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AFFINITY CHROMATOGRAPHY STUDY OF THE INTERACTION OF RIBONUCLEOTIDES WITH BOVINE PANCREATIC RIBONUCLEASE COVALENTLY BOUND TO SEPHAROSE 4B

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SUMMARY

The preparation of immobilized bovine pancreatic ribonuclease by covalent attachment to Sepharose 4B, with and without a spacer arm, is described. The coupling reaction was carried out at two different pH values, 8.5 and 10.5, and the different kinetic properties shown by the resulting preparations probably reflect the influence of the particular amino acid side-chains involved in the covalent coupling of the enzyme to the insoluble matrix. The strength of binding of mononucleotides, at 4°C, as deduced from the salt concentration at which they are eluted from an immobilized RNAase column, follows the order 5′-GMP > 5′-AMP > 3′-UMP > 3′-CMP. When binary mixtures of a 3′-pyrimidine nucleotide and a 5′-purine nucleotide are chromatographed jointly, a co-operative effect is found and the elution of either or both ligands is retarded. This behaviour can be explained in terms of the preferential binding of each kind of nucleotide to different sub-sites in the enzyme. The stoichiometry and association constant for 3′-CMP and 5′-AMP at pH 7.0 were also determined.

INTRODUCTION

It is well known that bovine pancreatic ribonuclease (E.C.3.1.27.5, RNAase) possesses several binding sub-sites which show different specificities depending on whether the ligand is a purine or a pyrimidine nucleotide and on the position (3′ or 5′) of the phosphate group[1–4]. It was thought that alkali-stable dinucleotides, that are not substrates of the enzyme, could be good models for the interaction between the enzyme and the substrate. The separation of the sixteen different alkali-stable dinucleotides in preparative amounts is a difficult task that has been partially solved by means of affinity chromatography on columns of RNAase covalently bound to Sepharose 4B[5]. However, during the preliminary checks with the immobilized enzyme preparation some striking differences in behaviour with respect to the soluble enzyme were found. For this reason a detailed study of the interaction of mononucleotides (a more easily available kind of ligand) with immobilized ribonuclease was undertaken.

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Special emphasis was placed on the behaviour of 3'-pyrimidine nucleotides and 5'-purine nucleotides as it has been shown that, in the soluble enzyme, they interact preferentially in different sub-sites, namely $B_1R_1p_1$ for 3'-CMP and 3'-UMP and $B_2R_2p_1$ for 5'-AMP and 5'-GMP[1]. On the other hand, as the presence of 5'-AMP enhances the activity of the soluble enzyme towards low-molecular-weight substrates such as 2',3'-cyclic pyrimidine nucleotides (that bind in $B_1R_1p_1$)[6], the behaviour of binary mixtures of the two types of nucleotide ligands was also studied. In the present work it has been found that, although many aspects of the enzyme–ligand interactions of the soluble enzyme apply to the immobilized preparation, there are some important differences. Moreover, the properties of the immobilized enzyme are strongly dependent on the conditions of coupling of the enzyme to the insoluble matrix.

MATERIAL AND METHODS

Immobilization of RNAase

Sepharose 4B (Pharmacia, Uppsala, Sweden) was activated with CNBr by the method of Axén *et al.*[7]. The coupling reaction was carried out as follows. To 20 ml of activated Sepharose 4B, 200 mg of bovine pancreatic RNAase (twice recrystallized) (Cambrian Chemicals, Croydon, U.K.) dissolved in 20 ml of 0.1 *M* sodium bicarbonate pH 8.5 (or pH 10.5 after adjustment with 0.5 *M* sodium hydroxide solution) containing 0.5 *M* sodium chloride solution were added. The mixture was gently stirred on a shaker for 16 h at 4°C. After washing with the same buffer, the unreacted activated groups were neutralized by reaction with 0.2 *M* glycine in 0.1 *M* sodium bicarbonate pH 8.5 (or 10.5). The gel was then washed and equilibrated with 10 m*M* ammonium acetate pH 5.5 or 7.0.

