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Studies on Conformation of Soluble and Immobilized Enzymes Using Differential Scanning Calorimetry. 2. Specific Activity and Thermal Stability of Enzymes Bound Weakly and Strongly to Sepharose CL 4B†

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ABSTRACT: Ribonuclease A (EC 3.1.4.22) and α-chymotrypsin (EC 3.4.21.1) have been covalently coupled, by a varying number of bonds, to Sepharose CL 4B which was activated with different amounts of CNBr. Upon increasing the number (1–8) of points of attachment between the enzyme and the matrix, the specific activities of immobilized ribonuclease A relative to its soluble counterpart decreased from 60 to 15% while the amount of protein coupled increased from 5 to 37 mg per g of sucked gel. Differential scanning calorimetry was used to determine whether the immobilization caused any changes in the physicochemical properties of the enzyme. Ribonuclease A, weakly bound to the matrix, showed almost the same behavior as the soluble enzyme. By contrast strongly immobilized enzyme exhibited a higher transition temperature (by about 5 °C) and a broader endotherm. Similar results were found for α-chymotrypsin.

Immobilized enzymes have received great attention in recent years since they represent valuable biological model systems (Srere and Mosbach, 1974). They also are of practical interest in enzyme technology (Pye and Wingard, 1974; Mosbach, 1976).

Such enzyme preparations usually have been characterized indirectly by kinetic methods and analyses such as enzyme activity determination. More direct characterization of their chemical and physicochemical properties has been neglected for the most part. Studies of this aspect have been hampered due to turbidity or opacity of such preparations and only few spectroscopic investigations have been reported to date. These include fluorometric examinations of immobilized trypsin (Gabel et al., 1971) and recent studies on Sepharose-bound proteins, such as α-lactalbumin (Barel and Prieels, 1975) and leucine aminopeptidase and carboxypeptidase A (Lasch, 1975). Preliminary reports with such preparations have appeared using circular dichroism (Zaborsky, 1974) and electron spin resonance (Reiner and Siebeneick, 1974). An important aspect of an immobilized enzyme is its conformation. Precise knowledge of this can give insights into its specific activity and thermal stability. We have therefore investigated an alternative method to spectral analysis: differential scanning calorimetry (DSC).[1] With this method an overall thermal behavior of the various enzyme preparations can be obtained easily and conclusions can be drawn as to enzyme conformation. Further information gained about the heat stability is of practical interest.

The chemical characterization of immobilized enzyme preparations has received little attention while major effort has been devoted to the nature of the support chosen. The kind of

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[1] Abbreviations used: DSC, differential scanning calorimetry; T_{tr}, transition temperature; ΔH_{tr}, enthalpy of transition; TNBS, 1-trinitrobenzenesulfonic acid; Polyox, poly(hydroxy ethylene glycol).

the amino acids involved in binding has also been studied. In this investigation we report on experiments carried out on preparations with different numbers of points of attachment between enzyme and matrix. We attempted to find a correlation between the number of linkages and the specific activity and heat stability of the enzymes.

Experimental Section

Materials

Ribonuclease A (bovine pancreas, type IA, 67 Kunitz units/mg protein), α-chymotrypsin (bovine pancreas, type II, 45 units/mg protein), *N*-acetyl-L-tyrosine ethyl ester, cyclic cytidine 2′,3′-monophosphate (sodium salt), and 1-trinitrobenzenesulfonic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.); Sepharose CL 4B was from Pharmacia (Uppsala, Sweden); cyanogen bromide was from Fluka AG (Buchs SG, Switzerland); and Polyox WSR 301 was from Union Carbide.

Methods

1. Preparation of Immobilized Ribonuclease A and α-Chymotrypsin. The enzymes were coupled to Sepharose CL 4B (the cross-linked form of the Sepharose was chosen as it shows better thermal stability) using the cyanogen bromide activation technique, modified as follows: 1.5 g of sucked Sepharose CL 4B (60 mg of dry weight) was added to 10 mL of a cold (4 °C) water solution containing 15, 30, 60, 120, 250, and 750 mg of CNBr, respectively. The activation took place at pH 10.8–11.0 for 5 min at 4 °C by titration with 0.2–4 M NaOH. Activated beads were thoroughly washed with 0.250–1 L of a cold 0.1 M $NaHCO_3$ solution, pH 8.4.

