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Thermodynamic Characteristics of Plasminogen Activation by Indirect Activators

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Abstract—Several indirect plasminogen (Pg) activators are known including streptokinase and the monoclonal antibody IV-Ic, whose mechanism of activation is well studied. To characterize thermodynamically the activation of Pg by streptokinase (SK) and the monoclonal antibody (mAB) IV-Ic, the activation energies were calculated for various reaction stages. Activation energy of 7.4 kcal/mol was determined for the interaction of the chromogenic substrate S-2251 with plasmin (Pm) and activated equimolar complexes Pm–SK and Pg*SK at the steady-state reaction stage, and 18.7 kcal/mol with the complexes Pg*IV-Ic. A 2.5-fold increase in the energy of activation for the Pg*IV-Ic complex suggests a more intricate mechanism of its interaction with the substrate. At the stage of increasing active center concentrations and the formation of activated complexes Pg*SK and Pg*mAB IV-Ic, the activation energy was found to be 10.5 and 38 kcal/mol, respectively. At this reaction stage the conformational rearrangement of Pg molecule with the formation of active center is the limiting stage determining the reaction rate. Unexpectedly high energy of activation at the second stage of interaction between mAB IV-Ic and Pg suggests several simultaneous reactions and complexity of conformation rearrangement in the Pg molecule in activated complexes, thus requiring large energy expense. Formation of the active center is probably accompanied by its transition within a narrow temperature range into another conformation state with the change in activation parameters of the reaction. Quantitative evaluation of the studied reactions from the perspective of thermodynamics of the enzymatic reactions gives more comprehensive characteristics of the activation mechanism.

Key words: energy of activation, plasminogen, plasmin, streptokinase, monoclonal antibody

The plasminogen (Pg)/plasmin (Pm) system is one of most important proteolytic systems in mammals. It is involved in regulation of multiple physiological and pathophysiological processes. Pg is the inactive precursor of plasmin, a fibrinolytic enzyme (EC 3.4.12.7). The mechanism of Pg activation is selective to multidomain molecular organization of Pg, and the generated Pm possesses specificity and high proteolytic activity. The main biological function of the Pg/Pm system is fibrinolysis, maintaining the blood in the fluid state.

Streptokinase (SK), a bacterial protein widely used in thrombolytic therapy, is an indirect plasminogen activator. SK interacts with Pg to form an equimolar complex and induces conformational changes in the proenzyme resulting in active center formation without cleavage of the activation peptide bond. The catalytically active complex (Pg*SK) effectively converts Pg into Pm via limited proteolysis [1, 2]. A quantitative kinetic analysis of Pm formation under the action of SK is given elsewhere [3]. Conformational changes in the Pg molecule are shown to be reversible, and the activation mechanism is a rapid equilibrium multistage process. The kinetic curve of chromogenic substrate hydrolysis is parabolic when Pg is activated by SK. The initial rate (v_1) characterizes the processes of both SK binding and conformation changes in the Pg molecule followed by increase in activation rate (v_2) [3, 4]. An open conformation of Pg Lys-form with exposed lysine-binding sites is most subject to interaction of SK with these sites during the activation process. The affinity of SK to Lys-Pg is 12-fold higher than to Glu-Pg because of the contribution of lysine-binding sites [4]. The rate of activation of Lys-Pg is slowed by 100%, and that of Glu-Pg by 25% in the presence of 10 mM 6-aminohexanoic acid. Lysine-binding sites of kringles 1-4 in plasminogen are necessary for providing high activation rate of plasminogen by SK and are important at all stages of the activation [5]. The interaction of kringle 5 with the B-domain of SK is necessary for rapid formation of Pg–SK complex, and kringle 5 is important for high-affinity binding [6, 7].

Abbreviations: SK) streptokinase; Pg) plasminogen; Pm) plasmin; mAB) monoclonal antibody; Pg*) activated plasminogen.

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Only native SK can provide complete activation of Pg. The dissociation constant of SK(1-378)–Pg complex increases by 30%, and amidolytic activity of the complex decreases by 80%, and the ability to activate Pg decreases by 80% [8]. The lack of C-terminal lysine in the SK molecule decreases its stability, and the activation rate of Glu-Pg is decreased threefold [5].

