# Urokinase: Basic and Clinical Aspects

Proceedings of the
Serono Symposia, Volume 48

Edited by

P.M. Mannucci and A.D'Angelo

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# Urokinase: Basic and Clinical Aspects

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## PREFACE

This volume is a record of papers presented at the second International Symposium on Urokinase held in Geneva, Switzerland, April 27-28, 1981. More than 200 participants attended this meeting at which there were sessions on the clinical use of urokinase in venous and arterial thromboembolism and on basic studies on urokinase.

Biochemists and pharmacologists have recently achieved new important results in this area and the first part of this volume includes a number of significant papers. On the other hand, clinicians are still working on urokinase in order to obtain the best results in thromboembolism and to avoid bleeding complications. No agreement has been reached either on the best treatment schedule or on the value of laboratory analysis for the steering of thrombolytic therapy toward success. The second part of this book illustrates the backgrounds of this controversy and is particularly dedicated to the clinicians fighting for therapeutic progress. It also explains why multicentre controlled trials are urgently needed.

The Editors are pleased to thank Professor F. Duckert for his invaluable help in the preparation of this Symposium and the Serono Institute who made it possible.

*September 1981*

P. M. MANNUCCI
A. D'ANGELO

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# UROKINASE. A SHORT REVIEW OF ITS PROPERTIES AND OF ITS METABOLISM

Ph. Schneider, F. Bachmann and D. Sauser

*Division of Haematology, University of Lausanne, School of Medicine, Lausanne and Hypolab S. A., Coinsins, Switzerland*

The first International Symposium on Urokinase was held in Rome in 1975. At that time, many investigators had started clinical trials with urokinase and results of the UPET and of the USPET trials had just been published. However, in the great majority of countries, urokinase had not yet been registered. At the Rome meeting, basic studies on the purification and the properties of urokinase took up half of the scientific programme. Today, urokinase is registered in many countries and more than three-quarters of the present scientific programme deal with the clinical aspects of thrombolytic therapy with urokinase. Therefore, we thought it would be appropriate to begin this Symposium with a short review on the properties and metabolism of urokinase. In doing this, we will concentrate on newer findings of interest.

## UROKINASE PRODUCTION SITES

Urokinase is not excreted into the urine as a waste product. It is produced by the kidneys and secreted into the urine to maintain patency of the urinary tract in case of haemorrhage. Barnett and Baron (1959) demonstrated that primary cultures of embryonic kidney cells were capable of producing urokinase. Bernik and Kwaan (1967) believe that this activity derives from endothelial cells of blood vessels which are present in embryonic kidney cells. Åstedt (1975) found that freshly-isolated human glomeruli were fibrinolytically inactive. How-

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ever, after organ culture of such glomeruli, marked release of plasminogen activator occurred. Paul *et al.* (1979) have demonstrated that urokinase is secreted by bleeding and vesiculation from a porcine kidney cell strain.

Over the past few years, different investigators discovered that many tumours such as carcinoma of the breast, the colon, the pancreas, the lung and of the ovary produce an urokinase-like plasminogen activator (Åstedt and Holmberg, 1976, Corasanti *et al.*, 1980, Markus *et al.*, 1980; Vetterlein *et al.*, 1980; Wu and Yunis, 1979), whereas other tumours, such as melanoma, produce a plasminogen activator resembling tissue activator or vascular activator (Wilson *et al.*, 1980; Rijken and Collen, 1981).

Shakespeare and Wolf (1979) claimed to have found significant quantities of urokinase in human whole blood. Using an anti-urokinase serum which gave at least five precipitin arcs against normal human serum, they found from 3 to 12 $\mu$g of protein in human plasma, cross-reacting with their anti-urokinase anti-serum. At a specific activity of high $M_r$ urokinase of approximately 100 000 IU/mg this would correspond to circulating urokinase level of 300 to 1200 IU/ml plasma! Åsted and Holmberg (1979), on the other hand, using antibodies obtained by injection of a highly purified urokinase to rabbits, found only 4 to 8 ng of plasma proteins/ml precipitating with their anti-urokinase and interpreted this result as due to non-specific precipitation. Holemans *et al.* (1965) could not find urokinase in renal veins, even after histamine stimulation. Most investigators were not able to quench tissue activator or vascular blood activator activity with an anti-urokinase serum (Kucinski *et al.*, 1968; Kok and Astrup, 1969; Åstedt, 1979; Binder *et al.*, 1979; Rijken *et al.*, 1980, 1981). These results demonstrate that the circulating fibrinolytic activators are clearly different from urokinase.

