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AMINO ACID–AMINO ACID INTERACTIONS STUDIED BY CHARGE-TRANSFER CHROMATOGRAPHY

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SUMMARY

The interaction between amino acids was studied by charge-transfer reversed-phase thin-layer chromatography. The dependence of the lipophilicity of Trp on the concentration of other amino acids in the eluent was considered to be linearly related to the strength of interaction. Arg, Asn, Glu, Met, Phe and Thr interacted with Trp; Ala, Gly and Ser showed no interaction. Stepwise regression analysis indicated that the pK value of the amino acid side-chain and the lipophilicity of the amino acid had the greatest impact on the interaction, suggesting the simultaneous presence of weak hydrophilic and hydrophobic bonding forces between amino acids. Sodium acetate in the eluent increased the interactive strength between Phe and Trp; acetic acid and sodium chloride did not influence the interaction significantly. No significant difference was found between the effects of L- and D-Asn.

INTRODUCTION

Interactions between pairs of amino acids influence the structure and association capacity of proteins [1]. It has been established that proline stabilizes solute proteins during freezing by interacting with them [2]. Lysine and cysteine residues were directly involved in the aggregation between ovalbumin and lysozyme; the aggregation is partially due to electrostatic attraction [3] and is inhibited by sodium chloride [4]. Free amino acids suppress the synthesis of extracellular proteinase, a single amino acid being less effective than a mixture of amino acids [5]. Tyrosine can interact with other amino acids in bovine α-lactalbumin [6]. The edge-to-face interaction of two aromatic side-chains makes an enthalpy contribution of between -1 and -2 kcal/mol to the energy stabilization of a protein [7,8]. However, the hydrophobic, hydrophilic or mixed character of in-

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teractions between the pairs of amino acids in proteins has been vigorously discussed. In many it is supposed that hydrophobic interactions are the most important physical forces that guide a polypeptide chain to its specific folded form in an aqueous environment [9–12].

In peptides Trp, Phe, Leu and Val have been involved in the formation of hydrophobic interactions [13]. In some instances hydrophilic interactions between polar amino acid residues have also been observed. Electrostatic interactions influence the association and conformation of proteins [14]. Charge transfer occurs between Trp and Tyr in peptides [15]. It was further established that the Valyl–tRNA synthetase of *Bacillus stearothermophilus* binds two Val molecules [16,17] and the binding is not selective [18]. The Val molecule binds with three hydrogen bonds to thermolyzine [19].

Amino acid interactions also have biological consequences. The peptides bound to the enzymes inhibit their activity [20]; this phenomena has also been observed with papain [21] and renin [22]. The immunogenic effect of some peptides has been enhanced by their hydrophobic bonding to proteosomes [23]; mastoparan, a peptide toxin from wasp venom, activates GTP-binding regulatory proteins [24].

The application of modifying agents mixed in the supports in order to improve separation was introduced in thin-layer chromatography (TLC) more than twenty years ago [25]. The relative difference in R_F values determined on supports with and without compounds suitable for charge-transfer interactions has been proposed as a measure of the strength of interaction [26]. Early studies applied charge-transfer chromatography in adsorptive TLC using non-polar solvents [27–29]. However, the interactive forces determined in organic eluents cannot be easily related to the interactions taking place in aqueous biological systems.

In recent research charge-transfer chromatography has been applied to study biologically important molecular interactions [30–32]. To avoid the application of non-polar organic solvents in the study of charge-transfer interactions, reversed-phase TLC (RP-TLC) was applied, using aqueous eluents [33,34]. When one of the interacting compounds also contains one or more dissociable polar substituents, the pH of the eluent [35,36] and the salt concentration modify its retention behaviour [37–39]. Therefore, it can be considered that with polar compounds such as amino acids he interactive strengths determined at different pH values and salt concentrations may differ from each other.

The objectives of this work were to study the interaction between amino acids by reversed-phase charge-transfer chromatography and to elucidate the role of various physicochemical parameters of amino acids in the interaction. We further studied the effect of pH and salt concentration on the strength of interaction.

