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Hydrophobic Chromatography and Fractionation of Enzymes from Extremely Halophilic Bacteria Using Decreasing Concentration Gradients of Ammonium Sulfate†

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ABSTRACT: Ammonium sulfate fractionation of proteins from extremely halophilic bacteria on Sepharose 4B, carboxymethylcellulose, diethylaminoethylcellulose, and hexamethylenediamine-Agarose is described. Halophilic proteins are adsorbed on these gels at 2.5 M ammonium sulfate and eluted by decreasing concentration gradients of this salt. The method has enabled the separation of malate dehydrogenase from glutamate dehydrogenase and aspartate aminotransferase on Sepharose 4B and the additional 15-fold purification of glutamate dehydrogenase on DEAE-cellulose. The technique is simple and convenient, operates at low cost, and possesses great power of resolution. The mechanism of adsorption is discussed and compared to previous instances of "hydrophobic chromatography". It is concluded that the retention of halophilic proteins on the polysaccharide gels at 2.5 M ammonium sulfate is due to hydrophobic interactions.

Enzymes from extremely halophilic bacteria constitute a fascinating example of biochemical adaptation. Thus, these enzymes which, both in vivo and in vitro, perform their catalytic functions at 4–5 M NaCl and KCl, lose their activity rapidly when exposed to low salt concentrations (Lanyi, 1974). They constitute therefore a unique system for inactivation–reactivation studies, which can shed light on the type of interactions involved in the maintenance of the integrity of native structures. In order to perform these studies it is indispensable to obtain pure enzyme preparations. However, the lability of these enzymes at low salt concentrations imposes many restrictions on the choice of purification techniques.

Recently, we have discovered that Agarose gels adsorb halophilic proteins from 2.5 M ammonium sulfate solutions. There were several previous attempts to use gradients of ammonium sulfate on solid supports for the fractionation of proteins—on Celite (King, 1972), on DEAE-cellulose[1] (Mayhew and Howell, 1971), on alkylamino-Agaroses (Rimerman and Hatfield, 1973; Doellgast and Fishman, 1974); and of transfer RNA species—on Sepharose (Holmes et al., 1975).

This communication deals with the chromatographic behavior of halophilic enzymes on several gels under the conditions of decreasing concentration gradients of ammonium sulfate. The results show the general utility of this technique, employing Sepharose and DEAE-cellulose as the solid supports, for the separation and purification of halophilic enzymes. The applications of this procedure to the separation of halophilic MDH from GDH and AAT on Sepharose 4B and to the purification of GDH on DEAE-cellulose are described.

Experimental Section

Chemicals. Oxaloacetic, α-ketoglutaric, and aspartic acids and NADH and NADPH were purchased from Sigma. All the salts employed were of analytical grade and their solutions were filtered through 0.45-μm Millipore filters before use. Sepharose 4B was obtained from Pharmacia, DEAE-cellulose (DE-52), and CM-cellulose (CM-52) were from Whatman. Celite 545 was a product of Fisher Scientific Co. and was used without further treatment. HMD-Agarose was prepared from Bio-Gel A-0.5m (a Bio-Rad Laboratories product) according to Jost and Yaron (1974) and contained 10.5 μmol of diamine/ml of gel.

Bacterial Extract. The bacteria of the species *Halobacterium* (Ginzburg et al., 1970) were a gift from Drs. M. and B. Z. Ginzburg. The bacteria were grown in 6-l. Erlenmyer flasks containing 1.5 l. with the following content (per l.): 208 g of NaCl, 46.6 g of $MgSO_4$ (anhydrous), 0.5 g of $CaCl_2$, 0.125 g of $MnCl_2$, and 10 g of Difco yeast extract. After 4 days of shaking the bacteria were harvested by centrifugation in a Sorvall GSA rotor. The yield was about 9 g of wet bacteria/l. of medium. The packed bacteria (50 g) were then resuspended in 4.3 M NaCl–0.01 M Na-phosphate, pH 7.3, and sonicated for 4 min at 0 °C in a Branson sonicator equipped with a microtip. The sonicated suspension was centrifuged for 1 h in a Spinco ultracentrifuge at 30 000 rpm at 25 °C. The supernatant was dialyzed against 1.6 M $(NH_4)_2SO_4$–0.05 M Na-phosphate, pH 6.6, and then centrifuged as above. Solid am-

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[1] Abbreviations used are: AAT, aspartate aminotransferase; CM-cellulose, carboxymethylcellulose; DEAE-cellulose, diethylaminoethylcellulose; GDH, glutamate dehydrogenase; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HMD-Agarose, hexamethylenediamine-Agarose; MDH, malate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; OD, optical density.