Sepharose 4B–carbonylaminoethylacetamide–RNAase was prepared from CNBr-activated Sepharose 4B by the method of Cuatrecasas[8] for the coupling of proteins to bromoacetyl Sepharose derivatives, using ethylenediamine and O-bromoacetyl-N-hydroxysuccinimide as reagents for forming the spacer arm. All reagents used were of analytical grade.

Determination of the amount of RNAase bound to Sepharose 4B

The amount of immobilized RNAase was determined by the method of Hartree[9]. The calibration curve was obtained by using a solution of activated Sepharose 4B containing different amounts of soluble RNAase.

Determination of Sepharose 4B–RNAase activity

This was measured by the pH-stat method[10] at pH values between 6.7 and 8.7 using cytidine 2',3'-phosphate obtained according to the method of Szer and Shugar[11] and RNA obtained according to the method of Glitz and Dekker[12] as substrates. In the kinetic experiments the final enzyme concentration used was in the range 0.18–11 μM depending on the individual preparation. All solutions were adjusted to ionic strength, $I = 0.1$ *M* with sodium chloride solution.

Interaction between immobilized enzyme and ligands

The strength of the interaction was studied by using a column (0.8 × 30 cm) containing the immobilized enzyme equilibrated with 10 m*M* ammonium acetate, pH

7.0 (or 5.5). Ligands were applied to the column dissolved in a volume of 0.2 ml of 10 m*M* ammonium acetate, pH 7.0 (or 5.5) and at a concentration of 2 mg ml^{-1} with a flow-rate of 6 ml h^{-1}. The column was washed with the same buffer, and eluted with a linear gradient between 10 m*M* and 0.2 (or 0.3) *M* ammonium acetate at the same pH. When mixtures of nucleotides were studied the individual peaks were identified by obtaining their UV spectra. In the case of RNAase coupled through the spacer arm, no salt gradient was applied as the affinity of the ligands was too low. Instead the reduced elution volume, V_e/V_t, was used, where V_e is the elution volume and V_t the bed volume. Control experiments were carried out by using CNBr-activated Sepharose 4B that was subsequently neutralized with glycine. No non-specific interactions were found except in the case of the nucleoside diphosphates 5'-ADP and 3'5'-ADP. Experiments were carried out at 4°C because of the higher stability of the enzyme preparation. However, the stability was checked every five or six runs by carrying out a control experiment with 5'-AMP. Changes in the elution pattern of 5'-AMP were interpreted as due to damage to the enzyme preparation and a fresh one was then used.

Stoichiometry of the immobilized enzyme–ligand interaction

The protein content of 4 ml of gel was determined. A solution of ligand (in 10 m*M* ammonium acetate, pH 7.0) at a concentration twenty times that of the enzyme was passed through the gel volume. When the column was saturated (the concentration of ligand was the same in the loading solution and in the eluate) the gel was washed with 10 m*M* ammonium acetate, pH 7.0, until no ligand was found in the eluate; the bound ligand was eluted with 0.2 *M* ammonium acetate pH 7.0. The amount of eluted ligand was measured spectrophotometrically and this was correlated to the amount of immobilized enzyme. Experiments were carried out at 4°C.

Determination of binding constants

Binding constants were determined in the case of the ligands 3'-CMP and 5'-AMP at pH 7.0 for the preparations of enzyme without spacer arm (coupling reactions at pH 8.5 and 10.5). The column of immobilized enzyme (4 ml of gel) was equilibrated with different concentrations of ligand in 10 m*M* ammonium acetate, washed with the same buffer and eluted with 0.2 *M* ammonium acetate. The amount of ligand eluted was measured in each case and the results were plotted according to the Scatchard equation[13]. A ligand:protein molar ratio range from 1:1 to 1:40 was used for each ligand. The Scatchard plot can also be used to obtain the stoichiometry between the immobilized enzyme and ligand in the case of 3'-CMP and 5'-AMP.

