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Use of Reversible Denaturation for Adsorptive Immobilization of Urease

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Abstract

Urease was chosen as a model multimeric protein to investigate the utility of reversible denaturation for immobilization to a hydrophobic support. Of the various procedures investigated, acidic denaturation provided the highest degree of immobilization and enzymatic activity with lowering of K_m (apparent). Exposure of hydrophobic clusters in the protein molecule induced by the acidic pH environment was confirmed by fluorescence studies using 8-anilino-1-naphtalene-sulfonate as a hydrophobic-reporter probe. The catalytic potential of the enzyme at low pH values was dramatically improved with significant heat and pH stability enhancement on immobilization. Furthermore, the immobilized preparation was used successfully in continuous catalytic transformations. Based on the results presented in this article and a recent report involving a relatively more simple monomeric protein, it is suggested that reversible denaturation may be of general utility for immobilization of proteins, which are not normally adsorbed on hydrophobic supports.

Index Entries: Adsorptive immobilization; urease; molten globule; 8-anilino-1-naphthalene-sulfonate; hydrophobic matrix.

Introduction

Jack bean urease (urea amidohydrolase, EC 3.5.1.5) is a homohexameric enzyme with a molecular mass of 545 kDa. It is made up of six identical subunits (mol wt = 90,770) with a total of 12 mol of nickel/mol of protein *(1,2)*. The enzyme occurs in many bacteria, several species of yeast, and a number of higher plants *(3)* and is important for the determination of urea. Urease hydrolyzes this substrate to form ammonia and

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carbamate, with the latter spontaneously degraded into CO_2 and a second molecule of ammonia *(4)*. Like a number of multimeric proteins, it has a more complex denaturation profile than the one observed for monomeric proteins *(5)*.

The protein can undergo dissociation from hexamer to a smaller, active dissociation product when submitted to either low or high pH *(6–10)*. Further denaturation in the form of unfolded monomers can be obtained by treatment with high concentrations of sodium dodecyl sulfate (SDS) or GdnHCl *(11)*, whereas active monomers have been obtained using low concentrations of SDS *(12)*. Under several mild conditions such as low concentration of GdnHCl, urease undergoes subtle conformational changes resulting in structures usually referred to as "molten globule" states. Such intermediate forms have been found to show greater affinity for 8-anilino-1-naphthalene-sulfonate (ANS), owing to increased accessibility of hydrophobic clusters, which are also known to be responsible for aggregation *(13–17)*.

Our previous studies indicated that carbonic anhydrase, which does not normally show any affinity for binding to hydrophobic supports, acquires such capacities while regaining most of its catalytic potential through the process of denaturation-renaturation *(18)*. The present article reports on immobilization of urease, a multimeric enzyme, using the same strategy. Various denaturation methods were explored, and using ANS as a fluorescence probe, the procedures were compared in terms of their potencies in exposing hydrophobic clusters in the protein structure for interaction with the hydrophobic ligand present on the matrix.

Materials and Methods

Materials

Jack bean urease and other chemicals were purchased from Sigma (St. Louis, MO). All chemicals were of analytical reagent grade.

Coupling of Palmityl Glycidyl Ether to Sepharose 4B

Preparation of palmityl glycidyl ether and its coupling to Sepharose 4B was carried out following the procedure reported previously *(19)*.

Protein Concentration

Protein concentration was determined by the method of Lowry et al. *(20)* and the turbidimetric tannin micromethod *(21)*.

Enzyme Assays

Urease activity was determined using a method based on urea hydrolysis and by coupling ammonia production to its consumption catalyzed by glutamate dehydrogenase *(22)*.

Preparation of Denatured and Renatured Urease

For acid denaturation, a solution of urease at a 0.3 mg/mL concentration was incubated in 0.024 *M* potassium phosphate and 0.013 *M* citric acid, pH 4.6, for 1 h. The pH was adjusted to neutral by adding NaOH. After 30 min of incubation at the new pH, the solution was diluted 120 times by the addition of 0.1 *M* phosphate buffer, pH 7.5, and its activity was measured. Percentage of activity recovery was determined by comparing the activity after renaturation with the original activity of the native enzyme.