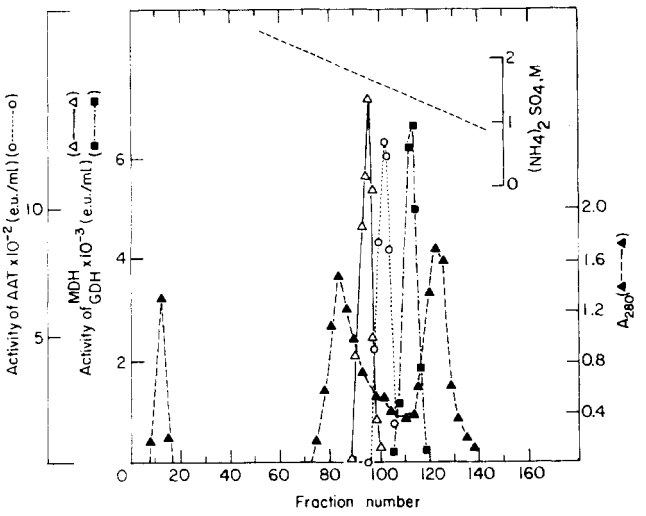

FIGURE 1: Ammonium sulfate fractionation of halophilic proteins on a column of Sepharose 4B. An 80-ml bacterial extract (see Experimental Section and Table I) was applied onto a 3.4 × 20 cm column of Sepharose 4B. A 1250-ml gradient of $(NH_4)_2SO_4$ decreasing from 2.5 to 0.75 M was applied to elute the enzymes at a flow rate of 66 ml/h. Fractions of 11 ml were collected and assayed for MDH, AAT, and GDH.

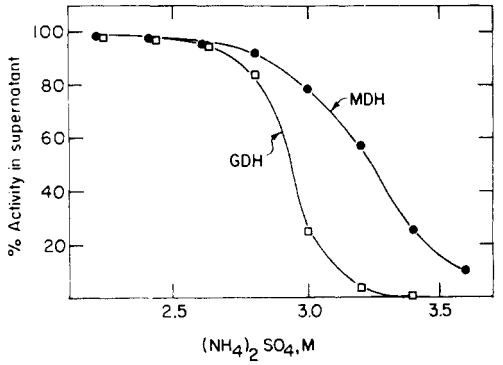

FIGURE 2: Solubility curves of GDH and MDH in ammonium sulfate. An extract of halophilic proteins was dialyzed against 2 M $(NH_4)_2SO_4$. Aliquots of this solution were diluted 1:3 with a saturated $(NH_4)_2SO_4$ solution to give concentrations of $(NH_4)_2SO_4$ ranging between 2 and 3.7 M. The precipitates were removed by centrifugation (at 12 000 rpm or at 20 000 rpm at the lower $(NH_4)_2SO_4$ concentrations) and redissolved in 4.3 M NaCl–0.05 M Na-phosphate, pH 6.6. Supernatants and precipitates were assayed for GDH and MDH.

monium sulfate was added to the supernatant to a final concentration of 2.5 M. After stirring for 1 h the suspension was centrifuged as above and the supernatant was collected and used for the experiments.

Column Chromatography. A column was packed with the gel and washed with several volumes of a solution of 2.5 M $(NH_4)_2SO_4$–0.05 M phosphate buffer, pH 6.6. A bacterial extract supernatant in 2.5 M $(NH_4)_2SO_4$ (see above) was applied onto the column and washed with about one column volume of 2.5 M $(NH_4)_2SO_4$. A linear gradient decreasing from 2.5 to 0.5 M $(NH_4)_2SO_4$ was then usually applied and the elution performed at flow rates between 18 and 35 ml/h. Alternate fractions were assayed for GDH and MDH. All operations were carried out at room temperature.

