Probing Enzymic Transition State Hydrophobicities†

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ABSTRACT: Hydrophobic interactions are important in numerous biological processes; however, the nature and extent of hydrophobic interactions in nonaqueous enzymology remain poorly defined. We have estimated the free energies of enzyme−substrate hydrophobic interactions for a model reaction catalyzed by subtilisin BPN′ (from *Bacillus amyloliquefaciens*) in various solvents. Transition state stabilization of subtilisin in water has contributions from both ground state destabilization of hydrophobic substrates and intrinsic enzyme−substrate hydrophobic interactions. Both contributions are evident even in hydrophobic organic solvents and can be modified by protein engineering of the enzyme's binding site, as well as by changing the hydrophobicity of the reaction medium. We have also developed a method to estimate the hydrophobicity of the enzymic transition state involving systematic variation of the substrate and solvent hydrophobicities. The observed binding pocket hydrophobicities were directly affected by replacing the Gly_{166} residue, located at the back of the hydrophobic S_1 binding pocket of subtilisin BPN′, with more hydrophobic amino acids such as alanine and valine. Thus, the observed S_1 binding pocket hydrophobicities of the wild-type, G166A, and G166V mutants were measured to be 1.2, 1.8, and 2.6 log *P* units, respectively. Our method of calculating effective binding pocket hydrophobicity was found to be applicable to other enzymes, including horseradish peroxidase and α-chymotrypsin. Measurements of the binding pocket hydrophobicities have significant implications toward tailoring enzyme function in aqueous as well as nonaqueous media.

The transition state stabilization and substrate specificity of enzymes depend on binding and catalytic components, both of which can be affected by the nature of the reaction medium. Under extreme conditions, the role of the reaction medium may be far from subtle. For example, the presence of organic solvents in an enzymic reaction mixture alters the fine balance of hydrophobic interactions that comprise binding and is known to drastically affect the ground state of the substrate. Hence, the free energy of substrate binding to the enzyme is affected. This in turn is at least partly responsible for the large changes in substrate specificities (Dordick, 1992; Klibanov, 1989), enantioselectivities (Tawaki & Klibanov, 1992), regioselectivities (Rich et al., 1994; Therisod & Klibanov, 1987), and chemoselectivities (Tawaki & Klibanov, 1993) that are often observed in different organic solvents. While the hydrophobic contribution toward solvent−substrate interactions can be determined through ground state (solvation) calculations (Ben-Naim, 1980; Ryu & Dordick, 1992; Wescott & Klibanov, 1993), hydrophobic contributions toward enzyme−substrate interactions are more difficult to assess directly due to the lack of a measure of the hydrophobic state of an enzyme's active site. Most definitions of hydrophobic interactions focus on the ordering of water molecules around nonpolar solutes (Frank & Evans, 1945; Kauzmann, 1959). However, unlike water, many organic solvents lack the ability to form hydrogen bonds. The magnitude and nature of the hydrophobic interactions in a nonaqueous environment, or in an enzyme reaction's transition state, therefore, remain poorly defined.

In this report, we address this problem by approximating the hydrophobicity of the enzyme's active site during the formation of the transition state. In such a manner, we are able to compare the magnitude of hydrophobic contributions in the transition state and, hence, those toward enzymic substrate specificity in different solvents. We have chosen subtilisin BPN', a serine protease from *Bacillus amyloliquefaciens* (M_r, 27 500), as a model enzyme to study hydrophobic interactions that affect the stabilization of an enzyme's transition state in aqueous and nonaqueous solvents. Subtilisin BPN' is structurally and kinetically well-characterized (McPhalen & James, 1987; Morihara & Oka, 1977; Gron et al., 1992) and is, therefore, an ideal enzyme for this study. Herein, we show that (i) enzyme−substrate hydrophobic interactions are important even in hydrophobic organic solvents for transition state stabilization, (ii) substrate specificities can be predictably modified in aqueous as well as nonaqueous solvents by protein engineering, and (iii) the hydrophobicity of the enzymic transition state can be estimated for different active site mutants.

EXPERIMENTAL PROCEDURES

Semipurified subtilisin BPN' wild-type enzyme and the G166A and G166V mutant enzymes were generously provided by Thomas Graycar at Genencor International. The site-directed mutations were introduced by a cassette mutagenesis method (Wells et al., 1985), and plasmids containing each mutant *B. amyloliquefaciens* subtilisin gene were expressed (Estell et al., 1985) at roughly equivalent levels

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in a protease deficient strain of *Bacillus subtilis*. Each enzyme was first purified by cation exchange chromatography (Estell et al., 1985), dialyzed against 20 mM sodium phosphate buffer (pH 7.8) for 24 h, and then lyophilized for 40 h. Unless otherwise mentioned, all of the enzyme preparations contained 70% (w/w) enzyme and 30% (w/w) sodium phosphate buffer in the final preparations. All reagents and solvents were purchased from Aldrich (Milwaukee, WI) and were of highest grade commercially available. The solvents were dried over molecular sieves for at least 24 h prior to use. α−Chymotrypsin and *N*-Ac-L-Phe-OEt were purchased from Sigma (St. Louis, MO), and all other substrates were synthesized from their respective L-amino acids by first esterifying with HCl/ethanol and then acetylating with acetyl chloride in ethyl acetate. Active sites of the enzymes were titrated in aqueous buffer with *N-trans*-cinnamoylimidazole as the titrant (Schonbaum et al., 1961). The wild-type, G166A, and G166V enzyme preparations contained 38, 48, and 40% active enzyme in water, respectively. In organic solvents, the active enzyme was ca. 15 ± 5% of the fraction active in water (Affleck et al., 1992).

Kinetic Measurements. Kinetics of hydrolysis of *N*-Ac-L-amino acid ethyl esters (0.1−1.0 mM) in water was followed using a Mettler DL21 pH-Stat as described by Zerner et al. (1964). A typical hydrolytic reaction mixture contained 0.5% (v/v) CH_3CN, 0.1 M NaCl, and 10−50 μg/mL enzyme at 30 °C. In organic solvents, subtilisin was lyophilized prior to use from a 5 mg/mL solution of enzyme in 20 mM sodium phosphate buffer (pH 7.8). Transesterification reactions of *N*-Ac-L-amino acid ethyl esters (0.1−250 mM) with 1-propanol (1 M) were performed in nearly anhydrous organic solvents with 1 mg/mL lyophilized enzyme powder. The reactions were shaken at 30 °C and 250 rpm. Gas chromatography [25-m capillary column with 530-μm fused silica gum (Hewlett-Packard), N_2 as carrier gas (30 mL/min), and injector and detector port temperatures of 250 °C] was used to measure the initial rate of formation of *N*-Ac-L-amino acid propyl esters. Reactions with α-chymotrypsin were performed in an analogous manner. Catalytic efficiencies (k_{cat}/K_m) were obtained by nonlinear regression of initial rate vs substrate concentration data.