## MOLECULAR FORMS OF UROKINASE

Urokinase appears to exist in several molecular forms. Very high molecular weight ($M_r$) forms of 100 to 200 kdaltons can be found in early tissue cultures and in freshly voided urine (Doleschel and Auerswald, 1967; Holmberg and Åstedt, this volume p.33; Bobbit *et al.*, 1980). There is some evidence that these forms represent a proactivator which can be converted into active urokinase upon incubation with small amounts of trypsin, plasmin, thrombin or Hageman factor fragments (Bernik and Oller, 1973; Maciag *et al.*, 1977). A few investigators believe that these forms represent dimers of urokinase or complexes with other proteins, but Bobbitt *et al.* (1980) upon repeat gel filtration of pig kidney cells culture high $M_r$ urokinase found substantial amounts in the 100 to 200 kdalton fractions and Markus (1980) observed that the 100 000 dalton forms cannot be disaggregated in 8M urea; these results represent a strong argument against complex or dimer formation. Several molecular forms of 46 to 60 kdaltons have been identified. Vetterlein *et al.* (1980) isolated a 60 000 and a 50 000 dalton urokinase from Detroit 562 (human pharyngeal carcinoma) and IMR-90 (human embryonic lung) cells. The former is clearly different from the classic high molecular weight urokinase, consisting of two chains and having a molecular weight of 54 000 (Lesuk *et al.*, 1965). The classic low molecular

form of urokinase (White *et al.*, 1966) consists probably of a family of degradation products with intact active sites and molecular weights around 32 000 (Lesuk *et al.*, 1967; Huber and Binder, 1980).

Husain, Gurewich and Lipinski (1981) have recently isolated from freshly voided human urine a single-chain, high $M_r$ urokinase, using affinity chromatography of fibrincelite. It has a molecular weight of approximately 56 kdaltons on SDS-polyacrylamid gel electrophoresis, remains intact after exposure to reducing agents and has a high affinity for fibrin. This single chain urokinase degrades into a double chain urokinase, unless protease inhibitors such as aprotinin or benzamidine are added to the urine (Fig. 1). It is not known, at the

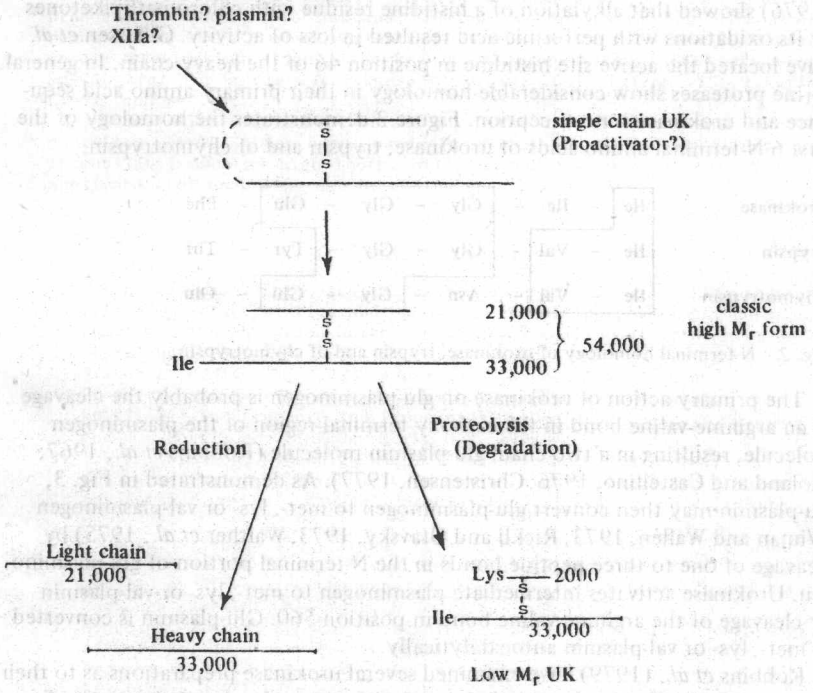


*Fig. 1.* Conversion of single chain into double chain urokinase and into different low $M_r$ forms.

present time, whether thrombin, plasmin and activated factor XII, which are known to convert an inactive proactivator in early tissue culture fluid into urokinase (Bernik and Oller, 1973; Maciag *et al.*, 1977) are also capable of splitting the 56 kdalton single chain into the classic double chain urokinase. There are two ways to obtain active low molecular weight urokinase of about 33 kdaltons. The classic double chain urokinase of 54 kdaltons can be reduced to two chains: a light chain of approximately 21 and a heavy chain of 33 kdaltons (Fig. 1).

