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Organic solvent changes the chymotrypsin specificity with respect to nucleophiles

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In α-chymotrypsin-catalyzed acyl-transfer reactions in water the specificity of the enzyme (the nucleophile reactivity of amino acid amides) is correlated with the substrate hydrophobicity and increases as the hydrophobicity of the side chain of the amino acid amides is increased. In a low water system (4% H_2O) bulky amino acid amides are less efficient nucleophiles. The specificity of α-chymotrypsin towards the amino acid amides in acyl transfer reactions in this case does not depend on the hydrophobicity of the amino acid side chains but correlates with their size. Therefore, different factors can be responsible for the specificity of enzymes in water and in a mainly organic medium.

α-Chymotrypsin; Low water system; Nucleophile specificity; QSAR

1. INTRODUCTION

There is now little question that enzymes can function in mainly organic media. Numerous examples of synthetic reactions catalyzed by enzymes in low water systems are available [1–6]. The specificity of enzymes is undoubtedly one of the most important characteristics influencing the product yields. In this context the compilation of all data accumulated in the literature concerning specificity in enzymatic reactions conducted in water is highly attractive. However, relatively few studies have been devoted to differences in enzyme specificity in reactions conducted both in water and in a mainly organic medium [7–11]: usually a comparison of different organic solvents is carried out ([12–16] and references therein). In this article we present the effects of the replacement of water with organic solvent on the S'_1 specificity [17] of α-chymotrypsin. The results show that the enzyme specificity in water and a mainly organic medium may be unrelated.

Abbreviations: QSAR, quantitative structure activity relationship; RS, relative nucleophile reactivity calculated according to Eqn. 1; v_w, normalized van der Waals volume; HPLC, high performance liquid chromatography; ε_{254}, molecular absorption coefficient at 254 nm; DMSO, dimethyl sulphoxide; DMF, dimethyl formamide; Tris, tris(hydroxymethyl)aminomethane; veronal, 5,5-diethyl-barbituric acid; OEt, ethyl ester; OMe, methyl ester; NH_2, amide; Ac, acetyl; Mal, maleyl; Xaa, amino acid residue; Acyl, acyl group to be transferred to $XaaNH_2$; Acyl-$XaaNH_2$, synthetic product; Nva, norvaline. If not otherwise stated, amino acid residues are of the L-configuration.

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2. MATERIALS AND METHODS

α-Chymotrypsin (EC 3.4.21.1) from bovine pancreas was obtained from Sigma and used without further purification. The enzyme was adsorbed on Celite using the published method [11] except that the α-chymotrypsin content was 100 mg of the enzyme per g of Celite. MalAlaAlaPhe OMe was synthesized as described elsewhere [18,19]. The amino acid amides (HCl-salts) were from Bachem; BzTyrOEt and trifluoroacetic acid (protein sequencing grade) were from Sigma, DMSO was from J.T. Baker Chemicals BV; acetonitrile (far UV) was purchased from Lab-Scan; Tris and 5,5-diethyl-barbituric acid were from Merck; $(C_2H_5)_3N$ was from Janssen; DMF and Celite were from BDH. If not otherwise stated, the chemicals were of analytical grade.

The relative specificity (RS) values in the water system were measured at pH 9.0 and 30°C. A solution (0.2 M) of MalAlaAlaPheOMe in DMSO (5 μl) was added to 0.5 ml of 0.1 M veronal buffer containing $GlyNH_2$ (0.2 M) and an amino acid amide ($XaaNH_2$). The concentration of the latter was selected to give approximately equal formation rates of the synthetic products Acyl-$GlyNH_2$ and Acyl-$XaaNH_2$. The mixture was kept at 30°C for 10 min and the reaction was initiated by adding 5 μl of the enzyme solution (about 0.1 mg/ml). Samples (100 μl) were collected at 5, 10, 20 and 45 min, added to 1 ml of DMSO, and the resulting mixture was analyzed by HPLC.

Determination of the RS values in a mainly organic medium were carried out in a mixture of 47.5% (v/v) acetonitrile, 47.5% (v/v) DMF, 1% (v/v) $(C_2H_5)_3N$ and 4% (v/v) H_2O. About 60 μmol of $XaaNH_2 \cdot HCl$ were dissolved in 80 μl of a 0.75 M solution of $GlyNH_2$ in water (pH 9.0), followed by the addition of 20 μl of $(C_2H_5)_3N$. Then, 0.95 ml acetonitrile was added to this mixture, followed by 0.95 ml of a 4 mM solution of BzTyrOEt in DMF and 100 mg of the adsorbed enzyme. The mixture was kept at 30°C for 1 week under constant shaking and analyzed by HPLC.