RESULTS AND DISCUSSION

The immobilization of bovine pancreatic RNAase on Sepharose has been reported by several authors as a means to solve some particular problem such as the isolation of labelled peptides from affinity-labelled proteins[14] or to study the conformation of immobilized enzymes using calorimetric procedures[15]. As a complete description of the Sepharose–RNAase preparations was not previously available, some kinetic properties of the immobilized preparation were checked before carrying out ligand-binding determinations.

Characterization of Sepharose 4B–RNAase preparations

The coupling of RNAase to Sepharose 4B was carried out at two pH values, 8.5 and 10.5, but as it is seen from Table I there is practically no difference in the yield of coupled enzyme. The yields of protein bound to the gel are lower than those found by the above-mentioned authors[14,15] (3 mg of protein per ml of Sepharose compared with 30–35 mg/ml). However, it should be noted that (i) in our preparation a lower protein:resin ratio was used (10 mg/ml Sepharose compared with 33–38 mg RNAase per ml Sepharose), and (ii) in the present work the amount of protein bound was determined by the modified Lowry method[9] whereas the other authors used the protein-balance method. On the other hand, the much lower coupling of enzyme to the preparation with the spacer arm may be a reflection of the lower number of active sites in the matrix.

The kinetic data show some interesting effects. Thus, when the coupling reaction takes place at pH 8.5 only 2.3–2.4% activity remains, whereas the enzyme preparation immobilized at pH 10.5 retains 36–47% activity (depending on the substrate used). In the case of the coupling of RNAase with a spacer arm, the retained activity varies between 57 and 67% for coupling at pH 8.5 and between 59 and 78% for coupling at pH 10.5.

TABLE I

CHEMICAL AND KINETIC PROPERTIES OF SEPHAROSE-BOUND RNAASE PREPARATIONS

The kinetic parameters were obtained by means of the pH-stat method at pH 7.0, I = 0.1 M, 25°C. K_M = Michaelis constant; k_{cat} = catalytic constant; V_{max} = maximum velocity; and E = enzyme concentration.

Preparation	*Protein content* ($\mu mol\ ml^{-1}$ *of gel*)	K_M		k_{cat}, $C > p$ (min^{-1})	V_{max}/E, *RNA* ($mg\ min^{-1}\ mol^{-1}$)	*% activity****	
		$C > p$* (*mM*)	*RNA*** ($mg\ ml^{-1}$)			$C > p$	*RNA*
Soluble RNAase	–	2.8 ± 0.3	1.00 ± 0.04	775 ± 51	173 ± 7	100	100
Soluble RNAase in the presence of Sepharose	–	3.1 ± 0.2	–	672 ± 61	–	87	
Soluble RNAase in the presence of activated and neutralized Sepharose	–	5.6 ± 0.4	1.45 ± 0.20	766 ± 69	193 ± 16	98	111
Sepharose 4B–RNAase (coupling reaction at pH 8.5)	0.16	3.7 ± 0.3	1.61 ± 0.10	24 ± 2	4.0 + 0.5	3.2	2
Sepharose 4B–RNAase (coupling reaction at pH 10.5)	0.14	3.0 ± 0.2	1.40 ± 0.20	376 ± 30	64 ± 7	48	36
Sepharose 4B–arm–RNAase (coupling reaction at pH 8.5)	0.05	3.3 ± 0.2	1.77 ± 0.15	521 ± 16	98 ± 8	67	57
Sepharose 4B–arm–RNAase (coupling reaction at pH 10.5)	0.04	2.9 ± 0.2	1.58 ± 0.14	606 ± 24	113 ± 9	78	59

* C > p = Cytidine 2',3'-phosphate; concentration range 1.6–16 mM.

** Concentration range: 0.56–8.41 mg ml^{-1}.

*** Referred to soluble RNAase.

It seems clear from the data on the protein content that the extent of the coupling reaction depends primarily on the number of active sites on the matrix, whereas the pH of the reaction may influence what amino groups from the enzyme show a preferential reactivity. It should be mentioned that extrapolation of the coupling data of Koch-Schmidt and Mosbach[15] to our conditions (375 mg CNBr per 1.5 g of swollen Sepharose) yields an average of seven points of enzyme–matrix attachment. This means that four amino groups of the enzyme remain free. It is likely that in the case of coupling at pH 8.5 some of the more catalytically important amino groups of RNAase, for example Lys-1, Lys-7 and/or Lys-41, would be involved in the attachment to the matrix, whereas in the case of coupling at pH 10.5 the reaction would take place preferentially through other amino groups not so important from the catalytic point of view.