Enzymic Assays (in 1 ml vol). MDH: 0.1 mM NADH, 0.15 mM oxaloacetate, 4.3 M NaCl, 0.01 M Na-phosphate, pH 7.3; AAT: 0.1 mM α-ketoglutarate, 4.5 mM L-aspartate, 300 units of purified halophilic MDH, 4.3 M NaCl, 0.01 M Na-phosphate, pH 7.3; GDH: 0.12 mM NADPH, 30 mM α-ketoglutarate, 1 M NaCl, 0.1 M NH_4Cl, 0.1 M Tris, pH 7.8.

TABLE I: Purification of Halophilic MDH on Sepharose 4B.

Fraction	Total Act. (units)	Total Protein (mg)	Sp. Act. (units/mg)	Yield (%)	Deg. of Purification
Sonicate	475 000	3570	133	100	1
Supernatant 1.6 M $(NH_4)_2SO_4$	475 000	3460	137	100	1
Supernatant 2.5 M $(NH_4)_2SO_4$	390 000	1510	259	82	2
Sepharose 4B	380 000	250	1510	80	11.4

The oxidation of NAD(P)H was followed at 340 nm with a Zeiss PMQ II spectrophotometer equipped with a linear-logarithmic converter and a recorder. One "enzyme unit" is defined as the amount of enzyme causing a change of 0.01 OD unit at 340 nm per min at 25 °C.

Protein Determination. Protein concentrations were determined by the modified biuret method (Koch and Putnam, 1971).

Determination of Salt Concentration. Salt concentration was determined by measuring conductivity with a Radiometer type CDM2d conductivity meter, after appropriate dilution.

Results

The chromatographic behavior of a crude extract of halophilic enzymes (see Experimental Section) on a Sepharose 4B column is shown in Figure 1. The enzymes in 2.5 M $(NH_4)_2SO_4$ were adsorbed onto the gel and eluted by a decreasing concentration gradient of ammonium sulfate (from 2.5 to 0.75 M). The order of elution of several enzymes is MDH, AAT, and GDH. The solubility curves of two of these enzymes, MDH and GDH, in $(NH_4)_2SO_4$ solutions are presented in Figure 2. It is clear that the enzymes are soluble at the higher ammonium sulfate concentration (2.5 M) used in this study. The solubility order MDH > GDH is retained in the elution patterns; i.e., more soluble enzymes are eluted first.

As can be seen in Figure 1, MDH is totally separated from GDH in the chromatogram. The recovery of the three enzymes and the degree of purification obtained by this technique are as follows: MDH (97.4%; 5.8-fold), AAT (72%; 3.6-fold), GDH (79%; 3.4-fold). The full protocol of MDH purification is given in Table I.

Several other solid supports, Celite 545, CM-cellulose, HMD-Agarose, and DEAE-cellulose were compared to Sepharose with respect to the fractionation of halophilic enzymes in ammonium sulfate. Celite did not adsorb these enzymes in the soluble form (at 2.5 M $(NH_4)_2SO_4$). However, the crude extract could be adsorbed onto Celite in the precipitated form at 4.1 M $(NH_4)_2SO_4$ (saturated ammonium sulfate solution) and desorbed by a decreasing concentration gradient from 4.1 to 1.6 M. On the other hand, CM-cellulose, HMD-Agarose, and DEAE-cellulose were able to adsorb the enzymes under the same conditions as Sepharose 4B. However, whereas GDH and MDH were eluted from CM-cellulose (Figure 3) and HMD-Agarose by decreasing the concentration of ammonium sulfate, in the case of DEAE-cellulose they were both desorbed only after applying an increasing concentration gradient of NaCl (Figure 4, for GDH only). Since MDH is very labile at the low ionic strength used for elution, it was

