Eur. J. Biochem. *195*, 691 – 697 (1991)
© FEBS 1991
0014295691000 83N

Efficient renaturation and fibrinolytic properties of prourokinase and a deletion mutant expressed in *Escherichia coli* as inclusion bodies

Gaetano ORSINI [1], Anna BRANDAZZA [1], Paolo SARMIENTOS [1], Antonio MOLINARI [2], Jacqueline LANSEN [2] and Gilles CAUET [1]

[1] Department of Biotechnology and [2] Cardiovascular Laboratory Farmitalia C. Erba, Milano, Italy

(Received August 8, 1990) – EJB 90 1029

Prourokinase is a plasminogen activator of 411 amino acids which displays a clot-lysis activity through a fibrin-dependent mechanism, and which seems to be a promising agent for the treatment of acute myocardial infarction.

The preparation of recombinant prourokinase in bacteria has been hampered by its insolubility and by difficulty in refolding the polypeptide chain.

In this paper we describe the renaturation process of two recombinant proteins expressed in *Escherichia coli* as inclusion bodies: prourokinase and a deletion derivative (*Δ*125-prourokinase) in which 125 amino acids of the N-terminal region have been removed. Deletion of this sequence brings to higher refolding yields and faster kinetics (first-order rate constant of renaturation of 0.57 h^{-1} for *Δ*125-prourokinase and 0.25 h^{-1} for prourokinase).

Our process involves sequential steps of denaturation, reduction and controlled refolding of the polypeptide chain. When applied to pure, non-glycosylated and active prourokinase, it gives a refolding yield of about 80%, demonstrating the efficiency of the renaturation procedure. Lower yields (15% and 30%, respectively, for prourokinase and *Δ*125-prourokinase) were obtained when the same refolding protocol was applied to inclusion bodies from bacteria.

After purification to homogeneity (as shown by HPLC and SDS/PAGE) specific activities were 160000 and 250000 IU/mg protein, respectively, for prourokinase and *Δ*125-prourokinase.

As with prourokinase, the deletion mutant *Δ*125-prourokinase displays a zymogenic nature, being activated by plasmin to the active two-chain form; however, this mutant is approximately fourfold more resistant than prourokinase to plasmin activation, and consequently shows a different fibrinolytic profile.

Prourokinase (proUK) is the single-chain precursor of urokinase-type plasminogen activator, a serine protease that plays a key role in the fibrinolytic system by converting plasminogen to plasmin [1]. The proUK molecule consists of 411 amino acids and 12 disulfide bonds and can be subdivided into three domains homologous with known serine protease structures, namely epidermal growth factor (EGF)-like, kringle and serine-protease domains [2]. ProUK is specifically converted to two-chain urokinase after limited proteolysis by plasmin at the Lys158-Ile159 bond; an additional site located at Lys135-Lys136 can also be cleaved by plasmin to generate a low-molecular-mass urokinase which displays the same activity as the high-molecular-mass form [3].

ProUK was found to be an effective thrombolytic agent for the treatment of acute myocardial infarction; however, due to its short half-life in circulation, high therapeutic doses are required [4]. On the other hand, it has been suggested that the EGF-like domain located at the N-terminus plays an important role in the clearance of proUK from the blood stream [5]. Mutants of proUK lacking this domain are potentially interesting as improved thrombolytics.

The expression of proUK in recombinant *Escherichia coli* cells results in the well-known phenomenon of inclusion body formation. In particular, following lysis of the bacterial cells, the recombinant polypeptide is found as inactive and insoluble aggregates.

The feasibility of *in vitro* renaturation of a protein, i.e. the transition from unordered structure to the correct native tridimensional conformation, has been established with pioneering experiments by Anfinsen [6]. However the renaturation of large, cysteine-rich and multidomain proteins is often a difficult exercise [7]. Indeed, the initial difficulties encountered in the reactivation of proUK from *E. coli* inclusion bodies has limited further development of this thrombolytic agent and the characterization of its structural and pharmacological properties.

In this paper we describe an efficient procedure for the renaturation of proUK in its active conformation. In addition, the same refolding process has been applied to a low-molecular-mass mutant of proUK, named *Δ*125-proUK, obtained by site-directed mutagenesis [8]. This mutant, which lacks the EGF-like and kringle domains, has been compared to the

Correspondence to G. Orsini, Farmitalia, Dept. of Biotechnology, Viale Bezzi 24, I-20146 Milano, Italy

Abbreviations. EGF, epidermal growth factor; proUK, single-chain urokinase-type plasminogen activator or prourokinase; S2444, chromogenic substrate pyroglutamylglycylarginyl *p*-nitroaniline; CA, casein unit; KIU, kallikrein inhibitor unit; IU, international unit.

