INFRARED STUDIES ON RIBONUCLEASE A–MONONUCLEOTIDE COMPLEXES

Ionization states and hydrogen bonding of the phosphate groups

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1. Introduction

The interaction of mononucleotide inhibitors with bovine pancreatic ribonuclease A (EC 3.1.4.22) has been studied by various methods (for a review, see [1,2]). Recently, Rüterjans et al. [3] and Gorenstein et al. [4] discussed in detail the ionization states and chemical environment around the phosphate groups in the RNAase nucleotide complexes using ^{1}H- and ^{31}P-NMR and X-ray crystallographic data. The different ionization states of phosphate ester groups can easily be determined by infrared spectroscopy [5,6]. In aqueous solution the characteristic bands of the vibrations of the nucleoside monophosphates are very intense and sensitive to protonation [6,7]. Fortunately there is only a slight interference of these bands with those of protein providing the opportunity to observe changes in ionization states in enzyme complexes.
In this paper we wish to report the application of infrared spectoscopy to the study of interaction of RNAase A with the phosphate groups of Cyd-2′-*P* and Cyd-5′-*P*. It is known that these two nucleotides show the largest differences in binding properties [2]. The infrared spectra of the nucleotides either 'free' in solution (i.e., in hydrated state) or bound to bovine pancreatic ribonuclease A are compared at three different pH values.

2. Materials and methods

The nucleotide inhibitors were obtained from Sigma. Bovine pancreatic ribonuclease A was purchased from Serva, Heidelberg, FRG and further purified on Sephadex G-25. This product was free of phosphate according to standard analytical procedures Deionized and double distilled H_2O was used for all solutions. Stock solutions of 0.022 M RNAase and 0.11 M nucleotides were prepared and aliquot amounts were mixed without additional salt in order to obtain 0.015 M 1:1 mixtures.

The pH was adjusted with 1 N HCl and measured on a Knick precision pH meter combined with an Ingold micro electrode assembly requiring only a sample volume of 20 μl. All solutions were mixed just before the pH measurement and the following infrared run.

The infrared spectra were recorded on a Perkin Elmer 283 grating spectrometer. A fixed cell with one germanium and one IRTRAN-2 window and wedge shaped sample space with 35 μm thickness was used in the sample beam. The interfering absorptions of H_2O were compensated by a second cell with variable thickness in the reference beam. Calibration of the baseline was checked at the H_2O vibration at 2120 cm^{-1} which is free from interfering absorptions of the solute. All spectra were taken at 25 ± 1°C.

3. Results and discussion

3.1. *Free nucleotides*

The two ionization states of the nucleotide phosphate groups free in solution can be distinguished from each other by the following bands (fig.1): Above pH 7.5 the free dianions exhibit a sharp band

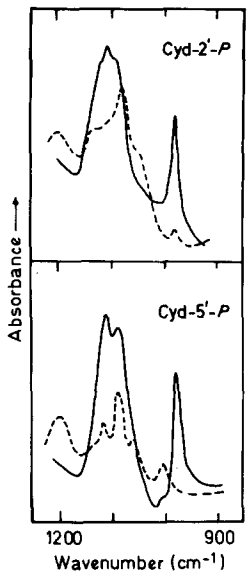


Fig.1. Infrared spectra of cytidine monophosphates, 0.015 M aqueous solution, ordinate expansion 2, (——) pH 7.5; (- - - -) pH 5.5.

($\nu_s PO_3^{2-}$) at 975 cm^{-1} which is attributed to the symmetric stretching vibration and a doublet of the antisymmetric stretching vibrations ($\nu_{as} PO_3^{2-}$) around 1100 cm^{-1} (table 1) [6,12]. The doublet[+] indicates that the pyramidal structure of the PO_3^{2-} group (pseudo-C_{3v} symmetry) is slightly disturbed [10,11]. The ratio of the intensities of the two bands of the doublet depends on the nucleobase and the substitution on the ribose ring (Haas, I. and Matthies, M. unpublished results). Below pH 5.5 the free monoanions show the symmetric stretching vibration ($\nu_s PO_2^-$) at about 1080 cm^{-1} [6,9] (table 1).

3.2. *Enzyme–nucleotide complexes*

In the wavenumber range 1200–900 cm^{-1} only one intense vibration of RNAase A at about 1082 cm^{-1} is found (presumably a C–N stretching vibration [9]) which does not change with pH (fig.2).