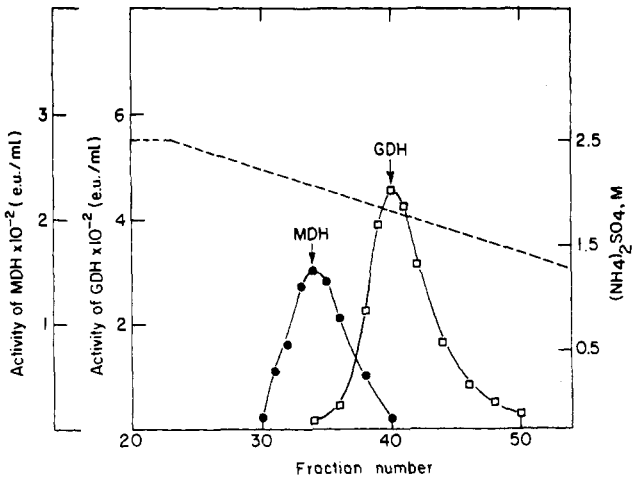

FIGURE 3: Ammonium sulfate fractionation of halophilic proteins on CM-cellulose. A bacterial extract (1 ml) in 2.5 M ammonium sulfate was applied onto a 1.6 × 11 cm column. The enzymes were eluted by a 100-ml gradient decreasing from 2.5 to 0.5 M $(NH_4)_2SO_4$. Fractions of 2 ml were collected and assayed for enzyme activity.

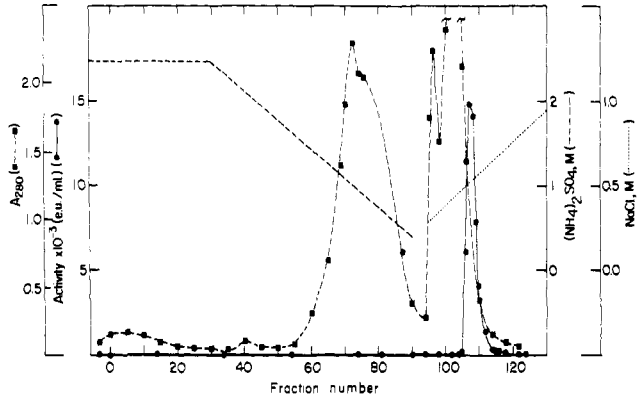

FIGURE 4: Purification of halophilic GDH on a column of DEAE-cellulose. The GDH-containing fraction (381 ml) after chromatography on Sepharose 4B (see Table II) was applied onto a 2.3 × 44 cm column of DEAE-cellulose. A 925-ml gradient of $(NH_4)_2SO_4$ decreasing from 2.5 to 0.3 M was then applied and thereafter the proteins were eluted with a 725-ml gradient of NaCl increasing from 0.3 to 1 M, buffered with 10 mM Hepes, pH 7.2. Fractions of 17 ml were collected in tubes containing 2 ml of 1.5 M NaCl (to prevent inactivation) and assayed for GDH.

necessary to protect its activity from inactivation with 1×10^{-4} M NADH.[2] Under these conditions MDH did elute from DEAE-cellulose slightly before GDH in the NaCl gradient (not shown). As can be seen from Figure 4 and Table II, GDH can be purified further by this method.

Table III summarizes the findings of this work with respect to MDH and GDH, by indicating the approximate concentrations of $(NH_4)_2SO_4$ at which the enzymes elute from various solid supports.

Discussion

The results described above clearly demonstrate the usefulness and the simplicity of the ammonium sulfate method for fractionation of enzymes on solid supports. On Sepharose 4B this procedure has enabled a facile separation of several enzymes, with purification factors ranging between four to six (Table I). In the case of DEAE-cellulose both MDH and GDH were retained on the column at the end of the ammonium

[2] Mevarech and Neumann, to be published.

TABLE II: Purification of Halophilic GDH on DEAE-Cellulose.

