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Kinetics and Mechanism of the Peptide Synthesis in Solution

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Abstract—The kinetics of the reaction of Boc-Xaa fluorophenyl esters (where Xaa = Ala, Val, Phe, Ser, Leu, Gly, Met, Pro, or Ile) with leucinamide was studied in order to measure changes in fluorescence emission at 375 nm of the fluorophenyl chromophore accompanying the reaction. It was found that the experimental kinetic data could not be described by a simple scheme of the second order reaction. Measurements of the kinetic parameters of the reaction at various initial concentrations of reagents indicated that the reaction rate can be expressed as: $v = kC_N^a C_{AE}^b$, where k is the reaction rate constant, C_N is the concentration of leucinamide, and C_{AE} is the concentration of fluorophenyl ester. The a and b reaction orders were close to 1/2 and 3/2 for Xaa = Ala, Val, Phe, Ser, or Leu, 1/2 and 1 for Gly, Met, or Pro, and 1 and 2 for Ile. The experimental equations for the reaction rate can theoretically be derived from a single scheme of chain reactions with various deactivation ways for active intermediates.

Key words: *fluorescence, mechanism of peptide bond formation, peptide fluorophenyl esters, peptide synthesis, reaction kinetics*

INTRODUCTION

Formation of the peptide bond between two amino acids is one of the fundamental chemical reactions studied in life sciences.[2] Nowadays, the mechanism of peptide bond formation during the ribosomal protein synthesis is the subject of particularly intensive studies. Recent advances in the area of ribosome spatial structure would undoubtedly rekindle interest in the more comprehensive studies of the mechanism of peptide bond formation [1].

The mechanisms of peptide bond formation, other than the ribosomal mechanism, are also studied. For example, Huber and Wachterschauser have recently demonstrated that amino acids can be activated for peptide bond formation under geochemically acceptable conditions (aqueous medium, 100°C, pH 7–10) with the use of carbon monoxide in the presence of a catalyst [2]. They directly associated the mechanism of peptide bond formation with the possibility of abiogenous protein synthesis and with the origin of life on Earth.

Knowledge of kinetics and the mechanism of the reaction of peptide bond formation is also very important from the practical point of view, especially for the combinatorial peptide chemistry, since an insufficient information in this area results in failures in formation of peptide libraries [3].

Unfortunately, there are few publications devoted to the kinetics of the peptide synthesis in solution. Emphasis is placed on the solid phase peptide synthesis and, in this connection, on the development of instrumental methods, such as the short-wavelength IR spectroscopy [4].

Here, we studied the mechanism of peptide bond formation in organic solvents. The use of organic solvents is connected with the chosen method of the amino acid activation (Pfp esters). In future studies, we plan to carry out similar experiments in aqueous medium using the hydrophilic *p*-sulfotetrafluorophenyl esters, which we proposed previously [5, 6].

Although active esters of protected amino acids are used widely in peptide synthesis, the mechanism of peptide bond formation by this method is not completely understood [7, 8]. Most kinetic studies in this area were performed on the basis of the presumption that this reaction proceeds the second order mechanism [7]. This approach was applied to most of the comparative studies when the solution required no changes in the starting reagent concentrations. For example, Kovacs *et al.* [9–11] obtained important information on the dependence of racemization on the relative parameter of the ester activation using the proposal about the second order of the reaction.

Possible mechanisms of peptide bond formation were comprehensively discussed by Kemp *et al.* [12]. The rate constants for 41 reactions of aminolysis of *p*-nitrophenyl esters of *N*-protected amino acids in DMF were obtained. All rate constants, except those for the reaction of proline derivatives used as nucleophiles, were approximated by the product of two partial rate constants. The aminolysis in DMF was shown to follow

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[2] Abbreviations: CHA, cyclohexylamine; Pfp, pentafluorophenyl; Tfc, *p*-chlorotetrafluorophenyl; and Tfp, tetraflorophenyl.

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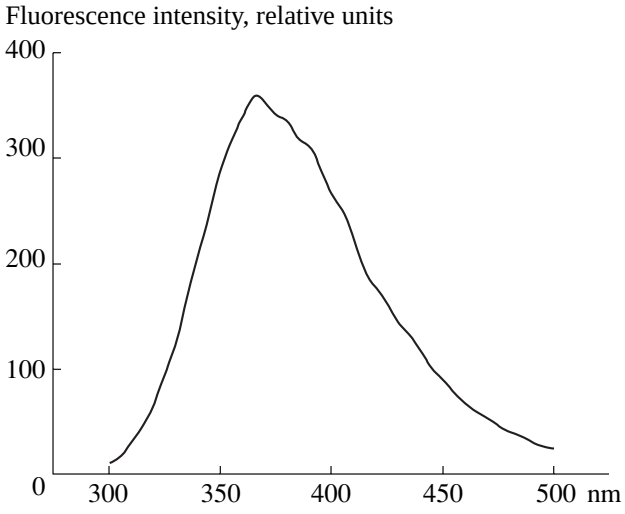

Fig. 1. Fluorescence spectrum of Boc-Ala-OPfp in DMF after excitation at 280.4 nm.

