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MINIREVIEW

ON THE PLASMINOGEN-ACTIVATING FUNCTION OF STREPTOKINASE

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Abstract—1. Several hypotheses have been advanced to explain the activating function of streptokinase. The predominant hypothesis suggests a stable equimolar streptokinase–plasmin(ogen) complex, activating free plasminogen by an active centre, which is located in the plasmin(ogen) part of the complex.

2. This hypothesis cannot explain a number of phenomena and certain accumulated experimental data, for example: rabbit and bovine plasminogen activation by streptokinase, not forming stable complexes with these plasminogens; possible activation with pH $\leqslant 2$, in the presence of urea, during modification of streptokinase tyrosine residues, i.e. when these two proteins cannot form a stable complex.

3. On the basis of acquired experimental data the following concept is suggested: the activating function of streptokinase is oxygen-dependent and is realised with the help of superoxide radical due to the $O_2^{\cdot}$-generating ability of plasminogen and the $O_2^{\cdot}$-converting ability of streptokinase.

INTRODUCTION

The plasminogen activation mechanism draws, at present, increasing attention due to the major role of fibrinolysis in realisation of numerous physiological and pathological processes for example: thrombogenesis and thrombolysis; tissue growth and differentiation; malignant and metastatic spreading of tumors; and embryo implantation.

We can distinguish two pathways of plasminogen (Pg) conversion into active serine proteinase i.e. plasmin: "proteinase" and "non-proteinase" (Jackson *et al.*, 1980). In the first pathway urokinase and tissue Pg activators, being serine proteinases with high specificity, directly act on the Pg molecule. The second pathway is realised by the addition to Pg of some specific proteins, devoid of proteinase and any hydrolase activity: streptokinase (SK), staphylokinase, and some other factors.

The clarification of the "non-proteinase" pattern is still evidently important for a full understanding of the fibrinolysis regulation pathway, in view of the possible existence of other Pg activation initiators beside SK and staphylokinase.

Proceeding from the above, it is imperative to decode SK activating function, as SK is one of the most effective activators of Pg, synthesized by a number of β-hemolytic streptococci strains of A, C and G serologic groups.

Different hypotheses have been advanced to explain SK activating function. The most well-known are: the hypothesis of direct SK action on Pg (Summaria *et al.*, 1969; Kline and Ts'ao, 1971); the hypothesis of SK interaction with specific protein–proactivator, leading to active Pg activator formation (Ablondi and Hagan, 1958; Takada *et al.*, 1970); the hypothesis of SK–Pg stoichiometric activating complex (Reddy and Markus, 1972; Brockway and Castellino, 1974; Castellino, 1979; Reddy, 1988).

At present, the activating complex hypothesis remains predominant and serves as a scientific explanation of SK action mechanism. The failure, as yet, to discover any enzymatic activity in SK, and its ability to activate very pure Pg samples, in which all impurities are excluded, cause this state of affairs.

This hypothesis is confirmed by the fact, that numerous researches using physical methods proved the formation of stable SK–Pg stoichiometric complex (De Renzo *et al.*, 1963; Davies *et al.*, 1964; Buck and Boggiano, 1971; Summaria and Robbins, 1971). Besides, it has been established, that addition of this complex to bovine, sheep or pig Pgs (poorly activated by SK), causes quick tranformation of zymogens into active plasmin (Castellino, 1979). Also it is already known, that inhibitor treatment (for example, with di-isopropyl fluorophosphate) of Pg part active centre disturbs the activating function of this complex (Buck *et al.*, 1968).

As a necessary pre-condition of Pg activation by SK, this hypothesis advances a stage of stable complex formation between these proteins, the complex itself being the activator of free Pg molecules.

