

## Thermodynamics of immobilized ribonuclease A

Ezio Battistel<sup>1</sup>, Daniele Bianchi<sup>1</sup> and Giovanni Rialdi<sup>2</sup>

- 1) Istituto G. Donegani, Via Fauser 4 - 28100 Novara, Italy
- 2) Centro Studi Chimico-Fisici Macromolecole Sintetiche e Naturali, Corso Europa 30, 16132 Genova, Italy

**Abstract** - Proteins can be bound to surfaces through chemical coupling reactions between the reactive groups of the protein and matrix. In this report, ribonuclease A immobilized on Silica beads, has been used as a model to study the modification of the properties of the enzyme after immobilization. The biological activity has been monitored by studying the binding of the inhibitor cytidine-3'-monophosphate to the free, chemically modified and immobilized RNase. The equilibrium constants and the thermodynamic parameters associated with the reaction were evaluated by calorimetric and spectrophotometric titration techniques. The thermal unfolding of free, chemically modified and immobilized RNase have been carried out by differential scanning calorimetry in various conditions. After immobilization, the process of unfolding of the protein is not comparable with that of the free enzyme. In general, a stabilizing effect was observed.

### INTRODUCTION

Since the exploitation of the unique catalytic properties of enzymes as technological tool for the production of commodities at industrial level, it has been of great interest to develop new technologies based on the immobilization of the biocatalysts (ref. 1), either enzymes or cells. The strategy of the immobilization techniques is to overcome the disadvantages inherent to the use of biocatalysts, i.e. stability, loss of activity, sensitivity to elevated temperatures and to organic solvents, which collectively limit their practical applications (ref. 2). If active supported enzymes are prepared, it becomes both possible and economically competitive to use them for industrial purposes because they can be easily removed from the reaction mixture and recycled several times, and often they show a marked increase in stability and may be used in bioreactors for continuous processing.

Not only the immobilization of enzymes is proved to be an essential technique for the practical manipulation of enzymes, but it can also provide a good model system for the actual study of enzyme function (ref. 3). Information can be obtained on fundamental aspects of enzyme physical chemistry such as heat stability and unfolding mechanisms, refolding pathways, subunit interactions and protein microenvironment. Moreover, enzyme immobilized on artificial supports can provide a more realistic systems to assess the properties of enzyme *in vivo* than the free state in dilute solution. Enzymes are quite often immobilized naturally to cellular components such as membranes or organelles like ribosomes inside the cells.

Although several immobilization techniques are already well documented (ref. 4-6), each biocatalytic system or class has to be studied independently since the success of the immobilization depends on the type of support chosen with respect to the biochemistry of the catalyst and the activity mechanism. These techniques can be generally divided into the following categories: i) covalent attachment to water-soluble or -insoluble synthetic or natural supports, involving a single or a multipoint linkage between biocatalyst and support; ii) adsorption on organic or inorganic surfaces; iii) entrapment or incapsulation in polymeric gels and microcapsules or hollow fibers and membranes, respectively.

The presence of the support may have a great influence on the properties of the enzyme (ref. 7). Thus, conformational changes, even though slight, are expected to take place after immobilization, particularly after covalent attachment (ref. 8). This effect itself may lead to changes in the stability towards temperature, pH and denaturing agents. Moreover, within an immobilized preparation, it is not sure that the conditions of the medium surrounding the enzyme would be identical to those in the pure solvent, in particular with supports bearing charged groups. Shifts in the pH of optimum activity (ref. 9), change in the reaction rates (ref. 10) and specificity (ref. 11) as well as stabilization effects are likely to occur depending on the physico-chemical properties of the supporting materials.

In general, immobilization is regarded as a successful method for increasing the stability of enzymes, intended either as an increase of the thermal stability or as a decrease of the rate constant of inactivation under denaturing as well as operating conditions (ref. 6). Since conformational changes are involved in the enzyme inactivation and unfolding (ref. 12), it is likely that the attachment has the consequence of preserving the active conformational geometry of the macromolecule. Several studies have supported the concept of increased stability which arises from an increased rigidity of the enzyme molecule (ref. 8,13), and also on the basis of restriction of the conformational entropy changes involved in the unfolding process (ref. 14). While several chemical events involved in the mechanism of enzyme inactivation have been investigated (ref. 15), attempts to explain the enhancement of the thermal stability after immobilization are rather incomplete with regards to the altered enzyme conformation or microenvironment. The explanations will remain incomplete until a quantitative description of the changes of the non-covalent forces which are responsible for protein folding after immobilization are available (ref. 3).