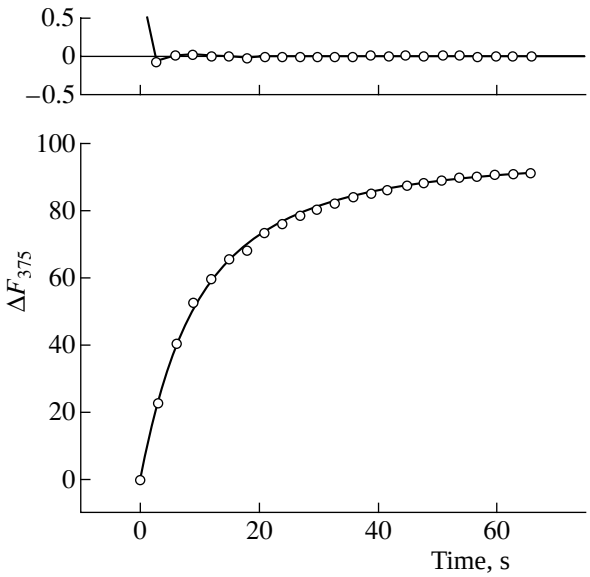

Fig. 2. Time dependence of fluorescence intensity at 375 nm in the course of the reaction between Boc-Ala-OTfp (1.5 mM) and $LeuNH_2$ (10 mM) in DMF in the presence of triethylamine (40 mM) at 20°C. Experimental data are marked by points. The theoretical curve was calculated using equations (1). The distribution of the differences between the experimental and theoretical values is given in the upper part of the figure.

the kinetics of the first order relative to amine. A linear dependence of the rate constant of the pseudofirst order on the amine concentration was observed in all cases, which suggests that the rates of these reactions depend on concentrations of both amine and active ester. A 10% increase in the rate constants of the second order was found when low concentrations of a nucleophile were used. This effect was explained by the possible presence of traces of dimethylamine in the reaction mixture.

Horiki and Murakami [13] studied the catalytic action of potassium salt of 1-hydroxybenzotriazole on the formation of peptide bond in THF when dimethylamine was evidently absent. They found a substantial deviation from the mechanism of the second order reaction for the coupling of 2,3,5-Tfp ester of Z-phenylalanine and alanine *p*-nitroanilide (without any catalyst) and concluded that this reaction follows a more complicated mechanism. Earlier, Menger and Smith [14] studied the reactivity of various aromatic esters and obtained similar results. The reaction orders of each of the components were not determined in this study. The complexity of the mechanism of amide bond formation and closely related reactions is well documented in organic chemistry (see review [15]).

When studying relative reactivities of fluoroaromatic esters with various levels of fluorine substitution, we failed to measure the rates of all reactions with the same reagent concentrations [6, 16], because we had to change the initial concentrations of both nucleophile and active ester to increase the reaction rate or to generate an appropriate level of fluorescent signal. A substantial dependence of the rate constant of the second order on the initial concentrations of reagents was observed. This circumstance required the measurement of the reaction orders for both components of the reaction [16]. We found that the reaction rate of the standard coupling of fluoroaromatic esters of Boc-Ala with leucinamide or valine methyl ester corresponds to the following equation:

$$v = kC_N^{1/2}C_{AE}^{3/2}, \quad (1)$$

where k is the reaction rate constant, C_N is the nucleophile concentration, and C_{AE} is the concentration of active ester. This equation suggests the complicated chain-reaction mechanism.

In this work, we present the results of our further studies in this area. These demonstrate that not only the rates but also the reaction orders depend on definite pairs of amino acid derivatives. Probably, several versions of peptide bond formation exist, and their choice depends on the nature of interacting amino acids.

RESULTS AND DISCUSSION

Active fluorophenyl esters have similar fluorescence emission spectra. The example of the emission spectrum of Boc-Ala-OPfp is given in Fig. 1. The reaction of an active fluorophenyl ester with amino acid derivatives causes a decrease in the fluorescence intensity at 375 nm. This fact was used for the kinetic study of the reaction [16]. The change in the fluorescence during the reaction between Boc-Ala-OTfp and $LeuNH_2$ is illustrated in Fig. 2. Pfp esters demonstrate the same changes, but their fluorescent signal is weaker. The theoretical curve calculated using equation (1) was fitted

to experimental points by modifying the *k* rate constant. The reaction orders in equation (1) (1/2 and 3/2) were obtained from logarithmic dependencies of the initial rate (v) on the reagent concentrations (Fig. 3) as described in our previous paper [16]. The following are the criteria for equation (1) correctness for the description of experimental data: a close correlation between the experimental data and the calculated curve (Fig. 2, upper panel) and the independence of the *k* rate constant and the reagent concentrations (Fig. 4).