EXPERIMENTAL

Amino acids (Ala, Arg, Asn, Glu, Gly, Met, Phe, Ser, Thr and Trp) were of analytical purity. Unless stated otherwise, L-amino acids were used in each experiment. Polygram UV_{254} plates (Macherey-Nagel, Düren, F.R.G.) were impregnated with paraffin oil as described [40]. Trp was dissolved in water–2-propanol (4:1, v/v) at a concentration of 1 mg/ml and 5 μl of the solution were

spotted on the plates. Distilled water was used as the eluent, containing the other amino acids in the concentration range 0.01–1 mol/l. To assess the possible differences between the interaction of D- and L-amino acids, the effect of D- and L-Asn (0–0.2 mol/l) on the retention behaviour of Trp was separately determined.

The influence of sodium chloride and the pH of the eluent on the strength of interaction was studied only for the pair Phe–Trp. The eluents were mixtures of 1 mol/l sodium chloride, 1 mol/l acetic acid and 1 mol/l sodium acetate in distilled water. Phe was added to the eluents in the concentration range 0–0.15 mol/l. The strengths of interaction determined in the different eluents were compared using Student's *t*-test. After development the plates were dried at 105 °C and Trp spots were revealed by their UV adsorption. The amino acid fronts were detected with ninhydrin.

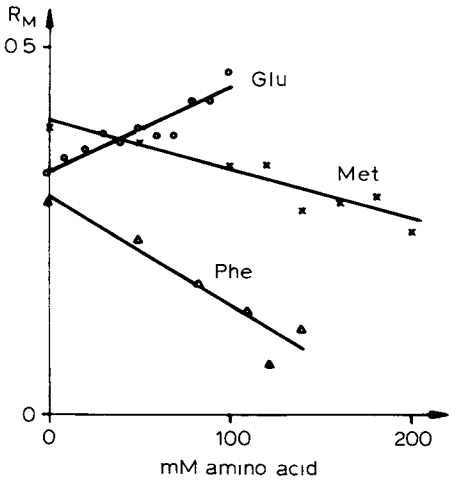
To obtain information about the effects of amino acids on the spot shape and spot compactness of Trp, some plates were evaluated with a Model CS-930 dual-wavelength TLC scanner (Shimadzu, Kyoto, Japan) at 280 nm. Each experiment was run in quadruplicate. The R_M values characterizing molecular lipophilicity in RP-TLC were calculated as described [41]. As amino acid fronts did not coincide with the eluent front the R_M values of Trp were corrected for the different mobility of amino acids (R_{MC}) as described [42]. Linear correlations were calculated between the corrected lipophilicity (R_{MC}) values of Trp and the concentration of amino acid in the eluent separately for each amino acid; the slope was considered to be the best estimate of the interactive strength [34]. Stepwise regression analysis [43] was applied to select the physicochemical parameters of amino acids that significantly influence the strength of interaction. The polarity parameters (pI and pK values) and the lipophilicity values were taken from refs. 44 and 45, respectively.

RESULTS AND DISCUSSION

Ala, Ser and Gly did not influence the R_M value of Trp. Hence, we infer that these amino acids did not interact with Trp. The other amino acids increased or decreased the retention of Trp (Figs. 1 and 2). This finding can be explained by the assumption that the amino acids interact with Trp and the molecular complex is either more lipophilic or more hydrophilic than the uncomplexed Trp, resulting in a modified retention.

The effect of Phe on the retention, peak shape and peak symmetry of Trp is shown in Fig. 3. The Trp peak is sharp and symmetric even in the presence of 0.15 mol/l Phe, that is, this amino acid did not modify the spot shape and compactness. Phe evenly covered the plate surface and its front was steep, that is, the R_F value of the amino acid front can be exactly determined and the correction of R_M values can be accurately carried out. Each amino acid behaved similarly and did not adversely affect the reproducibility of R_F determination even at their highest concentration.

The parameters of linear correlations between the R_M value of Trp and the amino acid concentration in the eluent are given in Table I. Ala, Gly and Ser did not influence significantly the lipophilicity of Trp. These amino acids probably



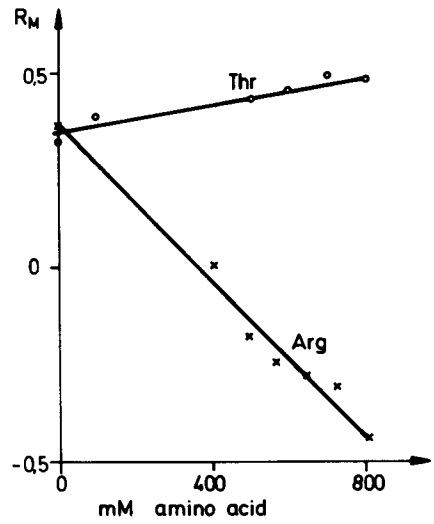


Fig. 1. Dependence of R_M value of Trp on the Glu, Met and Phe concentrations in the eluent.