In denaturation by GdnHCl and urea, enzyme solution was incubated in phosphate buffer containing the same denaturing agents at room temperature for 1 h. After overnight dialysis at 4°C, its activity was measured. Thermal denaturation of urease was followed by heating the enzyme solution at 58 and 65°C for 1 h. After incubation on ice (1 h), enzyme activity was determined.

Preparation of Immobilized Urease

Normally, immobilization was achieved by denaturation of the enzyme in the presence of 1 mL of palmityl-substituted Sepharose 4B (Sepharose-lipid) following one of the procedures described above. After renaturation, the suspension was centrifuged, and protein concentration and catalytic activity of the supernatant together with the activity of immobilized, denatured-renatured, and native urease were determined. When thermal denaturation was examined, the enzyme was preheated at the specific temperature followed by the addition of the hydrophobic matrix.

Catalytic Activity of Immobilized Preparations

Activity of the immobilized enzymes was determined by suspending a volume of the immobilized preparation corresponding to 0.1 mL of packed matrix in the assay mixture. The procedure was followed according to the method reported previously *(18)*.

Fluorescence Measurements

Extrinsic fluorescence studies were carried out using ANS as a fluorescence probe. Fluorescence measurements were done with a Hitachi MPF-4 apparatus. All measurements were carried out at 25°C, and final concentrations of protein and ANS were 0.3 mg/mL and 20 µ*M*, respectively. An excitation wavelength of 350 nm was used in this study.

Results

In a previous study *(18)*, we suggested a simple method for immobilization of carbonic anhydrase, which is not normally adsorbed on hydrophobic supports. In the present investigation, we report on a similar strategy for urease, which like carbonic anhydrase, does not show any affinity for binding to hydrophobic matrices in its native form.

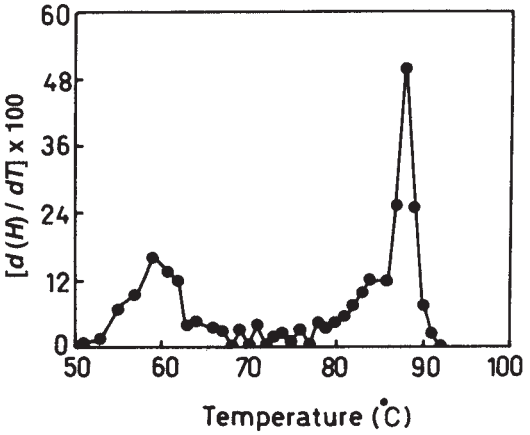

Fig. 1. Derivative thermal denaturation profile of urease: first derivative of hyperchromicity with respect to temperature vs temperature. The heating rate was approx 1°C/min. Enzyme concentration was 0.3 mg/mL in 0.1 *M* potassium phosphate buffer, pH 7.5.

Thermal denaturation of urease with a final concentration of 0.3 mg/mL in 0.1 *M* phosphate buffer, pH 7.5, was followed at 280 nm by continuous heating at a rate of 1°C/min using a Gilford spectrophotometer. The denaturation curve showed two phase profiles (Fig. 1) from which two melting temperatures, 59 and 88°C, were obtained. Because of the sensitivity of Sepharose-lipid to the higher temperature (88°C), heating at 58 and 65°C (near its first T_m) was tested using various incubation times. Thus, to immobilize urease, an enzyme solution was treated for 30 min at one of these temperatures. Preheated Sepharose-lipid was then added and kept for another 30 min, at the same temperature. Subsequently, the suspension was left on ice for 1 h, and the adsorbent containing the bound enzyme was separated from the unbound by centrifugation. Catalytic activities of the immobilized preparations were then determined. As shown in Fig. 2, 15% immobilization with 12% activity and 7% immobilization with 10% activity were observed at 58 and 65°C, respectively. In another experiment, a solution containing the enzyme was heated for 5 min at 80°C and then Sepharose-lipid was added and stirred for 30 min at 60°C. After cooling for 1 h on ice, the mixture was centrifuged, and protein concentration and enzyme activity were determined. In the conditions utilized, immobilization was improved, and about 50% of the enzyme could be adsorbed with a catalytic potential corresponding to 64% of the native enzyme (Fig. 2).