Like SK, the anti-plasminogen monoclonal antibody (mAB) IV-Ic possesses ability to activate Pg in indirect mode [9]. It is a suitable model for studies on non-enzymatic activation mechanisms of serine protease proenzymes.

Anti-plasminogen ABs are found in blood plasma of persons treated with SK. The appearance of autoantibodies similar to IV-Ic sometimes occurs in patients infected by streptococci and staphylococci. Pg–IV-Ic-like complexes are potentially dangerous for the human body [10]. Activation of Pg by mAB IV-Ic begins after a long lag-phase. The Pg–IV-Ic complex is formed in several minutes, but long activation time is required for additional interactions and conformational changes within the Pg–IV-Ic complex. It has been suggested that Pg exhibits amidase activity in the Pg–IV-Ic complex due to induction of catalytic activity in an active center locus under the action of IV-Ic. Pg*IV-Ic-active complex possesses activator activity and activates Pg–IV-Ic complexes via a feedback mechanism [11].

The V709-G718 epitope in mAB is shown to almost completely overlap one of six SK-binding sites in Pm B-chain localized in the site N711-E724 of the amino acid sequence of Pm [12]. Both SK and mAB IV-Ic can alter the catalytic activity of Pm [13].

The aim of present study was to give more complete characteristics of the mechanism of Pg activation by indirect activators and quantitative estimation of this process from the enzymatic thermodynamics point of view. We have calculated the energies of activation for the interaction of Pm and equimolar activated complexes Pm–SK, Pg*SK, and Pg*IV-Ic with chromogenic substrate S-2251 and the energy of activation for distinct stages of Pg activation by equimolar amounts of SK and mAB IV-Ic.

MATERIALS AND METHODS

Glu-Pg was isolated from plasma of volunteer blood taken with citrate by affinity chromatography on lysine-Sepharose (Pharmacia, Sweden) [14] followed by gel filtration on Sephacryl S-200 (Amersham Bioscience, USA).

Pm was produced by activation of Glu-Pg using urokinase (Sanofi Winthrop, France) immobilized on BrCN-Sepharose-4B (Pharmacia) [15]. The percentage of active centers in Pm and its derivatives was determined as described in [16].

SK (Kabikinase) (Pharmacia) was purified by affinity chromatography on Blue Sepharose CL-6B (Pharmacia) [17]. The purity of the protein was tested electrophoretically by SDS-PAGE [18].

Anti-plasminogen mAB IV-Ic was produced in the Department of Molecular Immunology of the Institute of Biochemistry of the Ukrainian National Academy of Sciences according with the routine technique using electrophoretically homogeneous human Glu-Pg as antigen [19]. Productivity of IV-Ic clones was 4.9 ± 0.051 μg/ml of nutritive medium. IV-Ic was isolated from culture medium after removal of the cells by two-step affinity chromatography on protein A-Sepharose (Amersham Bioscience) and Glu-Pg-Sepharose [9]. The IV-Ic was electrophoretically homogeneous. Immunochemical analysis showed that immunoglobulin IV-Ic belongs to IgG1 isotype. The characteristic feature of mouse immunoglobulins IgG1, IgG2a, and IgG2b of IgG class and human IgG1 class is the presence of C-terminal lysine in their γ- and κ-chains [20].

Amidolytic activity of complexes Pm–SK, Pg*SK, Pg*IV-Ic, and Pm was determined by the rate of *p*-nitroaniline release during hydrolysis of S-2251 substrate (H-D-Val-L-Leu-L-Lys-*p*-nitroanilide) (Chromogenix, Sweden), whose concentration was determined spectrophotometrically at 405 nm. The reaction was performed in the wells of plates for immunoenzyme analysis. The reaction volume was 0.25 ml. Glu-Pg and mAB IV-Ic were taken at equimolar concentrations of 100 nM; Glu-Pg, Pm, and SK at 10 nM; S-2251 substrate concentration was 0.3 mM.

Effect of temperature on pH in various buffer systems is presented in Fig. 1. The temperature effect on pH is negligible in Na-phosphate buffer system, so this buffer system was used for the reaction of Pg activation.