Surface Area Calculations. The X-ray structure of subtilisin BPN′ (Bott et al., 1988), Brookhaven Protein Data Bank entry 2ST1, was used for the calculations on wild-type and G166V mutant enzymes in the vicinity of the 166 position. All calculations were performed on a Silicon Graphics Indigo workstation running SYBYL 6.1. The calculations included all hydrogens (1908 in the wild type) and did not include the 154 water molecules present in the wild type. The G166V mutant was constructed using the "Mutate Monomers" command in SYBYL (Tripos Assoc.). Connolly surfaces (Connolly, 1983) were created within a 3-Å radius from the 166 position and included residues 153, 154, and 164−170. In such a technique, a continuous van der Waals surface of the aforementioned amino acid residues is constructed. A 2-Å-radius probe (to approximate the size of a methylene group) was then used to measure the total van der Waals surface area that is accessible.

RESULTS

Subtilisins are known to have an extended binding cleft comprising at least eight subsites, and the S_1 subsite of subtilisins prefers hydrophobic substrates (Gron et al., 1992; Morihara & Oka, 1977). Estell et al. (1986) and Bigler et al. (1993) have studied the nature of steric and hydrophobic effects on the substrate specificity of subtilisin BPN′ toward the P_1 amino acid residue of tetrapeptide substrates and polypeptide inhibitors, respectively. However, due to the interdependency of binding subsites in subtilisins, unfavorable S_1-P_1 interactions may be shielded by favorable interactions at other subsites for oligopeptide substrates/inhibitors (Gron & Breddam, 1992), and the true extent of S_1-P_1 hydrophobic interactions may not be observed for these substrates/inhibitors. Therefore, we have chosen to study the kinetics of hydrolysis and transesterification (with *n*-propanol as nucleophile) of *N*-acetyl-L-amino acid ethyl ester substrates, differing only in substrate side chain hydrophobicity, to elucidate specific S_1-P_1 hydrophobic interactions during the formation of the enzymic transition state. In order to compare the hydrophobic effects in organic solvents and in water, one has to compare the enzyme kinetics in these two types of media, and the underlying assumption for such a comparison is that transesterification reactions catalyzed by subtilisins in organic media follow a mechanism similar to that for hydrolysis in water (Kanerva & Klibanov, 1989; Adams et al., 1991).

Wild-Type Subtilisin BPN′ in Aqueous Buffer. Our experimental approach focused on the contributions of substrate and solvent hydrophobicities toward enzymic free energy of activation (i.e., values of k_{cat}/K_m). Increasingly hydrophobic amino acid-based substrates were chosen in the series Ala, 2-aminobutyric acid, norvaline, norleucine, and Phe, representing differential side chain hydrophobicities corresponding to one methyl group ($\pi = 0.5$, where π is defined in eq 1, and K_p is the partition coefficient of glycine or an amino acid between water and *n*-octanol) between consecutive substrates. These substrates are unbranched and are identical structurally except for different side chain lengths (and hence hydrophobicities), with the exception of the aromatic Phe. Thus, for the most part, and as long as steric effects are minimal, this group of substrates allows us to probe the hydrophobic interactions involved in subtilisin catalysis.

$$\pi = \log\left[\frac{(K_p)_{\text{amino acid}}}{(K_p)_{\text{glycine}}}\right] \quad (1)$$

k_{cat}/K_m for the hydrolysis of the amino acid esters in aqueous buffer increases as the hydrophobicity of the amino acid ester side chain increases (Figure 1). The positive slope ($\delta = 0.5$ for the wild-type subtilisin BPN′, where δ indicates the slope of log k_{cat}/K_m vs π) provides a value for the decrease in free energy of activation of subtilisin in water as compared to the free energy of transfer of the side chain alkyl groups from water to *n*-octanol as the side chain hydrophobicities are increased (Dorovska et al., 1972). In this case, specifically, the decreased free energy of activation is approximately one-half as large as the free energy of transfer of straight chain alkyl groups from water to *n*-octanol. The free energy of activation thus is lowered as substrate hydrophobicitiy is increased. Hydrophobic interactions between the enzyme and the transition state thus play an important role in lowering the activation energy of the reaction. These effects would be expected to become more favorable as the substrate hydrophobicity increases. Such a

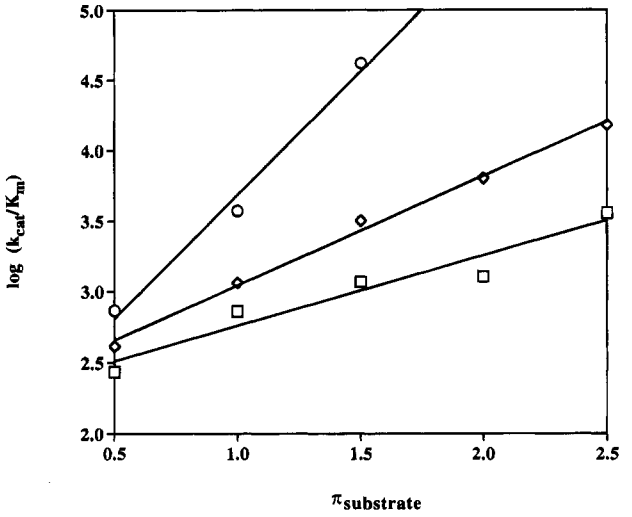

FIGURE 1: Effect of substrate hydrophobicity on the catalytic efficiency of subtilisin BPN′ and two hydrophobic mutants: (□) wild type; (◇) G166A; (○) G166V. Catalytic efficiencies were measured as described in the Experimental Procedures. The G166V mutant shows considerable steric hindrance for large hydrophobic substrates and, thus, was limited to *N*-Ac-L-Ala, *N*-Ac-L-aminobutyric acid, and *N*-Ac-L-norvaline as substrates. Values of k_{cat}/K_m are in M^{-1} s^{-1}.