Fig. 2. Dependence of R_M value of Trp on the Arg and Thr concentrations in the eluent.

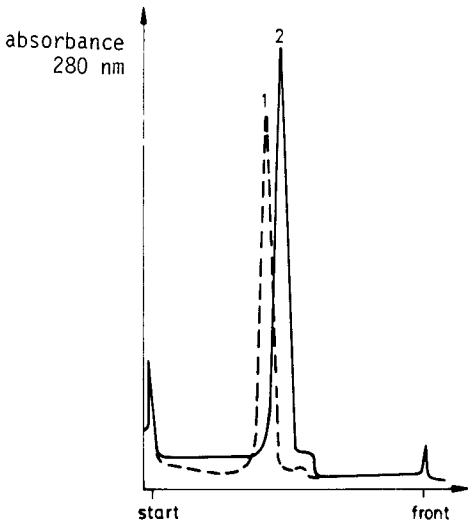


Fig. 3. Effect of phenylalanine on the retention and peak shape of tryptophan. (1) 75 mmol/l Phe in water; (2) 150 mmol/l Phe in water.

did not interact with Trp or the interaction was so weak that it was below the sensitivity limit of the method. Arg, Met and Phe decreased and Asn, Glu and Thr increased the retention of Trp. This result indicates that these amino acids formed complexes with Trp, but the opposite change in lipophilicity suggests that the character of the interaction may be different and it is composed of at least two effects, the first decreasing and the second increasing the lipophilicity of Trp.

We should point out that the strength of interaction between Trp and the other amino acids in very weak ($b = 0\text{–}1.11 \cdot 10^{-3}$) compared with the strengths of other

TABLE I

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE LIPOPHILICITY VALUE OF TRYPTOPHAN (R_{MC}) AND THE AMINO ACID CONCENTRATION IN THE ELUENT (C, mmol/l)

$R_{MC}=a+bC$.

Amino acid	n	a	$b\cdot10^{-4}$	r	Significance level (%)
Alanine (Ala)	8	Non-significant			
Arginine (Arg)	7	0.36	−9.98	0.9890	99.9
Asparagine (Asn)	8	0.29	3.91	0.8847	99.9
Glutamic acid (Glu)	11	0.33	11.10	0.9023	99.9
Glycine (Gly)	8	Non-significant			
Methionine (Met)	8	0.40	−6.66	0.9371	99.9
Phenylalanine (Phe)	6	0.30	−14.90	0.9472	99.0
Serine (Ser)	8	Non-significant			
Threonine (Thr)	6	0.34	1.85	0.9539	99.0

TABLE II

DEPENDENCE OF AMINO ACID-TRYPTOPHAN INTERACTION (b) ON THE pK VALUE OF THE AMINO ACID SIDE-CHAIN AND ON THE LIPOPHILICITY (R_M) OF AMINO ACIDS

Results of stepwise regression analysis. $b=a+b_1\text{p}K+b_2R_M^2$; $n=9$; $F=4.20$; $r^2=0.5817$.

Parameter	pK	R_M^2
$b\cdot10^{-4}$	−3.1	6.4
$s_b\cdot10^{-4}$	1.1	3.5
b' (%)	61.32	38.68

interactions determined under similar RP-TLC conditions, i.e., between non-ionic surfactants and dioleoylphosphatidylcholine ($b=1.19$–31.79) [33] and between nonylphenylnonylglycolate and some synthetic phospholipids ($b=0.47$–1.09) [34].

The results of stepwise regression analysis are given in Table II. Only two independent variables (pK value of the amino acid side-chain and the square of the lipophilicity value) influence significantly the strength of interaction. These two variables account for about 58% of the change in interactive strength ($r^2=0.5817$). The strength of interaction decreases linearly with increasing pK value of the amino acid side-chain and increases quadratically with increasing lipophilicity of amino acids. The path coefficients (b', %) show that the role of electrostatic interactions, i.e. the impact of the pK value, is higher in this interaction than that of hydrophobic interactions, i.e. the impact of the R_M value. The results of stepwise regression analysis support the assumption that other amino acids orient themselves with respect to the Trp molecule with their side-chain, the polar head group pointing towards the water. The side-chain can interact with Trp in two different ways: (a) hydrophilic–hydrophilic interaction between the polar groups of the amino acid side-chain and the most polar group in the side-chain of Trp