Several techniques have been used to investigate the change of the activity and stability of the immobilized enzymes with respect to the free enzymes (ref. 1). Usually these experiments are laborious, because of the enzyme and time consuming activity tests at various conditions. Moreover, all the techniques commonly used to study the protein conformation in solution cannot always be employed because of the interference of the supporting materials. Fluorescence (ref.16), IR (ref. 17), ESR (ref.18) as well as H-exchange (ref. 19) and specific antibodies (ref. 20) have been employed to study protein conformation after immobilization, when the specific conditions make their use possible.

Microcalorimetry (isothermal or temperature scanning) is a potentially powerful tool for studying the biochemical and thermodynamic properties of the immobilized protein systems (ref. 21). Since the matrices of the enzyme preparations are not expected to interfere with the thermal analysis, calorimetry provides rapid and precise techniques for obtaining information on immobilized enzyme. It has the advantage of being a non-invasive method which does not require a dissolved or optically transparent sample.

Isothermal microcalorimetry studies can be helpful to elucidate the changes in ligand binding affinity, intersubunit forces or linkage phenomena after immobilization (ref. 22). Thermal titration of the active site with a suitable ligand is also a tool to assess the integrity of the immobilised enzyme molecules. The free energy (derived from the equilibrium constants) and the enthalpy changes associated with the protein functions may be sensitive to the changes of the enzyme microenvironment or conformation brought about by the presence of the supporting materials.

Differential scanning calorimetry (DSC) allows a direct determination of the effect of the immobilization process on the thermal stability of the proteins. The thermodynamic parameters which are associated with the thermal unfolding of the protein molecule such as the enthalpy, entropy and free energy changes (ref. 23) give direct information on the properties of the unfolding mechanism and pathways, as well as on the changes of domain structures after immobilization. By comparison with the parameters characteristic of the enzyme in dilute solution, it is possible to gain an insight on the role of the non-covalent forces involved in the mechanism of enzyme stabilization.

## EXPERIMENTAL PART

Bovine pancreatic ribonuclease (RNase, from Sigma), 130 mg, was dissolved in 15 ml of phosphate buffer 0.1 M, pH 7. The solution was gently agitated in the presence of controlled-pore aminopropyl Silica beads (Fluka 500 Å pore size, 30-45 mesh, 5 g). The beads were previously activated with glutaraldehyde (10% v/v) for half an hour at 4 °C. During the immobilization, the temperature was kept at 20 °C with a water thermostat. The degree of attachment of the protein onto the support was followed spectrophotometrically by monitoring the disappearance of the absorbance at 278 nm of the supernatant ( $A=9800 \text{ M}^{-1} \text{ cm}^{-1}$ ). After 5-8 hours, the protein immobilised was 80-90% of the protein present. The final concentration of RNase was about 20 mg/g of dry Silica beads. The concentration of the immobilized protein was determined in three ways: 1) from the absorbance change during the immobilization; 2) by amino acid analysis, after acid hydrolysis at 110 °C, according to the method described by (ref. 24) (the support did not interfere with the analysis); 3) by the Lowry-Folin method (ref. 25), after dissolving the sample at a pH of about 12 at 100 °C. The last method gave an uncertainty on the concentration of about  $\pm 15\%$ , whereas with the first two methods the error was less than  $\pm 5\%$ . Valeraldehyde was allowed to react with RNase in the same conditions as those described for the immobilization to the Silica beads. The excess was removed by dialysis.

Isothermal binding experiments were performed with a Bio Activity Monitor, BAM, Thermometric AB, Jarfalla (S), equipped with a flow cell device for solution experiments and with a titration cell. The stability of the protein was studied with a MC2 Differential Scanning Calorimeter, Microcal Inc., Northampton, MA, USA (ref. 26).