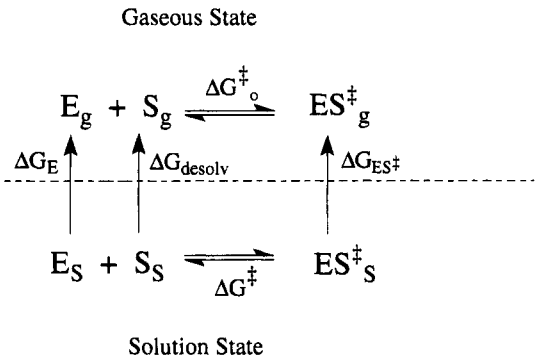

FIGURE 2: Hypothetical process path for subtilisin BPN′ catalysis in a given solvent, with subscripts g and S referring to gaseous and solution states, respectively. Thermodynamic variables are as described in the text.

solvation/transition state stabilization-driven process can be modeled through the use of a simple thermodynamic process path, as depicted in Figure 2. It should be noted that, when such process paths are written for two substrates differing by one methylene group, then $\Delta\Delta G$ expressions are used to indicate free energy changes per methylene group of substrate.

In such a process path, observed catalysis in a given organic solvent proceeds through the bottom path, while intrinsic, solvent-independent catalysis proceeds through the top path. This gives rise to a thermodynamic expression (eq 2) that relates changes in the intrinsic differential free energy of activation per methylene group of the substrate ($\Delta\Delta G_o^{\ddagger}$, i.e., in the hypothetical gaseous state) and the free energy of desolvation of the transition state from the solution state to the gas state ($\Delta\Delta G_{ES}^{\ddagger}$) to measurable quantities:

$$\Delta\Delta G^{\ddagger} + \Delta\Delta G_{ES}^{\ddagger} = \Delta\Delta G_{desolv} + \Delta\Delta G_E + \Delta\Delta G_o^{\ddagger} \quad (2)$$

Adding $\Delta\Delta G_g$ to both sides of eq 2, setting $\Delta\Delta G_E$ to zero (the differential free energy of enzyme desolvation per methylene group is independent of the substrate), and

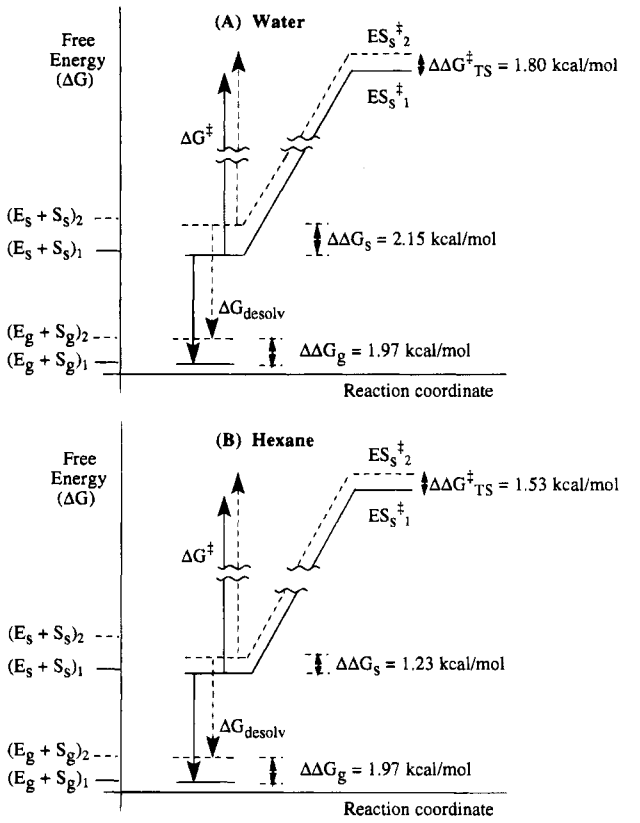

FIGURE 3: Free energy diagram for subtilisin BPN′ in (A) water and (B) hexane. Hypothetical free energy levels of the enzyme (E) and substrate (S) are depicted in solution (S subscripts) and in the gaseous state (g subscripts), whereas the transition state ($ES^{\ddagger}$) is depicted in the solution phase only. The subscripts 1 and 2 correspond to two substrates differing in size (and hence hydrophobicity) of one methylene group, with substrate 2 being the more hydrophobic one (indicated by dashed lines).

rearranging results in

$$\Delta\Delta G^{\ddagger} + \Delta\Delta G_g - \Delta\Delta G_{desolv} = \Delta\Delta G_o^{\ddagger} - \Delta\Delta G_{ES}^{\ddagger} + \Delta\Delta G_g = \Delta\Delta G_{TS}^{\ddagger} \quad (3)$$

The final equality in eq 3 comes through inspection of Figure 3, where the value of $\Delta\Delta G_{TS}^{\ddagger}$ is defined as the intrinsic transition state stabilization per methylene group. Calculation of intrinsic transition state stabilization per methylene group, therefore, requires knowledge of the variables on the left-hand side of eq 3.

The differential free energy of the substrate ground state ($\Delta\Delta G_S$) is calculated via two terms: the differential free energy of desolvation (of substrate out of the solvent and into the gaseous state) ($\Delta\Delta G_{desolv}$) and the differential free energy of a methylene group in the ideal gaseous standard state ($\Delta\Delta G_g$), as depicted in

$$\Delta\Delta G_S = \Delta\Delta G_g - \Delta\Delta G_{desolv} \quad (4)$$

Values of $\Delta\Delta G^{\ddagger}$ are obtained from Figure 1 (and calculated through the use of eqs 5 and 6):

$$\delta = \left[\frac{d\log(k_{cat}/K_m)}{d\pi}\right] = \frac{\Delta\ln(k_{cat}/K_m)}{2.3(\Delta(\pi))} = \frac{-\Delta\Delta G^{\ddagger}}{2.3(RT\Delta(\pi))} \quad (5)$$

$$\Delta\Delta G^{\ddagger} = -2.3RT\delta(\Delta(\pi)) \quad (6)$$

Table 1: Thermodynamic Parameters for Subtilisin Catalysis in Aqueous and Organic Media

