

Inhibitor-induced Enzyme Activation in Organic Solvents*

(Received for publication, May 9, 1988)

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The enzymatic activity of the protease subtilisin in anhydrous organic solvents can be dramatically increased by pretreating the enzyme before it is placed in the nonaqueous medium. For instance, lyophilization of subtilisin from aqueous solution containing competitive inhibitors (followed by their removal) created an enzyme which was up to 100 times more active than the enzyme lyophilized in the absence of such ligands. This phenomenon of ligand-induced "enzyme memory" also extends to the stability, affinity, and substrate specificity of subtilisin in organic solvents.

Recently, it has been discovered that enzymes can function as catalysts in anhydrous organic solvents (instead of their conventional aqueous reaction medium) (Klibanov, 1986; Deetz and Rozzell, 1988) and, when placed in this unnatural milieu, they exhibit novel catalytic properties such as greatly enhanced thermostability (Zaks and Klibanov, 1984), and radically altered substrate specificity (Zaks and Klibanov, 1986) and stereoselectivity (Margolin *et al.*, 1987). We now report a novel strategy to redesign predictably enzymic catalysts for use in organic solvents which exploits the phenomenon of "enzyme memory."

Catalysis by regulatory enzymes is tailored by the binding of ligands, resulting in activating or deactivating conformational changes in such allosteric enzymes (Hammes, 1982; Fersht, 1985). Some of these enzymes react slowly to the presence of a ligand, thus retaining its effect even after the ligand has been removed. Such hysteretic behavior (Frieden, 1979) depends on a high degree of protein rigidity (which in water most enzymes lack) in order to retain induced conformations in the absence of the ligand. In the course of our studies into nonaqueous enzymology, it has been found that enzymes are extremely rigid in anhydrous organic solvents (Zaks and Klibanov, 1984; Zaks and Klibanov, 1988). Consequently, we have endeavored to convert an enzyme (the protease subtilisin) which is nonallosteric in water into one which exhibits allosteric type activation in organic solvents by locking the enzyme into a ligand-induced permanently activated conformation before placing it in a nonaqueous solvent.

EXPERIMENTAL PROCEDURES

Subtilisin Carlsberg (EC 3.4.21.14) and the *N*-acetyl-L-amino acid derivatives were purchased from Sigma. Active site titration was performed on subtilisin using *N*-trans-cinnamoylimidazole (Polgar

and Bender, 1967). The organic solvents used in this study were of the highest purity commercially available (their purity being established by gas chromatography) and were dried by gentle shaking with 3-Å molecular sieves (Linde) overnight. The water concentration in organic solvents and in enzyme powders was determined using the optimized Fischer titration (Laitinen and Harris, 1975).

Subtilisin was preinactivated with phenylmethylsulfonyl fluoride by incubating an aqueous solution (0.1 M phosphate buffer, pH 7.8) of the enzyme (0.1 mM) in a molar excess of phenylmethylsulfonyl fluoride (0.5 mM) at room temperature for 30 min. Complete inactivation was verified by active site titration with *N*-trans-cinnamoylimidazole.

Freeze drying of aqueous solutions (0.01 M phosphate buffer, pH 7.8, containing 3% (v/v) dimethylformamide to enhance ligand solubility) of subtilisin (5 mg ml⁻¹), in the presence of ligand (typically 10 mM) where required, yielded powdered enzyme. The lyophilized powders were rinsed with a solvent which solubilized the ligand but not the protein (typically anhydrous acetonitrile) and recovered by filtration and vacuum drying. In this way the ligand was removed from the enzyme powders. Subtilisin lyophilized in the absence of ligands was treated identically to that lyophilized in the presence of ligands at all stages.