Denaturation by GdnHCl and urea was also examined. A solution of urease at 0.3–0.5 mg/mL in 0.05 *M* phosphate buffer containing 6 *M* GdnHCl or 8 *M* urea was prepared and incubated with the matrix for 1 h at room temperature. The suspension was then dialyzed against the buffer at 4°C overnight. The extent of immobilization and catalytic activities were determined following the procedure described under Materials

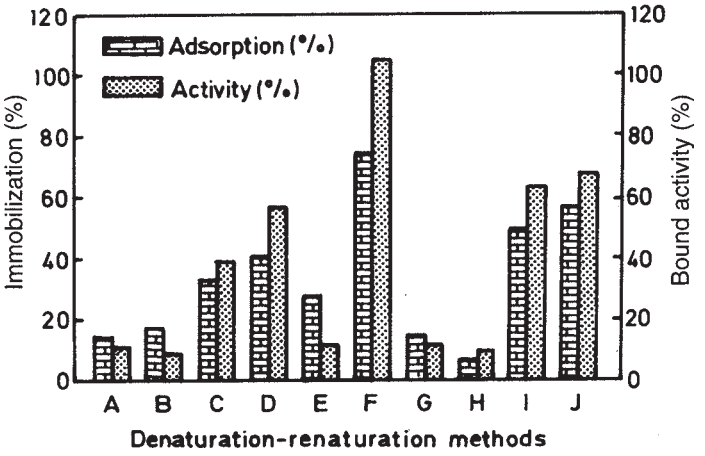

Fig. 2. Effect of various denaturation-renaturation methods on immobilization of urease on Sepharose lipid. Experimental details are given in the text. (A) 8 *M* urea; (B) 6 *M* GdnHCl; (C) 2 *M* GdnHCl; (D) 1 *M* GdnHCl; (E) acetate buffer, pH 3.5; (F) citrate-phosphate buffer, pH 4.6; (G) heating at 58°C; (H) heating at 65°C; (I) heating of the enzyme at 80°C for 5 min (in phosphate buffer, pH 7.5), followed by addition of the matrix and further incubation at 60°C for 30 min; (J) as for (I) with the difference that citrate-phosphate buffer, pH 4.6, was used.

and Methods. As shown in Fig. 2, a small proportion of the enzyme was found to be immobilized using these denaturing agents at the specified concentrations. Such observations were taken to be owing to irreversible denaturation *(23,24)*. Accordingly, to induce partial denaturation of urease, lower concentrations of GdnHCl were used. The unfolding experiments with 1 and 2 *M* GdnHCl in 20 m*M* phosphate buffer containing 1 m*M* EDTA and 5 m*M* dithiothreitol were carried out similar to the above procedure. The extent of immobilization in these conditions was higher than that using 6 *M* GdnHCl (the use of 1 *M* GdnHCl resulted in 41% immobilization with 57% activity, and 2 *M* GdnHCl resulted in 33% immobilization with 39% activity).

Denaturation of the enzyme by acidic pH was also performed following the procedure described under Materials and Methods and using different incubation times. As indicated in Fig. 3, best results were obtained with an incubation time of 1 h. Thus, on incubation for this time at pH 4.6, pH was raised to 7.5 and the mixture was kept at the new pH for various times. According to the data (not shown), 30 min was found sufficient for renaturation of the enzyme. Activity and concentration measurements showed that about 75% of urease could be immobilized with an enhanced catalytic activity of 105%, corresponding to the original native enzyme (Fig. 2). No detectable loss of activity was observed on washing columns containing such immobilized preparations with GdnHCl (1–5 *M*). At lower pH values such as 3.5 (0.1 *M* acetate), the extent of immobilization and activity decreased significantly (28% immobilization with 12% activ-