Rate constants for the formation of activated complexes of Pm/Pg with SK and Pg*mAB were calculated using the second-order equation for equimolar concentrations of the components:

$$k = 1/C_0 \cdot t_{1/2},$$

where C_0 is concentration of complexes; $t_{1/2}$ is half-transformation period determined from kinetics curves.

The period of the complex formation (t) was determined as the time point from which the rate of substrate cleavage becomes constant.

Rate of interaction of Pm and activated complexes with the substrate was calculated from the slope of the curve within the range of constant rate.

The rate of the first phase of the reaction was calculated using kinetic curves of Pg activation by mAB IV-Ic (dependence of absorption at 405 nm (A_{405}) on time) as the value which is reciprocal to lag-period, and the rate of the second phase was calculated as the rate of formation of Pm active centers per minute: $V = ([Pm_2] - [Pm_1])/(t_2 - t_1)$, nM Pm/min.

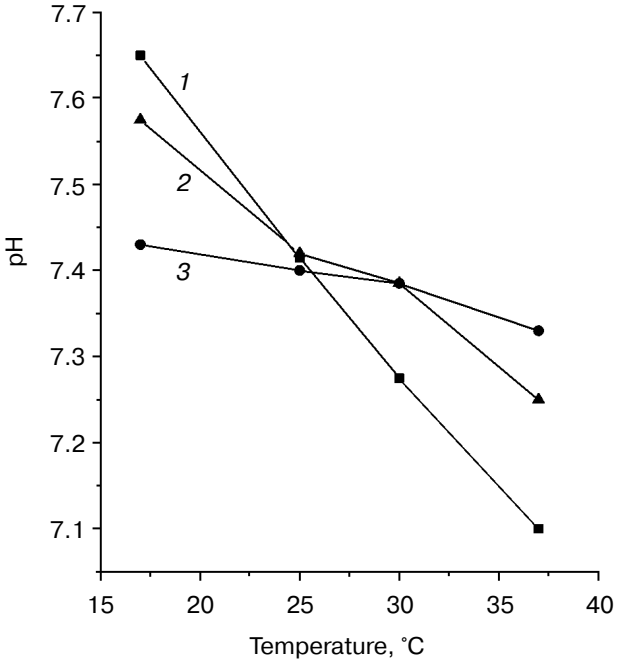

Fig. 1. Influence of temperature on pH in buffer systems: *1*) 0.1 M Tris-HCl; *2*) 0.1 M Tris-H_3PO_4; *3*) 0.1 M Na_2HPO_4/NaH_2PO_4.

Concentration of Pm active centers at time point t was quantitatively determined using a calibration curve drawn in coordinates {Pm concentration (nM), tanα}, where tanα is the tangent of the slope of the calibration curve for hydrolysis of S-2251 substrate by Pm with constant concentration and the tangent to the activation kinetic curve at time point t.

Activation energy was calculated using the Arrhenius equation:

$$\log k = A/T + B,$$

where k is a reaction rate constant; T is temperature, °K; and $\tan\beta = -A$.

From the straight lines $\log V$ (because $V = kc^n$) versus $1/T$ constructed from the experimental data, the tangent of slope was determined (tanβ) and the activation energy was calculated:

$$E = 2.303A \cdot R,$$

where R is the universal gas constant [21].

The data are presented as means; the experimental and calculation errors do not exceed 3%.

RESULTS AND DISCUSSION

The dependences of rate of S-2251 substrate hydrolysis by Pm, Pm–SK complex, and activated Pg*SK complex on temperature are given in Fig. 2. The shape of kinetics curves (*1-3*) suggest that conformational changes in the Pm/Pg–SK complex are not completed at 5°C. Amidolytic activity of Pm–SK complex is not increased in comparison with Pm, and activity of activated Pg*SK does not achieve the maximum. Starting with 10°C, the amidolytic activity of Pm–SK complex increases with respect to that of Pm, and the amount of active centers of Pg*SK complex achieves the maximum concentration, coinciding with that in Pm. The rate of S-2251 substrate hydrolysis by Pm and Pm–SK and Pg*SK complexes increases 3-fold with temperature increasing from 10 to 40°C. Reaction rate increases 6-fold with the temperature increase from 5 to 40°C at the stage of increase in active center concentration in the Pg*SK complex (Fig. 2, curve *4*).