The activated beads (400 mg) were transferred to 1 mL of the enzyme solution (15 mg of enzyme/mL in 0.1 M $NaHCO_3$). Another 400 mg of the activated beads were transferred to 2 mL of a 50 mM lysine solution, and the rest was kept in the same $NaHCO_3$ solution for use as blank gel. The coupling reactions proceeded for 14 h at 4 °C with gentle shaking after which the enzyme gels were carefully washed with 0.5 M NaCl, 1 mM HCl, distilled water, and finally with the assay buffer.

2. Determination of the Amount of Immobilized Ribonuclease A per Gram of Sucked Gel. The amount of enzyme bound to the Sepharose matrix was determined by two different methods. Due to the high concentration of coupled enzyme, it was possible to measure the absorbance at 278 nm (ribonuclease A, using $\epsilon_{278} = 8.1 \times 10^3$ cm^{-1} M^{-1}) from a spectrum run from 500 to 260 nm. About 30 mg of sucked enzyme gel was suspended in 2.5 mL of a 1% Polyox solution, dissolved in water, and correspondingly activated gels, without coupled enzyme, served as blanks. This method was checked by amino acid analysis (according to Spackman et al., 1958) of the immobilized ribonuclease A preparations and a complete correlation of the amount of bound protein per gram of sucked gel was obtained.

3. Determination of the Number of Potential Points of Attachment on the CNBr-Activated Sepharose CL 4B Matrix. The amount of active carboimidoesters, formed by activating the Sepharose matrix at pH 10.8–11.0 using 15, 60, and 750 mg of CNBr, respectively, was determined indirectly by amino acid analysis of lysine that had coupled. Under the conditions applied, lysine and/or homocitrulline, the latter containing a $-CO-NH_2$ group derived from the activated matrix, is found in the hydrolysate.

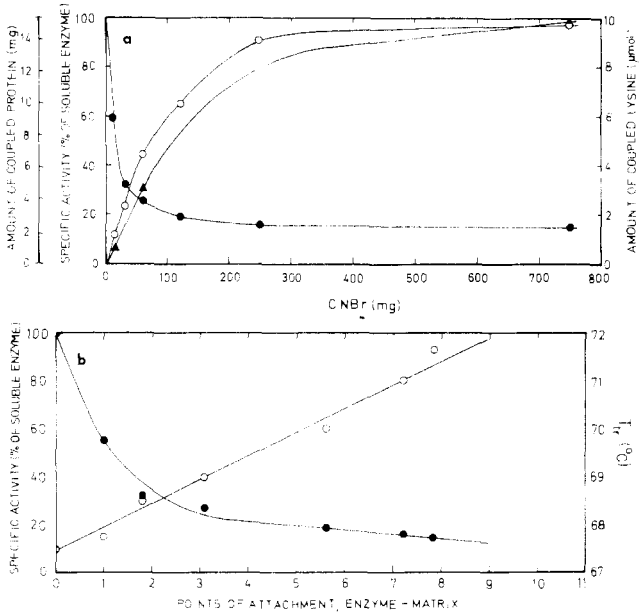

FIGURE 1: (a) The relative specific activity (●) expressed as a percent of the original soluble enzyme activity and the amount of coupled protein in mg/400 mg sucked gel (○) of ribonuclease A bound to Sepharose CL 4B. The gel was activated at pH 10.8–11.0 for 5 min using different amounts (15–750 mg) of CNBr in a total volume of 10 mL. The graph also shows the amount of lysine bound (▲) as a function of CNBr. (b) The relative specific activity of ribonuclease A (●) expressed as a percent of the original soluble enzyme activity, and T_{tr} (○) as a function of the number of points of attachment between the enzyme and the matrix.