solvent	$\Delta\Delta G_S$[a] (kcal/mol)	wild-type δ	wild-type $\Delta\Delta G^{\ddagger}$	wild-type $\Delta\Delta G_{TS}^{\ddagger}$[c]	G166A δ	G166A $\Delta\Delta G^{\ddagger}$	G166A $\Delta\Delta G_{TS}^{\ddagger}$	G166V δ	G166V $\Delta\Delta G^{\ddagger}$	G166V $\Delta\Delta G_{TS}^{\ddagger}$
water	2.15	0.50 ± 0.03	−0.35	1.80 ± 0.06	0.75 ± 0.04	−0.51	1.63 ± 0.05	1.75 ± 0.11	−1.18	0.97 ± 0.06
acetone	1.35	0.35 ± 0.04	−0.14	1.1 ± 0.1	0.43 ± 0.03	−0.29	1.06 ± 0.07	1.14 ± 0.13	−0.93	0.56 ± 0.11
diisopropyl ether	1.20[b]	−0.21 ± 0.02	0.14	1.34 ± 0.09	−0.04 ± 0.01	0.03	1.20 ± 0.08	0.20 ± 0.02	−0.13	1.07 ± 0.07
hexane	1.23	−0.43 ± 0.03	0.30	1.53 ± 0.07	−0.33 ± 0.02	0.24	1.47 ± 0.06	−0.30 ± 0.02	0.21	1.44 ± 0.06
octane	1.23	−0.49 ± 0.04	0.39	1.62 ± 0.08	−0.36 ± 0.03	0.28	1.51 ± 0.07	−0.46 ± 0.04	0.26	1.49 ± 0.08

[a] Free energy of desolvation per methylene group for solution → ideal gas (1 atm) (Abraham, 1982). [b] $\Delta\Delta G_S$ in diisopropyl ether is assumed to be the same as that in diethyl ether. [c] $\Delta\Delta G_{TS}^{\ddagger}$ is the total differential intrinsic free energy of activation per methylene group, where $\Delta\Delta G_{TS}^{\ddagger} = \Delta\Delta G^{\ddagger} + \Delta\Delta G_S$. Negligible error is assumed in the calculation of $\Delta\Delta G_S$. All experiments were performed in triplicate.

Thus, δ provides a direct measure (through eq 6) for the observed differential free energy of activation per methylene group. Values of $\Delta\Delta G_{desolv}$ can be calculated for transfer of a methylene group from the solvent to an ideal gas at 1 atm (Abraham, 1982). It should be noted that, in a given solvent, $\Delta\Delta G_{desolv}$ is independent of the enzyme and is used only to facilitate the calculation of $\Delta\Delta G_{TS}^{\ddagger}$. Values for $\Delta\Delta G_g$ (which is independent of *both* the enzyme and the solvent) can be estimated from standard free energies of formation of hydrocarbons. On the basis of the free energies of formation of alkanes in the range from ethane to octane, $\Delta\Delta G_g$ is 1.97 kcal/mol/methylene group (Liley & Gambill, 1973). It is important to stress that the values of $\Delta\Delta G_{desolv}$ are based on model hydrocarbon compounds because similar values for amino acid-based substrates would be difficult to measure experimentally. Similarly, the value of $\Delta\Delta G_g$ is based on model hydrocarbon compounds. Therefore, the thermodynamic analysis described in this work provides only an approximation of the absolute transition state stabilizations involving amino acid-based substrates. We shall demonstrate, however, that this analysis does provide a method by which to calculate the differences in the transition state stabilization of subtilisin BPN′ upon changes in the hydrophobicities of the solvent (via solvent engineering) and enzyme active site (via protein engineering).

For water, the values of $\Delta\Delta G_S$ and $\Delta\Delta G^{\ddagger}$ are 2.15 and −0.35 kcal/mol/methylene group, respectively. Thus, $\Delta\Delta G_{TS}^{\ddagger}$ is 1.80 kcal/mol/methylene group. This indicates that in water, with an observed lowering of the free energy of activation per methylene group ($\Delta\Delta G^{\ddagger}$) of 0.35 kcal/mol/methylene group, this value comprises the 2.15 kcal/mol/methylene group contributed by the ground state destabilization of a methylene group and the 1.80 kcal/mol/methylene group contributed by intrinsic transition state destablization. Because the destabilization of the transition state is less than the destabilization of the ground state, subtilisin catalysis in water results in a net lowering of the differential free energy of activation, as depicted in Figure 3A. Therefore, subtilisin catalysis proceeds more efficiently on more hydrophobic substrates.

Contributions from the enzyme and solvent can be altered either by changing the hydrophobicity of the enzyme's binding pocket (via protein engineering) or by changing the hydrophobicity of the reaction medium (via solvent engineering). To test this hypothesis and to gain a better understanding of the nature of hydrophobic interactions in different reaction media, we examined subtilisin catalysis with a variety of protein-engineered active site mutants in water and a number of organic solvents.

Protein Engineering. The hydrophobic S_1 binding cleft of subtilisin BPN′ contains a Gly_{166} residue, which occupies the back of the pocket. The small size of the Gly residue enabled us to use protein-engineered variants of subtilisin BPN′ differing in side chain hydrophobicities with minimal steric influence on k_{cat}/K_m. Specifically, two hydrophobic mutations were introduced at the 166 position: Gly_{166} → Ala (G166A) and Gly_{166} → Val (G166V). The values of δ were found to be 0.75 and 1.75 for G166A and G116V, respectively (Figure 1). Steric limitations become dominant for the G166V mutant for substrates larger than norvaline. Thus, the norleucine and phenylalanine derivatives are not included in our analysis for the G166V mutant.

The increased substrate partitioning from water into subtilisin's binding pocket via increased hydrophobicity of the S_1 binding pocket must contribute toward the increased values of k_{cat}/K_m for all amino acid ester substrates tested. In some cases, this catalytic activation is significant. For example, the G166A mutant was 1.5- and 3.5-fold more efficient than the wild-type BPN′ on *N*-Ac-L-Ala-OEt and *N*-Ac-L-norvaline-OEt, respectively. Similarly, the G166V mutant was 2.7- and 35.7-fold more efficient than the wild-type on *N*-Ac-L-Ala-OEt and *N*-Ac-L-norvaline-OEt, respectively.