CONTRADICTIONS WITH THE HYPOTHESIS OF ACTIVATING "STREPTOKINASE–PLASMIN(OGEN)" COMPLEX

A lot of experimental data have recently been produced, that contradict, and cannot be explained by this hypothesis. For example, rabbit Pg is known to be effectively activated by SK (Schick and Castellino, 1973). There is some evidence of bovine Pg slow activation, although the complex of the proteins has not been found (Müllertz and Lassen,

Table 1. Effect of urea on streptokinase activity (n-5; fibrin plate lysis method; Nikandrov *et al.*, 1986a)

Urea concentration (M)	Streptokinase activity		Urea concentration (M)	Streptokinase activity	
	10^3 IU/mg protein	%		10^3 IU/mg protein	%
0	120 ± 10	100	5	120 ± 6	100
1	102 ± 6	85	6	125 ± 3	104
2	97 ± 4	81	8	126 ± 7	105
3	102 ± 6	85			

1953). Using the method of lysis of fibrin plates, containing human plasminogen, specifically adsorbed on fibrin fibers, we have found out, that SK is able to activate Pg in the presence of 8 M urea (Table 1) and with pH $\leqslant$ 2 (Fig. 1; Nikandrov *et al.*, 1986a). At the same time, in these conditions SK–Pg stable complex is not formed (De Renzo *et al.*, 1963; Davies *et al.*, 1964; Summaria and Robbins, 1971). We also found, that after some of the tyrosine residue is iodinated, the SK molecule looses its ability (according to gel-chromatography) to form stable complexes with human Pg, but its ability to activate this zymogen is only weakly affected (Nikandrov and Kazyuchits, 1989).

Finally, it is worth pointing out, that the activator–complex hypothesis advances the active centre of Pg as a main functional mechanism within the complex (Buck *et al.*, 1968; Buck and Boggiano, 1971). Obviously, in this case, the activation mechanism should correspond to the serine proteinase catalytic mechanism, a major stage of which is acyl-enzyme formation with the establishment of a covalent bond. Nevertheless, all attempts to discover SK–Pg·Pg ternary complex failed. Only recently (Summaria *et al.*, 1987) has SK–plasmin light chain·Pg complex formation been described. But the last phenomenon does not correspond to the pattern which should have emerged if the case of "serine type" catalysis is to be realised: it must be possible to produce a ternary complex, in which the Pg molecule is not reduced.

All the above phenomena enabled us to assume, that SK–Pg stable complex formation is not necessary for zymogen activation, and that mechanism of this activation may be somewhat different, compared with serine proteinase action.

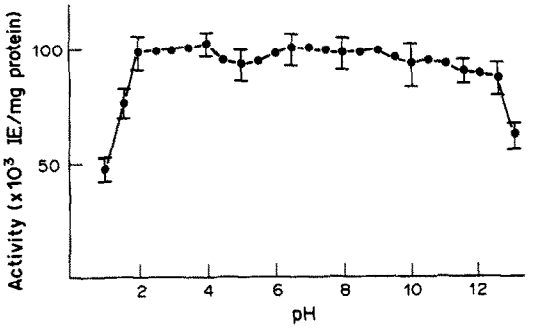

Fig. 1. Effect of solution pH on the activating function of streptokinase; fibrin plate lysis method (Nikandrov *et al.*, 1986a).

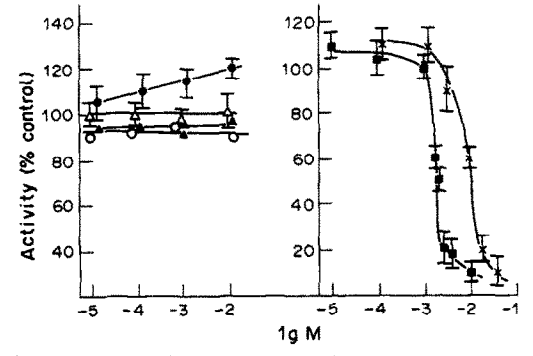

Fig. 2. Effect of singlet oxygen, ·OH or $O_2^{\cdot-}$-radicals scavengers on the activating function of streptokinase (sodium azide ●—●, L-histidine ○—○, L-tryptophan ▲—▲, D-mannitol △—△, nitrotetrazolium blue ■—■, adrenaline ×—×); fibrin plate lysis method (Nikandrov *et al.*, 1987c).

PREMISES OF THE NEW CONCEPT OF STREPTOKINASE-DEPENDENT PLASMINOGEN ACTIVATION

On the basis of accumulated experimental data we could suggest another SK-activating functional mechanism. These data are as follows:

(1) Pg activation by SK (not by urokinase, nor tissue Pg activator) is sharply inhibited by superoxide radical scavengers with 0.01 M concentration (Nikandrov *et al.*, 1987c). ·OH-radical scavengers (mannitol, formate) or singlet oxygen scavengers (sodium azide, histidine, tryptophan) have no inhibiting effect (Fig. 2).