(probably hydrogen bond formation); (b) hydrophobic–hydrophobic interaction between the apolar side-chain of amino acids and the corresponding Trp substructures. The effect observed is probably due to the result of the interplay of these interactions. The various eluent additives (sodium chloride, acetic acid, sodium acetate) influenced the retention of Trp and the mobility of the Phe front, but the character of the Trp–Phe interaction was similar in each eluent, i.e., the retention of Trp decreased in the presence of Phe (Fig. 4). The peak shape and compactness of Trp remained the same in distilled water (Fig. 3), in 1 mol/l sodium chloride (Fig. 5) and in 1 mol/l acetic acid (Fig. 6). In 1 mol/l sodium acetate Trp formed two incompletely separated peaks (Fig. 7). The second peak appeared at a Phe concentration of 75 mmol/l, increased at 97.5 and 127.5 mmol/l and the original Trp peak disappeared at 150 mmol/l. This result indicates that in an alkaline environment Trp forms two different complexes with Phe. As the new complex is more hydrophilic than the original one, it predominates at higher Phe concentrations and the Phe is more hydrophilic than Trp, therefore, we assume that this new complex has a different composition with a higher ratio of Phe, i.e., Phe/Trp = 2:1, instead of the original Phe/Trp = 1:1.

We should emphasize that the above explanation is hypothetical because the study of complex formation by charge-transfer chromatography does not give any information about the stoichiometry of the complexes. This phenomenon was not observed in less alkaline eluents such as 1 mol/l acetic acid–1 mol/l sodium acetate (13:7 and 7:13, v/v), which makes it probable that the extent of dissociation of the polar groups in the amino acids influence considerably the Trp–Phe interactions in an alkaline environment.

The various eluent additives did not affect the linear dependence of Trp lipophilicity on Phe concentration (Table III). The strength of interaction was greatest in sodium acetate, followed by sodium chloride and acetic acid. The complex

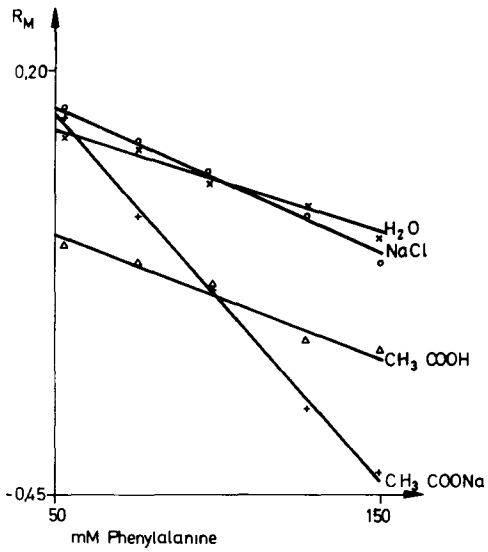


Fig. 4. Effect of various eluent additives on the Phe–Trp interaction.

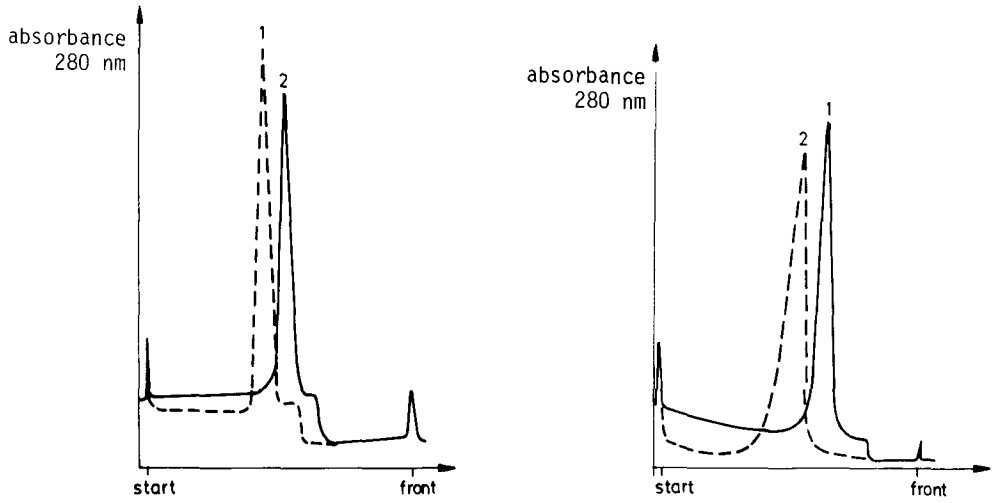


Fig. 5. Effect of Phe on the retention and peak shape of tryptophan. (1) 75 mmol/l Phe in 1 mol/l sodium chloride; (2) 150 mol/l Phe in 1 mol/l sodium chloride.