Pm, the protein with conformation of working active center, interacts with substrates at the active center. The enzymatic reaction proceeds with formation of *p*-nitroaniline, a hydrolytic product of the substrate; the kinetic parameters of the reaction are presented in [22].

The energy of activation of the given enzymatic reaction was calculated according the Arrhenius equation (Fig. 3, curve *2*; table) and does not exceed values typical for enzymatic reactions.

The Arrhenius equation is often valid for complex multistage enzymatic processes. In these cases, the energy of activation has no simple physical meaning and represents some function of activation energies of distinct

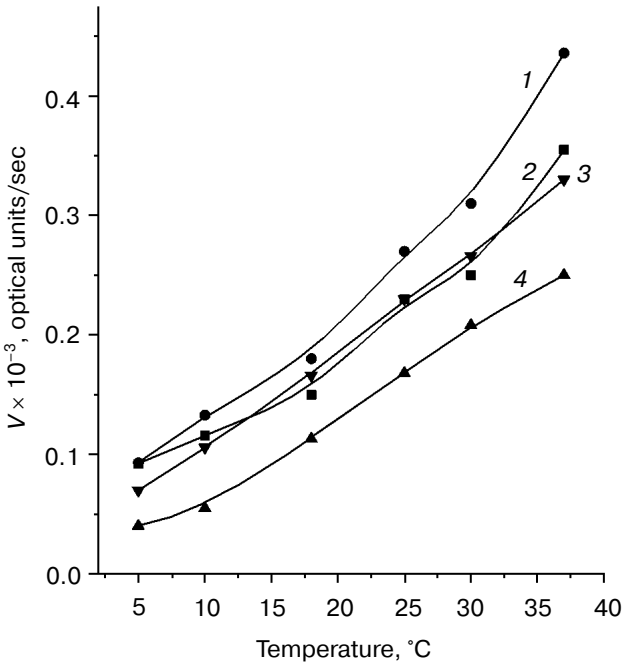

Fig. 2. Influence of temperature on the reaction rate (V) for the interaction between Pg/Pm and SK: *1*) Pm–SK; *2*) Pm; *3*) Pg*SK; *4*) Pg*SK at the stage of complex formation.

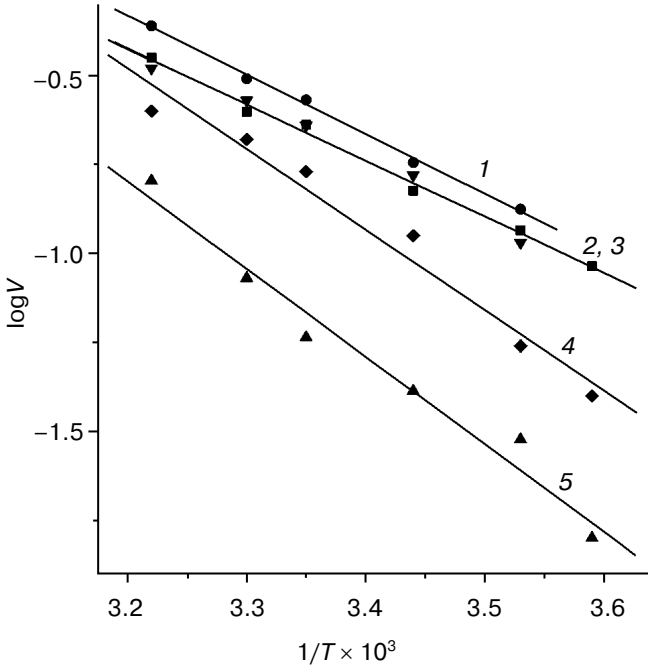

Fig. 3. Arrhenius plots for calculation of the energy of activation for the interaction of Pm and Pm/Pg–streptokinase complexes with the S-2251 substrate (*V* is reaction rate, *T* is temperature, °K): *1*) Pm–SK; *2*, *3*) Pm and Pg*SK; *4*, *5*) Pg*SK at the stage of active center formation in Pg with various amidolytic activity.

stages. Hence, it is correctly called "effective" or empirical energy of activation.

