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Thermodynamics of phenylacetamides synthesis: Linear free energy relationship with the pK of amine

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ABSTRACT

The effective equilibrium constants K'_C expressed through the total concentrations of the reagents for the synthesis of N-phenylacetyl-derivatives in aqueous medium from phenylacetic acid and various primary amino compounds have been determined with penicillin acylase as a catalyst. Broad specificity of penicillin acylase (EC 3.5.1.11) to amino components made possible to investigate the acylation of primary amines with different structures and physicochemical properties. Analysis of different components of the effective standard Gibbs energy change $\Delta G'^{o}_{C}$ has revealed favorable thermodynamics for the synthesis of phenylacetamides from unionized substrates forms, however the ionization of reactants carboxy and amino groups in aqueous solutions pushes the equilibrium position to the hydrolysis especially in case of highly basic amines. A linear correlation between the standard Gibbs energy change for amide bond formation from the unionized reagents species and the basicity of amino group was observed: $\Delta G^{o}_{T} = -3.56 \cdot \mathrm{pK}_{\mathrm{amine}} + 7.71$ (kJ/mol). The established linear free energy relationship (LFER) allows to predict the thermodynamic parameters for direct condensation of phenylacetic acid with any amine of known pK. Condensation of phenylacetic acid and amines with pK value within 1.5–8.5 was shown to be thermodynamically favorable in homogeneous aqueous solution.

1. Introduction

Biochemical equilibrium of enzyme-catalyzed transformations is one of the major factors which should be considered at rational bioprocess design. Nevertheless the state of the art in the area of thermodynamics of amide and peptide bond formation is rather limited and controversial [1,2]. The causes of this are mainly related to experimental problems. Slow attainment of equilibrium, difficulties to determine low equilibrium concentrations of products at some syntheses as well as side reactions stipulate application of indirect methods and non-standard experimental conditions. As a result the experimentally determined equilibrium constants are effective (apparent), and in many cases reliable only for given conditions.

The ionization of reagents in aqueous medium affects the equilibrium of condensation reactions more than other physical factors. In the early 60s Carpenter had introduced a "nonionized compound convention" by expressing the reactants concentrations in terms of all existing forms including their uncharged species [3]. This allowed to consider the contribution of the ionization of reagents and to compare the equilibrium constants of a wide range of reactions carried out in aqueous solutions at different pH. As a consequence it was found that the Gibbs energy components responsible for conversion of the nonionized reactants forms differ substantially in case of amide and ester hydrolysis, but vary within a narrow range for peptide and amide hydrolysis [3]. The following studies showed this convention to be insensitive to the nature of acyl moiety [4–6], except for formyl amides [7]. Other authors have revealed that the equilibrium constants referred to the neutral reactants species differ largely, and, moreover, with some exceptions correlate with the basicity of amino compounds [7–9]. However, recently most of the earlier published experimental data have been recalculated in terms of the "non-ionized compound convention", and a common, independent on the nature of reagents equilibrium constant K^{o}_{T}, $10^{3.6}$ M (ΔG^{o}_{T}, −20.5 kJ/mol) for hydrolysis of amide and peptide bonds has been established [10]. This discrepancy is quite a principal question as there is a need to set up a common model for quantitative estimation of the equilibrium parameters for amide bond hydrolysis/synthesis based on the physicochemical properties of reagents.

Abbreviations: IS, ionic strength; LFER, linear free energy relationship; PA, penicillin acylase.

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$$SH + Nu \overset{K_T}{\rightleftharpoons} P + H_2O \qquad (I)$$

$$SH \overset{K_{SH}}{\rightleftharpoons} H^+ + S^- \qquad (I\text{-a})$$

$$HNu^+ \overset{K_{HNu+}}{\rightleftharpoons} H^+ + Nu \qquad (I\text{-b})$$

Scheme 1. Direct condensation of an amine (Nu) with a carboxylic acid (SH) is characterized by the thermodynamic equilibrium constant K_T (I). The reaction (I) is complicated by ionization of the reagents (I-a and I-b); K_{SH} and K_{HNu^+} are the dissociation constants for reacting carboxylic and amino groups.