The isocratic HPLC was carried out using a Shimadzu HPLC system (LC-6A pumps, SPD-6A UV detector, SCL-6A system controller, C-R4A chromatopac integrator) equipped with a 25 cm LiChrospher 100 RP-18 (5 μ) column (Merck). The mobile phase contained different proportions of water and acetonitrile acid 0.5% (v/v) of trifluoroacetic acid. UV detection was at 254 nm. For all reactions except acyl transfer to $PheNH_2$, $TyrNH_2$ and $TrpNH_2$, ε_{254} of the peptide product was considered equal to ε_{254} of the initial ester and its hydrolytic product. In the synthesis of Acyl-$PheNH_2$, Acyl-$TyrNH_2$ and Acyl-$TrpNH_2$, ε_{254} of the peptide was considered the sum of ε_{254} of the

hydrolytic product and that of $XaaNH_2$. Measurements were carried out using a Shimadzu UV-260 spectrophotometer.

3. RESULTS

The height of the energy barrier between the acyl–enzyme intermediate and the synthetic product is a measure of the nucleophile specificity of the enzyme. To calculate this quantity from the experimental data, the activity of water must be measured. To avoid this problem, all data were normalized to the reactivity of $GlyNH_2$. With an excess of both $GlyNH_2$ and $XaaNH_2$ relative to the acyl group donor, the relative specificity (RS) calculated according to Eqn. 1 characterizes the difference between the heighs of the energy barriers.

$$RS = \ln\left(\frac{[\text{Acyl-XaaNH}_2][\text{GlyNH}_2]}{[\text{Acyl-GlyNH}_2][\text{XaaNH}_2]}\right) \quad (1)$$

In organic solvents, the synthetic reaction from MalAlaAlaPheOMe and amino acid amides results in the formation of $MalAlaAlaPheXaaNH_2$ and a number of unknown compounds. In water, the acyl transfer of the BzTyr-moiety to several amino acid amides was found to proceed in a more complex manner than is usually [19] proposed. Because of that, we used MalAlaAlaPheOMe for the analysis of the reactivity of the amino acid amides in water; the data in a mainly organic system were obtained using BzTyrOEt.

The specificity of α-chymotrypsin for amino acid amides as nucleophiles was completely different in water compared to a low water system (Fig. 1). The nucleophilic reactivity of the compounds tested in a mainly organic medium does not correlate with their nucleophile reactivity in water (Fig. 2).

By plotting the results obtained against different characteristics of the amino acid residues, a rather good correlation was found in two cases. The RS values obtained in water correlated with the hydrophobicity of the amino acid side chains (Fig. 3a), except for acyl-transfer to $ArgNH_2$ and D-$AlaNH_2$. (Hydrophobicity = $\log P(AcXaaNH_2) - \log P(AcGlyNH_2)$, where P is the partition coefficient of the compound in the water–octanol two-phase system [20].) Moreover, most experimental points were close to a straight line with a unit slope. Therefore, Fig. 3a shows that (i) the properties of the S_1'-subsite of α-chymotrypsin in many respects are similar to those of octanol and, (ii) the differences in the nucleophile reactivity of amino acid amides in chymotrypsin-catalyzed acyl-transfer reactions in water are mainly the result of the extraction of the nucleophile molecule from water by this subsite. The higher reactivity of $ArgNH_2$ appears to be the result of ionic interactions between two aspartic acid residues (Asp-64 and Asp-35) and the positively charged side chain of the arginine nucleophile [21]. Incorrect orientation of a

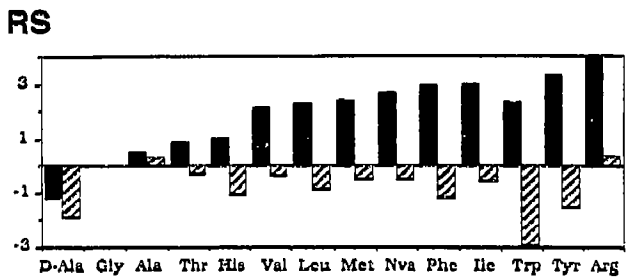

Fig. 1. Relative specificity (RS) of α-chymotrypsin in acyl-transfer reactions with respect to the amides of the listed amino acids in the water and the low-water systems. (Filled bars), water system: 0.1 M veronal buffer, pH 9.0, 30°C; (hatched bars), low-water system: 47.5% (v/v) acetonitrile, 47.5% (v/v) DMF, 1% (v/v) $(C_2H_5)_3N$, 4% (v/v) H_2O, 30°C.

methyl group in the complex of MalAlaAlaPhe-chymotrypsin with D-$AlaNH_2$ is probably the reason for the low reactivity of the latter compound.

