AFFINITY CHROMATOGRAPHY OF GUANYLORIBONUCLEASE FROM *Streptomyces aureofaciens*

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2′,3′-O-2-Carboxyethylideneinosine 5′-phosphate (*Ia*), 5′-phosphorothioate (*IIa*), 5′-O-carboxymethylinosine 2′(3′)-phosphate (*IIIa*) and 2′(3′)-phosphorothioate (*IVa*) were bound to 6-aminohexyl-Sepharose 4B by the method of mixed anhydrides with ethyl chloroformate. 5′-O-(2-Aminoethylamidocarbonylmethyl)inosine 2′(3′)-phosphorothioate (*Va*), O-(4-aminophenyl)-inosine 5′-phosphorothioate (*VIa*) and O-(4-aminophenyl)-O-phenyl phosphorothioate (*VIIa*) were bound to Sepharose 4B activated with cyanogen bromide. The resulting modified supports *IIa*—*VIb* bind the ribonuclease of *Streptomyces aureofaciens* within the pH range 6·5—7·5. The specific affinity bond manifests itself in derivatives with ligands of type *IVb* and *Vb*, on which a 50—70fold purification degree can be achieved, with respect to the crude preparation.

In preceding communications we described the preparation of derivatives of inosine 2′(3′)-phosphate and inosine 2′(3′)-phosphorothioate with a binding group in the position 5′ of the sugar residues[1], and the preparation of inosine 5′-phosphate and inosine 5′-phosphorothioate derivatives with a binding group in the position 2′,3′ of the ribonucleoside part of the molecule[2].

In this paper we study the binding of the mentioned potential inhibitors to the affinity support, determination of the properties of the polymers obtained, and mainly their application in affinity chromatography of guanylspecific ribonuclease of *Streptomyces aureofaciens*[3].

EXPERIMENTAL

The enzyme used (RNase of *Str. aureofaciens*) was isolated by J. Gašparík (Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava) according to the described procedure[3]. For the determination of the substrate and inhibitory activity an enzyme was used of specific activity 60000 EU/mg protein. For affinity chromatography a partially purified preparation of specific activity 875 and 1000 EU/mg of protein was used, strongly contaminated with brown coloured material. In order to eliminate denaturated proteins the enzyme was dissolved in 0·05M phosphate buffer of pH 7·0 and filtered through a Millipore filter. The determination of the enzyme activity was carried out according to Egami[4] with reference to macromolecular RNA at pH 7·0 (the EU unit is defined under the given conditions as the amount of enzyme increasing absorbance A_{260}

of the supernatant after precipitation with uranyl acetate by 1·0). Sepharose 4B and 6-Aminohexyl Sepharose 4B were preparations of Pharmacia Ltd., Sepharose 4B activated with cyanogen bromide was prepared fresh according to ref.[5]. Guanosine 2',3'-cyclophosphate (lithium salt) was prepared according to ref.[6], GpC (lithium salt) according to ref.[7].

Determination of Inhibitory Activity of Compounds *Ia* to *VIIa*

The reaction mixture (500 µl) contained lithium salt of guanosine 2',3'-cyclophosphate and the effector in concentrations $5 . 10^{-3}$M and 10000 EU in 0·05M-TRIS-buffer of pH 7·0; incubation was carried out at 37°C for 15 h, and aliquots 10 µl of the mixtures were analysed by HPLC for the extent of cleavage of the substrate to guanosine 3'-phosphate. The results are given in Table I.

Binding of Ligands onto Sepharose 4B

A) *On 6-Aminohexyl-Sepharose* 4B: Compound *Ia*—*IVa* (100 or 50 µmol, respectively) was dissolved in dioxane (2·0 or 1·0 ml, resp.) at 10°C, ethyl chloroformate (20 or 10 µl, resp.) was added and the mixture stirred at 10°C for 30 min. 6-Aminohexyl-Sepharose 4B (5 ml) in 10 ml of 50% dioxane was added and the mixture stirred at 4°C overnight. The suspension was filtered off and the precipitate washed with 50% dioxane and water. The product was suspended in 10 ml of 50% glycerol in 0·05M phosphate buffer of pH 7·0 and stored at −20°C. The determination of the bound ligand was carried out spectrophotometrically from the difference on the basis of its content in the filtrate of the reaction mixture. The amounts of ligand bound are given in Table II.