Fig. 6. Effect of Phe on the retention and peak shape of tryptophan. (1) 150 mmol/l Phe in 1 mol/l acetic acid; (2) 1 mol/l acetic acid.

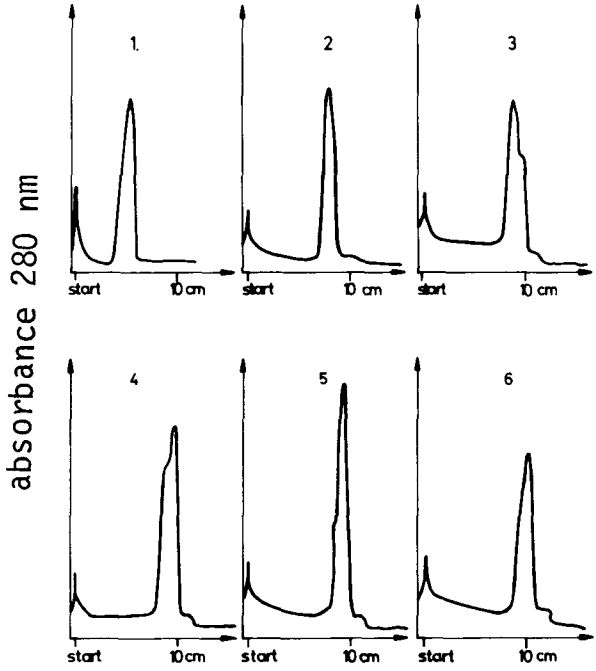


Fig. 7. Effect of phenylalanine on the retention and peak shape of tryptophan. Eluent, 1 mol/l sodium acetate. (1) No Phe; (2) 52.5 mmol/l Phe; (3) 75 mmol/l Phe; (4) 97.5 mmol/l Phe; (5) 127.5 mmol/l Phe; (6) 150 mmol/l Phe.

TABLE III

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE LIPOPHILICITY VALUE OF TRYPTOPHAN (R_{MC}) AND THE PHENYLALANINE CONCENTRATION IN THE ELUENT (C, mmol/l)

Parameter	Eluent composition (1 mol/l)			
	Water	Sodium chloride	Sodium acetate	Acetic acid
n	6	6	5	6
a	0.18	0.26	0.40	0.04
$b \cdot 10^{-3}$	−1.50	−2.28	−5.50	−1.88
$s_b \cdot 10^{-4}$	1.69	3.07	10.3	2.20
r	0.9755	0.9657	0.9514	0.9736

TABLE IV

SIGNIFICANCE TEST BETWEEN THE STRENGTH OF TRYPTOPHAN–PHENYLALANINE INTERACTION IN VARIOUS ELUENT SYSTEMS

Results of Student's t-test.

Eluent composition	Eluent composition		
	Acetic acid	Sodium chloride	Sodium acetate
Water	1.37	2.23	3.83
Acetic acid		1.06	3.44
Sodium chloride			3.00

TABLE V

DIFFERENCES BETWEEN THE EFFECT OF L- AND D-ASPARAGINE ON THE LIPOPHILICITY VALUE (R_{MC}) OF TRYPTOPHAN

Asparagine concentration (mmol/l)	L-Asparagine	D-Asparagine	R_{MC}
200	0.38	0.39	−0.01
180	0.39	0.33	0.06
160	0.36	0.32	0.04
140	0.35	0.30	0.05
120	0.34	0.34	0.00

formation was significantly stronger in sodium acetate than in the other eluents, which did not differ significantly from each other (Table IV). The L and D forms of Asn influenced the retention of Trp similarly (Table V). No significant difference was found between the R_M values, that is, the configuration of Asn did not influence the strength of interaction substantially.

It can be concluded that free amino acids interact with each other in aqueous media, the strength of interaction depending on the structure of the particular

amino acid pair. We assume that similar interactions may also exist in proteins, influencing the structure and the corresponding enzymatic activity.

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