[+] In molecules with C_{3v} symmetry the antisymmetric stretching vibration is doubly degenerated. The doublet is caused by a removal of the degeneracy [11]

Table 1
IR bands of phosphate vibrations of free and bound nucleotides

	pH 7.5		pH 5.5		pH 3.0	
Cyd-2'-*P*	1102 s	$\nu_{as} PO_3^{2-}$	1080 m	$\nu_s PO_2^-$	1080 m	$\nu_s PO_2^-$
	1094 sh					
	975 s	$\nu_s PO_3^{2-}$				
Cyd-5'-*P*	1105 s	$\nu_{as} PO_3^{2-}$	1085 m	$\nu_s PO_2^-$	1085 m	$\nu_s PO_2^-$
	1090 s					
	975 s	$\nu_s PO_3^{2-}$				
Cyd-2'-*P*	1132 m,br	$\nu_{as} PO_3^{2-}$	1132 m,br	$\nu_{as} PO_3^{2-}$	Masked	
+ RNAase A	1105 m					
	965 m	$\nu_s PO_3^{2-}$	965 m	$\nu_s PO_3^{2-}$	Masked	
Cyd-5'-*P*	1105 s	$\nu_{as} PO_3^{2-}$	Masked		Masked	
+ RNAase A	975 m	$\nu_s PO_3^{2-}$				

Abbreviations: s = strong, m = medium, br = broad, sh = shoulder

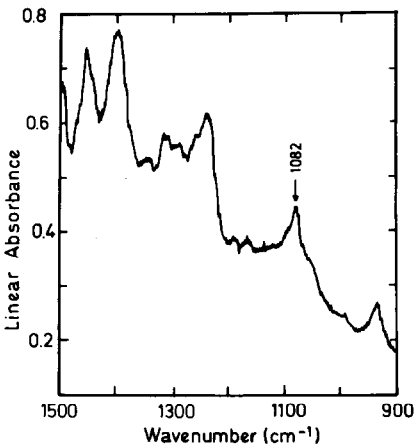


Fig.2. Infrared spectrum of RNAase A, 0.015 M aqueous solution, pH 7.4.

It masks the phosphate bands between 1100 and 1050 cm^{-1} (fig.3a,b) but does not overlap with the other phosphate bands above 1100 cm^{-1} and below 1050 cm^{-1}.

At pH 7.0 the infrared spectrum of Cyd-2′-*P*·RNAase A complex shows three new bands compared with that of the enzyme (table 1): 1132, 1105, and 965 cm^{-1}. It is known that the binding of inhibitors to RNAase strongly depends on pH [13,14]. Above and below pH 5.5 the dissociation constants increase. At pH 7.0 about 50% of Cyd-2′-*P* molecules are bound [13]. Therefore the spectrum reflects vibrations of a mixture of bound and free phosphate ester groups++. To decide which bands can be assigned to bound and which to free phosphate groups, spectra have been recorded at higher pH values. An increase in pH causes a further dissociation of the complexes. At pH 8.9 (fig.3a) only the bands at 1105 and 975 cm^{-1} can be observed which are found at actually the same position compared with the free nucleotides (fig.1). On the other hand, at the pH of maximum binding (pH 5.5) (fig.3a) almost all the nucleotides are bound and only the bands at 1132 and 965 cm^{-1} are seen which are therefore assigned to the bound phosphate groups. Hence in the complex one of the two antisymmetric PO_3^{2-} stretching vibrations is shifted

++ Due to the high concentrations used the enzyme will be completely saturated with nucleotides