B) *On cyanogen bromide activated Sepharose* 4B: a solution of ligand *Va*—*VIIa* (100 or 200 µmol, respectively) in 5 ml of 0·05M of sodium hydrogen carbonate buffer of pH 8·5 was added to a suspension of 5 ml of freshly prepared[6] activated Sepharose 4B in 10 ml of the same buffer and the suspension was stirred at 4°C for 24 h. It was filtered off under suction, the material on the filter washed with water and the product stirred with 25 ml 1M ethanolamine for 1 h.

TABLE I

The Inhibition of the Splitting of Guanosine 2',3'-Cyclic Phosphate by RNase of *S. aureofaciens*

Compound	Splitting ratio[a]	Compound	Splitting ratio[a]
Inosine 5'-phosphate	1·07	*Ia*	0·98
Inosine 5'-phosphorothioate	1·02	*IIa*	1·07
Uridine 5'-phosphorothioate	1·14	*IIIa*	0·95
Inosine 2'(3')-phosphorothioate	1·04	*IVa*	0·86
Inosine 2'(3')-phosphate	0·95	*Va*	1·02
Inosine 2',3'-O,O-cyclophosphorothioate	0·98	*VIa*	0·98

[a] Splitting in the presence of an equimolar concentration of the effector, referred to the control experiment.

Filtration was repeated, the material washed with water and stored in 10 ml of 50% glycerol in 0·2M TRIS-HCl of pH 7·5 at −20°C.

Dependences of the Enzyme-to-Support Binding on pH

An enzyme solution (0·5 ml; 2000 EU) in 0·05M-TRIS-HCl buffer of pH 6·5—8·0 (in 0·5 pH steps) was pipetted into 0·2 ml of affinity support and the suspension was allowed to stand with occasional stirring at 4°C for 2 h. After centrifugation an aliquot (50 µl) of the supernatant was pipetted into 100 µl of GpC solution (lithium salt) (5 mg/ml pf 0·05M-TRIS-HCl buffer of pH 7·5). After 15 h incubation at 37°C 20 µl samples were analysed by HPLC and the cleavage was determined from the area of the cytidine peak, in comparison with a control experiment without the enzyme. Optimum values of pH for the enzyme binding are given in Table II.

Binding of the Enzyme on Sepharose 4B

A column (0·6 × 3·6 cm) of 1 ml of Sepharose 4B was equilibrated with 0·05M phosphate buffer of pH 7·0 and 3 ml of enzyme solution (25550 EU) in the same buffer were introduced into the column. The column was washed first with 3 ml of starting buffer and then with 3 ml of 3M sodium chloride. Activity was determined in individual eluates: in the first 6 ml of eluate 25270 EU were recovered. The contaminating coloured material is not retained.

The Binding of the Enzyme on Modified Sepharoses 4B

This was carried out on 1 ml (column dimension 0·6 × 3·6 cm) of support equilibrated with 0·05M phosphate of pH 7·0; after application of 3 ml (25550 EU) of the enzyme the column was washed with 2 ml of the same buffer and the activity of the eluate (4·5 ml) was determined according to Egami[4]. The column was eluted with 5 ml of 2M sodium chloride and the activity of the enzyme set free was determined in the same manner. The results are given in Table II.

TABLE II
Properties of Modified Supports

Support	Bound ligand (µmol/ml)	pH_{opt}	Capacity EU/ml[a]	Spec. activity EU/mg[b]	Degree of purification
Ib	10·0	7·0—8·0	12 800	5 830	7·7
IIb	12·0	7·0—8·0	21 200	7 300	9·7
IIIb	8·0	6·5—8·0	19 100	5 830	7·7
IVb	8·0	6·5—8·0	23 200	37 500	50·0
Vb	[c]	7·0—7·5	20 850	51 670	69·0
VIb	[c]	6·5—7·5	10 700	5 700	7·6
VIIb	[c]	6·5—7·0	—	2 400	3·2

[a] 25 500 EU/ml of support were applied; [b] starting enzyme: 750 EU/mg of protein; [c] not determined.

Operation Capacity of the Support

The determination of the capacity was carried out with the support *Vb* on a 1 ml column (0·6 × × 3·6 cm) by gradual application of a solution of the enzyme (8 500 EU/ml, in 0·3 ml portions) in 0·05M phosphate buffer of pH 7·0 until the activity of the eluate was equal to the activity of the solution applied (in EU/ml). The value found was 23 000 EU/ml of support *Vb*.