By undertaking a similar analysis for the two protein-engineered hydrophobic mutants, as was done with the wild type earlier, the nature of intrinsic hydrophobic interactions becomes clearer. Specifically, while $\Delta\Delta G_S$ remains 2.15 kcal/mol/methylene group, the value of $\Delta\Delta G_{TS}^{\ddagger}$ decreases to 1.63 and 0.97 kcal/mol/methylene group for the G166A and G166V mutants, respectively. Compared to the wild type, the magnitude of destabilization of the mutant enzymes is smaller per substrate methylene group. This results in an additional lowering of the differential free energy of activation for the mutants as compared to the wild type and, hence, increased values of k_{cat}/K_m for increasingly hydrophobic substrates. The results for the wild type and the two hydrophobic mutants are summarized in Table 1. It should be noted that, while the absolute values of $\Delta\Delta G_{TS}^{\ddagger}$ for a given enzyme in a given solvent depend on the value of $\Delta\Delta G_g$, relative transition state stabilizations do not. Thus, although absolute transition state stabilizations may be somewhat speculative due to the calculated value of $\Delta\Delta G_g$, relative transition state stabilizations do provide an accurate measure of differences between the wild-type and mutant enzymes. For example, the relative stabilization of the transition state of subtilisin in water brought about by increasing the hydrophobicity of the active site is −0.17 and −0.83 kcal/mol/methylene group for the G166A and G166V mutants, respectively.

Solvent Engineering. A complementary approach to protein engineering for altering the partitioning of substrate from the reaction medium to the enzyme's binding pocket

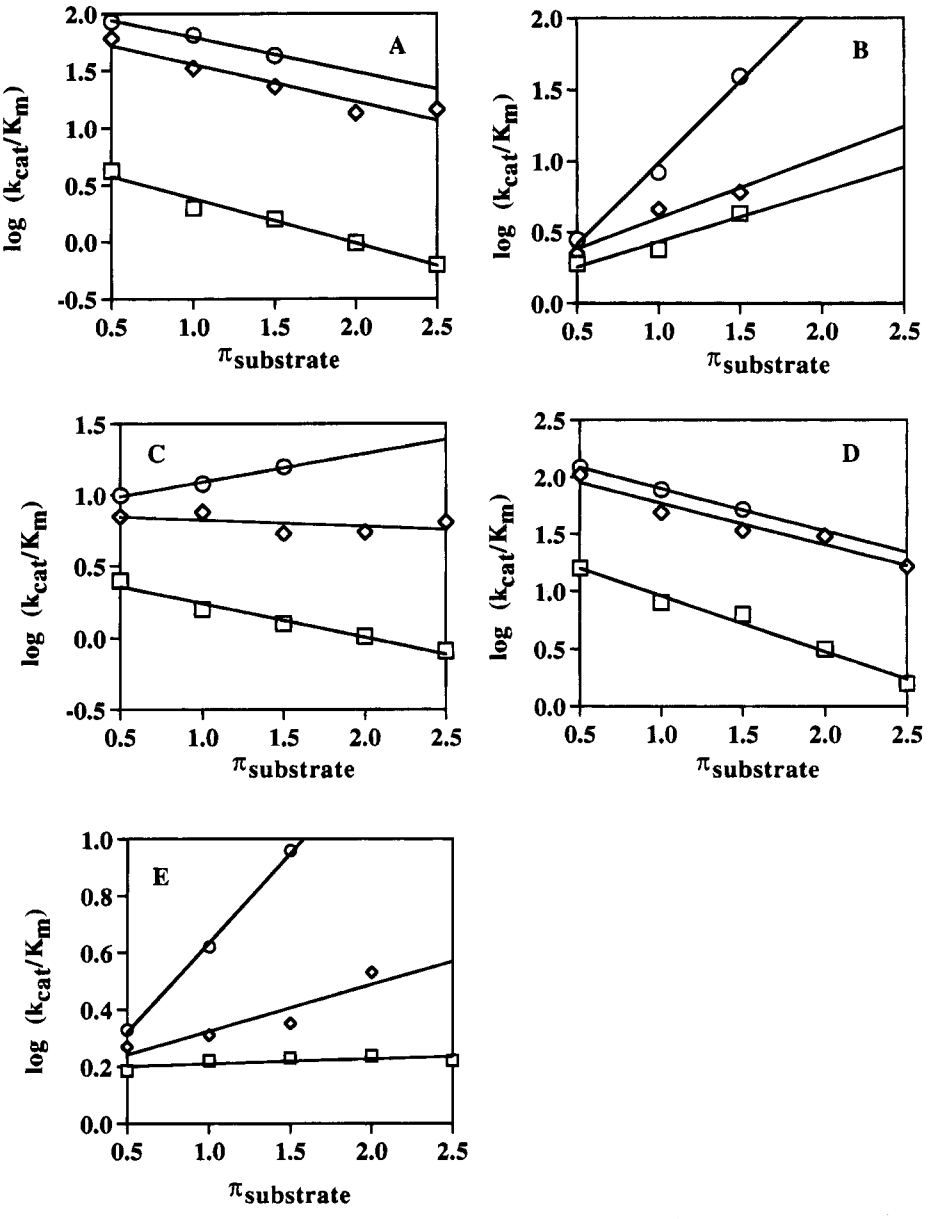

FIGURE 4: Effect of substrate hydrophobicity on the catalytic efficiency of subtilisin BPN′ and two hydrophobic mutants [(□) wild-type; (◇) G166A; (○) G166V] in different organic solvents: (A) hexane; (B) acetone; (C) diisopropyl ether; (D) octane; (E) *tert*-amyl alcohol. Values of k_{cat}/K_m are in M^{-1} s^{-1}.

is to change the hydrophobicity of the solvent. A series of experiments was performed with the wild type and the two mutant enzymes in *n*-hexane as the solvent for transesterification reactions between *N*-Ac-L-amino acid-OEt and *n*-propanol. The results in hexane were far different from those in water. The k_{cat}/K_m of subtilisin catalysis *decreased* as the substrate hydrophobicity increased for the wild type and both hydrophobic mutants (Figure 4A). Moreover, unlike in water, the values of δ were only slightly different for the wild type and two mutant enzymes and ranged from −0.43 for the wild type to −0.30 for the G166V mutant (Table 1). The negative values of δ can be rationalized if we consider substrate partitioning between the solvent and the active site where the S_1 binding pocket of subtilisin BPN′ is more hydrophobic than water, but not as hydrophobic as *n*-hexane. Thus, partitioning of hydrophobic substrates out of hexane into the enzyme's active site is unfavorable.