## Binding studies with free and immobilized RNase

The binding reaction between the inhibitor cytidine-3'-monophosphate (3'CMP) and RNase is an exothermic process. The heat evolved from the specific interaction of the two molecules can be monitored by an isothermal flow microcalorimeter by titrating a fixed amount of enzyme with different concentrations of the inhibitor. The enthalpy change per mole of enzyme, after subtraction of the heat of dilution of both protein and ligand, is shown as a function of the free inhibitor concentration in Fig. 1. The mathematical fitting of the experimental curve according to the method described by Bolen et al. (ref. 27) allows the best estimate of the enthalpy change,  $\Delta H$ , the equilibrium constant of the reaction,  $K$ , and the number of binding sites,  $n$ . The calculated  $\Delta H$  and  $\Delta G$  ( $=-RT \ln K$ ) of the binding reaction with the free enzyme are shown in Tab. 1, and compared with the literature values. The observed discrepancies may be due to the different experimental conditions, since both  $K$  and  $\Delta H$  are quite sensitive to ionic strength and buffer composition (ref. 27,28). The stoichiometry of the reaction is one mole of 3'CMP per mole of RNase ( $n=1$ ).

TABLE 1. Thermodynamic parameters of the reaction of 3'CMP with Ribonuclease A in 0.1 M acetate buffer, pH 5.0, 25 °C.

	$K$ $\text{mol}^{-1}$ $\times 10^4$	$-\Delta H$ $\text{J/mol}$ $\times 10^3$	$-\Delta G$ $\text{J/mol}$ $\times 10^3$	$-\Delta S$ $\text{J/mol.deg}$	$n$ exp.
free $\alpha$	5.06( $\pm 0.2$ )	37.4( $\pm 0.4$ )	26.9( $\pm 0.1$ )	35.8	7+2
free $\beta$	5.05( $\pm 1$ )	38.7( $\pm 0.8$ )	26.9( $\pm 0.2$ )	40.2	5
free $\gamma$	----	38.3( $\pm 0.8$ )	----	----	2
	3.5 $\delta$	48.6 $\delta$	25.9 $\delta$	75.9 $\delta$	
VA-modified $\alpha$	8.5( $\pm 0.4$ )	49.5( $\pm 0.3$ )	28.1( $\pm 0.1$ )	71.6	7+4
VA-modified $\beta$	8.5( $\pm 0.5$ )	47.4( $\pm 1$ )	28.1( $\pm 0.1$ )	65.1	3
immobilized $\beta$	7.60( $\pm 1$ )	35.6( $\pm 1$ )	27.7( $\pm 0.3$ )	26.3	4
immobilized $\epsilon$	5.01( $\pm 0.5$ )	----	26.0( $\pm 0.3$ )	----	4

$\alpha$  flow calorimeter,  $\beta$  batch calorimeter,  $\gamma$  free enzyme in presence of silica beads,  $\delta$  ref. 34,  $\epsilon$  spectrophotometer.

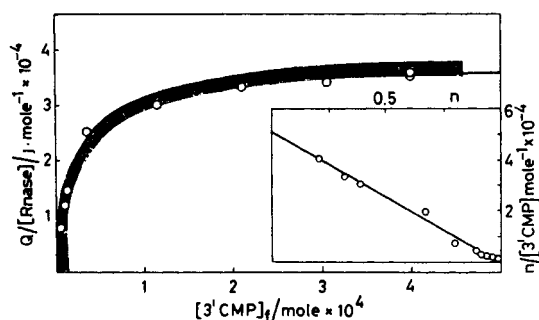


Fig.1. The molar heat of the reaction,  $Q$ , between 3'CMP and native RNase as a function of the free ligand concentration at 25 °C. The solid line was obtained with the flow calorimeter. The dashed line is the range obtained with 5 independent measurements with the batch calorimeter. The concentration of RNase was 0.15 mM, in 0.1 M acetate buffer, pH 5.