4. Determination of the Number of Covalent Linkages between Ribonuclease A and the Matrix. Sucked enzyme gel (10–30 mg) was suspended in 2 mL of a 1-trinitrobenzenesulfonic acid, TNBS, solution. The concentration of TNBS was high, 1 mg/mL in 4% $NaHCO_3$, in order to avoid diffusion problems due to the presence of the matrix. The mixture was allowed to react with gentle shaking for 2 h in the dark at 40 °C (Habeeb, 1966). The beads were washed on glass filter using a 4% $NaHCO_3$ solution and suspended in 2.5 mL of a 1% Polyox solution. Comparably activated blank gels were treated with TNBS and served as blanks. Spectra were run from 600 to 300 nm in order to get a reliable baseline. The absorbance was read at 348 nm.

The soluble enzyme (10–100 mg) was treated with 2.5 mL of a TNBS solution (0.33 mg/mL in 4% $NaHCO_3$) in a way similar to that described for immobilized enzyme.

5. Determination of the Enzymic Activity of Soluble and Immobilized Enzymes. The activity of ribonuclease A was assayed titrimetrically using a titrator–titrigraph assembly (ABU 12, Radiometer, Copenhagen, Denmark). Five to ten milligrams of sucked enzyme gel were assayed at 25 °C using 8.8 μmol of cyclic cytidine 2′,3′-monophosphate in 1.2 mL of 0.1 M NaCl solution at pH 7.25 by titration with 10 mM NaOH under nitrogen atmosphere.

The activity of α-chymotrypsin was assayed spectrophotometrically using a flow system described earlier (Mosbach and Mattiasson, 1970; Johansson and Mosbach, 1974). Approximately 10 mg of sucked gel was suspended in 10 mL of a 0.1 M Tris buffer–0.1 M in NaCl, pH 8.1, and assayed at 237 nm using 10 μmol of *N*-acetyl-L-tyrosine ethyl ester at 25 °C. The respective soluble enzymes were assayed in the same way.

6. Investigation of the Regained Enzymic Activity of Soluble and Immobilized Ribonuclease A after Heat Treatment. About 10 mg of sucked enzyme gel was suspended in 0.25 mL

TABLE I: Correlation between Specific Activivity, T_{tr}, ΔH_{tr}, and Regained Activity of Weakly and Strongly Immobilized Ribonuclease A Preparations.

Sample	Amount CNBr (mg/1.5 g sucked Sepharose)	Points of Attachment, Enzyme–Matrix	Spec Act. (in % of soluble enzyme)	T_{tr} (°C)	ΔH_{tr} (kcal/mol (immob. enzyme)	Recovered Peak Area (%)[a]	Regained Act. (%)[b]
Ribonuclease A, pH 7.25, 1% solution			100	67.5	100	80	78
Ribonuclease A-Sepharose, pH 7.25 (R_{15})	15	1.0	59	68	106	85	84
Ribonuclease A-Sepharose, pH 7.25 (R_{30})	30	1.8	33	68.5	76	89	86
Ribonuclease A-Sepharose, pH 7.25 (R_{60})	60	3.1	25	69	74	72	70
Ribonuclease A-Sepharose, pH 7.25 (R_{120})	120	5.6	19	70	81	50	45
Ribonuclease A-Sepharose, pH 7.25 (R_{250})	250	7.2	16	71	80	32	30
Ribonuclease A-Sepharose, pH 7.25 (R_{750})	750	7.8	14	72	74	27	18

[a] After heating at 97 °C for 10 min in the calorimeter and in percent of the first transition peak area. [b] After heating at 97 °C for 10 min in a water bath.

of the assay buffer (0.1 M phosphate buffer, pH 7.25) and then kept at 97 °C in a water bath for 10 min. The samples were then cooled on ice and washed with 0.1 M NaCl. The regained activity was assayed at 25 °C. Soluble ribonuclease A was treated in the same way using a 1% solution of enzyme.

7. Thermal Analysis of Soluble and Immobilized Enzymes Using DSC. Thermal analysis of the soluble and immobilized enzymes was performed using a DSC-2 differential scanning calorimeter (Perkin-Elmer) equipped with a cooling system. Aluminum pans, made for water solutions, were used exclusively.