The nature of subtilisin catalysis in hexane can be ascertained through the use of thermodynamic analysis. Figure 3B depicts the free energy diagram for catalysis in hexane. The desolvation of methylene groups from hexane to the gaseous state results in a value of $\Delta\Delta G_S$ = 1.23 kcal/mol/methylene group. Although unfavorable, this represents a relative stabilization of the ground state of a methylene group as compared to water. The value of $\Delta\Delta G_{TS}^{\ddagger}$ in hexane is also smaller than that in water, indicating that the transition state is less destabilized per methylene group in a more hydrophobic solvent. For example, the relative stabilization of the transition state of subtilisin brought about by increasing the hydrophobicity of the solvent is −0.27 kcal/mol/methylene group for hexane as compared to water (solvent 1).

The values of $\Delta\Delta G_{TS}^{\ddagger}$ are similar for the wild type and both mutant enzymes (Table 1). Thus, the three enzymes have virtually identical intrinsic transition state stabilizations per methylene group—a situation different from that in water. However, in hexane the hydrophobic mutants activate subtilisin BPN′ for all substrates compared to the wild type.

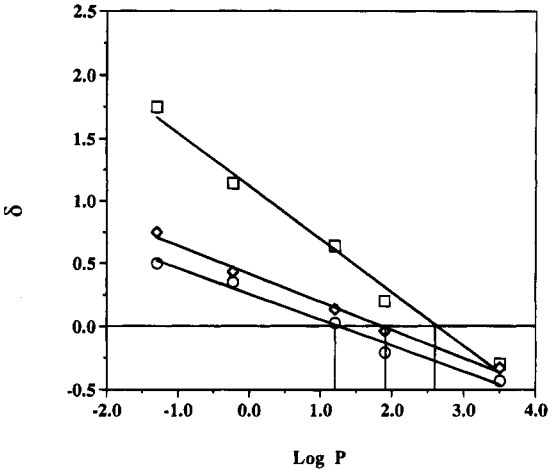

FIGURE 5: Determination of isophobic points for (O) wild-type, (◇) G166A, and (□) G166V subtilisins BPN′. δ represents the slope of $\log(k_{cat}/K_m)$ values vs $\pi_{substrate}$ curves for Figures 1 and 4. Isophobic points are 1.2, 1.8, and 2.6 log P units for the wild-type, G166A, and G166V subtilisins BPN′, respectively. Solvents are (from left to right) water, acetone, *tert*-amyl alcohol, diisopropyl ether, and hexane.

Inspection of Figure 4A reveals that the G166A and G166V mutants are 13- and 18-fold more active, respectively, than the wild type on *N*-Ac-L-Ala-OEt as substrate in hexane. For more hydrophobic substrates, the effect is magnified still further, such that the G166A and G166V mutants are 18- and 33-fold more active, respectively, than the wild type toward *N*-Ac-L-norvaline-OEt as substrate.

Isophobic Point. It was of interest to examine the nature of subtilisin catalysis in solvents with hydrophobicities intermediate between those of water and hexane. To that end, wild type and the two mutant subtilisins were studied in acetone and diisopropyl ether. Plots of log (k_{cat}/K_m) vs π for subtilisin BPN′ in these solvents are shown in Figure 4. The value of δ for acetone is positive, yet the magnitude is lower than that in water. Conversely, the value of δ in diisopropyl ether is negative, albeit less so than in hexane. Indeed, the values of δ progress from positive (in water) to negative (in hexane) with a semilogarithmic dependence. An interesting manifestation of this phenomenon is the existence of a solvent hydrophobicity at which δ is zero. At this point, which we shall call the *isophobic point*, the apparent hydrophobicity of the transition state is proposed to be equivalent to the hydrophobicity of the organic solvent and, therefore, there is no hydrophobic driving force for substrate partitioning between the solvent and the enzyme's binding site (Figure 5). For the wild-type enzyme, the isophobic point is reached at a solvent log $P = 1.25$ (where P equals the partition coefficient of the solvent between *n*-octanol and water), which is close to the hydrophobicity of *tert*-amyl alcohol. In fact, catalysis of wild-type BPN′ in *tert*-amyl alcohol was found to be essentially independent of the substrate hydrophobicity ($\delta = +0.03$) (Figure 4E). Similar analysis of the hydrophobic G166A and G166V mutants in different reaction media also results in discrete isophobic points, yet more hydrophobic than that of the wild type (Figure 5). Specifically, G166A and G166V give isophobic points of 1.8 and 2.6 log P units, respectively.

The increase in hydrophobicity from the G_{166} to A_{166} enzymes, representing one methylene group (with a $\pi = 0.5$), shows an internal hydrophobicity gain of 0.55 log P unit, which is consistent with the predicted gain in transition state hydrophobicity for an additional methylene group in the binding pocket (i.e., $\pi_{Ala} - \pi_{Gly} = 0.5$). Similarly, the increase in hydrophobicity from G166 to V166, representing three methylene groups (with $\pi = 1.5$), shows an internal hydrophobicity gain of 1.35 log P units, which is close to the increase of 1.5 log P units expected on the basis of the increased hydrophobicity of Val166 compared to the wild type (i.e., $\pi_{Val} - \pi_{Gly} = 1.5$). Thus, the isophobic points reflect the change in hydrophobicity of the S_1 binding pocket upon protein engineering.

The responses of the wild type and mutants to solvent hydrophobicity are not identical, as evidenced by the different values of δ shown in Figure 5. Indeed, the mutants and the wild type merge to give similar values of δ in hydrophobic solvents, such as hexane. Moreover, in solvents even more hydrophobic than hexane, the mutants and wild type exhibit values of δ (and hence $\Delta\Delta G^{\ddagger}$) that are relatively insensitive to binding pocket hydrophobicity. Thus, δ values in octane are similar to those in hexane (Table 1).

Diisopropyl ether presents us with an interesting phenomenon, whereby inverted substrate specificities result from a site-directed mutation in the binding site of subtilisin. For example, the wild type is 2.4-fold more active toward *N*-Ac-Ala-OEt than *N*-Ac-norvaline-OEt. Conversely, the G166V mutant is 1.6-fold more active toward *N*-Ac-norvaline-OEt than *N*-Ac-Ala-OEt. This phenomenon is predicted by isophobic point analysis whereby, compared to the wild type's binding pocket, the more hydrophobic S_1 binding pocket of the G166V mutant is better suited to solvate the more hydrophobic norvaline substrate in diisopropyl ether.