(2) Human Pg activation can be produced in the absence of SK by treating zymogen with H_2O_2 ($\geqslant$0.5 M) (Fig. 3), or with superoxide radical generation systems (Nikandrov *et al.*, 1986d; Nikandrov and Pyzhova, 1989). This activation is less effective than that with SK. Besides, Pg activation by H_2O_2 is inhibited by superoxide radical scavengers (nitroblue tetrazolium, for example), but is not affected by ·OH-radical or singlet oxygen scavengers (Table 2; Nikandrov and Pyzhova, 1989).

Recently, oxidative inactivation of a number of proteinase inhibitors has been described, including that of α_2-antiplasmin and tissue Pg activator inhibitor (Stief and Heimburger, 1988; Stief *et al.*, 1988; Lawrence and Loskutoff, 1986). This phenomenon demonstrates the theoretical possibility of increased proteolytic activity of Pg samples (if they contain trace impurities of tissue Pg activator) by inactivating the named inhibitors. However, an important feature of proteinase inhibitors oxidative inactivation is its inhibition and, consequently, Pg activation inhibition by singlet oxygen scavengers: sodium azide and methionine (Stief and Heimburger, 1988). At the same time, the described phenomenon of Pg activation in the presence of active forms of oxygen is similarly unaffected by these scavengers, like the Pg activation process.

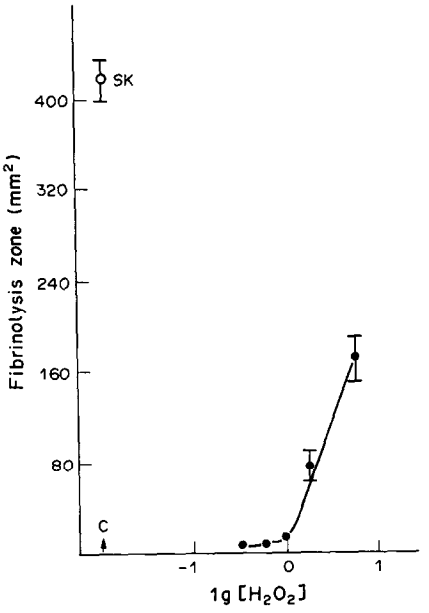

Fig. 3. Effect of H_2O_2 addition to human plasminogen samples on their fibrinolytic activity (C–control, without H_2O_2; SK–plasminogen samples with addition of streptokinase, 750 IU); fibrin plate lysis method (Nikandrov and Pyzhova, 1989).

(3) Pg is able to generate oxygen radicals during H_2O_2 decomposition (Fig. 4; Nikandrov *et al.*, 1986c), or spontaneously, it seems, during slow autooxidation (Fig. 5). Concentration of radicals is weak, and the adrenaline oxidation rate constant with adrenochrom formation in presence of Pg equals $5 \cdot 10^{-5}\,min^{-1}$ (Nikandrov and Pyzhova, 1988b). Human and rabbit Pgs (well-activated by SK) demonstrate the above ability, in contrast to bovine Pg which is more poorly activated by SK.

Table 2. Effect of scavengers of active oxygen species on the human plasminogen activation by H_2O_2 (n-5; fibrin plate lysis method; Nikandrov and Pyzhova, 1989)

Experimental condition	Fibrinolysis zones (mm^2)
Plasminogen	0
Plasminogen + streptokinase, 750 IU	468 ± 20
Plasminogen + H_2O_2, 5.6 M	250 ± 15
Plasminogen + mannitol, 0.01 M + H_2O_2, 5.6 M	240 ± 15
Plasminogen + sodium azide, 0.01 M + H_2O_2, 5.6 M	309 ± 23
Plasminogen + L-tryptophane, 0.01 M + H_2O_2, 5.6 M	282 ± 15
Plasminogen + nitrotetrazolium blue, 0.01 M + H_2O_2, 5.6 M	0

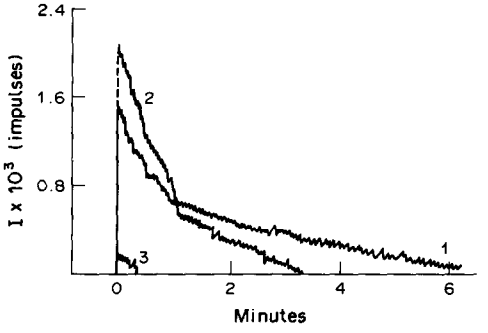

Fig. 4. Chemiluminescence kinetics in "luminol-H_2O_2" system under additions of human (1), rabbit (2) or bovine (3) plasminogens. Protein concentration in reaction mixture is 54 μg/ml (Nikandrov *et al.*, 1986c).