The decreased activity of the preparations obtained at pH 10.5 and those with the spacer arm with respect to the soluble enzyme would be the result of two factors: (i) the shift in pH optimum (Fig. 1) and (ii) the increased lack of flexibility of the protein as a consequence of its attachment to the gel at several points. In the case of the preparations with the spacer arm, effect (i) would be almost wholly responsible for the diminished activity. As is seen in Fig. 1, the pH dependences of the activity show a pH optimum shifted towards more basic values, in accordance with previous reports on other enzyme systems[16–18].

It is seen in Table I that the K_M value for cytidine 2′,3′-phosphate increases significantly only in the preparation coupled at pH 8.5 without the spacer arm. A large increase in the K_M value of soluble RNAase is found when activated and neutralized Sepharose 4B is present in the reaction mixture, although the increase is practically non-existent when soluble RNAase is measured in the presence of Sepharose 4B. This difference may be due to the presence of the additional carboxylate groups of the glycine used to neutralize the activated groups on the matrix. In the case of a high-molecular-weight substrate such as RNA, significant increases in K_M

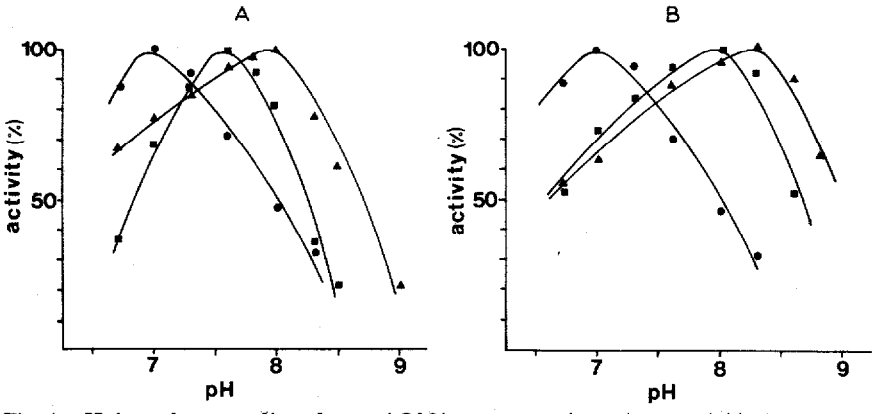

Fig. 1. pH-dependence profiles of several RNAase preparations. A, ●, soluble RNAase; ■, Sepharose 4B–RNAase (coupling reaction at pH 8.5); ▲, Sepharose 4B–RNAase (coupling reaction at pH 10.5); B, ●, soluble RNAase; ■, Sepharose 4B–spacer–RNAase (coupling reaction at pH 8.5); ▲, Sepharose 4B–spacer–RNAase (coupling reaction at pH 10.5). Results are expressed as percentage of the maximum. The concentration of cytidine 2′,3′-phosphate was 15 m*M* (five times the value of K_M at pH 7.0). Determinations were carried out with the pH-stat method at 25°C, in 0.1 *M* sodium chloride solution.

are found in all cases, indicating the existence of a clear steric hindrance to the accessibility of the polymeric substrate. This interpretation is supported by the fact that, at least in the soluble enzyme, K_M equals the dissociation constant, K_s, at pH 7.0 and 25°C[19] and thus is a true measure of the affinity between enzyme and substrate. However, other factors must also play some rôle as the K_M with RNA is still higher in the preparation with the spacer arm than in those without it. In this sense it is plausible to think of non-specific interactions between the enzyme and the matrix, as in the case of soluble RNAase in the presence of activated and neutralized Sepharose.