In this paper the thermodynamics of amide bond formation between phenylacetic acid and various primary amino compounds has been studied. The effective equilibrium constants for the synthesis of N-phenylacetyl-derivatives in aqueous medium have been determined with penicillin acylase EC 3.5.1.11 (PA) as a catalyst. PA-catalyzed acylations in aqueous medium are widely used for antibiotic synthesis [11] as well as for chiral resolutions [12–14], therefore thermodynamic characterization of these biocatalytic conversions is very important for process optimization.

2. Equations

The thermodynamic equilibrium constant for direct condensation of unionized forms of a carboxylic acid and an amine (reaction I, Scheme 1) is expressed in terms of the activities of reaction components:

$$K_T^o = \frac{a_P \cdot a_W}{a_{SH} \cdot a_{Nu}} \qquad (1)$$

If we take into account the ionization reactions I-a and I-b (Scheme 1) as well as the water activity (a_W) being equal to 1, the equilibrium constant can be represented as:

$$K_T^o = \frac{K_{SH}^o}{K_{HNu^+}^o} \cdot \frac{a_P}{a_{S^-} \cdot a_{HNu^+}}$$

Here we consider the ideal-dilute systems only:

$$K_T^o = \frac{K_{SH}^o}{K_{HNu^+}^o} \cdot \frac{1}{\alpha_{S^-} \cdot \alpha_{HNu^+}} \cdot \frac{c_P}{c_S \cdot c_{Nu}} \qquad (2)$$

Hence the experimentally determined and expressed through the total concentrations of the reagents effective equilibrium constant makes up

$$\begin{aligned} K_C' &= K_T^o \cdot \frac{K_{HNu^+}^o}{K_{SH}^o} \cdot \alpha_{S^-} \cdot \alpha_{HNu^+} \\ K_C' &= K_T^o \cdot \frac{[H^+]\cdot[Nu]}{[HNu^+]} \cdot \frac{[SH]}{[H^+]\cdot[S^-]} \cdot \frac{1}{1+([SH]/[S^-])} \cdot \frac{1}{1+([Nu]/[HNu^+])} \\ K_C' &= K_T^o \cdot \frac{1}{([S^-]/[SH])+1} \cdot \frac{1}{([HNu^+]/[Nu])+1} \\ K_C' &= K_T^o \cdot \frac{1}{1+(K_{SH}^o/[H^+])} \cdot \frac{1}{1+([H^+]/[K_{HNu^+}^o])} \\ K_C' &= K_T^o \cdot \frac{1}{(1+10^{(pH-pK_{SH})})\cdot(1+10^{(pK_{HNu^+}-pH)})} \end{aligned} \qquad (3)$$

Assuming designations

$$\Delta G_{ion}^{o'} = R \cdot T \cdot \ln\left[(1+10^{(pH-pK_{SH})})\cdot(1+10^{(pK_{HNu^+}-pH)})\right] \qquad (4)$$

Eq. (3) can be expressed in terms of Gibbs energy change:

$$\Delta G_C^{o'} = \Delta G_T^o + \Delta G_{ion}^{o'} \qquad (5)$$

This equation combines true thermodynamic and effective equilibrium parameters of amide bond formation. Evidently, the pH-independent component of standard Gibbs potential ΔG_T^o characterizes the thermodynamic equilibrium of the reaction for unionized species; the contribution of ionization $\Delta G_{ion}^{o'}$ takes into account the acid–base properties and reflects Gibbs energy change related to the neutralization of the reagents charged functional groups at given conditions, i.e. protonation of carboxylic group and deprotonation of amino group in aqueous solution.