(4) $O_2^{\cdot-}$ generation by Pg is 60% increased with EDTA and effectively inhibited by o-phenantroline (Table 3; Nikandrov and Pyzhova, 1988b). Phenantroline and sodium diethyldithiocarbamate enhance native SK activating function by 20–25%, while EDTA reduces it by 25% (Table 4). After

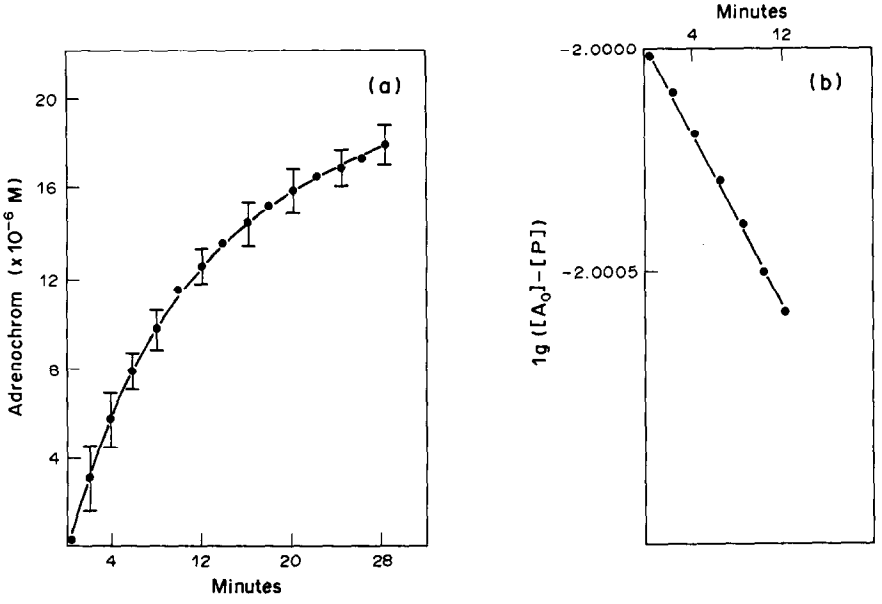

Fig. 5. Adrenochrom formation kinetics under the presence of human plasminogen (a) and semilogarithmic anamorphose of this kinetics (b). Final concentration of adrenaline—0.01 M; plasminogen in the reaction mixture is 2.6 mg, pH 7.0, 25°C, air atmosphere (Nikandrov and Pyzhova, 1988b).

Table 3. Effect of some active oxygen species scavengers and chelating agents on the adrenaline oxidation by human plasminogen (n-5; Nikandrov and Pyzhova, 1988b)

Experimental conditions	Adrenochrome formation ($\times 10^{-7}$ M·min^{-1})
Control (plasminogen)	15.0 ± 1.4
+ L-histidine, 0.02 M	13.5 ± 1.5
+ NaN_3, 0.01 M	12.8 ± 1.2
+ Ethanol, 1 M	15.0 ± 0.9
+ D-mannitol, 0.01 M	14.4 ± 0.9
+ Catalase, $2 \cdot 10^{-7}$ M	16.4 ± 1.0
+ Superoxide dismutase,	8.0 ± 0.9*
+ KCN, 0.001 M	15.8 ± 1.2
+ EDTA, 0.01 M	24.0 ± 1.7*
+ o-Phenantroline, 0.01 M	7.1 ± 0.4*

*$P < 0.05$.