Similar measurements were performed for the reaction of leucinamide with another type of active esters, Boc-Xaa-OPfp, i.e., Pfp esters of the following Boc-protected amino acids: alanine, glycine, methionine, phenylalanine, isoleucine, proline, serine, and leucine. PfpOAc was also used. The reaction orders and rate constants for these reactions (measured using the method described above) are given in the table. One can see that the reaction orders are 1/2 and 3/2 in most cases (Xaa = Ala, Val, Phe, Ser, Leu, or Ac). In some cases, the reaction orders can be 1/2 and 1 (Xaa = Gly, Met, and Pro) and even 1 and 2 (Ile). Usual reaction orders (1 and 1) were found when leucinamide was replaced by stronger nucleophile such as CHA, which can be regarded, to a first approximation, as a topological analogue of most amino acids (see the two examples in the lower part of the table).

Note that the regularities observed in our experiments did not depend on the nature of fluoroaromatic ester. For example, the Pfp, Tfp, and Tfc esters of Boc-Ala display similar reaction orders in their reaction with $LeuNH_2$, although they had different reaction rates due to the different electrophilicity of the leaving group.

The half-integer orders of reaction are characteristic of chain reaction processes with various mechanisms of chain termination [17]. Such chain reactions usually imply the participation of free radicals that cannot exist in our systems. Previously, we proposed the following mechanism for reactions with the 1/2 and 3/2 orders:

$$\text{(a) } N + AE \longrightarrow (AE \times N)^*,$$
$$\text{(b) } N + (AE \times N)^* \longrightarrow (N \times AE \times N)^*,$$
$$\text{(c) } (N \times AE \times N)^* + AE \longrightarrow AN + (AE \times N)^* + E^- + H^+, \quad (2)$$
$$\text{(d) } 2(N \times AE \times N)^* \longrightarrow 2AN + 2N + 2(E^- + H^+).$$

Here: (a) The formation of the activated tetrahedral complex (AE × N)* between the active fluorophenyl ester AE and amino acid N.

(b) The interaction of N with the complex (AE × N)* that follows the chain initiation and leads to the formation of the triple activated complex (N × AE × N)*; the possibility of the formation of such a complex was repeatedly discussed by Johnson [15].

(c) The interaction of the (N × AE × N)* triple activated complex with the active fluorophenyl ester (AE) results in the formation of the reaction product (AN), intermediate (AE × N)*, phenolate E^-, and free proton H^+.

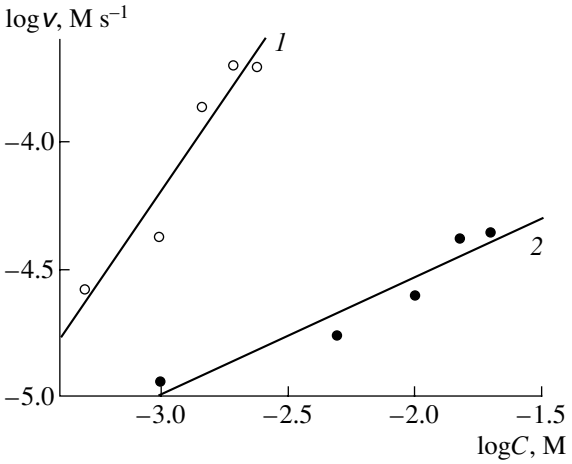

Fig. 3. The logarithmic dependence of the initial rate of the peptide bond formation in the reaction of Boc-Ala-OTfc with $LeuNH_2$ on the concentration of (*1*) $LeuNH_2$ (C_{AE} 0.5 mM) and (*2*) Boc-Ala-OTfc (C_N 20 mM). The reaction was carried out in DMF in the presence of triethylamine (40 mM) at 20°C.

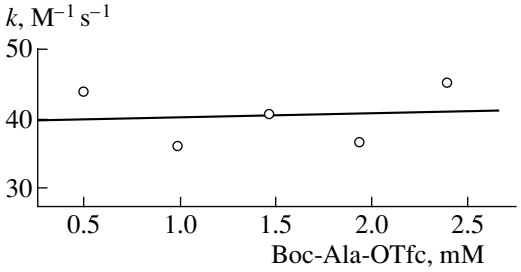

Fig. 4. The dependence of the rate of the reaction of Boc-Ala-OTfc with $LeuNH_2$ (20 mM) in DMF in the presence of triethylamine (20 mM) on the concentration of Boc-Ala-OTfc.