The sample was filled with 8–12 mg of well-sucked gel after which 5 μL of the buffer solution (0.1 M phosphate buffer, pH 7.25, for the ribonuclease A preparations, and 0.1 M Tris buffer–0.1 M in NaCl, pH 8.1, for α-chymotrypsin gels) was added to obtain a homogeneous gel without air bubbles between the beads. To study the soluble enzyme, 15 μL of a 1% enzyme solution was used. The pans were filled, pressure sealed, and weighed. All reference pans contained 15 μL of the corresponding buffer solution. The gel itself did not show any transition in the temperature interval studied. The thermograms were run from 290 to 370 K using a heating rate of 10 °C/min.

In order to determine the enthalpy of the transition, ΔH_{tr} (proportional to the area under the peak), a straight line was drawn under the thermogram connecting the baseline at the temperature of initial deflection with the baseline at the temperature where the transition ceased. The recorder range used was 0.05 mcal/s. Temperatures were reproducible to within 1 °C and the peak areas to 8%.

Results and Discussion

The treatment of a carrier containing hydroxyl groups, like Sepharose, with CNBr in alkaline solution results in the formation of reactive carboimidoesters. When a protein is allowed to react with such an activated matrix, the ε-amino groups of its lysines are involved primarily in the coupling process (Axén et al., 1967).

By varying the concentration of CNBr in the activation step differing numbers of potential attachment points are formed on the matrix. This is substantiated by the different coupling yields found using the compound lysine as a model (Figure 1a).

Sepharose CL 4B was activated with different amounts of CNBr and coupled with ribonuclease A. More linkages were formed between the enzyme and the matrix when more carboimidoesters were available on the gel (Table I). The immobilized preparations of ribonuclease A prepared by adding the same amount of enzyme to differently activated Sepharose CL 4 B showed a decrease in specific activity as the CNBr concentration in the activation mixture was increased (Figure 1a and Table I). The total activity, as well as the amount of protein coupled per gram of sucked gel, increased with the CNBr concentration. This indicates that no steric hindrance exists within the matrix. Similar results were also obtained when α-chymotrypsin was coupled to a similar matrix.

Figure 1b shows that the relative specific activity of immobilized ribonuclease A in percent of the original activity of the soluble enzyme is a function of the mean value of the estimated number of attachment points between enzyme and matrix. The higher the number of attachment points, the lower the specific activity obtained. Similar results were also found for ribonuclease A coupled in phosphate buffer and for immobilized α-chymotrypsin preparations. As can also be seen from this figure and discussed later, the transition temperature, T_{tr}, rises with increasing number of attachment points. The number of linkages per enzyme molecule was calculated from the titration (with TNBS) of the free amino groups remaining on the immobilized enzyme. We assumed that only primary amino groups (11 are available per ribonuclease A molecule) are involved in binding. CNBr-activated Sepharose CL 4B itself also showed some reaction with TNBS. Similarly activated gels, treated with TNBS, were used as blanks. The blank values, however, were very low with weakly activated gels and never exceeded 10% with even the most strongly activated samples. We like to stress that the number of attachment points given between enzyme and matrix may represent average values for those preparations in the range of 2–7 linkages. Such heterogeneity is probably present only to some degree since the thermograms of such intermediary preparations (Figure 4) do not cover a wide temperature range. They show shifts in T_{tr}

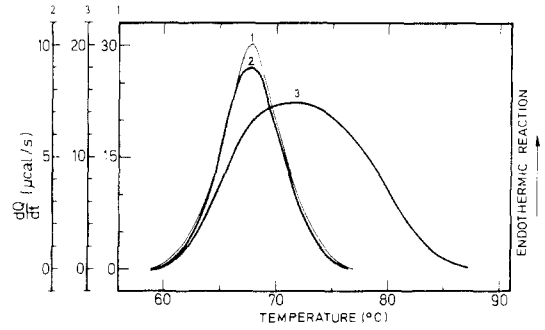

FIGURE 2: Thermograms of the endothermic process of heat transition of 15 μL of a ribonuclease A solution (1% in 0.05 M phosphate buffer, pH 7.25) (No. 1, —), ribonuclease A bound through 1 (No. 2, ▬), and 8 (No. 3, ▬) bonds to Sepharose CL 4B, at pH 7.25. The amount of sucked gel used was about 12 and 8 mg, respectively. Heating rate: 10 °C/min.