TABLE II

ELUTION IN AMMONIUM ACETATE, pH 7.0 AND 4°C, OF DIFFERENT NUCLEIC ACID DERIVATIVES, ALONE AND IN BINARY MIXTURES, FROM A SEPHAROSE 4B–RNAASE COLUMN

I = Sepharose 4B–RNAase (coupling reaction at pH 8.5); II = Sepharose 4B–RNAase (coupling reaction at pH 10.5); III = Sepharose 4B–spacer–RNAase (coupling reaction at pH 8.5); IV = Sepharose 4B–spacer–RNAase (coupling reaction at pH 10.5).

		I (mS)	*II (mS)*	*III (V_e/V_t)*	*IV (V_e/V_t)*
Purine		0.86	0.86	1	1
Ribonucleosides*		0.86	0.86	1	1
3′-CMP		4.7	3.1	2.24	1.40
3′-UMP		6.0	–	–	–
5′-AMP		6.6	3.8	3.08	1.82
5′-GMP		8.0	–	–	–
3′-CMP + 5′-AMP	3′-CMP	6.3	3.3	2.66	1.68
	5′-AMP	8.5	4.0	4.07	1.68
3′-CMP + 5′-GMP	3′-CMP	5.8	–	–	–
	5′-GMP	8.4			
3′-UMP + 5′-AMP	3′-UMP 5′-AMP	7.6	–	–	–
3′-UMP + 5′-GMP	3′-UMP 5′-GMP	6.6	–	–	–
2′-CMP + 3′-CMP	2′-CMP 3′-CMP	4.5	–	–	–
5′-AMP + 5′-GMP	5′-AMP 5′-GMP	7.0	–	–	–
5′-ADP		7.5**	–	–	–
		14.4			
3′5′-ADP		7.2**	–	–	–
		15.6			
3′-CMP***		4.7	–	–	–
5′-AMP***		5.5	–	–	–
3′-CMP + 5′-AMP***	3′-CMP 5′-AMP	5.6	–	–	–

* Nucleosides appear as contaminants of nucleotides. The identification was done by means of UV spectroscopy and thin-layer chromatography (TLC). Although the elution of the base purine occurs at the same conductance as the nucleosides, the latter have an elution volume larger than the former.

** The peak of 5′-ADP eluting at 9.8 mS and that of 3′5′-ADP eluting at 7.2 mS are due to non-specific interactions of the nucleoside diphosphate with the matrix.

*** Experiments carried out under the same chromatographic conditions except that the pH was 5.5.

Strength of the interaction between Sepharose 4B–RNAase preparations and several ligands

The results in Table II indicate the strength of the interaction of several ligands with the enzyme covalently bound to Sepharose 4B. The binding strength, measured as the conductance at which the ligands are eluted, follows the same order in the preparations obtained by coupling of the enzyme at pH 8.5 and 10.5. In the case of RNAase attached to the matrix through a spacer arm, a much weaker interaction is found. In fact no salt gradient can be applied because the ligands are already eluted with the starting buffer (10 m*M*). For this reason the binding strengths can be compared only by using the V_e/V_t elution indices. In this case too, 5'-AMP binds more strongly than 3'-CMP and in the preparation in which the enzyme was coupled at pH 8.5, but not in that coupled at pH 10.5, a co-operative effect is found. In both types of immobilized enzymes (with and without spacer arm) the binding is stronger in the preparations obtained at pH 8.5 than in those obtained at pH 10.5.

Although the order of binding strength coincides with that found for the soluble enzyme with respect to bases, nucleosides and nucleotides, the strength of interaction for the different nucleotides differs from that found previously[20,21]. In this context it should be mentioned that the reported dissociation constants were obtained from the inhibition index[21] or Lineweaver-Burk plots[20], always assumming that the ligand was a competitive inhibitor. However, this is not a valid assumption, at least for 5'-AMP, which is an activator of RNAase when cytidine 2',3'-phosphate is used as substrate[6]. Other values reported in the literature (*e.g.*, refs. 2, 6, 22) have been obtained with only a few ligands, with different methods and under different experimental conditions so it is not possible to establish reliable comparisons. A compilation of the existing data can be found in ref. 23.