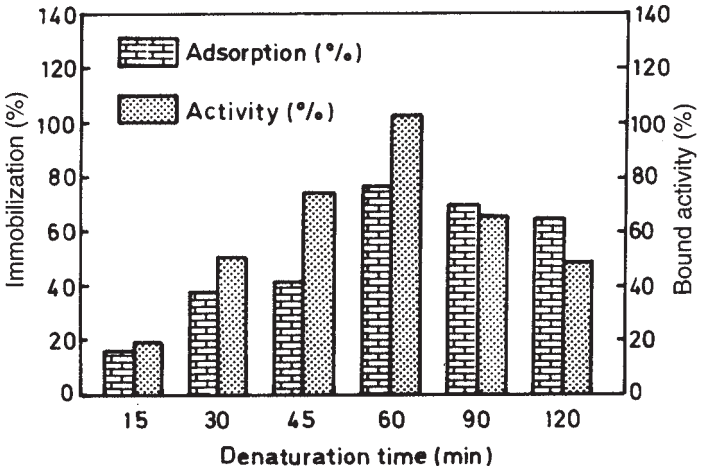

Fig. 3. Effect of duration of denaturation on the extent of immobilization and catalytic activity of immobilized urease.

ity). A combination of thermal and acid denaturation was also studied, which provided 57% immobilization and 68% activity (i.e., less than the acid denaturation alone as already described).

In none of the experimental conditions utilized did we observe any detectable adsorption of the protein on the original unsubstituted Sepharose 4B. This is in line with a previous conclusion *(19)* that immobilization of the protein occurs as a result of its interaction with the alkyl residues of the substituted support.

Binding of ANS to Urease in Different Denaturation Conditions

ANS was used as a fluorescence probe to investigate exposure of hydrophobic clusters in the protein molecule induced by various denaturation methods used in the present investigation. Figure 4 represents the effect of low pH (4.6), GdnHCl, and urea on fluorescence of ANS in the presence of the enzyme. Solutions of urease in 0.1 *M* phosphate buffer, pH 7.0, containing 1, 2, or 6 *M* GdnHCl or 8 *M* urea were prepared and incubated for 1 h at room temperature. The extent of ANS binding when using 2 *M* GdnHCl was higher than that using 6 and 1 *M* concentrations. However, in all conditions utilized, interaction of ANS with the denatured forms of urease was higher than that for the native enzyme (0.3 mg/mL in 0.1 *M* phosphate buffer, pH 7.0). Denaturation of urease by acidic pH (0.024 *M* potassium phosphate, 0.013 *M* citric acid, pH 4.6) was performed with an incubation time of 1 h. On introduction of ANS and further incubation at the same pH for an additional 1 h, the affinity of ANS for urease was found to increase significantly as reflected by a more than fivefold fluorescence enhancement in comparison with the situation in which the native protein was used. Concomitantly, a blue shift of 45 nm was observed. Also, in a control experiment, no such fluorescence enhancement of ANS

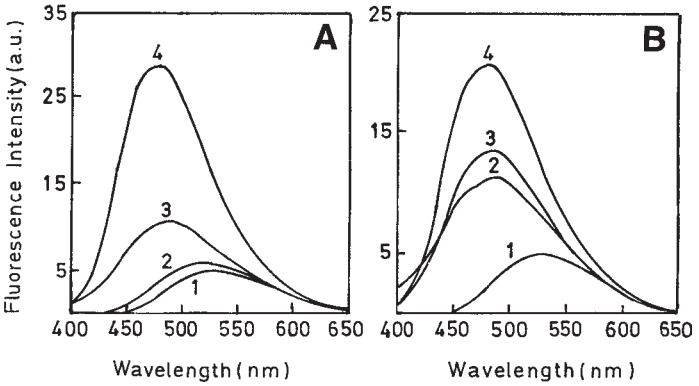

Fig. 4. Fluorescence spectra of 20 µ*M* ANS in the presence of the following: **(A)** 1 = no addition, 2 = native urease, 3 = unfolded urease (8 *M* urea), 4 = partially unfolded urease (0.024 *M* potassium phosphate; 0.013 *M* citric acid, pH 4.6); and (B) 1 = native urease, 2 = unfolded urease (6 *M* GdnHCl), 3 = partially unfolded urease (1 *M* GdnHCl), 4 = partially unfolded urease (2 *M* GdnHCl). For further details, see Materials and Methods.

was observed when incubation with the previously denatured enzyme was carried out at pH 7.0 (i.e., with "native" enzyme).