Three stages can be distinguished in the activation reaction, and reaction rates can be calculated from kinetics curves for each of them. The first stage is complex formation and beginning of active center formation and amidolytic activity is exhibited—this is the lag-phase of the reaction. The second phase is increase in active center concentration and, respectively, reaction rate increase up to a constant value—the activation rate. Substrate cleavage continues with a constant rate in the third stage because the active center concentration achieves maximum for the given reaction.

SK has high affinity to Pm [23, 24] and forms Pm–SK complex in several seconds (table). As a result of conformational rearrangement of Pm active center, its amidolytic activity is enhanced (Fig. 2, curve *1*). However, the energy of activation does not change for the interaction between the formed complex and substrate (Fig. 3, curve *1*; table).

More complex conformational alterations with formation of active center take place during activation of Pg by SK. An active complex possessing amidolytic and activator activities is formed as a result of interaction between equimolar amounts of Pg and SK [3, 22]. The affinity of SK to Pg is substantially lower than to Pm [4, 23], and the rate of active Pg*SK complex formation is one order of magnitude less (table). This enables distinguishing a section of the kinetic curve where the concentration of reaction product continuously increases approaching a constant level, i.e., to a linear section of kinetic curve. The tangent to this straight line is equal to the stationary rate. The energy of activation for the interaction of the formed activated complex with the substrate was calculated using stationary reaction rate and from the rate constant of the activated complex formation. The data obtained agree

Kinetic and thermodynamic characteristics of the interaction between plasmin and activated complexes with the S-2251 substrate

Enzymatic complex	Stage of reaction	*A*	E_a, kcal/mol	K_d, M[a]	K_m, M[a]	k, $M^{-1}\cdot sec^{-1}$ [b]
Pm		1.58	7.2		$2.1\cdot10^{-4}$	
Pm–SK	3	1.67	7.6	1.1×10^{-11}	$7.8\cdot10^{-4}$	$1.1\cdot10^{7}$
Pg*SK	2	2.3	10.5	6.2×10^{-7}		
	3	1.58	7.2		$3.2\cdot10^{-4}$	$1.0\cdot10^{6}$
Pg*IV-Ic	1	4.3	19.5	5.0×10^{-8}		
	2	8.1	38.0			
	3	4.1	18.7			$0.02\cdot10^{6}$
Pm–IV-Ic					$0.5\cdot10^{-4}$	

[a] Data from the literature: K_d [23]; K_m [22]; K_m for Pm–IV-Ic [11].
[b] k, at 25°C.

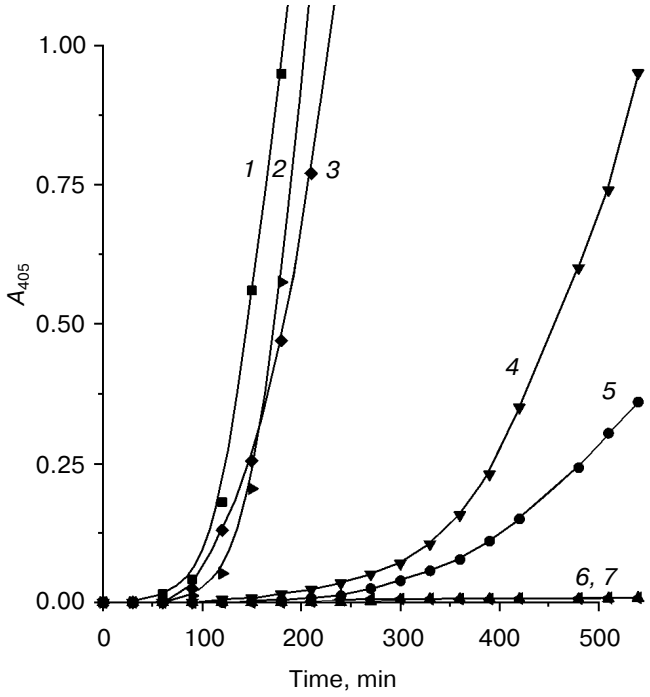

Fig. 4. Kinetic curves for activation of Pg by monoclonal antibody IV-Ic at various temperatures (°C): *1*) 37; *2*) 40; *3*) 34; *4*) 30; *5*) 25; *6*) 19; *7*) 10 (A_{405}, absorption at wavelength 405 nm).

with each other and are equal to the energy of activation in the reaction with Pm (Fig. 3, curves *2* and *3*; table).