The RS values obtained in a mainly organic medium correlated neither with the data for the water system (Fig. 2) nor with the amino acid side chain hydrophobicity. However, a good correlation between the reactivity of different nucleophiles and the normalized van der Waals volumes was observed (Fig. 3b). The normalized van der Waals volume (ν_v) characterizes the size of the amino acid side chain and was calculated [20] according to the following equation $\nu_v = [V(\text{side chain}) - V(H)]/V(CH_2)$, where V is the van der Waals volume. The differences in the reactivity of different amino acid amides (except $TrpNH_2$) in a mainly organic medium were smaller than in water. D-$AlaNH_2$ and $ArgNH_2$ were the exceptions both in water and organic systems, probably due to the same reasons in both media.

4. DISCUSSION

The bulk of a molecule can be defined in several ways. The polarizability (and molar refractivity, which is related to the polarizability by simple arithmetic) of a substrate is a popular parameter reflecting the size of a substituent of a molecule [20,22]. However, for amino acid amides polarizability depends mainly on the molecular weight of the substituent because other quantities used in the calculation of polarizability (index of refraction, density) vary to a smaller extent than the molecular weights of these compounds. This fact results in a high degree of correlation between the normalized van der Waals volume, polarizability and the molecular weight of amino acid side chains (Fig. 4). Therefore, the choice of the bulk parameter for the correlation presented above is mainly a matter of convenience. In our opinion the normalized van der Waals volume better reflects the size of a molecule.

The comparison of the specificity of enzymes in water with their specificity in mainly organic media is not a straightforward task. The pronounced effects of organic

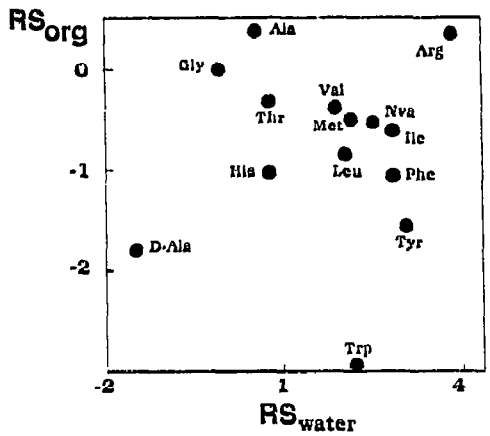

Fig. 2. Relationship between relative specificity (RS) of α-chymotrypsin in an acyl-transfer reaction with respect to the added nucleophiles in the water system (RS_{water}) and that in the low-water system (RS_{org}). Water system, 0.1 M veronal buffer, pH 9.0; low-water system, 47.5% (v/v) acetonitrile, 47.5% (v/v) DMF, 1% (v/v) $(C_2H_5)_3N$, 4% (v/v) H_2O. Temperature, 30°C. The points correspond to the amides of the listed amino acids.

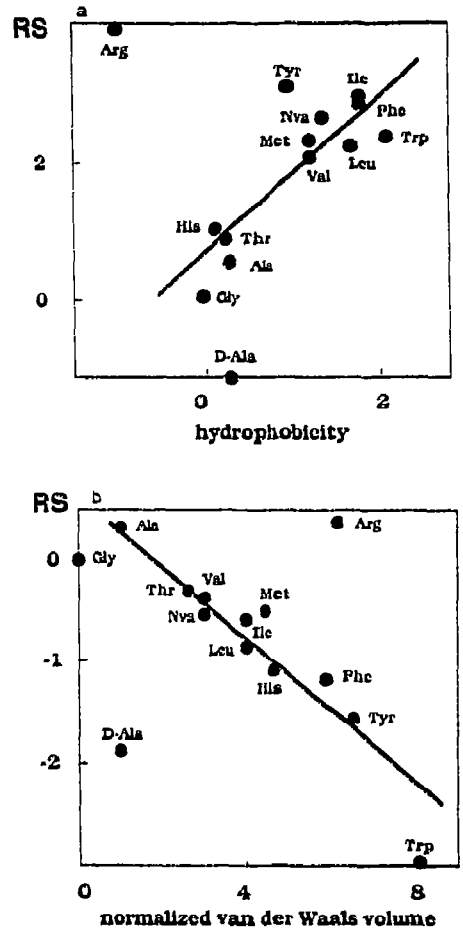

Fig. 3. Correlation between different amino acid side chain parameters and the relative specificity (RS) of α-chymotrypsin in acyl-transfer reactins with respect to the added nucleophiles. The points correspond to the amides of the listed amino acids. (a) Water system, 0.1 M veronal buffer, pH 9.0, 30°C. The straight line corresponds to the 'ideal' model, when only hydrophobicity of the amino acid side chains is responsible for the differences between the reactivity of different added nucleophiles. The points correspond to the amides of the listed amino acids. (b) Low water system, 47.5% (v/v) acetonitrile, 47.5% (v/v) DMF, 1% (v/v) $(C_2H_5)_3N$, 4% (v/v) H_2O, 30°C. The straight line was drawn to show better the correlation between RS and the normalized van der Waals volume (ν_v) for all amino acid amides except $ArgNH_2$ and D-$AlaNH_2$.