All enzymatic transesterifications in organic solvents were assayed using gas chromatography to follow the accumulation of the new ester, as described previously (Zaks and Klibanov, 1988). Typically, enzyme (1 mg ml⁻¹ organic solvent) was added to the ester substrate in the presence of the nucleophile propanol (1 M). After 10 s of sonication, the vial was placed on an orbital shaker and shaken at 30 °C and 250 rpm. Periodically, aliquots were withdrawn and assayed for the accumulation of the propyl ester product. Measurement of k_{cat}/K_m for transesterification of various substrates in organic solvents was performed as described previously (Zaks and Klibanov, 1988), as was the determination of rates of subtilisin-catalyzed hydrolysis of the ester substrates in aqueous solution.

The binding constant of subtilisin for *N*-acetyl-L-tryptophan amide in acetonitrile was measured by suspending the enzyme (10 mg ml⁻¹) in acetonitrile containing varying concentrations of *N*-acetyl-L-tryptophan amide (0.1–5.0 mM) and, after a 10-s sonication, shaking the resultant suspensions at 25 °C and 250 rpm. The decrease in concentration of ligand was then followed by high performance liquid chromatography until binding equilibrium was reached (typically 1–2 h).

The time course of irreversible enzyme thermoinactivation was determined by suspending subtilisin (10 mg ml⁻¹) in octane in a sealed vial which was incubated at 110 °C. At various time points enzyme was recovered from the ampules, separated from the solvent by centrifugation, dried under vacuum, and redissolved in aqueous buffer (0.1 M Tris-HCl, pH 8.6). The activity of the resultant solution was measured with the substrate succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide, as described previously (Russell and Fersht, 1987). The stability of subtilisin toward the denaturant dimethyl sulfoxide was measured by determining the rate of transesterification in *tert*-amyl alcohol in the presence of varying concentrations of the denaturant (0–50%).

RESULTS AND DISCUSSION

Subtilisin Carlsberg (EC 3.4.21.14) is a nonhysteretic serine endoprotease which has a broad specificity and catalyzes the hydrolysis of not only peptide but also ester bonds (Philipp and Bender, 1983). Powdered subtilisin suspended in organic solvents (enzymes are insoluble in nearly all nonaqueous solvents (Singer, 1962)) readily catalyzes the transesterification reaction between *N*-acyl-L amino acid esters and alcohols in a variety of organic solvents (Zaks and Klibanov, 1986 and 1988; Riva *et al.*, 1988). For this reaction, k_{cat}/K_m (the specificity constant) exceeds the bimolecular rate constant for the nonenzymatic process by a factor of up to 10¹¹. Comparisons can also be made between the k_{cat}/K_m for hydrolysis of an ester and that for its transesterification (Zaks and Klibanov,

* This work was funded by National Science Foundation Grant CBT-8710106. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1988). In some cases the activity is much greater in hydrolysis, but in others the situation is reversed (see Table I in Zaks and Klibanov, 1986). Since catalysis in organic solvents leads to reactions which do not take place in water and substrate specificities in the two reaction media are distinct (Zaks and Klibanov, 1986), we will avoid drawing conclusions from comparisons of catalytic constants from different processes. Significantly, the transesterification reaction requires the catalytically competent enzyme, since subtilisin preinactivated with phenylmethylsulfonyl fluoride (an active site-directed reagent) is completely inactive in organic solvents (Zaks and Klibanov, 1986, 1988; Riva *et al.*, 1988). Furthermore, it has been demonstrated by active site titration that the number of active sites available for catalysis is similar in aqueous and organic solvents (Zaks and Klibanov, 1988). Hence aggregation of the enzyme is not a handicap in the latter system, that is in the particles of powdered enzyme in anhydrous organic solvents the active sites are not obscured from the substrate.

