]_t \tag{7}$$

where v and v_0 are the enzyme rates in the presence and in the absence, $v_0 = V_{max.} \cdot [S]/(K_m + [S])$, of inhibitor respectively. Thus v' is the relative enzyme rate in the presence of inhibitor.

In order to express this last equation as a function of measurable variables, free inhibitor concentration should be expressed as $[RI] = K_1 \cdot [i/(1-i)]$ (Segel, 1975), where K_1 is the overall inhibition constant and i is the relative inhibition degree, $[E-RI]/[E]_t$. Total inhibitor concentration can also be expressed as a function of i. For such a purpose, it is convenient to dispose of a standard curve of inhibition degree at saturating substrate concentration, i versus total inhibitor concentration, like the one shown in Fig. 7. Considering total inhibitor concentrations in the linear range of this curve, it can be assumed that $i = p \cdot [RI]_t$, and thus:

$$v' = 1 - i/p \cdot [E]_t + (K_1/[E]_t) \cdot [i/(1-i)] \tag{8}$$

This expression will be valid at any substrate concentration in the assay medium. We have fitted the experimental data (i, v') to this equation by non-linear-regression analysis. Thus a K_1 value of 3.22×10^{-12} M $\pm$ 0.67×10^{-12} M was found, thereby indicating its smallness compared with the K_m value of the RNAase A (0.583×10^{-3} M). Therefore it was not surprising to obtain a practical K_1 value of zero when analyses of tight binding inhibition were tried. This value is very low in comparison with the ones previously reported for RI from other species (Table 2). This discrepancy should be explained as a consequence of the different methods employed for the K_1 determinations rather than resulting from differences in the molecular designs of the inhibitors studied. Thus the classical Michaelis–Menten treatments carried out by Blackburn et al. (1977) and Burton et al. (1980) give questionable kinetic conclusions, as discussed by Turner et al. (1983) on the basis of the non-considered

Table 2. Comparison of the results obtained for the RNAase A inhibition by different endogenous RNAase inhibitors studied

References: [1] Blackburn et al. (1977); [2] Burton et al. (1980); [3] Turner et al. (1983); [4] present results.

Source of RI	$10^{12} \times K_i$ (M)	Mechanism of inhibition proposed
Human placenta[1]	300	Non-competitive*
Bovine brain[2]	700	Non-competitive*
Pig liver[3]	400	Competitive*
Pig thyroid[3]	100	Competitive*
Rat testis[4]	3	No competition†

* From a classical point of view.
† From a functional point of view (see the text).

tight-binding character of the inhibition. A more rigorous treatment must give a lower K_i value. In fact, when we used the same classical treatment for our results on rat testis RI, a K_i value of 9×10^{-10} M was obtained which is of the same order as the ones obtained by the Blackburn group.

On the other hand, the results reported by Turner et al. (1983) for different pig RNAase inhibitors seem to indicate a different behaviour of these RIs, as revealed by the more adequate methods employed for determining the K_i values. However, they employed inhibitor concentrations up to 15 times higher than the molar concentration of RNAase, and even so they were able to measure enzyme activity. This constrasts with the normal behaviour observed by Blackburn and co-workers (Blackburn & Jailkhani, 1979; Burton et al., 1980; Blackburn & Gavilanes, 1980, 1982) and by ourselves, where inhibitor concentrations 3 times higher than the molar concentration of RNAase yield a practically total inhibition of the enzyme activity.

An alternative explanation of the above results could lie in the non-pure character of the inhibitor preparations reported by Turner et al. (1983). Thus it could be concluded that the true K_i values of pig RI have to be lower than that reported. Moreover, this reasoning could explain the similarity among the results that those authors obtained when both tight-binding analysis and the Baici (1981) method were applied to this inhibition, despite the fact that this latter method is not valid for a tight-binding situation.

Shapiro & Vallee (1987) have described a K_i value much lower than 1×10^{-10} M for the interaction of angiogenin, an RNAase protein, and human placental RI. The exact K_i value cannot be calculated, although it is reported to be 2.8-fold higher than that obtained for the RNAase A–RI as model system (Shapiro & Vallee, 1987). This would agree with our results.

A K_i value as small as the one herein reported would explain the non-competitive mechanism early found for the RNAase inhibitors (Blackburn & Gavilanes, 1980, 1982). It would result from the absence of competition between substrate and inhibitor in the assay conditions. However, the study of the RNAase A residues involved in RI binding allows one to classify this inhibitor as competitive as a result of the involvement of lysine-41 in

the binding site of the enzyme (Blackburn & Jailkhani, 1979). In a less formal discussion, it has no meaning to classify this inhibition within the categories usually employed for low-M_r ligands with binding constant similar to K_m. In the present case the macromolecular nature of the inhibitor as well as its small K_i value allows one to discard any discussion in this sense, because a competitive character, if present, would not be effective. It is better to consider that the regulation of the inhibitory activity in vivo would be exerted through some modulation system rather than as resulting from competition with the substrate. Such a modulation system would control the reversibility of the inhibition equilibrium. These requirements become obvious if inhibitor concentrations found in vivo are considered. In fact, RI concentrations normally exceed by several times the RNAase concentration of the cell (Blackburn & Moore, 1982). Therefore the functioning of RI would imply the formation of a dead complex if some modulation system were not present. In this sense the involvement of the GSH system in the regulation of the RNAase–RI system could be proposed. This observation is based on a structural property found for the RNAase inhibitors studied: all of them are proteins containing essential free thiol groups, and their inactivation by thiol-blocking reagents is currently employed to assay latent RNAase activity (Blackburn & Moore, 1982). Taking these facts into consideration, one may consider the possible involvement of such thiol groups in the regulation of the RNAase–RI system in vivo. In fact, we have found that thiol-blocking reagents, other than the conventionally used p-hydroxymercuribenzoate, release RNAase activity in RNAase/RI preparations (García-Segura et al., 1985). One of such reagents was the GSSG/GSH system. Taking into account the role attributed to this system as cytoplasmic thiol-group buffer, its influence on the inhibitor protein would give an interesting perspective to the regulation of the intracellular RNAase activity by the cellular redox state. The possible existence of a modulator for the RNAase–RI system becomes more plausible after the present study on the mechanism of inhibition by RNAase inhibitor proteins, which is only possible if the described treatment of the experimental data is performed.

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