solvent on thermodynamics often makes the choice of a reaction which occurs both in water and a mainly organic medium difficult. The acyl-transfer peptide synthesis catalyzed by proteases is an exception. This reaction is favourable in both water and organic solvents; operating in the latter medium suppresses the hydrolysis of the peptide synthesized [23]. However, apart from the thermodynamic restrictions, kinetic problems exist. The activity and other properties of enzymes in mainly organic mixtures are undoubtedly strongly influenced by the thin water layer around the protein [13,24,25]. Characteristics of such a water layer should depend on the enzyme form used: insoluble suspended enzyme, soluble poly(ethylene glycol)-modified enzyme, adsorbed enzyme, etc. The properties of the surface that the enzyme is adsorbed to undoubtedly influence the properties of the catalyst [26]. These circumstances could probably explain the fact that the S_1 specificity of α-chymotrypsin dissolved in water is the same as that of the Celite-adsorbed enzyme in a mainly organic medium [11], but the S_1 specificity of poly(ethylene glycol)-modified chymotrypsin dissolved in benzene is different from that in water [8,9]. Alternatively, the discrepancy may depend on the choice of different model reactions in these studies. The data obtained in the present work can be directly compared to the results of only one work [11]. In both cases enzyme adsorbed on Celite was used. The results show dramatic differences between the effects of the organic mixture on the S_1- and the S'_1 specificity of α-chymotrypsin. In contrast to the S_1 specificity of this enzyme, the S'_1 specificity of α-chymotrypsin in a mainly organic medium is absolutely different from that in water.

Previously, similar comarisons between water and low water s[illegible]as were carried out for suspended enzymes. A negative effect of substrate hydrophobicity on enzyme specificity in mainly organic media was observed by Dordick [10] using peroxidase-catalyzed oxidation of phenols. In the latter case, however, substrate hydrophobicity was shown to have a very slight effect on the reaction rate of the peroxidase-catalyzed reactions in water. In our case, the hydrophobicity, which primarily affects the S'_1 specificity of α-chymotrypsin in water, has no influence on the S'_1 specificity in the organic medium.

The reactivity of amino acid amides is mainly affected by the size of the nucleophile. Kise et al. [27] observed this phenomenon, but the data available at that time did not allow them to make quantitative conclusions. Our

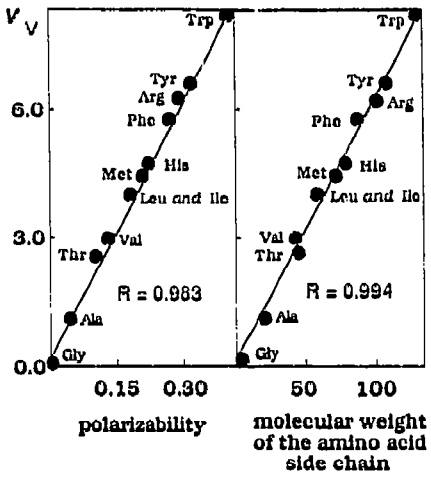

Fig. 4. Correlation between different amino acid side chain parameters characterizing the size of the amino acid substituent. The straight lines were drawn using the least-squares method; R is the correlation coefficient. Data were taken from the literature [20]. The points correspond to the amides of the listed amino acids.

data shows that the influence of the organic solvent on the specificity may be even more complicated than that claimed by Zaks and Klibanov [7]. They showed that the presence of an organic solvent caused the inversion of the S_1 specificity of α-chymotrypsin and subtilisin Carsberg. The weakening of hydrophobic interactions responsible for enzyme specificity in water was proposed as the rationale of this phenomenon. Our data shows that the specificity in organic solvent is not simply reversed but can be unrelated to that in water (Fig. 2). The substrate hydrophobicity becomes an unimportant parameter. Instead, the reactivities of the nucleophiles depend on their size. D-AlaNH_2 has the lowest reactivity in water and one of the lowest in a mainly organic medium. In our opinion, these effects are difficult to explain in terms of the simple extraction model, which is very useful in many cases [16]. The simplest explanation of this phenomenon may be as follows: the S'_1 specificity of α-chymotrypsin is affected by both the hydrophobicity and the size of the nucleophile. In water, the hydrophobic effect is stronger than that of the size, so in water the effects of size are diminished. In organic solvent, hydrophobic interactions are weakened and the size becomes a more important parameter.

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