Based on the results presented, it is suggested that among various procedures examined, denaturation of urease at pH 4.6, following the specified experimental conditions, provided the highest degree of association between palmityl residues on the matrix and the hydrophobic side chains in the protein molecule during its conformational transitions. This procedure is therefore suggested as a useful method for immobilization of urease. Accordingly, we examined the catalytic function of the immobilized urease prepared in this manner.

Kinetic Properties of Immobilized Urease

As shown in Fig. 5, all the three enzyme preparations (native, denatured-renatured, and immobilized) had the same pH optimum of about 7.5. Also, the overall shapes of the pH profiles presented were similar to the immobilized preparation showing significantly higher activities at low pH values.

We also determined the kinetic parameters for the three forms of urease using urea as substrate. The K_m (apparent) values for the native, denatured-renatured, and immobilized forms were 2.85, 2.47, and 1.11 m*M*, respectively. Clearly, immobilization caused a decrease in K_m value for urea by about 2.5-fold (Fig. 6).

Stability of Immobilized Urease

Thermal stability of urease in native, denatured-renatured, and immobilized forms was investigated. Samples were incubated at 65°C for

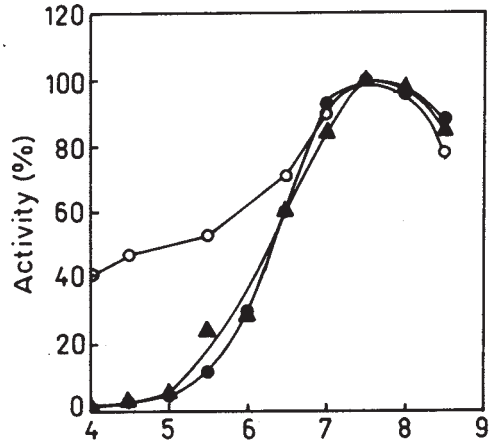

Fig. 5. Activity of native (●), denatured-renatured (▲), and immobilized (○) urease at various pH values. Further details are described in Materials and Methods.

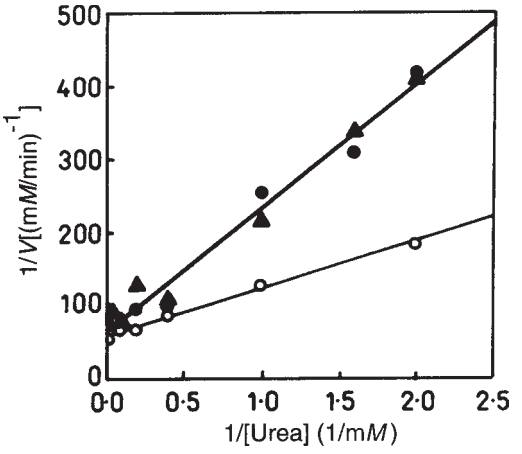

Fig. 6. Double reciprocal plot for native (●), denatured-renatured (▲), and immobilized (○) urease at several concentrations of urea. Further details are described in the text.

various times and then withdrawn at intervals. After cooling on ice, enzyme activity was determined. No loss of activity was observed in the case of the immobilized preparation during a total incubation time of 2 h (Fig. 7).

The effect of pH on stability of urease was also determined in the pH range 3.0–9.0 using a mixture of MES, Tris, HEPES, and citrate each at a 10 m*M* concentration. The enzyme samples were incubated at various pH values for 30 min followed by measuring activity at pH 7.5. According to the results presented (Fig. 8), the immobilized enzyme showed significantly higher pH stability than the free forms in acidic pH.