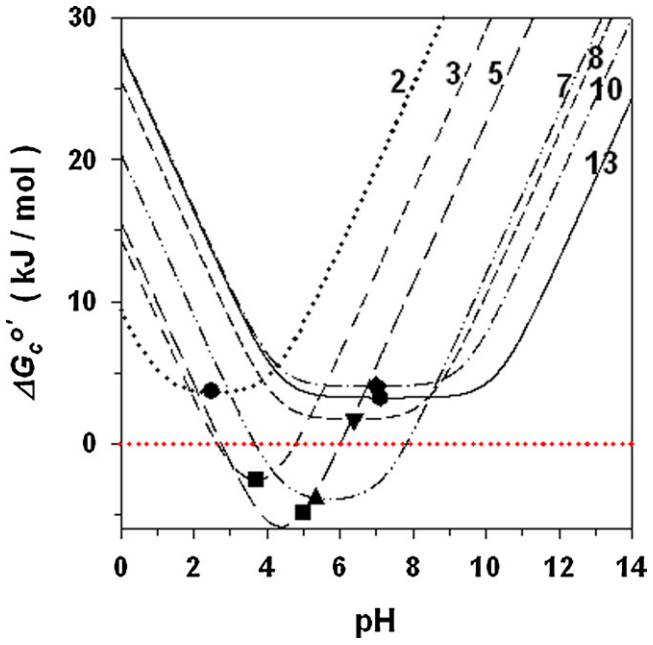


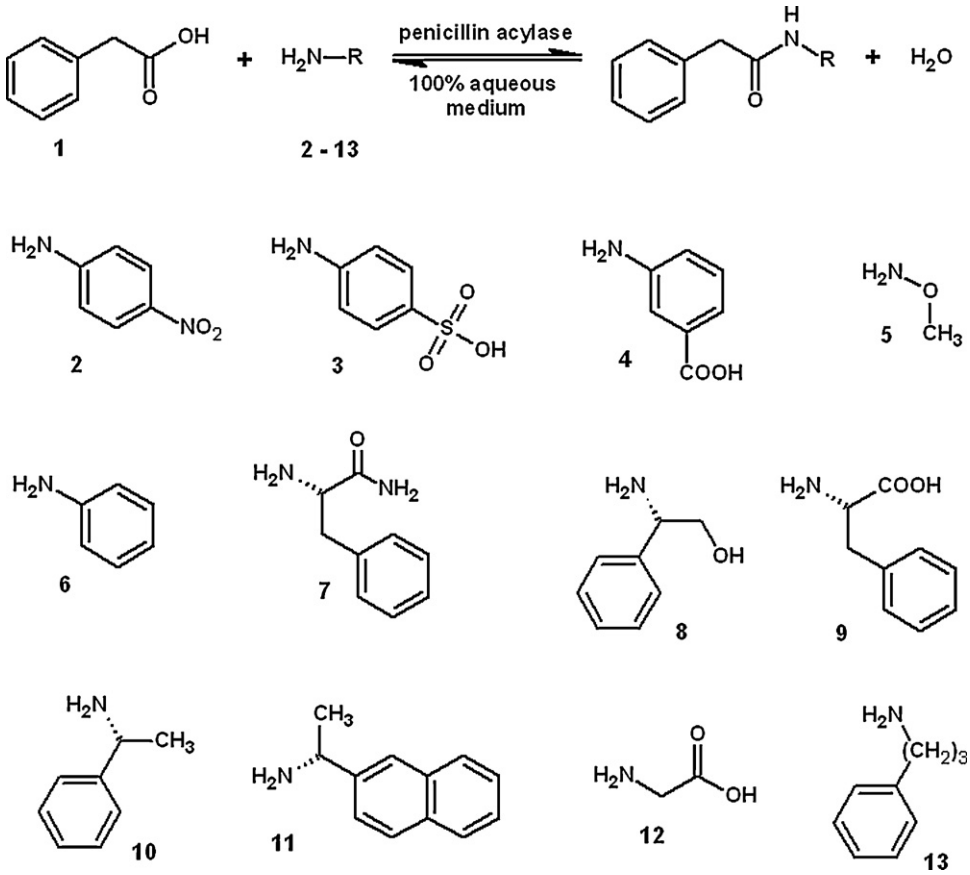
Fig. 1. Thermodynamics of direct condensation of phenylacetic acid and amines (Scheme 2, experimental conditions: 298 K, IS 0.1 M. Theoretical curves were calculated according to Eqs. (4) and (5) using the experimental data from Tables 1 and 2.