Proteolysis appears to affect primarily the light chain and results in products with molecular weights of 30 to 34 kdaltons, whose heavy chain remains mostly intact. Gillessen *et al.* report in this symposium (see p. 17) on sequence studies of such a low molecular weight from urokinase which still has a remnant of some 21 amino acids in its light chain. The N-terminal of the heavy chain consists of isoleucin.

## STRUCTURE AND ACTIVITY OF UROKINASE

Urokinase (EC 3.4.99.26) is a serine protease. Landmann and Markwardt (1970) have demonstrated that the active site contains serine, and Ong *et al.* (1976) showed that alkylation of a histidine residue with chloromethylketones or its oxidations with performic acid resulted in loss of activity. Gillessen *et al.* have located the active site histidine in position 46 of the heavy chain. In general, serine proteases show considerable homology in their primary amino acid sequence and urokinase is no exception. Figure 2 demonstrates the homology of the first 6 N-terminal amino acids of urokinase, trypsin and of chymotrypsin.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Urokinase | Ile | – | Ile | – | Gly | – | Gly | – | Glu | – | Phe |
| Trypsin | Ile | – | Val | – | Gly | – | Gly | – | Tyr | – | Thr |
| Chymotrypsin | Ile | – | Val | – | Asn | – | Gly | – | Glu | – | Glu |

*Fig. 2.* N-terminal homology of urokinase, trypsin and of chymotrypsin.

The primary action of urokinase on glu-plasminogen is probably the cleavage of an arginine-valine bond in the carboxy terminal region of the plasminogen molecule, resulting in a two chain glu-plasmin molecule (Robbins *et al.*, 1967; Violand and Castellino, 1976; Christensen, 1977). As demonstrated in Fig. 3, glu-plasmin may then convert glu-plasminogen to met-, lys- or val-plasminogen (Wiman and Wallén, 1973; Rickli and Otavsky, 1973; Walther *et al.*, 1975) by cleavage of one to three peptide bonds in the N-terminal portion of glu-plasminogen. Urokinase activates intermediate plasminogen to met-, lys- or val-plasmin by cleavage of the arginine-valine bond in position 560. Glu-plasmin is converted to met-, lys- or val-plasmin autocatalytically.

Robbins *et al.*, (1979) have examined several urokinase preparations as to their steady state kinetic parameters of activation of native glu-plasminogen and of lys-plasminogen. All four preparations had apparent Michaelis constants of the same order of magnitude (1.7 – 7.4 $\mu$M). Catalytic rate constants ranged from 28 to 78 per minute. Second order rate constants, a parameter which best indicates enzymatic efficiency, were 9 to 27 $\mu M^{-1}\ min^{-1}$. Although all these values are similar, each individual urokinase preparation had slightly different kinetic constants, suggesting structural differences in the enzyme species of each preparation.

Rijken (1980) compared the amidolytic activity of urokinase and of tissue activator on various synthetic substrates (Table I). The chromogenic tripeptide

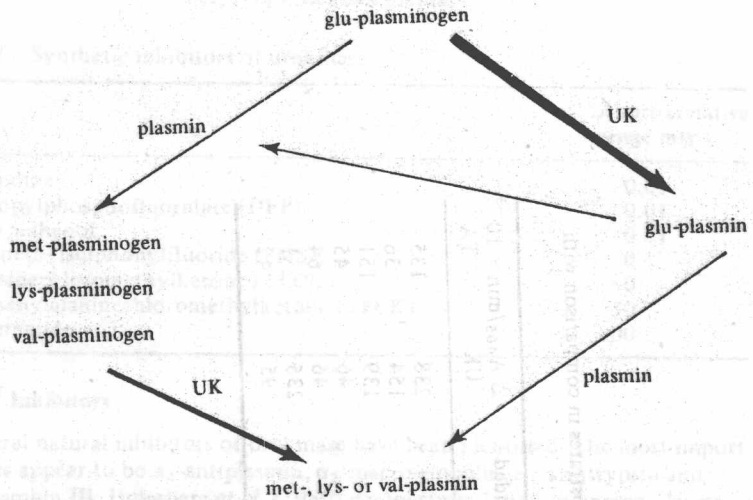


*Fig. 3.* Activation of glu-plasminogen to plasmin.