In the present study the order of the strength of interaction between the two groups (3'-pyrimidine and 5'-purine) is nucleotides is clearly established. The elution results show that in the immobilized enzyme obtained at pH 8.5, which was the most thoroughly studied, the strength of the interaction, at pH 7.0 and 4°C, follows the order 5'-GMP > 5'-AMP > 3'-UMP > 3'-CMP. Of all ligands tested, the strongest interactions occur with the nucleoside diphosphates 3'5'-ADP and 5'-ADP, although in these two cases very important non-specific interactions also occur as could be seen by chromatography of 5'-ADP and 3'5'-ADP on a column of Sepharose previously activated and neutralized with glycine.

Although the comparison between the soluble and immobilized forms of the enzyme is subject to limitations, the co-operative behaviour of mixtures of 3'-pyrimidine and 5'-purine nucleotides can be explained in the light of the known behaviour of the soluble enzyme. It has been shown[1] that 3'-pyrimidine nucleotides bind preferentially to $B_1R_1p_1$ whereas 5'-purine nucleotides bind to $B_2R_2p_1$; the binding of either of them induces a conformational change in the protein making it more compact[24,25] and, as a consequence, the binding of either or both ligands becomes tighter. Haffner and Wang[6] showed that a ligand in $B_2R_2p_1$ activates the soluble enzyme and it is likely that a conformational change of the protein is also involved, as shown by Arús *et al.*[26]. This is substantiated by the fact that a mixture of the 2' and 3' isomers of CMP (both bind to $B_1R_1p_1$) shows no co-operative effect. These facts together with the very strong binding of 5'-ADP and PP_i to the soluble enzyme[20] and of 5'-ADP with the immobilized preparation show that the p_1 site can accom-

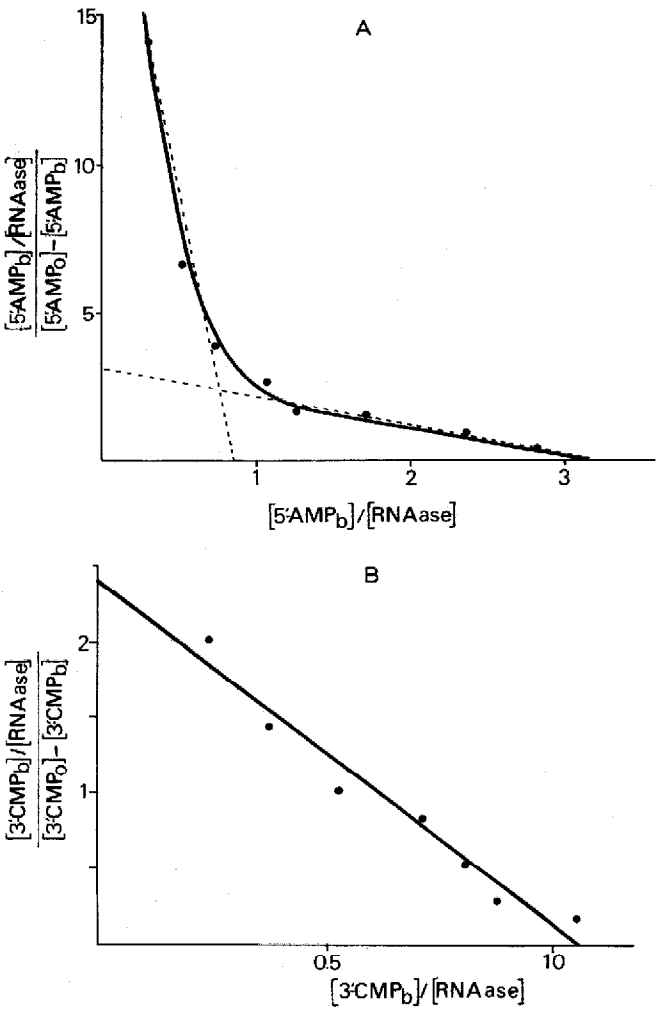

Fig. 2. Scatchard plots of the interaction of nucleotides with Sepharose 4B–RNAase. A, 5'-AMP; B, 3'-CMP. In both cases the immobilized preparation was obtained using pH 8.5 for the coupling reaction. The experiments were carried out at pH 7.0 and 4°C. The subscript b refers to bound ligand and the subscript o to initial ligand. See Methods for more details.