3. Results and discussion

The effective equilibrium constants K_C' for the synthesis of N-phenylacetyl-derivatives from phenylacetic acid and different primary amino compounds in aqueous medium have been determined at 298 K and ionic strength (IS) 0.1 M using PA from *Escherichia coli* as a catalyst. Broad specificity of PA to amino components made possible to investigate the equilibrium parameters of acylation of various primary amines (Scheme 2), which differ largely by structure and basicity of their amino group. The effective equilibrium constants K_C' have been determined by the most rigorous method, when the equilibrium position is attained from both sides: the side of enzymatic synthesis and the side of enzymatic hydrolysis (Table 1).

The values of the thermodynamic equilibrium constant K_T^o and the corresponding standard Gibbs energy change ΔG_T^o related to direct condensation of unionized reagents forms for each of the investigated reactions were calculated on the basis of the experimental data at given pH according to Eqs. (3)–(5) (Table 2). When considering amines containing additional ionogenic group the corresponding microconstants for their zwitterions were taken into account according to the procedure described earlier (Table 3) [10,15]. Further, based on the determined intrinsic value ΔG_T^o of each condensation reaction the pH-profile of the effective Gibbs energy change $\Delta G_C^{o'}$ has been calculated. According to the obtained profiles (Fig. 1) the condensation of phenylacetic acid with many of the amino compounds appears to be thermodynamically unfavorable.

It is interesting to consider how much each of the both components of $\Delta G_C^{o'}$ (according to Eq. (5) depends on the nature of amino



Scheme 2. Penicillin acylase-catalyzed condensation between phenylacetic acid (**1**) and various amino compounds: p-nitroaniline (**2**), 4-aminobenzenesulfonic acid (**3**), 3-aminobenzoic acid (**4**), O-methylhydroxylamine (**5**), aniline (**6**), (S)-phenylalanine amide (**7**), 2-amino-2-phenylethanol (**8**), phenylalanine (**9**), (R)-1-phenylethylamine (**10**), (R)-1-(2-naphthyl)ethanamine (**11**), glycine (**12**), 3-phenylpropanamine (**13**).

Table 1
Determination of the equilibrium position of phenylacetamides hydrolysis/synthesis using penicillin acylase as a catalyst (298 K, IS 0.1 M).

Amine	pH	Equilibrium concentration, mM			Method	K'_C M^{-1}
		Acyl donor	Amine	Amide		
(**2**)	3.6	98.0	3.5	1.43	Synth.	0.20
		55.0	5.0	1.57	Hydr.	0.25
(**3**)	3.7	7.90	8.10	0.16	Synth.	2.5
		17.3	17.0	0.81	Hydr.	2.8
(**4**)	4.7	5.60	5.50	0.062	Synth.	2.0
	4.3	2.90	2.90	0.015	Hydr.	1.8
(**5**)	5.0	35	35	7.7	Synth.	6.3
		33.5	33.5	8.25	Hydr.	7.4
(**6**)	4.3	3.92	3.56	0.052	Synth.	3.7
	4.4	2.20	2.30	0.02	Hydr.	3.9
(**7**)	5.23	10.9	9.40	0.48	Synth.	4.6
	5.47	10.0	9.20	0.42	Hydr.	4.6
(**8**)	6.4	45.6	42.8	0.96	Synth.	0.49
		45.7	47.1	1.14	Hydr.	0.53
(**9**)	7.2	52.0	60.0	1.08	Synth.	0.35
	6.67	43.0	50.7	1.15	Hydr.	0.53
(**10**)	7.0	59.5	54.6	0.62	Synth.	0.19
		56.2	55.0	0.60	Hydr.	0.19
(**11**)	7.5	9.58	9.57	0.020	Synth.	0.22
		10.2	10.9	0.020	Hydr.	0.18
(**12**)	7.0	11.4	11.4	0.0167	Synth.	0.13
	7.05	11.4	11.4	0.0144	Hydr.	0.11
(**13**)	7.1	46.1	55.6	0.75	Synth.	0.29
		4.31	8.50	0.010	Hydr.	0.27

Table 2
Thermodynamics of N-phenylacetamides synthesis by direct condensation at 298 K, IS 0.1 M.