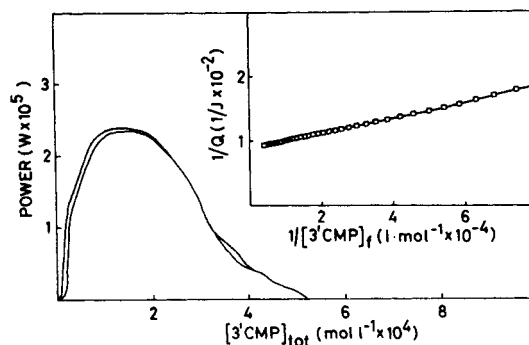


Fig.2. The molar heat of the reaction,  $Q$ , between 3'CMP and immobilized RNase as a function of the total ligand concentration as studied by the batch calorimeter. The curves were corrected for the Tian effect. The inset is a double reciprocal plot of the same data. The RNase concentration was 20 mg/g Silica beads in 0.1 M acetate pH 5, 25 °C.

The shaded curve shown in Fig. 1 represents the range of 5 binding isotherms obtained with a batch microcalorimeter. A solution of 3'CMP was pumped to a RNase solution of known concentration at 25 °C, until 5-10 fold molar excess was reached with respect to the protein. At the end of the titration, the heat evolved from the reaction as a function of time allows the calculation of the enthalpy change as a function of the 3'CMP concentration; this is shown in Fig. 1 as dashed area. The curve has been corrected for the Tian effect (ref. 29), due to the delay in the instrument time response, using the method described in ref. 30 and the algorithm of Kirchhoff, (NBS, Gathersburg, MD, USA). It can be seen that the binding isotherms, and consequently the  $\Delta H$  and  $K$ , obtained with the two independent techniques are similar.

The enthalpy of binding of 3'CMP to the immobilized RNase as a function of the inhibitor concentration as recorded by the batch calorimeter and corrected for the Tian effect is shown in Fig. 2. The thermogram allowed the calculation of the  $\Delta H$  and  $K$  of the binding reaction, which are listed in Tab. 1. The  $\Delta H$  is similar to that of the free enzyme, suggesting that the binding activity of RNase is fully preserved and that the enzyme molecule does not undergo any significant conformational change after immobilization. The inset of Fig. 2 shows the double reciprocal plot of the data of  $1/Q$  (the experimental heat of reaction) versus  $1/[3'CMP]_f$  (the free ligand concentration). The intercept at the ordinate of the best linear fit gives the reciprocal of the  $Q_{max}$  and from the slope the equilibrium constant of the reaction,  $K$ , is estimated. The apparent  $K$  calculated in this way is higher and is affected by a larger uncertainty than that calculated for the native enzyme, but it is more similar to that of the valeraldehyde modified RNase (see below).

The binding reaction of 3'CMP and immobilized RNase has also been studied by spectrophotometry. A solution of 3'CMP has been progressively added to a preparation of immobilized enzyme of known concentration and the concentration of the free inhibitor has been monitored by measuring the absorbance of the supernatant at 271 nm ( $A=9100$ , pH 5), after centrifugation at 3,000 rpm. The experimental results are shown in Fig. 3 curve a, where the concentration of the bound 3'CMP is plotted as a function of the free ligand. Curve b has been calculated by measuring the absorbance of the supernatant in the presence of Silica beads, with and without glutaraldehyde activation. By subtraction of curve b from curve a the actual amount of 3'-CMP bound is calculated as a function of the free ligand concentration. The analysis of the two sets of data according to Scatchard is shown in the inset of Fig. 3. The intercept of the best linear fitting through the experimental points with the ordinate gives the apparent  $K$  of the reaction. An average value of  $5.0 \pm 0.5 \times 10^4$  has been obtained, which is in agreement with the calorimetric results for the native enzyme, but slightly different from that obtained for the immobilized RNase with the batch calorimeter. The average number of the binding active site,  $n$ , is

0.97±0.05, as in the case of the free enzyme. This result suggests that all the enzyme molecules bound to the support are competent and active towards the correct binding of 3'CMP and that the presence of covalent link(s) between support and enzyme molecule does not involve the binding site region.