Fraction	Total Act. (units)	Total protein (mg)	Sp. Act. (units/mg)	Yield (%)	Deg. of Purification
Sonicate	1 600 000	3460	400	100	1
Sepharose 4B	1 456 000	1710	850	91	2
DEAE-cellulose	1 018 000	75	13 600	64	31

TABLE III: Elution of Two Halophilic Enzymes by Decreasing Concentration Gradients of Ammonium Sulfate.[a]

Column	GDH (M)	MDH (M)
Sepharose 4B	1.44	1.70
CM-cellulose	1.84	2.06
HMD-Agarose	1.18	
DEAE-cellulose	ne[b]	ne[b]
Celite	3.11	3.53
Solubility[c]	3.01	3.32

[a] Ammonium sulfate concentration at which the enzymes eluted from the various solid supports. [b] Not eluted: the enzymes did not elute until 0.4 M $(NH_4)_2SO_4$ (even at 0.3 M $(NH_4)_2SO_4$ in the case of GDH). They could, however, be eluted by a NaCl gradient. [c] Ammonium sulfate concentration at which 50% of the activity was found in the supernatant.

sulfate gradient. The two enzymes were thus separated from a large peak of contaminating protein and nucleic acids which elute around 1.2 M $(NH_4)_2SO_4$ (Figure 4). The elution of MDH and GDH could be accomplished by applying a NaCl gradient. An additional 15-fold purification for GDH was thus obtained (Table II). Due to the large amounts of NADH required for the protection of MDH against low-salt-induced inactivation this was not a practical method for the purification of this enzyme.

It is also worth mentioning that these columns can be used repeatedly without repouring, after reequilibration with 2.5 M ammonium sulfate, and that fairly high-flow rates (20 ml h^{-1} cm^{-2}) were achieved without any compression of the gels. This procedure can be applied with very dilute solutions of proteins, since at 2.5 M $(NH_4)_2SO_4$ almost all the halophilic proteins are retained on Sepharose or DEAE-cellulose. This property can also be exploited to achieve fast concentration of halophilic proteins, even in solutions containing 1.6 M NaCl as well as 2.5 M $(NH_4)_2SO_4$ (unpublished results). Another advantage of this method is based on the fact that, during adsorption and elution, the proteins remain in solution and adverse effects, such as irreversible loss of activity, that can be caused by precipitation, are avoided.

The ammonium sulfate concentrations at which the enzymes GDH and MDH are eluted from various solid supports are compared in Table III with the ammonium sulfate concentration at the midpoint of the solubility curve (Figure 2), i.e., where 50% of the total activity is found in the supernatant. It is obvious that the elution from Celite is governed mainly by the solubility properties of the two halophilic enzymes in $(NH_4)_2SO_4$, as was originally proposed for other proteins (King, 1972). As for the other solid supports, the ammonium sulfate concentrations at which the two enzymes are eluted are much lower than those at which they are precipitated, although

the order of elution is related to that of decreasing solubility. Since Sepharose gels are essentially uncharged and since the proteins are adsorbed in 2.5 M ammonium sulfate, it is clear that no electrostatic forces are involved in the binding. Moreover, this method requires high concentration of strong structure-forming anions, such as sulfate, and does not operate with anions such as chloride, which are only mildly structure forming. The interactions involved in the adsorption of halophilic enzymes on Sepharose are therefore most probably of "pure" hydrophobic nature, as it is known that this type of interaction is favored by high concentrations of structure-forming anions (von Hippel and Schleich, 1969).

Several workers (Rimerman and Hatfield, 1973; Doellgast and Fishman, 1974) have utilized amino acid and aromatic amine derivatives of Sepharose with decreasing concentration gradients of structure-forming anions (sulfate or phosphate salts) to fractionate proteins. In these cases, the retention of the proteins appeared to be related to the hydrophobic character of the substituted Sepharose. Presumably, unsubstituted Sepharose is sufficiently nonpolar with respect to the retention of halophilic proteins at 2.5 M ammonium sulfate. It has been recently reported that decreasing concentration gradients of ammonium sulfate enabled the fractionation of transfer RNA species on unsubstituted Sepharose (Holmes et al., 1975). To date, no proteins other than halophilic proteins could be retained on unsubstituted Sepharose under these conditions.[3]