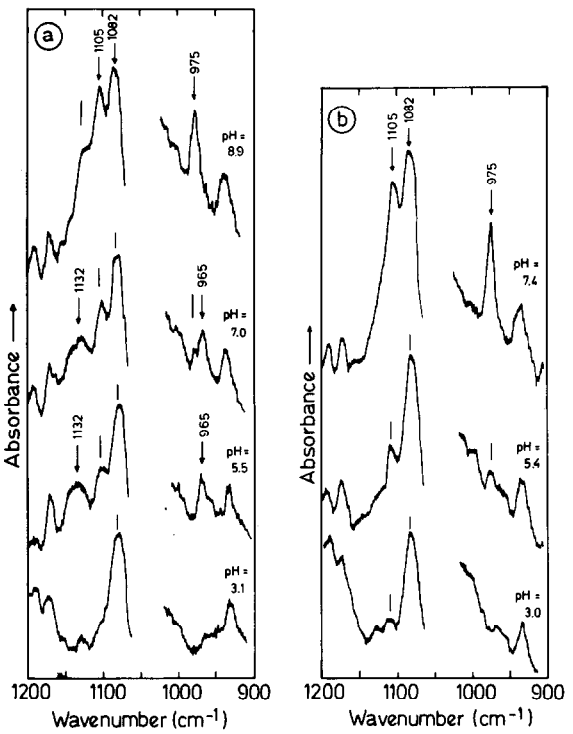


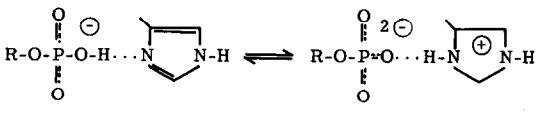
Fig.3. Infrared spectra of (a) RNAase A·Cyd-2′-*P* complexes, and (b) RNAase A·Cyd-5′-*P* complexes, ordinate expansion 4, abscissa expansion 2. The steep slope of the protein band between 1060 and 1020 cm^{-1} was omitted and the spectra below 1030 cm^{-1} were graphically displaced on the absorbance scale.

from 1105 to 1132 cm^{-1}, i.e., 27 cm^{-1} to higher wavenumbers, and the symmetric PO_3^{2-} stretching vibration from 975 to 965 cm^{-1}, i.e., 10 cm^{-1} to lower wavenumbers. The second antisymmetric PO_3^{2-} stretching vibration is masked by the protein band at 1082 cm^{-1}.

The band shifts reflect the lowering of the symmetry of the phosphate groups which can be taken as evidence for the involvement of the phosphate groups of Cyd-2′-*P* in binding to the enzyme. From X-ray and NMR studies it is assumed that His 12* form hydrogen bonds with the phosphate moiety of Cyd-2′-*P* [2,3,8]. These hydrogen bonds can be represented by the following equilibrium**

* Note the reversal of the ^{1}H-NMR peak assignments of His 12 and His 119 [15]

** Additional H-bonds, e.g., to water, have been omitted



I II

Considering the phosphate bands in Cyd-2′-*P*·RNAase A complexes we are able to determine the ionization state of the phosphate group of Cyd-2′-*P* and thus the position of the proton in the hydrogen bond. At pH 5.5 the infrared spectrum only reflects vibrations of bound phosphate groups. Compared to the infrared spectra of the monoanionic and the dianionic Cyd-2′-*P* in the absence of RNAase A (fig.1) the corresponding spectrum of the complex (fig.3a) resembles more the spectrum of the dianionic state (fig.1). Both the antisymmetric and the symmetric stretching vibrations of the PO_3^{2-} group are shifted and broadened but do not vanish. Hence the proton in the hydrogen bond is not attached at the phosphate group and the proton-transfer complex II is strongly favoured. The potential energy function of the hydrogen bond can be described by an asymmetrical double minimum potential curve [5,16] with the higher well at the phosphate group and the deeper well at the imidazolium residue of His 12.

Beside these hydrogen bonding interactions the remaining P–O bonds can still form hydrogen bonds with other proton donor groups, e.g., water molecules or the ϵ-amino group of Lys 41 as discussed by Rüterjans et al. [3]. The replacement of H-bonds between the PO_3^{2-} group of free nucleotides and water by those with amino acid side chain groups in the complex causes the strong decrease of the symmetry of the phosphate groups which is indicated by the band shifts of the ν_{as} and ν_s PO_3^{2-} vibrations.

A further decrease of the pH to 3.1 causes the disappearance of the bands at 1132 and 965 cm^{-1} indicating the protonation of the phosphate group. Since the wavenumber range of the $\nu_s PO^{2-}$ is masked by the protein band at 1082 cm^{-1} it is not possible to obtain information about the interactions of the phosphate group with the enzyme at low pH.

In contrast to the observations with Cyd-2′-*P*·RNAase A complexes no band shifts are found with Cyd-5′-*P*·RNAase A complexes (fig.3b). At pH 7.4 one $\nu_{as}PO_3^{2-}$ vibration occurs at 1105 and the $\nu_s PO_3^{2-}$ vibration at 975 cm^{-1} is observed at actually the same position as found with free Cyd-5′-*P* (dianionic ionization state (fig.1)). With decreasing pH (5.4) these bands disappear. Hence, the phosphate group of Cyd-5′-*P* seems to be in the monoanionic state at the pH of maximum binding. From the similar pH dependence of the phosphate groups of free and bound nucleotides it may be concluded that they are even still hydrated throughout the whole pH range of the titration, i.e., that they do not (or only slightly) interact with the enzyme.

Acknowledgements

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