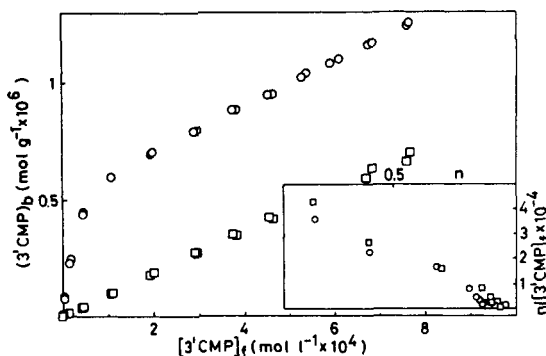


Fig.3. The amount of 3'CMP bound to immobilized RNase as a function of the free ligand concentration as studied by spectrophotometry. Curve A was obtained by adding a solution of 3'CMP stepwise to a preparation of immobilized RNase. Curve B was obtained in the absence of the protein. The inset is a Scatchard plot of the same data after subtraction of curve B from curve A.

In order to mimic the effect on the enzyme molecule of the covalent attachment to the support mediated by the bifunctional glutaraldehyde, RNase has been chemically modified with valeraldehyde. The enzyme has been allowed to react with the monofunctional valeraldehyde under the same conditions (phosphate buffer 0.1 M, pH 7, 25 °C, 7 hours) of the immobilization reaction. The extent of the modification has been measured by titrating the remaining free primary amino groups with trinitrobenzenesulfonic acid (ref. 31). It has been found that 1.7 ±0.2 groups were acylated per protein molecule. Since the reactivity of the amino groups towards aldehydes is higher as the pH approaches their ionization pKs (ref. 32) (around 10.2 for the lysyl side chains in proteins), it is possible that the terminal amino group is the most reactive since it has a pK of 7.8 (ref. 33). The valeraldehyde modified RNase is still able to bind the inhibitor 3'CMP. The thermodynamic parameters associated with the binding reaction are shown in Tab. 1. A change of both K and  $\Delta H$  of the binding reaction is observed with respect to the unmodified enzyme, suggesting that an increase of the affinity towards the ligand is accompanied by a slight change in the protein-substrate interactions, which are dominated at pH 5 by Wan der Walls forces rather than electrostatic effects (ref. 34). The modified group(s) may be located close to the binding site, but they do not prevent the protein-ligand interactions.

#### Thermal stability of ribonuclease A

The thermal stability of the free, acylated and immobilized RNase have been studied by DSC. For all the enzyme forms, a cooperative transition associated with the unfolding of the protein structure has been observed. From the heat sorption peak of the excess heat capacity as a function of temperature the overall enthalpy change,  $\Delta H_C$ , which is associated to the transition, can be derived by integration of the peak area. The equilibrium constant of the unfolding process as a function of temperature and, hence, the vant'Hoff enthalpy,  $\Delta H_{VH}$ , can be calculated from the same thermogram by using the vant'Hoff relationship. If the ratio  $\Delta H_C / \Delta H_{VH}$  is close to unity, the observed process can be approximated to a two-state transition, otherwise the presence of intermediate states may be postulated in the unfolding pathway. For the free enzyme a single sharp cooperative transition is observed as a function of temperature at all the pHs studied. The deconvolution of the thermogram according to the mathematical treatment of Freire and Biltonen (ref. 35) allows the best estimate of both  $\Delta H_C$  and  $\Delta H_{VH}$ . The results for all the enzyme forms are summarised in Tab. 2. In the case of the free enzyme, by varying the pH, the calorimetric enthalpy change is linearly related with the middle point transition temperature,  $T_m$ . The best linear fit of the experimental points is in agreement with the literature data for RNase (ref. 36). The data for the overall unfolding of the immobilized enzyme do not fit the same line, but they lie on a curve parallel to it. In the case of the valeraldehyde modified RNase, the transition enthalpy changes fitting the curve of the native enzyme well, indicating that the  $\Delta C_p$  of unfolding, given by the slope of the linear fit of the data, is similar with regards to the two RNase forms. As shown in Tab. 2, the presence of the 3'CMP bound to the immobilized RNase brings about a shift of 3 °C of the  $T_m$  with respect to the apoenzyme. A similar

shift of  $T_m$  towards higher values in the presence of ligands has also been observed for the liver alcohol dehydrogenase (ref. 21). The overall  $\Delta H$  increases because the enthalpy of ligand released during unfolding has to be added to the transition enthalpy change, as reported (ref. 28). It is interesting to note that the  $T_m$  of both transitions are increased, suggesting that both the domains of the protein (see below) are equally stabilized by the presence of the ligand and therefore that the two domains are 'coupled' to some extent.