Chromatography of RNase of *S. aureofaciens* on Supports *Ib*—*VIIb*

An enzyme solution (4 ml; 30 000 EU, spec. activity 1 000 EU/mg) was introduced into a column of the support (1 ml, 0·6 × 3·6 cm) equilibrated with 0·05M phosphate buffer of pH 7·0 and the column was washed with the basic buffer until the absorbance at 280 nm decreased, and finally with a linear gradient (0—2M) of sodium chloride (in ten 1 ml steps) in the same buffer. The elution rate was 12 ml/h. Enzyme activity was determined in the fractions (2 ml) according to Egami (see above) and on the basis of A_{280}. The graphs of these separations are given in Fig. 1. The degree of purification of the enzyme is given in Table II.

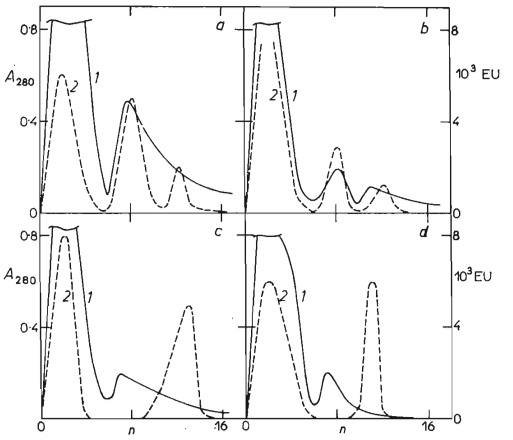


FIG. 1

Affinity Chromatography of RNase from *S. aureofaciens*

A support *IIb*, *B* support *IIIb*, *C* support *IVb*, *D* support *Vb*; ——— A_{280}, ----- enzyme activity (EU). The beginning of the sodium chloride gradient is in fraction No 6.

RESULTS AND DISCUSSION

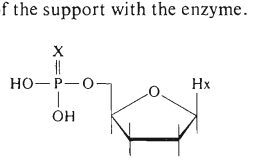
A condition of successful affinity chromatography of the enzyme is the inhibitory effect of the corresponding ligand in the enzyme-catalysed reaction. Therefore we first studied the effect of individual ligands, phosphorothioates and phosphates of inosine[1,2] on the cleavage of guanosine 2',3'-cyclic phosphate with the ribonuclease of *S. aureofaciens*. The results of these experiments, shown in Table I, demonstrated that under the conditions used the inhibition of the cleavage takes place merely in the case of compound *IVa*. In view of the fact that ribonuclease has a limited hydrolytic step, *i.e.* it cleaves primarily the internucleotidic bonds by the transfer reaction, a very high degree of inhibition cannot be expected in 2',3'-cyclic phosphate either. Therefore we decided to check the bond of the enzyme onto the modified supports directly.

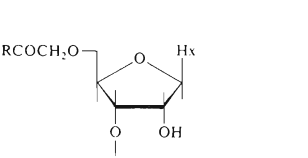
Depending on the structure of the ligand these supports can be divided into three groups: derivatives of nucleotides and thionucleotides of inosine with the bonding group having the character of a carboxyl group (*Ia*–*IVa*), a derivative with a bonding group containing an amino group (*Va*), and finally compounds with a *p*-aminophenolic function (in addition to the ester of inosine-5'-phosphorothiate *VIa* (see[2]) O-(4-aminophenyl)-O-phenylphosphorothioate (*VIIa*) was also used as a reference substance[7,8]).

The first group of compounds was condensed with 6-aminohexyl-Sepharose 4B using the mixed anhydride method with ethyl chloroformate. It was shown paralelly that under these conditions desulfuration of the P=S bond of phosphorothioate[1,2] does not take place. However, it may be expected that while in the case of compounds *Ia*, *IIa* the phosphate or the phosphorothioate group in the position 5' will not be affected, in the case of the bonding to the support by this method, the grouping of the cyclic 2',3'-diester will be formed from 2'(3')-phosphate and 2'(3')-phosphorothioate. A bonding different from that involving the carboxyl group can also be excluded. The second group of substances is structurally favoured for the binding onto epoxy- or cyanogen-bromide-activated Sepharose 4B. We used the second method of activation. (While we were able to determine the degree of the binding of the ligand onto the support (8–10 μmol per ml) for the first group, for technical reasons these data are not available for the second group of supports with the ligands *Vb*–*VIIb*).