Table 4. Effect of chelating agents on the activating function of streptokinase before and after treatment by 80% (v/v) dimethylsulfoxide (n-5; fibrin plate lysis method; Nikandrov *et al.*, 1986b)

Experimental conditions	Fibrinolysis zones (mm^2)
Streptokinase in 0.06 M phosphate buffer, pH 7.4 (control)	465 ± 6.5
+ EDTA, 0.01 M	349 ± 9.8*
+ Sodium diethyldithiocarbamate, 0.01 M	581 ± 15.6*
+ 8-Hydroxyquinoline, 0.01 M	507 ± 27.2
+ o-Phenanthroline, 0.01 M	558 ± 14.1*
Streptokinase in 80% dimethylsulfoxide (control)	437 ± 14.1
+ EDTA, 0.01 M	265 ± 20.0*
+ Sodium diethyldithiocarbamate, 0.01 M	391 ± 10.0*
+ 8-Hydroxyquinoline, 0.01 M	326 ± 13*
+ o-Phenanthroline, 0.01 M	391 ± 20*

*$P < 0.05$.

pre-treatment of SK with 80% DMSO, o-phenenthroline and diethyldithiocarbamate decrease the activating function by 15–30%, and EDTA halves it (Nikandrov *et al.*, 1987c). These facts enable us to assume, that the above Pg and SK functions are preconditioned by the presence of transition metals in these proteins (Nikandrov and Pyzhova, 1988a).

(5) In model systems of superoxide radical generation SK is able to inhibit nitroblue tetrazolium reduction (Nikandrov *et al.*, 1986b). This phenomenon is very important for medical care, as new approaches for the use of SK preparations arise. SK itself neither decomposes H_2O_2, nor forms oxygen radicals (Nikandrov *et al.*, 1986c).

The above experimental data suggest an oxygen-dependent Pg activation pathway, not linked with serine proteinase function (Nikandrov and Pyzhova, 1988a).

According to this suggestion, Pg activation is realised due to zymogen's ability of slow $O_2^{\cdot}$ generation (as an initial stage), and to SK's ability to react with both this radical and Pg, resulting in zymogen activation.

THE NEW INTERPRETATION OF STREPTOKINASE ACTIVATING FUNCTION RAISES NEW PROBLEMS

It is obvious, that superoxide radical exclusion from the Pg–SK system completely halts the process. But it is worth pointing out, that even weak concentration of this radical is sufficient for effective activation. A sharp increase in its concentration not only enhances the activation process, but even slows it down due to unspecific damaging of protein molecules. More than that, high $O_2^{\cdot}$ concentration increases its transformation into $^{\cdot}OH$-radical and singlet oxygen.

It is only logical to conclude, that, if absolute $O_2^{\cdot}$ concentration is not involved, then during Pg activation by SK catalytic mechanism must be realised, which hampers the dissipation of radicals and ensures their specific action on certain Pg molecule sites. All the above presupposes realisation of the catalytic mechanism by SK.

The following questions arise: how is the activation process realised and what is the SK catalysis mechanism? It seems, that the initial stage is a SK–superoxide radical reaction. Besides, affinity between SK and $O_2^{\cdot}$ is very high, because radical scavengers inhibit the process in relatively high concentration. SK much more effectively activates Pg, when water is substituted with organic solvents: aliphatic alcohols and such aproton solvents as dioxane, dimethylsulfoxide, and dimethylformamide (Fig. 6; Nikandrov *et al.*, 1987a). These conditions favourably influence free $^{\cdot}OH$-radical-fixation and increase $O_2^{\cdot}$ life-time.

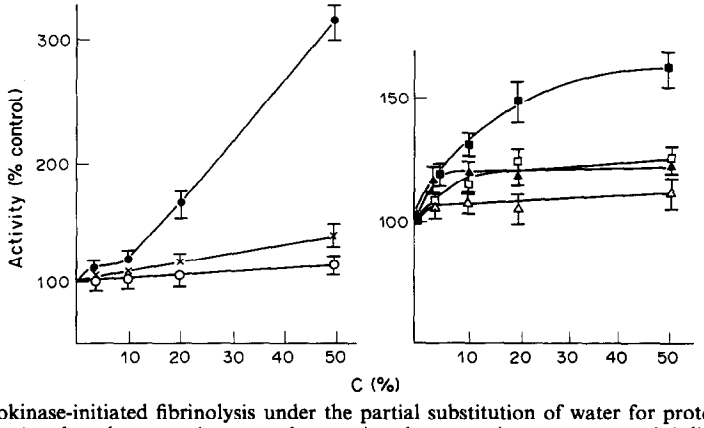

Fig. 6. Streptokinase-initiated fibrinolysis under the partial substitution of water for proton developing (methanol ○—○, ethanol ×—×, 1-propanol ●—●) and aproton (acetone △—△, 1,4-dioxane ▲—▲, dimethylsulfoxide □—□, N,N-dimethyl-formamide ■—■) solvents; fibrin plate lysis method (Nikandrov *et al.*, 1987a).