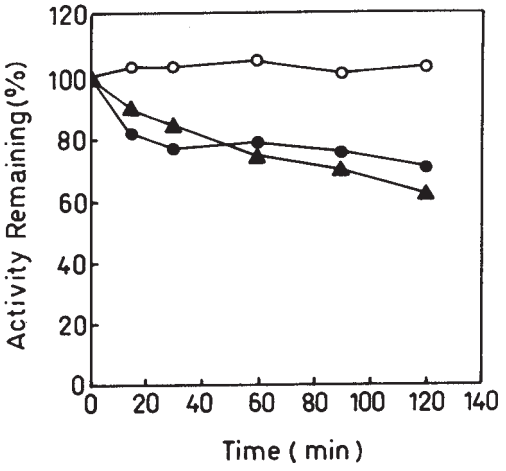

Fig. 7. Stability of native (●), denatured-renatured (▲), and immobilized (○) urease in 0.1 *M* potassium phosphate buffer, pH 7.5, at 65°C. Samples were withdrawn at various times, cooled at 4°C for 30 min, and assayed at 25°C in the usual manner.

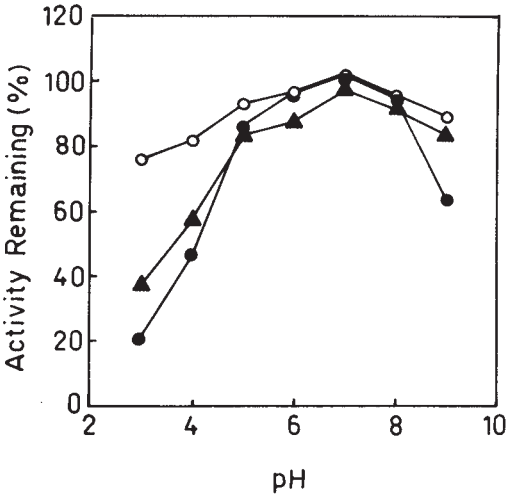

Fig. 8. Stability of native (●), denatured-renatured (▲), and immobilized (○) urease at different pH values. The enzyme was incubated for 30 min in a mixed buffer containing MES (2-N-morpholinoethanesulfonic acid), HEPES, Tris, and citrate each at a 10 m*M* concentration adjusted at various pH values. Samples were placed in 0.1 *M* phosphate, pH 7.5, in which they were kept for 30 min on ice before being assayed.

Continuous Catalytic Operations with Immobilized Urease

Using a small packed-bed reactor, activity of immobilized urease was investigated following continuous hydrolysis of urea at 22°C. As shown in Fig. 9, after prolonged pumping of 60 m*M* urea in 30 m*M* Tris, pH 7.5, through the column, about 100% of activity had remained. Similar results were obtained at 4°C.

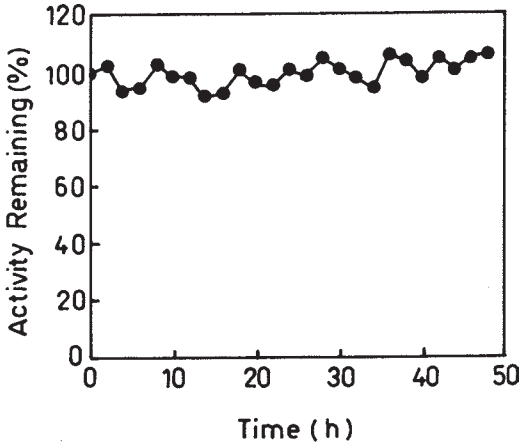

Fig. 9. Immobilized urease in continuous catalytic operation at 4°C. The column (0.7 × 10 cm) was washed with a solution of the reactants using a flow rate of 0.3 mL/min.

Discussion

In a previous study, we reported on immobilization of carbonic anhydrase, a monomeric enzyme, through its denaturation-renaturation pathway *(18)*. The present article describes the use of the same strategy for adsorptive immobilization of urease, which, like carbonic anhydrase, does not show any affinity for binding to a hydrophobic support in its native form *(19)*.