N-phenylacetyl-derivative	Macroscopic pK values[a]		pH	Effective (IS 0.1)	Thermodynamic (IS 0)		Contribution
	Amine[b]	Product		$\Delta G_C^{o'}$, kJ/mol	$K_T^o \cdot 10^{-3}$ M^{-1}	ΔG_T^o, kJ/mol	$\Delta G_{ion}^{o'}$, kJ/mol
(**2**)	1.0 [23]	–	3.6	3.71	0.00027	3.23	0.481
(**3**)	3.3 [24]; 1.0[c]	1.0[c]	3.7	−2.43	0.00462	−3.79	1.36
(**4**)	4.4, 3.3 [25]	3.9[c]	4.2	−2.57	0.00948	−5.57	3.00
(**5**)	4.6 [26]	–	5.0	−4.76	0.0574	−10.0	5.27
(**6**)	4.6 [27]	–	4.35	−3.31	0.0223	−7.69	4.38
(**7**)	7.2 [28]	–	5.35	−3.80	4.10	−20.6	16.8
(**8**)	8.5[c]	–	6.4	1.76	5.755	−21.4	23.2
(**9**)	9.3, 2.2 [29]	3.5[c]	7.25	2.59	34.99	−25.9	28.5
(**10**)	9.5	–	7.0	4.07	20.87	−24.4	28.7
(**11**)	9.6	–	7.6	3.99	36.43	−26.0	30.0
(**12**)	9.8 [30], 2.4 [24]	3.7[c]	7.0	5.08	41.29	−26.3	31.4
(**13**)	10.3 [31]	–	7.1	3.27	265.67	−30.9	34.2

[a] pK values were recalculated for IS 0 M.
[b] First value corresponds to amino group.
[c] The pK values were calculated using the computer programs developed by ACD Labs (www.acdlabs.com) and SPARC calculator (http://ibmlc2.chem.uga.edu); the pK 4.2 for 1 was from [32].

Table 3
Experimental and estimated[a] microscopic and macroscopic pK values.

Amine	Microscopic constants				Macroscopic constant		
	$pK_{acid}^{o,m}$	$pK_{base}^{o,m}$	$pK_{acid}^{\pm,m}$	$pK_{base}^{\pm,m}$	pK_{acid}^{o}	pK_{base}^{o}	pK_{prod}^{o}
(**3**)	1.6	2.7	1.0	3.3	1.0	3.3 [24]	1.0
(**4**)	4.0	3.7	3.1	4.6	3.3 [25]	4.4 [25]	3.9
(**9**)	4.1	7.4	2.1	9.4	2.2 [29]	9.3 [29]	3.5
(**12**)	4.4 [33]	8.0	2.4	9.8	2.4	9.8 [30]	3.7

[a] The pK values were calculated using the computer programs developed by ACD Labs (www.acdlabs.com) and SPARC calculator (http://ibmlc2.chem.uga.edu).

compound and what impact it brings to the overall Gibbs energy change at amide bond formation.

The consideration of the equilibrium position of reaction I for unionized species (Scheme 1) is reasonable because the kinetically reactive species in the formation of an amide bond during direct condensation are the neutral forms of reagents [6,16]. Surprisingly, the obtained results demonstrated that the thermodynamic parameters for the synthesis of N-phenylacetamides (Scheme 2) referred to unionized species of reaction components linearly depend on the pK of amino group (Fig. 2):