Whereas the interactions between halophilic proteins and the agarose gels at 2.5 M $(NH_4)_2SO_4$ are purely hydrophobic, in the cases of the ion exchangers CM-cellulose (Figure 3), HMD-Agarose, and DEAE-cellulose (Figure 4) electrostatic interactions are involved as well. As a result of the repulsion forces between the anionic CM-cellulose and the negatively charged GDH and MDH, their elution pattern is shifted to higher ammonium sulfate concentrations than required for Sepharose (Table III). The opposite effect, i.e., a shift to lower ammonium sulfate concentration, occurs in the elution patterns from the cationic HMD-Agarose[4] and DEAE-cellulose (Table III). Moreover, in the case of DEAE-cellulose, which has a higher charge density than HMD-Agarose, the electrostatic forces become dominant before the hydrophobic interactions have subsided. Thus, GDH and MDH cannot be eluted at all by a decreasing concentration gradient of ammonium sulfate, but are readily released when ammonium sulfate is replaced by a salt such as NaCl which reduces electrostatic attraction forces, without promoting hydrophobic interactions. The purification of halophilic GDH on DEAE-cellulose exploits both hydrophobic chromatography of the contaminating proteins eluting in the ammonium sulfate gradient and ion-exchange chromatography of GDH using a NaCl gradient.

The described method provides examples of hydrophobic interaction between the virtually uncharged unsubstituted Agarose gels (Sepharose 4B) and the halophilic proteins and of mixed ionic-hydrophobic adsorption to anionic and cationic ion exchangers in 2.5 M ammonium sulfate. Usually, only mixed ionic-hydrophobic adsorption of proteins to substituted agaroses has been observed and their elution was obtained by increasing salt (usually NaCl) concentrations (Shaltiel and Er-El, 1973; Hofstee, 1973; Hjerten, 1973; Jost et al., 1974; Jennissen and Heilmeyer, 1975). This has been the case because the substituents were coupled to CNBr-activated Agarose, and the positive charges present on the gel after coupling (Porath, 1968; Svensson, 1973; Jost et al., 1974) induce electrostatic interactions. However, with uncharged substituted Agaroses, such as benzylated Sepharose (Porath et al., 1973) and alkylhydrazide-Sepharose (Jost et al., 1974), "pure" hydrophobic absorption has been observed, and the proteins could not be eluted by increasing salt concentrations. Similarly, in the case of alkylamino-Sepharoses with long aliphatic chains (usually more than six methylenes), no elution occurred even at high NaCl (Er-El, 1975; Hofstee, 1973). In the case of charged substituted Agaroses, hydrophobic chromatography will prevail if one uses decreasing concentration gradients of a structure-forming salt such as ammonium sulfate (Rimerman and Hatfield, 1973; Doellgast and Fishman, 1974).

In summary, chromatography of halophilic proteins with decreasing concentration gradients of a structure-forming salt (ammonium sulfate) on unsubstituted and charged polysaccharide gels has achieved several goals. In addition to being a useful and convenient technique for the separation and purification of halophilic proteins, it has enabled a better understanding of the forces involved in the adsorption process of proteins to charged and uncharged gels.

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[3] It is interesting that (similarly to transfer RNA) halophilic proteins are, in general, highly negatively charged (Larsen, 1967), due to an excess of acidic groups as high as 17–18 mol % (Reistad, 1970).

[4] In the case of HMD-Agarose the shift to increased concentrations of ammonium sulfate to elute GDH might be due in part to increased hydrophobic interactions with the hexamethylene backbone. However, halophilic GDH can also be adsorbed on HMD-Agarose in the absence of ammonium sulfate and eluted by NaCl gradients, as in ion-exchange chromatography (Werber and Kalif, 1975).