(d) The interaction of two triple activated complexes (N × AE × N)* results in their decomposition with the formation of reaction product (AN), free amino acid (N), E^-, and H^+.

This hypothetical reaction scheme leads to equation (1) for the reaction rate. The activated intermediate states indicated by asterisks can be the charged dipole activated complexes.

Various reaction orders can be derived when changing the last reaction in scheme 2 (the deactivation of the intermediate state). For example, if the reaction (d) were replaced by the reaction

$$\text{(e) } 2(N \times AE \times N)^* + AE \longrightarrow 3AN + N + 3(E^- + H^+),$$

the equation for the reaction rate would be:

$$v = kC_N^{1/2}C_{AE}, \quad (3)$$

Orders and rate constants of the reactions of peptide synthesis in DMF in the presence of 40 mM triethylamine*

AE	N	*a*	*b*	*k*, $M^{-1}s^{-1}$
BocAlaOPfp	$LeuNH_2$	0.70 (1/2)	1.53 (3/2)	33.6 ± 8.3
BocGlyOPfp	"	0.55 (1/2)	0.92 (1)	0.86 ± 0.12**
BocMetOPfp	"	0.52 (1/2)	1.04 (1)	0.36 ± 0.06**
BocValOPfp	"	0.51 (1/2)	1.60 (3/2)	0.32 ± 0.04
BocPheOPfp	"	0.67 (1/2)	1.31 (3/2)	16.9 ± 3.0
BocIleOPfp	"	0.79 (1)	1.83 (2)	28.2 ± 4.8
BocProOPfp	"	0.46 (1/2)	1.11 (1)	0.18 ± 0.01**
BocSerOPfp	"	0.56 (1/2)	1.31 (3/2)	8.2 ± 1.0
BocLeuOPfp	"	0.57 (1/2)	1.36 (3/2)	10.6 ± 1.6
AcOPfp	"	0.58 (1/2)	1.49 (3/2)	7.7 ± 1.5
BocIleOPfp	CHA	0.72 (1)	1.13 (1)	2.1 ± 2.0
AcOPfp	"	0.90 (1)	0.89 (1)	19.3 ± 3.5

* The experimental kinetic data were approximated using the following equation: $v = kC_N^a C_{AE}^b$, where *k* is the reaction rate constant, C_N is the concentration of a nucleophilic reagent, and C_{AE} is the concentration of active ester. The values of *k*, *a*, and *b* were determined by the method described in [16].

** $M^{-1/2}\ s^{-1}$.

whereas the replacement of reaction (d) by the reaction

$$\text{(f) } (N \times AE \times N)^* \longrightarrow AN + N + (E^- + H^+),$$

gave the equation of the reaction rate

$$v = kC_N C_{AE}^2. \quad (4)$$

Thus, the experimental results can be described by one and the same kinetic scheme (2), to which at least two additional reactions [(e) and (f)] of the deactivation of activated intermediate states should be added. One can expect that the reactions of the active esters Boc-Xaa-OPfp with $LeuNH_2$ mainly proceed via the deactivation pathway (d) (for Ala, Val, Phe, Ser, Leu, and PfpOAc); the pathway (e) is valid for Xaa = Gly, Met, and Pro; and the (f) pathway for Xaa = Ile.

The important conclusion from this study is that the direct comparison of various reactions of peptide synthesis with the use of virtual rate constants is often inadequate, because the reaction mechanism may be unique in each special case. One possible solution to this problem may be a comparison of the initial rates of these reactions.

EXPERIMENTAL

Fluorophenols of 99% purity (Perm division of the Institute of Applied Chemistry), Boc-protected amino acids, and leucinamide hydrochloride (Reanal, Hungary) were used in this study. Fluorophenyl esters of the Boc-protected amino acids were synthesized according to procedures described previously [6, 10, 18, 19]. DMF was distilled in a vacuum with the addition of ninhydrin (0.5 g per liter) and stored at 4°C. Triethylamine was distilled over potassium hydroxide.

Fluorescence spectra were recorded at 20°C on a laboratory spectrofluorimeter whose construction was described in [20]. The fluorescence of active esters was excited at 280.4 or 296.7 nm and measured from the front surface of a quartz cell. The fluorescence intensity was measured at 375 nm as a time function after mixing the solutions in order to study the kinetics of the reactions. Virtual rate constants of the reactions of active esters were determined according to the reaction mechanisms described above with the use of scheme of nonlinear regression [21].

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