$$\Delta G_T^o = -3.56 \cdot pK_{amine} + 7.71 \text{ (kJ/mol)} \quad (6)$$

This correlation is an example of linear free energy relationship (LFER). Earlier satisfactory linear correlations of the Gibbs energy of reaction with the basicity of the nucleophile as well as of the leaving group were disclosed for transacylation reactions [17–19]. A surprising result of the obtained LFER is that the steric and so-called "α-effect" of substituents, which are significant for kinetic properties [20], apparently are not manifested. So, O-methoxyamine as well as anilines, amino alcohols, amino acids, amides and nonfunctionalized amines sufficiently obey this correlation (Fig. 2). The value of thermodynamic Gibbs potential ΔG_T^o for amide bond formation according to Eq. (6) could be within the range +2 to −35 kJ/mol depending on the acid-base properties of amines. According to the obtained results, the recently supposed common value ΔG_T^o, −20.5 kJ/mol [10] appear to be rather averaged one and does not reflect the situation in case of amines with low as well as high pK values.

The ΔG_{ion}^o is minimal when pH is equal to half of the sum of pK_{SH} and pK_{HNu^+}, and $\Delta G_C^{o'}$ is equal to ΔG_T^o when $pK_{SH} > pK_{HNu^+}$, that is a seldom case in real systems. As a rule $pK_{HNu^+} > pK_{SH}$, and when the difference between pK values of two functional groups is increased it leads to higher ΔG_{ion}^o and correspondingly to higher total Gibbs potential. The ΔG_{ion}^o for condensation of phenylacetic acid and amines at optimal pH exponentially increases with the pK_{amine}, so for highly basic amines its value reaches +25 to +50 kJ/mol (Fig. 2).

So, increase of the pK of the amino group of acyl acceptor leads, on the one hand, to the linear decrease of ΔG_T^o favoring synthesis, but, on the other hand, to the exponential growth of ΔG_{ion}^o

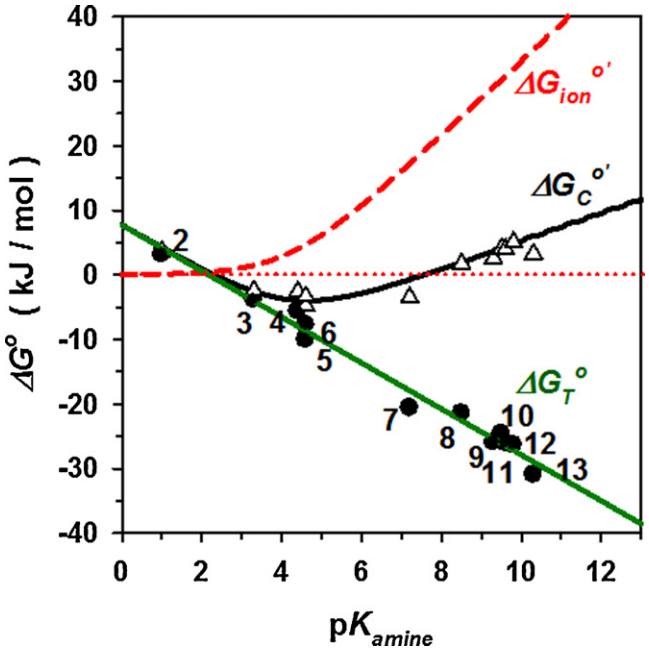


Fig. 2. Dependence of the effective standard Gibbs energy change $\Delta G_C^{o'}$ (solid line, Δ) and of its components: the thermodynamic unionized convention ΔG_T^o (dash-dotted line) and the neutralization contribution $\Delta G_{ion}^{o'}$ (dash line) for direct condensation between phenylacetic acid and amino compounds (Scheme 2 on the pK of their aminogroup at optimal pH, 298 K, IS 0.1 M. The curves were calculated according to the Eqs. (4)–(6) using experimental data (Tables 1 and 2).

that finally determines the increased total Gibbs potential $\Delta G_C^{o'}$. Evidently, a large difference in the pK values of the carboxylic and amino groups makes direct condensation thermodynamically unfavorable. Thus, the equilibrium is shifted to the synthesis for condensations of carboxylic acid with weakly basic amines [6,9], and, on the contrary, the condensation of carboxylic acid with highly basic amines is unfavorable [3,21].