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Resonance Raman Evidence for Substrate Reorganization in the Active Site of Papain†

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ABSTRACT: Resonance Raman spectra were obtained for the acylenzyme 4-dimethylamino-3-nitro(α-benzamido)cinnamoyl-papain prepared using the chromophoric substrate methyl 4-dimethylamino-3-nitro(α-benzamido)cinnamate. These spectra contained vibrational spectral data of the acyl residue while covalently attached to the active site and could be used to follow directly acylation and deacylation kinetics. Spectra were obtained at pH values ranging from those where the acyl-enzyme is relatively stable (pH 3.0, $\tau_{1/2} \simeq 800$ s) to those where it is relatively unstable (pH 9.2, $\tau_{1/2} \simeq 223$ s). Throughout this range acyl-enzyme spectra differed completely from that of the free substrate or the product (4-dimethylamino-3-nitro(α-benzamido)cinnamic acid), indicating that a structural change occurred on combination with the active site. The spectra are consistent with rearrangement of the α-benzamido group in the bound substrate, -NH—C(=O)Ph becoming —N=C(—OX)Ph, where the bonding to oxygen is unknown. Superimposed on these large differences, small changes in acyl-enzyme spectra also occurred as pH was raised to decrease the half-life. All of the above spectral perturbations are consistent with a structural change in the acyl-enzyme which precedes the rate-determining step in deacylation. Thus, deacylation proceeds from an acyl residue structure differing from that of the substrate in solution. Upon acid denaturation the spectrum characteristic of the intermediate reverts to one closely resembling the substrate, demonstrating that a functioning active site is necessary to produce the observed differences. Spectra in D_2O of native acyl-enzyme were identical with those in H_2O, indicating that the observed differences in rate constant were not due to solvent-induced structural changes. Activated papain purified by crystallization or by affinity chromatography formed the acyl-enzyme. However, the kinetics of formation and deacylation differed between these materials, as did the spectral properties. Small differences in active-site structure are considered to be responsible for this effect, and it is suggested that such spectral perturbations may be useful in directly relating small differences in structure of the substrate in the active site with corresponding differences in kinetics.

A knowledge of the events which occur in submolecular groupings of a substrate during catalysis would greatly extend the experimental basis for theories of enzyme mechanisms. A potentially powerful way of obtaining such data is by using chromophoric acylating groups which yield resonance Raman vibrational spectra when specifically combined with enzyme active sites (Carey and Schneider, 1974, 1976). The utility of this method (Carey et al., 1972) arises from its ability to monitor the vibrational spectrum of the substrate alone during enzymolysis and from the wealth of detail resonance Raman spectra contain about submolecular groupings in selected compounds. Since vibrational spectra are sensitive to the kind of chemical effects thought to be important during catalysis (e.g., bond strain and changes in charge distribution), they are ideal monitors for catalytic processes.

Papain-catalyzed hydrolysis of esters proceeds through the formation of a covalent acyl-enzyme intermediate linking the thiol group of the enzyme with the acyl residue of the substrate (Glazer and Smith, 1971). Several stable acyl-enzymes have been prepared (Lowe and Williams, 1965; Brubacher and Bender, 1966; Hinkle and Kirsch, 1970). The present paper describes the preparation and enzymatic and resonance Raman properties of 4-dimethylamino-3-nitro(α-benzamido)cinnamoyl-papain. The aim of the present study was to see if changes occurred in particular submolecular groupings of the acylating group during the course of the enzymatic reaction, and then to determine the nature of such changes.

Papain was chosen because of the known ability of sulfhydryl enzymes to produce large red shifts in the absorption spectra of acylating groups. The resonance Raman technique requires, at present, acylating groups with suitable absorption properties above 400 nm, and synthesis of such compounds is difficult. It was expected that the strong absorption band of the substrate at 350 nm (Figure 1) would be sufficiently red shifted on combining with papain so that a resonance Raman spectrum of the bound substrate could be obtained at low concentrations using 441.6-nm laser excitation. This expectation was born out experimentally. The particular substrate used (II, Figure 2) was chosen and synthesized because, in addition to its suitable chromophoric properties, the α-benzamido side chain introduces a degree of specificity into a synthetic substrate (Brocklehurst and Williamson, 1966; de Jersey, 1970).

The main thrust of the results was unexpected, and not apparent from examination of the absorption spectra alone. The

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