To lock subtilisin, employed as a model system in our studies, into a conformation which resembles the enzyme-substrate complex, we lyophilized the enzyme from an aqueous solution containing the competitive inhibitor *N*-acetyl-L-tyrosine amide. This ligand is soluble in organic solvents, and it was subsequently washed out of the inhibited enzyme resulting in the production of a dry enzyme powder which no longer contained the ligand. However, the enzymatic activity of this powder in the reaction between *N*-acetyl-L-alanine methyl ester and propanol, in anhydrous octane, was 55 times greater than that of subtilisin lyophilized without ligand and washed in the same way (see Table I). In another anhydrous solvent, *tert*-amyl alcohol, the rate enhancement of the same reaction as a result of lyophilizing in the presence of *N*-acetyl-L-tyrosine amide was even higher, 63-fold. Importantly, the two enzyme preparations displayed identical activities toward the hydrolysis of the same ester in water when redissolved in aqueous solution. Hence the "ligand activation" effect (i) is not the result of the amino acid amide protecting the enzyme from inactivation during the freeze-drying process (indeed, no appreciable inactivation of subtilisin occurs during lyophilization with or without ligand) and (ii) is displayed only in organic solvents but not in water.

It was found that the magnitude of the aforementioned rate enhancement increased with increasing concentration of the ligand in aqueous solutions of subtilisin prior to lyophilization, indicating that the effect is dependent on the proportion of inhibited enzyme before lyophilization. Also, addition of the ligand to the enzyme prelyophilized in the absence of ligand had no activating effect. This observation demonstrates that ligand activation requires the enzyme to be in a flexible enough state to bind ligand effectively; clearly, this is not the case in organic solvents. Subtilisin lyophilized in the presence of ligand retained its ligand-induced activation even after prolonged incubation in anhydrous octane (48 h at room temperature), that is the new conformation of enzyme is kinetically stable and remains once placed in the rigidifying medium of a nonaqueous solvent. As can be seen in Table I, the ligand-induced activation phenomenon is rather general; a number of different ligands are able to trigger "memory" in subtilisin toward a variety of similar substrates.

Fig. 1 depicts the effect of water content in octane on subtilisin activity in that solvent. There is a marked increase in the rate of reaction for the enzyme lyophilized in the absence of ligand as the water content increases. The activity of the ligand-induced enzyme, however, is not greatly affected by water content. Furthermore, the addition of water, which loosens up protein molecules (Finney and Poole, 1984), irre-

TABLE I
Enhancement of subtilisin activity in octane induced by various ligands

Subtilisin was dissolved (4 mg ml^{-1}) in 10 mM aqueous phosphate buffer (pH 7.8) containing 3% dimethylformamide and the corresponding ligand, and lyophilized. The washed lyophilized powder (1 mg) was placed in 1 ml of anhydrous octane containing 5 mM *N*-acetyl-L-amino acid ester and 1 M *n*-propyl alcohol, and in order to homogenize the suspension it was sonicated for 10 s. Next, the reaction mixture was shaken (250 rpm) at 30 °C; periodically, aliquots were withdrawn and assayed for the propyl ester product by gas chromatography. No transesterification reaction was observed without enzyme.

Ligand ^a	Substrate ^a	Rate enhancement ^b
N-Ac-Tyr-NH ₂	N-Ac-Ala-OMe	55
	N-Ac-Tyr-OEt	11
	N-Ac-Phe-OEt	3
	N-Ac-Trp-OEt	25
N-Ac-Trp-NH ₂	N-Ac-Ala-OMe	96
	N-Ac-Tyr-OEt	12
	N-Ac-Phe-OEt	3
	N-Ac-Trp-OEt	16
2-Naphthol	N-Ac-Ala-OMe	24
	N-Ac-Tyr-OEt	21
Indole	N-Ac-Ala-OMe	8
	N-Ac-Tyr-OEt	15
Phenylboronic acid	N-Ac-Ala-OMe	6
	N-Ac-Tyr-OEt	10

^a Abbreviations: Ac = acetyl, Tyr = L-tyrosine, Trp = L-tryptophan, Ala = L-alanine, Phe = L-phenylalanine, NH₂ = amide, OMe = methyl ester, OEt = ethyl ester. The ligand concentrations in aqueous solutions of the enzyme prior to lyophilization were 10, 20, 100, 100, and 5 mM for N-Ac-Tyr-NH₂, N-Ac-Trp-NH₂, 2-naphthol, indole, and phenylboronic acid, respectively.