Using Arrhenius plots for the second stage of the reaction (Fig. 3, curves *4* and *5*), the energy of activation was calculated; this energy is necessary for complete conformational rearrangement and formation of activated complex with maximum concentration of active centers. The active center formation in the Pg–SK complex requires great energy, and the process is limiting at this reaction stage. The energy of activation 1.4-fold exceeds the values for the interaction between the substrate and Pm– and Pm/Pg–streptokinase complexes at the third stationary reaction stage (table).

The energy of activation is independent of amidolytic enzymatic activity but depends on temperature coefficient of the given reaction. In Fig. 3 the Arrhenius plots for Pg with various activity have virtually the same tangent (curves *4* and *5*), and the energies of activation are 10.5 and 10.9 kcal/mol.

The influence of temperature on Pg activation by mAB IV-Ic is shown in Fig. 4. Activation is not observed at temperatures below 20°C, and the rates of the first and second phases achieve maximum at 37°C. A drastic increase in the rate of active center appearance is observed when the temperature is increased from 30 to 34°C. The rates of the first and third reaction stages increase 4-fold and the rate of the second stage 18-fold when the temperature is increased from 25 to 37°C. The second stage of the reaction is the most influenced by temperature.

Electrophoretic analysis of the reaction mixture containing Glu–Pg and mAB IV-Ic showed that neither the Lys-form nor Pm were formed from Glu-Pg for 2 h after beginning of the activation. This transformation began after 6 h, and a major portion of Pg was transformed into Pm after 20 h [11]. Pg has high affinity to the mAB IV-Ic (table), but amidolytic activity appears after a long lag-period. The rate of the first reaction phase can be considered as the rate of conformational changes resulting in the formation of active center and appearance of amidolytic activity. The energy of activation was calculated from the rate of the first phase of the reaction (Fig. 5, curve *1*; table). The 2.0- and 2.5-fold increase in the energy of activation with respect to the values obtained at the second and third stages of the reaction of interaction between Pg and SK is evidence for more complex mechanism of conformational rearrangements in the Pg molecule for the formation of the active center. It was established that two-center interaction of the paratope IV-Ic with the serine-proteinase domain of Glu-Pg and C-terminal lysine with one of lysine-binding sites on Pg is necessary for manifestation of the catalytic activity in the Pg–IV-Ic complex. An equimolar complex of IV-Ic antibody with V709-G718 antigen is formed first, and then Glu-Pg converts into the open Lys-form Pg conforma-

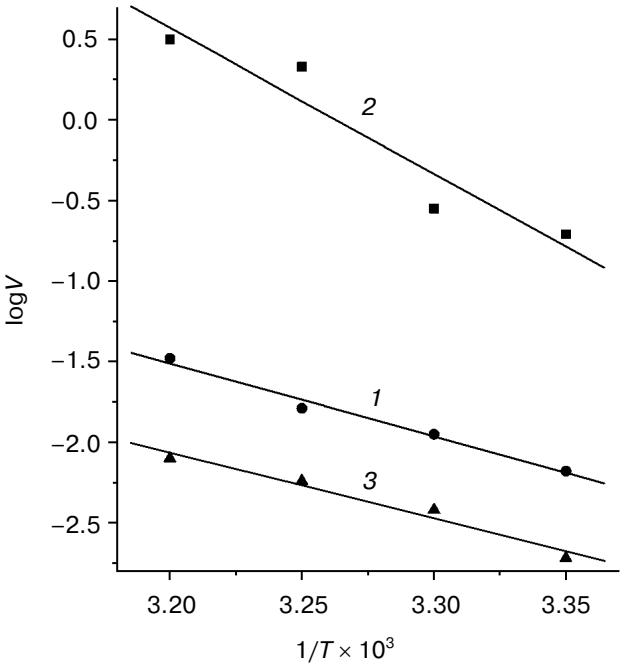

Fig. 5. Arrhenius plots for calculation of activation energy for the interaction between Pg*mAB IV-Ic and S-2251 substrate at three stages of the reaction in the Pg activation process by monoclonal antibody IV-Ic (V, reaction rate; T, temperature, °K): *1*) first stage; *2*) second stage; *3*) third stage of the reaction.