Further detailed research is needed on the Pg activation mechanism, if possible at the electron level.

At present, taking into account the above, we can presume the following concept. It is probable, that SK is able to transfer an electron from $O_2^{\cdot}$ to certain sites on the Pg molecule, namely, on the carbonyl carbon atom of the peptide bond Arg_{560}-Val_{561} (the reason may be specific structure of Pg). As a result, the peptide bond may become destabilized and broken. Naturally, in case this mechanism exists, one should assume the possibility of both intramolecular transfer of the electron into Pg (from the $O_2^{\cdot}$ generation site) and intermolecular one. It ensures realisation of slow Pg autoactivation process.

The mechanism of peptide bond destabilization, through electron transfer on the carbonyl carbon atom, is described based on experiments with excited tryptophan molecules (Kayushin *et al.*, 1976). The report on activation process of ribonucleotide-reductase from *E. coli* should also be remembered (Eliasson *et al.*, 1986). This enzyme possibly functions only after tyrosine radical formation in the active centre of the enzyme. Such a radical is formed in the presence of superoxide radical and superoxide dismutase (Eliasson *et al.*, 1986). In the absence of superoxide dismutase active ribonucleotide-reductase does not appear. The mechanism of this process is still unclear. In this case we can compare ribonucleotide-reductase activation with Pg activation by SK: in both cases the active enzyme form is produced in the presence of superoxide radical and superoxide dismutase. That is why, we hope, that all the above mentioned assumptions have some real foundation.

CONCLUSION

Stemming from the above, the suggested concept for SK activating function is based on oxygen(superoxide)-dependent Pg activation. To discover this activation mechanism we need to carry out a comprehensive study of the submolecular and electron processes in the Pg–SK system. Our suggestions raise, at least, four important questions:

(1) Is SK a catalyst? According to publications, SK differs from other catalyst-proteins in one respect: SK splits during activation (Markus *et al.*, 1976). But we must take into account that SK action on Pg results in plasmin–active proteinase. For human and animal organisms SK is a xenogenic protein, whose evolutionary purpose (unlike enterokinase, which is often used as an analogy when considering SK activating function—Wohl *et al.*, 1978) does not, most probably, include activation of animal proteinase zymogens. Apart from this phenomenon, there is no evidence to consider SK as devoid of a catalytic function.

(2) Much more important is the question of SK catalytic function mechanism, considering its ability to react with superoxide radical. The discovery of this mechanism demands extensive studies on the submolecular and electron levels in the Pg–SK complex. In this respect, it seems important to study Pg and SK active centers, conducting "operations" with superoxide radicals: state of metals, their coordination by ligands, etc.

This issue has a clear practical importance in the treatment, with SK preparations, of pathological processes unrelated to hemostatic system disturbances.

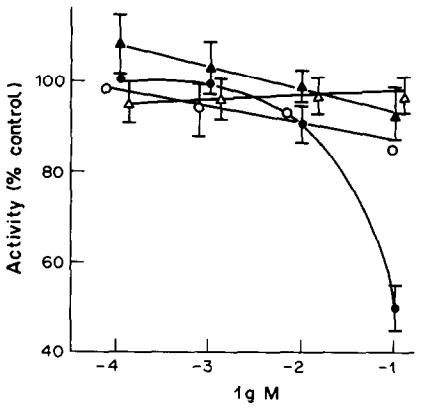

Fig. 7. Effect of ATP (●—●), ADP (○—○), 5′-AMP (▲—▲), GTP (△—△) additions on the streptokinase-initiated fibrinolysis (Nikandrov *et al.*, 1987b).