^b Defined as the ratio of enzymatic transesterification of a given ester catalyzed by ligand-activated subtilisin to that by subtilisin lyophilized without ligand ($4.2, 14.2, 7.0$, and $1.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for the alanine, tyrosine, phenylalanine, and tryptophan ester substrates, respectively). Experimental error for initial rate determinations was typically $\pm 10\%$. (It should be noted that there was no detectable enzymatic hydrolysis of the amide ligands under the conditions used in this study prior to lyophilization of the aqueous solutions.)

ocably destroys subtilisin's memory; when the water which was added to the activated enzyme is subsequently removed (by repeated washing of the enzyme with anhydrous tetrahydrofuran), the catalytic activity returns to the low level of the nonactivated enzyme.

In order to establish unequivocally that the active site of ligand-activated subtilisin possesses a conformation which binds substrates and ligands more effectively, we have investigated the binding capacity of ligand-activated subtilisin in organic solvents with respect to the ligand with which the enzyme was lyophilized. In acetonitrile, there is no detectable binding of *N*-acetyl-L-tryptophan amide to subtilisin. However, subtilisin lyophilized in the presence of this ligand and subsequently washed to remove all the ligand, does bind *N*-acetyl-L-tryptophan amide with a respectable binding constant of 0.6 mM. (The binding constant of subtilisin to *N*-acetyl-L-tryptophan amide in aqueous solution is about 100 mM (Philipp and Bender, 1983).) Crucially, phenylmethylsulfonyl fluoride-inactivated subtilisin lyophilized in the presence of *N*-acetyl-L-tryptophan amide exhibits no ligand binding in acetonitrile. Thus, the ligand-induced binding must be the result of changes at the active site of subtilisin and not the induction of general nonspecific binding on the protein molecule. Interestingly, the presence of 0.4% water in the

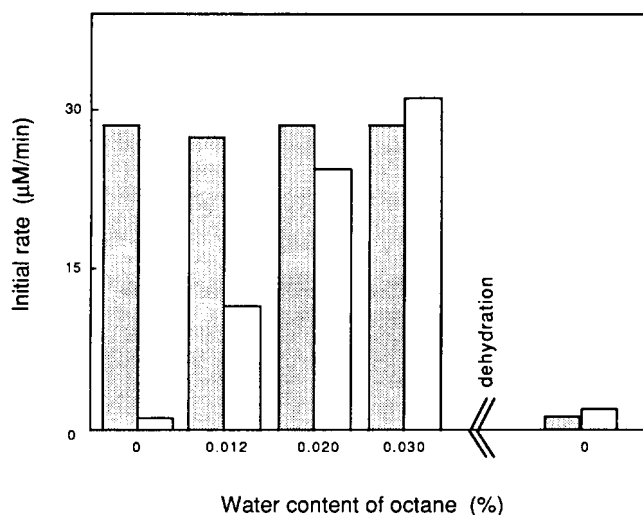


FIG. 1. The dependence of subtilisin activity in octane upon the water content of the solvent. Solid bars correspond to the enzyme lyophilized in the presence of 10 mM *N*-acetyl-L-tyrosine amide; open bars correspond to subtilisin lyophilized without ligand. Water was added to octane before the enzyme. Dehydration of both preparations of subtilisin from octane containing 0.05% (v/v) water was achieved by recovering the solid enzyme by centrifugation and washing with anhydrous tetrahydrofuran to bring their water content down to 3.7% (w/w). For experimental conditions, see Footnote *a* to Table I. The enzyme lyophilized in the presence of ligand contains no more water than that lyophilized in the absence of ligand (4.0 versus 4.7% (w/w), respectively). Thus ligand activation is not the result of differences in water concentration of protein powders.