It has been shown that several proteins may be immobilized on hydrophobic supports via nonionic interactions *(19,25)*. As indicated *(19)*, some proteins such as carbonic anhydrase and urease do not show any affinity for interacting with these adsorbents owing to the unavailability of their hydrophobic side chains for interaction in their native structures. However, our more recent observations suggested that heat denaturation of carbonic anhydrase may provide a convenient method for making such sites available for binding with retention of enzymatic activity *(18)*. Having encountered this new and exciting opportunity, we tried to extend the approach for other proteins whose immobilization through hydrophobic adsorption did not otherwise deem possible. Accordingly, urease, a multimeric enzyme characterized by a more complex denaturation process than that observed for a monomeric protein such as carbonic anhydrase, provided the subject of the present investigation.

It has been shown that treatment of urease with GdnHCl, SDS, and high temperatures results in aggregation followed by precipitation of the enzyme *(13)*. It has been further suggested that partial or transient exposure of hydrophobic clusters in the protein is responsible for this phenomenon *(15)*.

Using ANS as a hydrophobic fluorescent probe, it has been concluded that during denaturation by various procedures, the tertiary structure of a

protein may loosen up, providing hydrophobic sites for this probe *(26–28)*. Often, proteins in their native and fully unfolded states have a modest effect on ANS, whereas folding intermediates having high fluidities (referred to as molten globule states) promote a dramatic increase in its fluorescence intensity *(29–31)*. It has been reported that ANS, like polyethylene glycol or chaperonins that bind to exposed hydrophobic clusters *(32–35)*, can decrease the size or number of urease aggregates in partial denaturation conditions *(36)*. Formation of ion pairs with the cationic group of protein *(37)* and change in protein conformation *(38)* have also been reported in relation to ANS binding.

On the basis of the nature of Sepharose-lipid and the properties of urease we have described, it was expected that binding of the protein to the matrix during its denaturation-renaturation pathway would occur. Accordingly, various procedures such as denaturation by heat, low pH, GdnHCl, and urea were used. Acidic denaturation provided the highest degree of immobilization and catalytic activity as compared to the other procedures. The fluorescence studies reported here confirmed a higher degree of exposure of the hydrophobic pockets in the protein structure brought about by acid denaturation as compared with the other denaturation conditions utilized (Fig. 4).

The existence of structural transitions in acidic pH has been demonstrated by several criteria. It has been found that at low pH, a protein may lose its tertiary structure while maintaining its secondary structure. This sort of structural change has been described for proteins such as carbonic anhydrase *(27,28)*, α-lactalbumin *(39)*, interleukin-2 *(40)*, and β-lactamase *(41)* and is characterized as the molten globule state. According to the present results, it is suggested that similar structural transitions occurring in urease may be the reason for such observations. Thus, it may be concluded that the appearance of relatively more hydrophobic binding sites in urease in acidic conditions, as compared to other procedures, provides greater opportunities for hydrophobic interactions to occur. Compared with other denaturation procedures, acidic denaturation has been proven more useful in this connection, which is in accordance with the fluorescence data we have discussed.

The dramatic enhancement of stabilization of urease observed on immobilization (Figs. 5 and 7–9) warrants further discussion. It has been suggested that low concentrations of a detergent such as SDS results in the formation of active monomers of urease *(15)* and, by forming an amphiphilic interface, stabilizes the protein *(13)*. A similar mechanism has been suggested for stabilization by ANS *(15)*. Based on such reports and the results presented here, it is suggested that the palmityl residues present on the surface of the hydrophobic matrix may interact with the same or similar hydrophobic sites on the intermediate structures discussed. Such interactions ultimately result in an immobilized preparation with enhanced catalytic activity, possibly owing to the lowering of K_m (apparent) discussed previously. The latter occurs in spite of the mass transfer and diffusion

limitations expected to occur in such a heterogeneous system. Because the hexameric form and the individual dissociated subunits have similar catalytic activities *(42,43)*, we cannot make any suggestion about the size of the protein in the immobilized form.

Finally, according to the results presented herein using a multimeric protein and a previous report utilizing a relatively more simple monomeric protein *(18)*, it is suggested that reversible denaturation may be of general utility for adsorptive immobilization by hydrophobic interactions. The strategy described is especially useful for those proteins that do not show any affinity for binding to hydrophobic supports in their native forms.

Acknowledgment

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