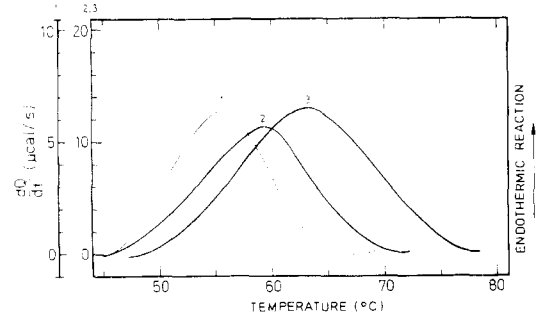

FIGURE 3: Thermograms of the endothermic process of heat transition of an α-chymotrypsin solution (1% in 0.1 M Tris buffer, 0.1 M in NaCl, pH 8.1) (No. 1, —), and α-chymotrypsin weakly bound (15 mg CNBr/10 mL; no. 2, ▬), and strongly bound (750 mg CNBr/10 mL; No. 3, ▬) to Sepharose CL 4B using 11 and 8 mg of sucked gel, respectively. Heating rate: 10 °C/min.

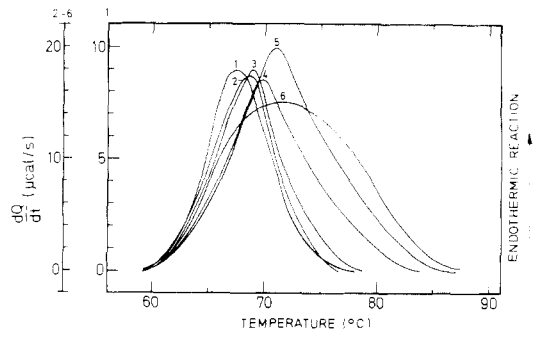

FIGURE 4: Thermograms of immobilized ribonuclease A. The enzyme was bound through between 1 and 8 estimated points of attachment to the matrix. The various preparations scanned were 1 = R_{15}, 2 = R_{30}, 3 = R_{60}, 4 = R_{120}, 5 = R_{250}, and 6 = R_{750}. The designations R_{15}, R_{30}, etc. refer to the CNBr concentration used (see also Table I). The thermograms were run using from 12 down to 8 mg of sucked gel, pH 7.25, against a blank containing buffer. Heating rate: 10 °C/min.

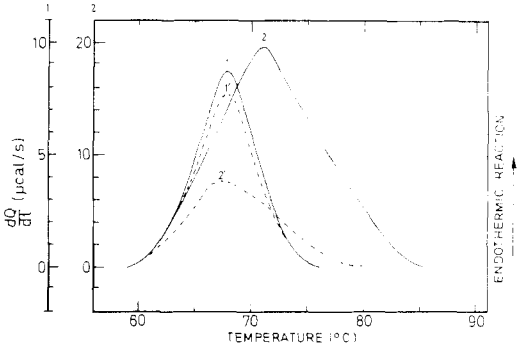

FIGURE 5: The first (1, 2, —) and the recovered transition profile of a second run (1′, 2′, - - - -) of two immobilized ribonuclease A preparations (1 = R_{15}, 2 = R_{250}) after heat treatment of the sample at 97 °C for 10 min in the calorimeter. The amount of sucked gel used was about 12 and 9 mg, respectively. Heating rate: 10 °C/min.

to higher temperatures the more activated the gels are that are used.

We ascribe the correlation of number of linkages and specific activity to the possibility that through immobilization the enzyme becomes fixed in a partly unfolded state (Lasch, 1975) or to restricted flexibility of the immobilized enzyme molecule. It might also be due to the binding of lysine, located in (e.g., Lys-41 in ribonuclease A) or near the active site, which results in inactivated enzyme or decreased possibility of the substrate reaching the active site. Since derivatization of 8 (excluding Lys-7, -37, and -41) of the 11 primary amino groups of ribonuclease A with poly(DL-alanine) in phosphate buffer has little effect on catalytic and physical properties of the enzyme (Cooke et al., 1963), modification per se of the ε-amino groups located far from the active site seems not responsible for the observed changes in specific activity. In the various immobilized preparations assayed, the matrix itself did not cause diffusional hindrance for substrate or product.