pyroglutamyl-glycylarginine-paranitroanilide S-2444 was most efficiently hydrolysed by urokinase. However, several other synthetic substrates are also hydrolysed by urokinase at varying efficacy. Urokinase split Chromozyn-Pl, a substrate developed for the determination of plasmin activity, surprisingly well.

## UROKINASE INHIBITORS

### Synthetic Inhibitors

Strong inhibitors of urokinase (Table II) are benzamidine, DFP and mercaptoethanol (Markwardt, 1978). The property of benzamidine to bind reversibly to urokinase has been successfully employed in purification procedures, using benzamidine-Sepharose as an affinity chromatography medium (Holmberg *et al.*, 1976). The chloromethylketones TLCK and TPCK, which block the histidine group in the active site of urokinase, are rather weak inhibitors, as is iodoacetamide (Ong *et al.*, 1976; Rijken, 1980). The ε-aminoacids EACA (epsilon-aminocaproic acid), 6AHA (6-aminohexanoic acid) and AMCHA (tranexamic acid) are antifibrinolytic agents. However, they do not block the activation of plasminogen to plasmin by urokinase in concentrations up to 10 - 100 mM. On the contrary, they stimulate conversion of glu- and of lys-plasminogen to plasmin by urokinase, in concentrations of 0.01 - 10 mM for tranexamic acid, of 0.1 - 100 mM for aminohexanoic acid and of 1 - 1000 mM for lysine (Takada and Takada, 1980). This stimulation is thought to be due to conformational changes of the plasminogen molecule through interaction of its lysine binding sites with ε-aminoacids.

*Table I.* Amidolytic activity of urokinase (UK) on synthetic substrates in comparison with tissue activator (TA).

| Substrate | Formula | Recommended for | $\Delta A_{405}$/min x $10^{-3}$ UK | TA |
|---|---|---|---|---|
| S-2444 | Pyro-Glu-Gly-Arg-pNA | UK | 238 | 135 |
| S-2227 | H-Glu-Gly-Arg-pNA | (UK) | 154 | 36 |
| S-2322 | H-D-Val-Gly-Arg-pNA | TA | 139 | 151 |
| S-2238 | H-D-Phe-Pip-Arg-pNA | Thrombin | 46 | 45 |
| Chromozym-Try | Cbz-Val-Gly-Arg-pNA | Trypsin | 46 | 64 |
| Chromozym-Pl | Tos-Gly-Pro-Lys-pNA | Plasmin | 235 | 1 |
| Chromozym-Th | Tos-Gly-Pro-Arg-pNA | Thrombin | 95 | 42 |

From : Rijken (1980).

*Table II.* Synthetic inhibitors of urokinase.

| | Approximative range mM |
|---|---|
| Benzamidine | 0.01 |
| Diisopropylphosphofluoridate (DFP) | 0.01 |
| Mercaptoethanol | 0.01 |
| Phenylmethylsulphonylfluoride (PMSF) | 0.1 |
| Tosyllysinechloromethylketone (TLCK) | 50 |
| Tosylphenylalaninechloromethylketone (TPCK) | 50 |
| Iodoacetamide | 100 |