DISCUSSION

The catalytic efficiency of subtilisin BPN′ is strongly influenced by substrate, solvent, and binding site hydrophobicities. Hydrophobic effects on both the ground state and the intrinsic transition state play an important role in catalysis. In the present approach, ground state effects are independent of the enzyme. Specifically, values of $\Delta\Delta G_{TS}^{\ddagger}$ are based on the value of $\Delta\Delta G_E$ always being zero for the differences in free energies between two substrates for a given enzyme in a given solvent. Thus, it would be interesting to compare hydrophobic effects on the transition state of subtilisin to that of other enzymes. For example, cytochrome P-450 catalysis utilizes nonbinding hydrophobic interactions between enzyme and substrate (Backes et al., 1993). Specifically, for hydrocarbon substrates in water, values of $\Delta\Delta G^{\ddagger}$ and $\Delta\Delta G_S$ are -0.68 and 2.15 kcal/mol/methylene group, respectively. Thus, $\Delta\Delta G_{TS}^{\ddagger}$ is 1.47 kcal/mol/methylene group, which is lower than that for wild-type subtilisin BPN′. However, because cytochrome P-450 reacts with hydrophobic hydrocarbon substrates, it is expected to have more hydrophobic surface area in its transition state than subtilisin and will be able to stabilize a methylene group in the transition state better than subtilisin. Furthermore, we have chosen to probe only the S_1 binding site of subtilisin BPN′ and its specific contribution toward lowering the free energy of activation of the enzymic reaction. In reality, the other S and S′ subsites will also contribute toward transition state stabilization for larger, more natural substrates of subtilisin.

Likewise, making the binding pocket of subtilisin more hydrophobic, via protein engineering, will increase the

hydrophobicity of the transition state and will increase the observed transition state stabilization relative to the wild type toward hydrophobic substrates. This indicates that the hydrophobicity of the enzymic binding site can influence the stabilization of the enzymic transition state, a finding that is consistent with previous observations (Fersht et al., 1980; Murphy & Benkovic, 1989; Krebs et al., 1993). Similarly, the hydrophobicity of the reaction medium can influence observed transition state stabilizations, because observed effects incorporate substrate desolvation. Such ground state effects can be calculated per methylene group, a convenient normalization to the simplest organic hydrophobic unit.

Hydrophobic mutations in the S_1 binding pocket of subtilisin BPN′ significantly affect values of k_{cat}/K_m on given amino acid-based substrates, as well as values of δ in aqueous solutions. Thus, protein engineering of the binding pocket is an effective strategy for altering the reactivity and specificity of subtilisin BPN′ in water and in hydrophilic organic solvents. Conversely, in hydrophobic media such as hexane or octane, mutation of the S_1 binding pocket does little to change δ (i.e., the slopes in Figure 5 merge). Why is this observed? We may speculate that the S_1 mutants may be unable to impart their relative stabilizing effects on the transition state of subtilisin BPN′ in some organic solvents because transition state desolvation negates intrinsic transition state stabilization. This hypothesis can be described mathematically as follows for catalysis in a given hydrophobic solvent such as hexane or octane:

$$(\Delta\Delta G_S)_{WT} = (\Delta\Delta G_S)_{mutant} \quad (7)$$

$$(\Delta\Delta G^{\ddagger})_{WT} \approx (\Delta\Delta G^{\ddagger})_{mutant} \quad (8)$$

Equation 7 holds because substrate desolvation is independent of the nature of the enzyme. Equation 8 approximately holds because the values of δ are nearly identical for the wild-type and hydrophobic mutant enzymes in hexane or octane (see Figure 5), within experimental error. Thus, combination of eqs 2, 4, 7, and 8 results in

$$(\Delta\Delta G_o^{\ddagger})_{WT} - (\Delta\Delta G_o^{\ddagger})_{mutant} = (\Delta\Delta G_{ES}^{\ddagger})_{WT} - (\Delta\Delta G_{ES}^{\ddagger})_{mutant} \quad (9)$$

We may speculate that the equality represented in eq 9 suggests that any relative intrinsic stabilization of the enzymic transition state brought about by enhanced hydrophobic binding energy of the mutant enzymes is balanced by more favorable relative desolvation of the transition state. The latter is due to the smaller S_1 cavities of the mutant enzymes that are available to be desolvated as a result of the space occupied by the hydrophobic side chains of A166 or V166. Indeed, computer-generated surfaces of the residues surrounding the 166 position of the wild-type and V166 mutant enzymes yielded a 2-Å probe radius accessibility of 162.82 and 127.68 Å^2, respectively. Thus, the calculations predict that the V166 mutant has ca. 22% *less* surface area accessible to a methylene group and, hence, a smaller effective S_1 cavity.

In hydrophobic solvents like hexane, a smaller hydrophobic surface area favors desolvation of the transition state into the hypothetical gaseous state as compared to a larger hydrophobic surface area. Thus, with smaller hydrophobic surface areas needed to be desolvated out of the organic solvent into the gaseous state, the transition states of the mutant enzymes are more favorably desolvated relative to the wild type. Hence, no incremental increase in stabilization of the transition state per methylene group is observed in hydrophobic organic solvents upon engineering hydrophobic residues into the binding pocket of subtilisin BPN′.

This analysis can also be used to understand the independence of δ from solvent hydrophobicity for solvents more hydrophobic than hexane. By using eqs 7−9 and replacing WT and mutant with hexane and octane, respectively, it is seen that any increased stabilization brought about by catalysis in the more hydrophobic octane [enzymes in organic media typically are more active in hydrophobic solvents than in their hydrophilic counterparts (Dordick, 1992)] is lost by decreased desolvation of the transition state in the more hydrophobic octane.

It is important to keep in mind that this thermodynamic analysis is based on the incremental transition state stabilization afforded per methylene group. For comparison of the values of k_{cat}/K_m for the wild-type and mutant enzymes in a given solvent, however, the added relative stabilization of $\Delta\Delta G_o^{\ddagger}$ afforded by the hydrophobic mutant is critical. In such a manner, the hydrophobic mutants will have lower values of $\Delta\Delta G_o^{\ddagger}$ and higher catalytic activities on hydrophobic substrates than the wild-type enzyme.