solvent results in the loss of binding capacity in the ligand-activated subtilisin. Hence, addition of water erases the enzyme's ligand-induced memory both toward catalysis (as already discussed) and binding. Ligand-induced alteration of subtilisin also affects other fundamental enzymatic properties in organic solvents, such as substrate specificity and stability. For subtilisin lyophilized without ligand, the k_{cat}/K_m values for *N*-acetyl-L-alanine methyl ester and *N*-acetyl-L-phenylalanine ethyl ester were similar, whereas in the case of subtilisin lyophilized in the presence of *N*-acetyl-L-tyrosine amide the specificity factor for the former substrate was almost 10 times greater than for the latter. The two forms of subtilisin had distinct sensitivities toward the denaturant dimethyl sulfoxide; the relative activity of the ligand-induced form in *tert*-amyl alcohol containing 20% (v/v) dimethyl sulfoxide was 70% lower than that of the noninduced form. Finally, the half-life of the ligand-induced form of the enzyme at 110 °C in octane was 10 min versus 80 min for the nonin-

duced form (note the remarkably high resistance of both enzymes against irreversible thermoinactivation due to the removal of water from the system (Zaks and Klivanov, 1984)).

The only plausible explanation of the described phenomena which is consistent with all the experimental data is that ligands cause an activating conformational change in the subtilisin molecule. Even after removal of the ligand, this new conformation is retained due to the rigidity of the enzyme in the absence of water. Indeed, it has been reported (Genest and Ptak, 1982) that substrate binding to subtilisin causes a conformational change which increases the nucleophilicity of the active site's serine residue by strengthening the hydrogen bond network at the active site. Subtilisin which is locked into this conformation could function more efficiently in the rigidifying environment of an organic solvent.

There is a striking phenomenological resemblance between our data and those in a recent report on the conversion of an allosteric inhibition to activation by means of site-directed mutagenesis (Lau and Fersht, 1987). The modification of enzymatic properties is accepted as a major goal on the path to the greater understanding and use of proteins. Ligand-induced changes of enzymatic properties in organic solvents, as described herein, provide an alternative and complementary strategy to approaches based on site-directed mutagenesis (Knowles, 1987; Russell and Fersht, 1987).

REFERENCES

- Deetz, J. S., and Rozzell, J. D. (1988) *Trends Biotechnol.* **6**, 15-19
- Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, pp. 263-292, W. H. Freeman and Co., New York
- Finney, J. L., and Poole, P. L. (1984) *Comm. Mol. Cell. Biophys.* **2**, 129-151
- Frieden, C. (1979) *Annu. Rev. Biochem.* **48**, 471-489
- Genest, M., and Ptak, M. (1982) *Int. J. Pept. Protein Res.* **19**, 420-431
- Hammes, G. G. (1982) *Enzyme Catalysis and Regulation*, pp. 152-186, Academic Press, Orlando, FL
- Klibanov, A. M. (1986) *CHEMTECH* **16**, 354-359
- Knowles, J. R. (1987) *Science* **236**, 1252-1258
- Laitinen, H. A., and Harris, W. E. (1975) *Chemical Analysis* pp. 361-363, McGraw-Hill Book Co., New York
- Lau, F. T. K., and Fersht, A. R. (1987) *Nature* **326**, 811-812
- Margolin, A. L., Tai, D. F., and Klibanov, A. M. (1987) *J. Am. Chem. Soc.* **109**, 7885-7887
- Philipp, M., and Bender, M. L. (1983) *Mol. Cell. Biochem.* **51**, 5-32
- Polgar, L., and Bender, M. L. (1967) *Biochemistry* **6**, 610-620
- Riva, S., Chopineau, J., Kieboom, A. P. G., and Klibanov, A. M. (1988) *J. Am. Chem. Soc.* **110**, 584-589
- Russell, A. J., and Fersht, A. R. (1987) *Nature* **328**, 496-500
- Singer, S. J. (1962) *Adv. Protein Chem.* **17**, 1-68
- Zaks, A., and Klibanov, A. M. (1984) *Science* **224**, 1249-1251
- Zaks, A., and Klibanov, A. M. (1986) *J. Am. Chem. Soc.* **108**, 2767-2768
- Zaks, A., and Klibanov, A. M. (1988) *J. Biol. Chem.* **263**, 3194-3201