The thermal stabilities of both acylated and native enzyme are similar in the whole pH range studied, despite the modification of almost two groups per protein molecule. The acylated enzyme shows a single cooperative transition as a function of the temperature. The chemical modification of 1-2 amino groups with valeraldehyde does not seem to significantly alter the thermal stability of the protein molecule, but somehow changes the binding properties. It should be noted the ratio  $\Delta H_c/\Delta H_{vH}$  is very close to unity for both native and modified enzymes, indicating that the protein apparently unfolds as a single cooperative domain in a two-state process. However, by inspection of the X-ray diffraction study of the protein structure, it is possible to observe the presence of two structural domains, which are separated by a deep cleft (ref. 37). The two structural domains cannot be distinguished by the thermodynamic analysis of the DCS data probably because they are strongly interacting and 'coupled' or have the same thermal stability, giving an apparent 'single' transition (ref. 38).

TABLE 2. DSC calorimetric results for Ribonuclease A

	pH	$\Delta Hc1$	$Tm1$	$\frac{\Delta Hc1}{\Delta H_{vH}}$	$\Delta Hc2$	$Tm2$	$\frac{\Delta Hc2}{\Delta H_{vH}}$	$\Delta H_{tot}$	$T_{tot}$
		KJ/M	°C		KJ/M	°C		KJ/M	°C
		$\pm 2$	$\pm 0.5$	$\pm 0.05$	$\pm 2$	$\pm 0.5$	$\pm 0.05$	$\pm 5$	$\pm 0.5$
free	3.0	300.7	47.9	0.99					
	4.3	361.3	56.9	1.01					
	5.1	392.2	60.3	1.00					
	7.0	463.0	65.6	1.01					
VA-modif.	3.0	336.3	53.3	1.01					
	4.0	405.0	55.8	0.99					
	5.0	394.0	59.3	1.02					
	7.0	441.6	63.6	1.01					
Immobilize	3.0	297.6	47.0	0.99	289.7	60.4	1.01	588	55.3
	4.0	266.1	59.1	1.05	266.2	69.2	1.01	492	60.5
	5.0	270.5	60.0	1.02	229.5	67.8	1.00	499	61.4
	7.0	292.3	62.8	1.00	170.6	66.3	1.00	463	62.3
" +3'CMP	5.0	332.0	62.0	1.1	285.5	70.0	1.2	617	64.1

The covalent attachment to the activated Silica beads brings about significant changes in the thermal stability and thermodynamics of unfolding of the immobilized enzyme molecule. The deconvolution of the thermograms reveals that the unfolding single transition is given by the sum of two overlapping unfolding processes. An example is shown in Fig. 4, where the deconvolution analysis was carried out in the case of immobilized RNase at pH 5. A general stabilizing effect of the protein molecule is obtained after immobilization. The same effect has been studied as a function of the number of linkages between support and protein (ref. 21). The presence of two transitions in the immobilized enzyme preparations were observed in the whole pH range studied (Tab. 2). At low pH the stabilization effect becomes larger: for example at pH 4 the  $T_m$  of the immobilized enzyme has an average value of about 60 °C, six degrees higher than that of the native enzyme at the same pH. The change in pH does not induce a concurrent change in the  $T_m$  and  $\Delta H$  of unfolding as large as in the case of the free enzyme. To a first approximation, this effect may be due to a decrease in the charged amino groups, since almost two of these groups may be involved in the linkage between protein and support. Moreover, it may imply that the change in the protein ionisation state upon unfolding, and therefore the enthalpy

associated with it, is smaller, at least between pH 4 and 7, after immobilization. This may also reflect the peculiarity of the protein microenvironment inside the pores of the silica beads, which may be somehow different from that of the bulk solution.