The "real", practically important value of Gibbs potential change $\Delta G_C^{o'}$ can be obtained by summarizing ΔG_T^o (pK_{amine}) (Fig. 2, dash-dotted line) and ΔG_{ion}^o (pK_{amine}) (dash line) (see Table 2). The resulting effective $\Delta G_C^{o'}$ calculated at optimal pH for amide bond synthesis, represents a turned bell-shaped dependence upon the pK of amino group of acyl acceptor with a narrow minimum equal to −6.3 kJ/mol at pK 4–5 (Fig. 2, solid line). According to the obtained dependences the condensation of phenylacetic acid (**1**) and amines with pK value within range of 1.5–8.5 is thermodynamically favorable ($\Delta G_C^{o'} < 0$) at 298 K, IS 0.1 M. However even for the most favorable synthesis a high degree of conversion in a homogeneous reaction mixture could be attained only by using significant excess of phenylacetic acid. The precipitation driven synthesis [34,35] can be also an efficient tool to improve the conversion via direct condensation. Systematic investigation of the effect of physicochemical characteristics of both carboxylic acid and amino compound, and the target product on the equilibrium apparently could allow to estimate thermodynamic parameters of amide bond synthesis and to find out optimal reaction conditions on the basis of accessible properties of initial reagents.

4. Conclusions

Analysis of the different components of standard Gibbs energy change has revealed thermodynamically favorable synthesis of N-phenylacetamides from unionized species. However the ionization of a carboxy group of phenylacetic acid and amino group of amino compound in aqueous solution seriously influences the overall Gibbs potential pushing equilibrium in the aqueous reaction systems to hydrolysis. This effect is especially remarkable in case of highly basic amines. A linear correlation between the standard Gibbs energy change for amide bond formation from the unionized reagents species and the basicity of amino group was observed, which allows to predict the thermodynamic parameters for direct condensation of phenylacetic acid with any amine of known pK. In homogeneous aqueous solution condensation of phenylacetic acid and amines with pK values within 1.5–8.5 is thermodynamically favorable and can be used for preparative synthesis using penicillin acylase as a catalyst.

5. Materials and methods

5.1. Materials

Phenylacetic acid and 3-phenylpropylamine were products of Aldrich; O-methylhydroxylamine-hydrochloride, (S)-phenylglycinol and (R)-1-(2-naphtyl)ethylamine were products of Fluka; glycine, (S)-phenylalanine and 3-aminobenzoic acid were products of Acros; (R)-1-phenylethylamine and (S)-phenylalaninamide were products of Sigma; aniline, p-nitroaniline, 4-aminobenzenesulfonic acid and phenylacetamide were products of Reakhim. N-phenylacetyl-derivatives of amines were prepared as described [12]. Wild type penicillin acylase from *E. coli* was purified as described earlier [22].

5.2. HPLC analysis

Concentrations of the reactants were determined on a Perkin Elmer HPLC-system Series 200 at 210 nm using a reversed phase column: Chrompack Nucleosil 100 C-18 5 μ, 150 mm × 4.6 mm, Phenomenex Luna C-18 5 μ, 250 mm × 4.6 mm, or Kromasil Ethernity C18 5 μ, 250 mm × 4.6 mm. Mobile phase consisted of 7 mM phosphate buffer pH 3.0, acetonitrile (26–50%, v/v) and 0.7 g/l of sodium dodecylsulphate. Retention time was (in min): (a) for the eluent with 50% CH_3CN (Phenomenex column, flow rate 0.5 ml/min) –phenylacetic acid (9.3), N-phenylacetyl-O-methylhydroxylamine (7.0); 3-aminobenzoic acid (7.5), N-phenylacetyl-3-aminobenzoic acid (14.5); phenylalanine (7.8), N-phenylacetyl-phenylalanine (18); aniline (7.9), N-phenylacetyl-aniline (19); p-nitroaniline (12), N-phenylacetyl-p-nitroaniline (45); (b) for the eluent with 40% CH_3CN (Kromasil column, flow rate 0.7 ml/min) –phenylalanine (5.8), phenylacetic acid (7.3); N-phenylacetyl-phenylalanine (8.7); (c) for the eluent with 20% CH_3CN (Phenomenex column, flow rate 0.5 ml/min) –4-aminosulfonic acid (3.9), phenylacetic acid (4.7), N-phenylacetyl-4-aminosulfonic acid (10.4); (d) for the eluent with 26% CH_3CN (Chrompack column, flow rate 1.0 ml/min) –phenylacetic acid (7.8), N-phenylacetyl-phenylglycinol (13.8), phenylglycinol (18); (e) for the eluent with 40% CH3CN (Chrompack column, flow rate 1.0 ml/min) –phenylacetamide (2.9), phenylacetic acid (4.4), 3-phenylpropylamine (10), N-phenylacetyl-3-phenylpropylamine (20), 1-phenylethylamine (10.5), N-phenylacetyl-1-phenylethylamine (19), 1-(2-naphthyl)ethylamine (16.6), N-phenylacetyl-1-(2-naphthyl)ethylamine (33.4).