The determination of optimal pH for the binding of the enzyme to the support (Table II) showed that this range is rather broad and therefore we operated at neutral pH. Carrying out an experiment for comparison with unsubstituted Sepharose 4B we found that no sorption of the enzyme onto it takes place. The capacity of individual supports for the enzyme (Table II) is dependent on the type of the ligand. From these data it follows that the enzyme is affinity-bonded to the support with the ligand bearing the structure of inosine 2'(3')-phosphorothioate (*Vb*) and also inosine 5'-phosphorothioate (*IIb*). In these supports the capacity is approximately equal

(however, the degree of substitution is different). The operational capacity of the support, determined in the case of support *Vb*, is approximately 23000 EU/ml, *i.e.* similar to the value found under conditions which do not guarantee the saturation of the support with the enzyme.



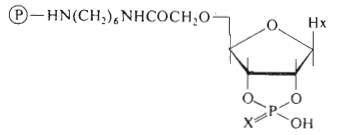


Ia, X = O, R = OH
Ib, X = O, R = $NH(CH_2)_6NH$—Ⓟ
IIa, X = S, R = OH
IIb, X = S, R = $NH(CH_2)_6NH$—Ⓟ

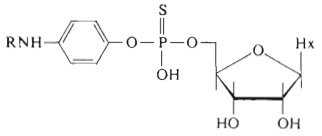
IIIa, X = O, R = OH
IVa, X = S, R = OH
Va, X = S, R = $NH(CH_2)_2NH_2$
Vb, X = S, R = $NH(CH_2)_2NHC(=NH)O$—Ⓟ

The final evaluation of the suitability of the supports prepared for affinity chromatography of the ribonuclease from *S. aureofaciens* was carried out under analytic conditions simulating preparative chromatography. A low concentration solution of an enzyme of low specific activity and contaminated with coloured substances from the fermentation medium was used expressly in an amount exceeding the capacity of the support. The experiments were evaluated according to the yield of the recovered enzyme, the degree of its purification, and the possibility of the elimination of coloured material. These data are presented in Fig. 1 and in Table II. The highest degree of purification was achieved on the supports *IVb* and *Vb* (50–70 fold) in which the activity of the enzyme is eluted only after the fractions of inactive protein. In the case of both remaining supports (*IIb*, *IIIb*) the enzyme is retained on the support, but this sorption is not specific, because the enzyme is released from the support simultaneously with the ballast protein. The adsorption of the coloured material on the support follows the order: $V = VI < II < III < IV < I$. On supports *Vb* and *VI* coloured material is not adsorbed at all, but it is eluted together with ballast proteins with the starting buffer. The penetration of a part of the enzyme activity through the column is caused by purposeful overloading and it also may be a consequence of the contamination by other nucleolytic activities in the crude preparation.

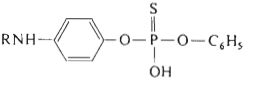
The results of this study demonstrate that the affinity support of the type of Sepharose 4B with bound ligands containing the groupings inosine 2',3'-O,O-cyclophosphorothioate (*IVb*) and inosine 2'(3')-phosphorothioate (*Vb*) bind specifically guanyl-



IIIb, X = O
IVb, X = S



VIa, R = H
VIb, R = —C(=NH)—O—Ⓟ



VIIa, R = H
VIIb, R = —C(=NH)—O—Ⓟ

In formulae *I*—*VII*: Hx ... hypoxanthin-9-yl residue, Ⓟ ... Sepharose 4B polymer unit.

specific ribonuclease from *S. aureofaciens*; the specificity of the binding decreases in ligands of the type of inosine 5'-phosphorothioate (*IIb*) and 2',3'-cyclophosphate (*IIIb*). The binding is weak and unspecific in derivatives containing a ligand of the type of inosine 5'-phosphate (*Ib*) and of aromatic diester of inosine 5'-phosphorothioate (*VIb*). From the viewpoint of practical application the support of type *Vb* seems most suitable for use, because on it a high degree of purification can be achieved together with the elimination of the contaminating coloured material. It is probable, that the carriers with the ligand of type *Vb* and *IVb* could also be applied in affinity chromatography of other guanylspecific ribonucleases capable of interacting with nucleotide derivatives of inosine.

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