Although intrinsic transition state stabilization is critical for elucidating the effect of hydrophobicity on enzyme function, observed stabilization is useful for providing a measure of the magnitude of transition state hydrophobicity. Hence, observed hydrophobicities of the enzymic transition state can be estimated through the procedure depicted in Figures 1, 4, and 5. To examine the generality of such an approach, we have applied this method to estimate the hydrophobicity of other enzymes. For example, horseradish peroxidase (HRP) in *both* aqueous and organic solvents shows *decreased* values of V_{max}/K_m as a function of increased hydrophobicity of phenolic side chains (Ryu & Dordick, 1989), yet the slopes of V_{max}/K_m vs π become increasingly negative as the solvent hydrophobicity increases. Recalculation of the value of δ as a function of the log P of the solvent results in an observed transition state hydrophobicity of −1.9 log P units (Figure 6). Hence, the transition state of HRP is even more hydrophilic than water and suggests that sufficient ionic character must be present in HRP's transition state. Indeed, HRP contains a protoporphyrin IX group that is held in place by electrostatic interactions between one of the heme's propionic acid side chains and the apoprotein's Lys_{174} group (Yeoman & Hager, 1980). While the crystal structure of HRP has not been determined, the active site structure is believed to be similar to that of cytochrome *c* peroxidase, with the Arg_{183} residue in HRP corresponding to Ser_{185} in cytochrome *c* peroxidase (Welinder, 1979; Ortiz de Montellano, 1987; Sakurada et al., 1986). Both the Lys_{174} and Arg_{183} residues are charged and may provide significant electrostatic interactions in the enzymic transition state.

α-Chymotrypsin, an enzyme of more direct relevance to this work, was also investigated in a manner similar to that of subtilisin BPN′, α-Chymotrypsin is known to have a very hydrophobic S_1 binding pocket (Dorovska et al., 1972). This was confirmed in our investigation of the isophobic point of the enzyme (Figure 6), which is calculated to be 5.8 log

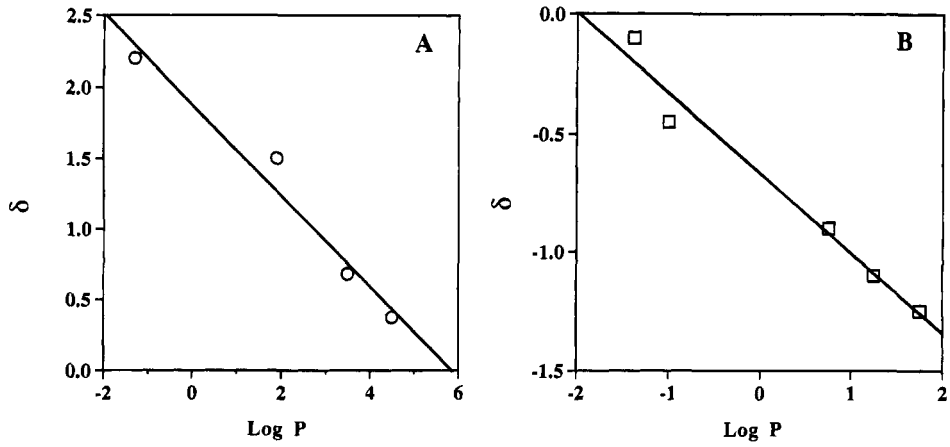

FIGURE 6: Determination of isophobic points for (A) α-chymotrypsin and (B) horseradish peroxidase. Data for horseradish peroxidase adapted from Ryu and Dordick (1992).

P units. In aqueous buffer, the value of $\Delta\Delta G^{\ddagger}$ is −1.52 kcal/mol/methylene group. Thus, $\Delta\Delta_{TS}{}^{\ddagger}$ is 0.63 kcal/mol/methylene group, which is 1.17 kcal/mol/methylene group more stabilized than subtilisin BPN′. Such a situation is even more significant for valyl-tRNA synthetase (Fersht et al., 1980), for which the $\Delta\Delta G_{TS}{}^{\ddagger}$ is calculated to be 2.85 kcal/mol/methylene group more stabilized than subtilisin BPN′. How can such high degrees of hydrophobicity exist in the transition state of certain enzymes? This question may be answered if one considers the extremely hydrophobic nature of enzymic binding pockets and the large dispersion energies afforded by unoccupied cavities in the hydrophobic binding pocket. The additivity of dispersion energies quickly results in very high degrees of hydrophobicity for the binding pockets of enzymes. The greater ability of an enzyme to maintain a hydrophobic binding pocket in an organic solvent would be represented by an increase in the value of the isophobic point.

CONCLUSIONS

The kinetic analysis described in this work provides further insight into hydrophobic interactions in enzymatic catalysis. It is possible to gain a quantitative measure for the effective hydrophobicity of the transition state of an enzymic reaction and to predict the effectiveness of hydrophobic mutations in the binding site of an enzyme for stabilizing hydrophobic transition states in aqueous and organic media. Our results with subtilisin BPN′ indicate that site-directed mutations in the S_1 binding pocket will strongly influence observed enzyme specificity (i.e., $\Delta\Delta G^{\ddagger}$) in hydrophilic media (i.e., water and hydrophilic organic solvents), whereas identical mutations have very little influence on enzyme specificity in hydrophobic organic solvents. Instead, the reaction medium becomes a dominant factor that affects enzyme function (via substrate and transition state desolvation) in such hydrophobic media. For reactions where substrate hydrophobicity is important, the isophobic point acts as a dividing line between the effectiveness of solvent or protein engineering to tailor reaction specificities. In hydrophilic media, protein engineering is capable of altering the nature of transition state stabilization, while in hydrophobic media, protein engineering is not effective and only changing the physicochemical nature of the solvent (i.e., through changes in hydrophobicity) will be effective in altering the nature of transition state stabilization.

Values of $\Delta\Delta G_{TS}{}^{\ddagger}$ in organic solvents are comparable to that in water, indicating that hydrophobic interactions that stabilize enzymic transition states do exist in even the most hydrophobic of organic solvents. This observation cannot be explained on the basis of the most widely accepted mechanism of hydrophobic interactions, which implies that such interactions must originate from the ordering of hydrogen-bonded water molecules around a hydrophobic solute (Hecht et al., 1993). Unlike water, hydrophobic organic solvents (such as hexane or octane) do not have hydrogen-bonding capabilities, yet hydrophobic contributions to transition state stabilization clearly exist.

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