**Natural Inhibitors**

Several natural inhibitors of urokinase have been identified. The most important ones appear to be $\alpha_2$-antiplasmin, $\alpha_2$-macro-globulin, $\alpha_1$-antitrypsin and antithrombin III. Holmberg *et al.* (1980) demonstrated that, in human plasma, urokinase, but not DFP-inactivated urokinase, bound preferentially to $\alpha_2$-antiplasmin. Complex formation with $\alpha_2$-macroglobulin occurred equally well with native as with DFP-inactivated urokinase. These authors concluded, therefore, that an intact active site of urokinase is not necessary for complex formation with $\alpha_2$-macroglobulin. Vahtera and Hamberg (1978) demonstrated that $\alpha_2$-macroglobulin is capable of binding urokinase in a non-specific, probably electrostatic attachment that can be disrupted by isoelectric focusing, but not by gel filtration. Clemmensen and Christensen (1976) showed that $\alpha_1$-antitrypsin forms stochiometric complexes with urokinase. The inhibition of urokinase by antithrombin III appears to be accelerated in the presence of heparin (Clemmensen, 1978). This type of behaviour of antithrombin III has been observed with a number of other serine proteases, particularly with the active form of coagulation factors XI, IX, X and with thrombin. Many natural inhibitors, which are less well defined and may in part be different from those listed above, have been described in various body fluids and tissues. The subject has been recently reviewed by Hedner (1979) and Gallimore (1979). Hedner (1973, 1979) found an $\alpha_2$-globulin plasma inhibitor of urokinase which did not cross-react with $\alpha_2$-macroglobulin, $\alpha_2$-antiplasmin or $\alpha_1$-antitrypsin. Campbell *et al.* (1980), Brakman and Astrup (1963) and Åstedt *et al.* (1972) reported on an urokinase inhibitor, whose activity was markedly increased in plasma of pregnant women and suggested that it is produced by the placenta. Kawano *et al.* (1968), Holmberg and Åstedt (1979) and Wijngaards (1979) were able to extract large quantities of an urokinase inhibitor from human placenta and Aznar *et al.* (1976) and Duthie *et al.* (1979) isolated it from aminotic fluid.

## UROKINASE UNITS

Unfortunately, three different activity units are still in use. The first unit was described by Plough and Kjeldgaard (1957) and is still used by the manufacturers of Leo urokinase. In 1969, the Committee on Thrombolytic Agents

proposed the CTA unit, defined by the urokinase activity in three different assay systems: comprising a fibrinolytic, a caseinolytic and an esterolytic method (Johnson *et al.*, 1969). One CTA unit is roughly equivalent to 0.74 Plough units. In 1969, the WHO established the international unit (IU), based on an International Reference Preparation, containing a mixture of high and low $M_r$ urokinase. The IU is nearly identical with the CTA unit. Activity standardization of urokinase remains problematic because of the different behaviour of low and high $M_r$ urokinase in different test systems (see below). However, these differences appear to be no larger than the inter-laboratory variation in relative potency estimation of urokinase, even when calibrating assays against the same standard (Gaffney *et al.*, 1981). The international reference preparation of urokinase can be obtained from Dr Gaffney at the National Institute for Biological Standards and Control in Holly Hill, London. The authors strongly suggest that all manufacturers and researchers henceforth use the IU.

## HIGH $M_r$ VERSUS LOW $M_r$ UROKINASE

For the last several years investigators and manufacturers of urokinase tried to solve the puzzle, which of the two commercially available forms of urokinase was more effective as a thrombolytic agent, the high $M_r$ form, also called UK II or the low $M_r$ form, also designated as UK I.

Samama and his group (1978), and other investigators as well, have observed that equal concentrations of UK II and UK I, as determined by amidolytic assays, yielded different lysis times when incorporated into human plasma clots. Using a plasma clot lysis time, UK I was less efficient and yielded in the average 1.5 times longer lysis times than an equivalent amount of UK II.

Suyama *et al.* (1977) compared the activity of high and low $M_r$ urokinase in the Chandler's loop method. For the standardization of these two forms of urokinase they used an *in vitro* two-stage lysis time method. They observed that thrombus dissolution time was much shorter when using high $M_r$ urokinase than with an equivalent amount of low $M_r$-urokinase. On the other hand, Marder *et al.* (1978), in patients with pulmonary embolism, observed the same plasma biochemical responses after administration of the activator, either purified from urine (40% high $M_r$ and 60% low $M_r$ urokinase) or from tissue culture (100% low $M_r$ weight urokinase).

## UROKINASE CLEARANCE

Plasma clearance rate studies have been performed after injection of human urokinase into animals or human beings (Tajima *et al.*, 1974; Ueno *et al.*, 1979; Woodard *et al.*, 1970; Bachmann and Kulapongs, 1979 and 1980; Bachmann and Schneider, 1980; Matsuo *et al.*, 1978; Matsuo and Mihara, 1977, Som *et al.*, 1975; Barlow and Marder, 1980). The subject of urokinase metabolism has already been reviewed at the first Urokinase Symposium (Alkjaersig and Fletcher, 1977).

It is evident that the methodology used to determine urokinase in plasma