(3) It is extremely important to reveal SK's physiological role in streptococcus. This aspect is completely enigmatic. We can only point out, that SK is found in hemolytic streptococcus membranes (cellular membranes), while active SK is absent in cellular cytoplasm (Nikandrov *et al.*, 1990). Besides, SK activating function is inhibited by ATP and 3′,5′-AMP (Figs 7 and 8; Nikandrov *et al.*, 1987b). With regard to proteolytic processes, all these facts are unusual. As all the described nucleotide effects on proteolytic enzyme activity were realised in proteolysis activation and, as a rule, depended on the number of phosphate residues in the nucleotide molecule (Pillai *et al.*, 1983; Watabe and Kimura, 1985); i.e. they could be explained by the presence of energy-rich bonds; and do not manifest themselves with 3′,5′-AMP action, it is possible to presume that SK has some connection with the streptococcus cell-energy process. The discovery of a mechanism of reaction between SK and superoxide radical will help to specify these concepts more precisely.

(4) Finally, the last task is to determine the frequency of endogenous oxygen radicals participating in zymogen activation of other proteinases and in the

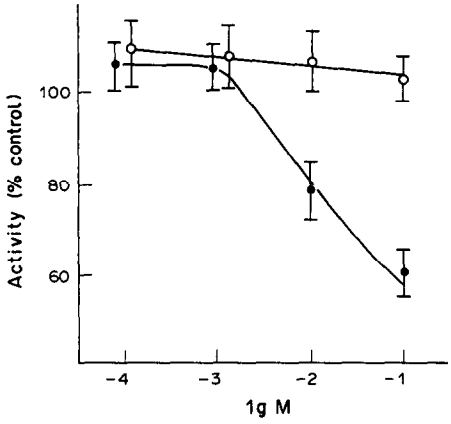

Fig. 8. Changes of activating function of streptokinase after addition of 3′,5′-AMP (●—●) or 2′,3′-AMP (○—○) (Nikandrov *et al.*, 1987b).

catalytic action of proteolytic enzymes. This problem is more significant than SK action and its solution will seriously influence proteolysis theory. During proteolytic action of pepsin and papain we found an analogous inhibiting effect of superoxide radical scavenger (nitroblue tetrazolium) (Nikandrov, 1988). The catalytic mechanism of serine proteinases is especially interesting and is explained by a number of hypotheses, but every one of them has its limitations.

As a whole, the found phenomenon in the first case of endogenously formed superoxide radical participation in limited proteolysis process mechanism.

SUMMARY

The mechanism of Pg activating function of SK is now most often explained from the standpoint of an activator–complex hypothesis. This complex is a stable equimolar complex between SK and plasmin(ogen). According to this hypothesis, a stage of the above complex-formation is a necessary condition for Pg activation by SK, and the very process is realised with the help of an active centre of serine proteinase type, which functions in the plasmin(ogen) part of the complex.

However, this hypothesis cannot explain the very slow activation of bovine Pg and activation of rabbit Pg, which do not form stable complexes with SK. Adsorbed on fibrin fibers human Pg is possibly activated in the presence of 8 M urea or with pH $\leqslant$ 2.0; i.e. in the conditions preventing stable activator-complex formation. Besides, SK activating function does not disappear after partial iodination of its tyrosine residues, which makes impossible the formation of stable complex with human Pg.

A new hypothesis of SK activating function is suggested on the basis of following data. Pg activation by SK is inhibited by superoxide radical scavengers. Human Pg can be partially activated during its treatment with superoxide radical generating systems or H_2O_2 ($\geqslant$0.5 M). In the last case, activation is completely inhibited by the addition of nitroblue tetrazolium, but not by sodium azide, nor other singlet oxygen or OH-radical scavengers. Human or rabbit Pg is able to generate active oxygen species during H_2O_2 decomposition, or superoxide radical during slow auto-oxidation. Bovine Pg manifests this ability very weakly. It is presumed that SK has a catalytic function, hampering superoxide radical dissipation and transferring electrons from these radicals to certain site of the Pg molecule. This process can cause formation of carbonyl carbon radical of the bond Arg_{560}-Val_{561} and destabilize this bond. A concept of an oxygen-dependent pathway of Pg activation is suggested. From the point of view of this hypothesis the following problems are outlined; mechanism of SK catalytic function on electron level (along with disclosing metal-containing site topography); its physiological function in the streptococcus cell (taking into account inhibition of activating function by ATP and 3′,5′-AMP); the place of endogenous active oxygen species in activation of other zymogens and in proteinase catalytic function.

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