modate more than one phosphate and so the phosphates of a 3'-pyrimidine nucleotide and of a 5'-purine nucleotide both fit in p_1 despite the likely charge repulsions. The described behaviour applies to preparations obtained both by coupling at pH 8.5 and 10.5.

Stoichiometry of the interaction and binding constants

Fig. 2A and B shows the Scatchard plots corresponding to the interaction of Sepharose 4B–RNAase (coupling reaction at pH 8.5 without spacer arm) with 5'-AMP and 3'-CMP respectively at pH 7.0 and 4°C. Table III lists the binding constants, K_a, and number of binding sites for 3'-CMP and 5'-AMP with the two im-

TABLE III

INTERACTION OF 3′-CMP AND 5′-AMP WITH TWO DIFFERENT SEPHAROSE 4B–RNAASE PREPARATIONS AT pH 7.0, 4°C.

Figures were obtained by using the Scatchard plot. I, Coupling reaction at pH 8.5; II, coupling reaction at pH 10.5.

Preparation	*Ligand*	K_a ($M^{-1} \times 10^{-4}$)	*Stoichiometry (mol ligand/mol enzyme)*	
I	3′-CMP	0.28 ± 0.02	1	
I	5′-AMP	2.54 ± 0.30	1*	3.3**
II	3′-CMP	0.27 ± 0.02	1	
II	5′-AMP	1.49 ± 0.16	1*	1.5**

* Strong binding.
** Total (strong + weak) binding.

TABLE IV

STOICHIOMETRY OF THE INTERACTION BETWEEN SEPHAROSE 4B–RNAASE AND SEVERAL LIGANDS CALCULATED BY USING A SINGLE SATURATION POINT

Coupling reaction at pH 8.5. The ligand:enzyme molar ratio was always 20:1. Experiments were carried out at Ph 7.0, 4°C.

Ligand	*Stoichiometry*
3′-CMP	1
5′-AMP	2.9
5′-GMP	3.8
5′-CMP	1.6
5′-UMP	1.6

mobilized-RNAase preparations (without spacer arm). It is seen that whilst the K_a for 3′-CMP does not change, that for 5′-AMP decreases by a factor of two when the coupling is carried out at pH 10.5 instead of 8.5. However, in both cases the K_a for 5′-AMP is significantly larger than that for 3′-CMP (nine and five times respectively). It is also very interesting that whereas there is always only one binding site for 3′-CMP, in the case of 5′-AMP there is one strong binding site and several weak binding sites. The dependence of K_a on the pH of the coupling reaction demonstrates the influence of the groups involved in the covalent attachment with the matrix on the general flexibility of the protein. The results shown in Table IV, obtained by another method, also demonstrate the binding of only one molecule of 3′-CMP and the multiple binding of 5′-nucleotides. The binding of several molecules of 5′-AMP to RNAase has also been shown for the soluble enzyme[27].

The K_a values obtained for 3′-CMP and 5′-AMP are of the same order of magnitude as those for the soluble enzyme, even taking into account the already mentioned difficulties in the comparison of results. Several methods have been proposed for the determination of K_a values by affinity chromatography[28–30]. In all these methods the soluble enzyme binds to an immobilized ligand and it is later eluted with another ligand (in a competitive manner), whereas in the present work a soluble ligand was bound to an immobilized enzyme preparation and was then eluted with

a salt gradient. This difference in methodology, as well as the different ligands and experimental conditions used, again prevent any comparison of results.

Finally, it should be mentioned that preliminary studies show that the behaviour of immobilized ribonuclease A, purified from the twice recrystallized commercial preparation by the method of Taborsky[31], is completely analogous to that described in the present work.

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