tion. Owing to this transition, the interaction of Lys-binding sites of the first or fourth kringles with the C-terminal Lys on one of the IV-Ic chains becomes possible, thus inducing the formation of the active center in the protease domain [11].

For the second reaction stage the rate of Pm active center formation has been calculated, and using this value the energy of activation was determined, which was 3.5-fold higher than that at the corresponding stage of Pg activation by SK and 2-fold higher than the activation energy value for the enzymatic reactions (table). As seen from Fig. 5, curve *2*, a drastic change is observed between the points at 30 and 34°C. The straight line connecting these points smoothes the tangent and, hence, the activation energy value at this step. The presence of sudden changes like these on the Arrhenius plot can be explained by the changing of the limiting reaction stage, as well as by the transition of the active center of the enzyme molecule over a narrow temperature range into other conformational state with the change in the activation parameters of the reaction.

The energy of activation for the interaction between the activated Pg*IV-Ic complex and substrate was calculated from the stationary reaction rate at the third stage and from the constant of the active complex formation rate; the values are equal and almost 2-fold higher than the corresponding value for the Pg*SK complex (Fig. 5, curve *3*; table).

The energy of activation at all stages of the interaction between plasminogen and mAB IV-Ic exceeds the corresponding values in the reaction of Pg with SK. The kinetic parameters of the interaction of Pm and Pm/PgSK with the substrate significantly differ from corresponding parameters for the Pg*IV-Ic complex. The rate constant for the formation of activated Pg*IV-Ic complex is two orders of magnitude lower than for Pg*SK. The affinity of the substrate to Pm–IV-Ic complex is virtually one order of magnitude higher than it is to the Pm–SK and Pg*SK complexes (table). As stated above, we can only discuss the effective value of the energy of activation, which is, however, the thermodynamic characteristic for this reaction. It confirms the complexity of the mechanism of Pg activation by monoclonal antibody IV-Ic—the need for major energy expenses for conformational changes, active center formation, and interaction between the activated Pg*IV-Ic complex and substrate.

It is known that if one enzyme interacts with several substrates, the energy of activation remains the same in all cases. And vice versa, if one reaction is catalyzed by several enzymes, each enzyme is characterized by its own energy of activation. Thus, the activation energy value is mostly determined by the enzyme rather than substrate characteristic [25]. From the perspective of thermodynamics the enzyme–substrate interaction is not contravened for Pm and Pg/Pm–SK complexes. The activation energy values for their interactions with the substrate remain constant and do not exceed the values for enzymatic reactions. Pm and Pm–SK complexes and Pg*SK can be regarded as the same enzyme. In contrast, the state of active center of the Pg molecule in the complex with mAB IV-Ic is so altered that the Pg*mAB IV-Ic complex can be considered as another enzyme that differs from Pm. The activation energy elevation at all stages of the Pg activation process by monoclonal antibody IV-Ic is evidence for complex mechanism of the reaction. The energy expenses spent on conformational changes in the Pg molecule and on the interaction between the complex and substrate are elevated.

Thus, from the comparison of the obtained values of the energy of activation for the reactions considered here, evaluation of the complexity of the reaction mechanisms from their quantitative thermodynamic characteristics can be made. The conformational rearrangement in Pg molecule and the formation of active center is the most complex and energy consuming process. The rate of this process is limiting. The energy of activation at this stage of the reaction is higher than that at other stages. Unusually high energy of activation in the second phase of the reaction between the mAB IV-Ic and Pm suggests several simultaneous reactions and complexity of the active center formation in the proenzyme molecule. The active center formation is possibly accompanied by transition of the active center of the enzyme molecule into another conformational state over a narrow temperature range with the change in activation parameters of the reaction.

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