5.3. Solubility measurements

The solubility of amides in water was determined by stirring an excess of amide suspension during 1 h in a thermostatted cell of a pH-stat (Titrino 718, Metrohm, Switzerland) at corresponding pH, 298 K, 0.1 M KCl. Then suspensions were centrifuged and supernatants were taken for HPLC-analysis.

5.4. Determination of the ionization constants of amino groups

The pK value of amino groups of analyzed amino compounds was determined by both acidic and alkaline titration of 0.1 M aqueous solution of amine (total volume, 10 ml) containing 0.1 M KCl. The titration was carried out in a thermostatted cell of a pH-stat (Titrino 718, Metrohm, Switzerland) at 298 K using 2.32 M KOH or 2.61 M HCl solution as a titrant. The pH was changed in the range of pH 2–12. The titration curves were simulated in MathCad 7Pro. The ionization constants determined from both acidic and alkaline titration curves differed from each other no more than by 0.1.

5.5. Determination of the equilibrium position for amines condensation with phenylacetic acid

Determination of the effective equilibrium position at the condensation of amines with phenylacetic acid was performed by monitoring the concentration change of all reaction components in reaction mixtures of different composition created to achieve equilibrium from synthesis and hydrolysis side. Each of the reactions was carried out in a thermostatted cell of a pH-stat (Titrino 718, Metrohm, Switzerland) at pH-value close to the half of the sum of the pK values of the carboxylic and amino groups, at 298 K, IS 0.1 M in aqueous medium (total volume 5 ml) in the presence of 10 μM PA under permanent stirring. The ionic strength (IS) value for each reaction mixture was adjusted to 0.1 M by 3 M KCl solution. The enzymatic activity in each reaction mixture was monitored until the end of conversion in order to be sure that enzyme inactivation did not take place until equilibrium position was reached. The equilibrium was assumed to be reached when the concentrations of all of the reaction components stayed constant during last 30 h of the

reaction time. In order to exclude errors in determination of equilibrium concentrations two two samples of the liquid phase of each reaction mixture were prepared by withdrawing the aliquots via a Chromafil 0.45 μm filter (Bester, Amstelveen, The Netherlands) and were diluted by the eluent in order to obtain samples with the concentrations of the analyzed components in the range 5 μM to 0.5 mM, preferably 0.02–0.1 mM. When the equilibrium concentration of the amide was more than 10 times lower than that of the amine and phenylacetic acid the withdrawn samples were analyzed in the following way: the concentration of the amide was determined after lower dilution, whereas the concentrations of amine and acyl donor were determined after higher dilution; in both cases the analyte concentration was in the range 0.02–0.1 mM. The obtained samples were subjected to HPLC-analysis to determine the average concentrations of each reaction component based on corresponding calibration graphs and to calculate the effective equilibrium constant values (Table 2). Each of the calibration graphs was linear through origin in the concentration range 5 μM to 0.5 mM with the correlation coefficient not less than 0.9985.

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