Differential scanning calorimetry was used to further investigate the conformational state of immobilized ribonuclease A. With this method the endothermic unfolding process occurring in the protein molecule on heating can be traced. The transition temperature, T_{tr}, and the enthalpy of the whole transition, ΔH_{tr}, are obtained from the thermogram. Figure 2 shows the thermograms of soluble ribonuclease A obtained in a buffered solution, pH 7.25, as well as those of ribonuclease A bound through 1 and 8 primary amino groups to Sepharose CL 4B, respectively. Figure 3 shows the corresponding thermograms for α-chymotrypsin. These thermograms substantiate the results from enzyme activity assays and show that a loosely bound molecule retains its native properties. A strongly immobilized molecule with a relatively low specific activity behaves in a different manner.

Figure 4 depicts thermograms of a series of ribonuclease A preparations. They were obtained by using different amounts of CNBr in the activation process and are designated R_{15} to R_{750} (15–750 mg of CNBr/1.5 g of sucked gel). The more bonds between enzyme and matrix, the more the profile is displaced to a higher temperature (Figure 1b and Table I), which could be a result of a reduced configurational entropy of the unfolded state (Kauzmann, 1954).

The broadening and the displacement of the profile illustrate that there exist immobilized protein molecules which show higher thermal stability than the native enzyme as a result of the introduction of covalent bonds between enzyme and matrix. A mixture of soluble enzyme and blank gels gave the same original profile and T_{tr}. This proves that the matrix itself does not cause the broadening.

The ΔH_{tr} values (Table I) of ribonuclease A decreased from 0 to about 30% below that of the native enzyme when the enzyme was immobilized on a matrix. This decrease can partly be correlated with the number of covalent bonds formed between enzyme and matrix and indicates the presence of unfolded (= inactive), immobilized enzyme molecules. We would like to point out that these data do not represent absolute values. The highest sensitivity of the apparatus had to be used and the weighing of small samples presented technical difficulties.

The reversible character of the unfolding process of a protein is easily followed using the DSC technique. Figure 5 illustrates the thermograms obtained from two different immobilized ribonuclease A preparations, R_{15} and R_{250}, which were scanned a second time after a first run up to 97 °C. A third run gave exactly the same result as the second, indicating that a given fraction of the immobilized enzyme molecules retains the ability to reversibly fold and unfold. The recovered peak areas are given in Table I in percent of the original areas of these and other preparations.

These calorimetric studies were correlated with enzyme activity tests in which the ribonuclease A preparations were kept at 97 °C for 10 min and then cooled. Afterward the regained activity was assayed. From Table I it can be seen that the percentage of the regained activity was in accordance with the percent of recovered peak area from the thermogram. It can be seen also that the introduction of a few bonds does not adversely affect the reversibility of the refolding process. Several bonds caused less reversibility.

Corresponding studies on α-chymotrypsin revealed that the unfolding process appeared irreversibly for both soluble and immobilized enzyme as no endotherm was obtained in a second run.

The thermograms and enzyme activity studies of the different ribonuclease A preparations revealed that immobilization of the enzyme by multiple points of attachment changed the reversibility of the refolding process. Generally a protein is kept in a native conformation by a large number of low energy interactions (Weber, 1975). Some of these are probably affected when linkages are introduced between enzyme molecule and matrix. This leads to great tensions within the enzyme molecule and thus to a changed conformation. The flexibility of the enzyme in the immobilized state may also be impaired. A thermal treatment causes large successive deformations of the protein which can be reversible for some proteins like native ribonuclease A (Anfinsen and Scheraga, 1975) and irreversible for others like α-chymotrypsin. In the case of immobilized ribonuclease A, the introduction of a few bonds did not affect the reversible process. More bonds did have an effect which is due probably to a larger influence of simultaneous movements of the matrix chains. The latter prevent the enzyme from obtaining its catalytically active original conformation.

In conclusion the DSC technique is a useful tool in characterizing the thermal behavior of various immobilized enzyme preparations. The number of linkages between the enzyme and the matrix is of importance in relation to the specific activity and heat stability of the immobilized enzyme. Since the transition point, or point of heat denaturation, is raised for strongly immobilized enzyme preparations by several degrees, immobilization allows enzymic reactions to be carried out at higher temperatures.

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