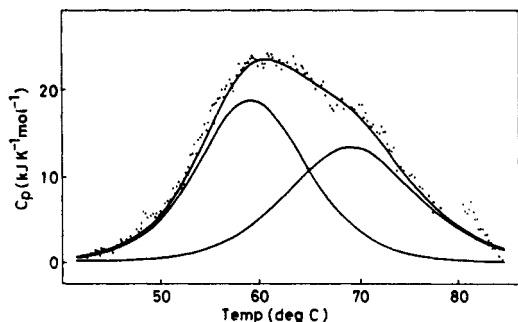


Fig.4. Temperature dependence of the specific heat capacity,  $C_p$ , of immobilized RNase (about 3 mg) in 0.1 M acetate buffer pH 5. Two overlapping transitions have to be considered in order to obtain the best fit of the overall experimental transition.

The presence of two overlapping transitions during the unfolding process of RNase may be ascribed to the following reasons: i) the presence of two thermodynamically distinct populations of enzyme molecules, for example one linked to the supporting matrix with one covalent bond and the other with two bonds or partially unfolded and not-native like molecules; ii) the consecutive unfolding of two domains of the same molecule. The first hypothesis may be ruled out on the basis of the following reasoning. The ratio  $\Delta H_C / \Delta H_{VH}$  obtained from the deconvolution analysis is close to unity for each of the two transitions analysed independently. This would not be the case if point i) is correct because the concentration of the protein molecules unfolding in each of the two transitions would only be a part of the total. Consequently, also the ratio  $\Delta H_C / \Delta H_{VH}$  for each transition would be less than one. Other evidence, although less relevant, is that the binding studies confirmed the presence of a homogeneous population of molecules as far as the affinity of the binding site is concerned.

The second hypothesis (point ii)) assumes that the two observed processes may be due to the unfolding of two domains of the immobilized RNase. Each transition can be approximated by the all-or-none two-state model, which does not hold for the overall process. The structure of RNase shows the presence of two structural domains separated by a groove where the binding site is located. The two domains behave as a single cooperative unit in the native enzyme as studied by DSC. After immobilization, the presence of one or two covalent bonds with the support may change the relative intrinsic stability by stabilization of one domain over the other, which leads to the splitting of the unfolding transition. On the other hand, the domains may be partially 'uncoupled', i.e. the favorable interaction free energy lowered and the interdomain interactions weakened. Both effects may also superimpose each other. A similar 'decoupling' of the RNase structural domains during protein unfolding as revealed by DSC has been observed as a function of methanol concentration (ref. 38). The acylated enzyme does not behave as the immobilized enzyme, because it shows a single unfolding transition as the native enzyme. Therefore it does not represent a good model for studying the thermal stability of supported RNase. This is not surprising, because the length and type of the support is an important factor on thermal stability (ref. 39). It is difficult to know which one of the two domains of RNase has been more stabilized than the other after immobilization. Assuming that the terminal amino group is the most reactive, the linking of the protein to the support through that group should stabilize the N-terminal domain. This is in agreement with the NMR studies (ref. 40) which showed that the most thermal labile part of the protein is that with the carboxyl terminal group.

#### CONCLUDING REMARKS

In conclusion, the present study on a model protein may give suggestions for the practical exploitation of immobilized enzymes as biocatalysts. In our experimental conditions, the attachment to the support through few (one or two) chemical links does not significantly alter the biological activity (as studies by the binding of 3'CMP) and affords a general stabilizing effect towards irreversible thermal denaturation. Multipoints attachment further enhances the stability, but it may be accompanied by a severe loss of catalytic activity (ref.21). Changes of pH result in an even greater

stabilization with respect to the free enzyme. This information, in the case of a weak dependence on pH of the activity, can be quite useful when the biocatalyst is used in a continuous process and the greatest long-term operational stability is required. Details of the unfolding process were evaluated by deconvolution analysis of the DSC thermograms. Two subprocesses are present during the unfolding of immobilized RNase. The analysis of the enthalpy changes associated with these processes, gives an insight on the unfolding mechanism and pathway. The results are consistent with the hypothesis of a different stabilising effect on the two structural domains of the protein after immobilization.

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