Enzymes. Plasmin (E.C. 3.4.21.7); thrombin (E.C. 3.4.21.5); trypsin (E.C. 3.4.21.4); Klenow DNA polymerase (E.C. 2.7.7.7); DNA ligase (E.C. 6.5.1.2).

intact molecule in terms of refolding kinetics and fibrinolytic activity.

EXPERIMENTAL PROCEDURES

Construction of the expression plasmids

A detailed procedure for the construction of the two expression vectors pFC16 and pFC45 has already been described [9]. Briefly, we first isolated pFC16 through the insertion of the following restriction fragments into the plasmid vector pDS20 [10]: an *Eco*RI – *Hin*dIII fragment, obtained from the pDR720 (Pharmacia), coding for the *E. coli* tryptophan promoter; a *Hin*dIII – *Taq*I synthetic oligonucleotide coding for the MS-2 Shine-Dalgarno sequence; the ATG initiator codon and the first 25 nucleotides of the coding sequence for mature proUK; a *Taq*I – *Nco*I carrying the rest of the proUK cDNA sequence.

The gene coding for Δ125-proUK was obtained by site-directed mutagenesis after subcloning the *Hin*dIII – *Bam*HI restriction fragment from pFC16 into a M13 vector (mp18). A specific synthetic oligonucleotide (GATGAAGTTCCATCGAAGAAGCCCTCCTCTCCTCCA) coding for the desired deletion was first hybridized to the recombinant M13, then elongated using the Klenow DNA polymerase (Boehringer). Following ligation (DNA ligase, Boehringer) and transformation of bacterial cells, screening of the positive clone was carried out by colony hybridization to the synthetic oligonucleotide previously used for mutagenesis. Successively, the expression plasmid for the new mutant was constructed by inserting the mutagenized *Hin*dIII – *Bam*HI fragment in pFC16. Both plasmids were then introduced into an *E. coli* type-B strain and grown in a fermentor at high biomass.

Protein assay

Protein determination was performed according to the Bradford method, with bovine serum albumin as standard [11].

SDS/polyacrylamide gel electrophoresis

Discontinuous SDS/PAGE in 12.5% or 15% polyacrylamide separating gels was performed according to the procedure of Schaegger and Von Jagow [12] or Laemmli [13]. Samples were boiled in 2% SDS, 0.25 M Tris, pH 6.8, with or without 5% 2-mercaptoethanol.

Amidolytic activity

Dilution buffer was 0.05 M Tris, 38 mM NaCl, 0.01% Tween 80, pH 8.8. Samples were converted to the active two-chain form by a 15-min incubation at 37 °C with 10 μg/ml porcine plasmin (Sigma). 20-μl plasmin-activated samples were then incubated with 180 μl diluting buffer containing 0.5 mM chromogenic substrate S-2444 (KabiVitrum) and 100 KIU/ml aprotinin (Sigma) at 37 °C for 30 min. Absorbance at 405 nm was measured after stopping the reaction with 50 μl 50% acetic acid. Enzymatic activities were calculated in international units (IU) from a urokinase working standard (specific activity 130900 IU/mg protein) calibrated against an International Reference Preparation.

Purification of recombinant proUK from E. coli

Centrifuged cells from 200 ml high biomass fermentation were diluted to the original volume with 0.15 M NaCl and 20 mM phosphate, pH 7.5 ($NaCl/P_i$) and disrupted by sonication (2 × 3 min at 200 W). The suspension was centrifuged (30 min at 5000 *g*), and the collected pellet washed with 0.1% Triton X-100, 10 mM Tris, pH 8, and centrifuged. The washed pellet was solubilized after overnight incubation at 4 °C in 6 M guanidine hydrochloride, 10 mM Tris and 50 mM 2-mercaptoethanol, pH 8.5, then renaturated by 20 – 30-fold dilution to decrease the protein concentration to about 0.2 g/l in 2.5 M urea, 10 mM Tris and 5 mM EDTA, pH 8. Maximal activity was obtained after 20 – 24 h at 15 °C.

The volume of the refolded solution was reduced by ultrafiltration (Minitan Ultrafiltration apparatus, 10-kDa cut-off membrane), diluted with 2.5 M urea, 10 mM Tris and 5 mM EDTA, pH 7.6, to decrease the ionic strength and loaded on a Sepharose-S (Pharmacia) column (80 ml) equilibrated with dilution buffer. A single peak of enzymatically active proUK was eluted with 0.5 M NaCl and 10 mM Tris,

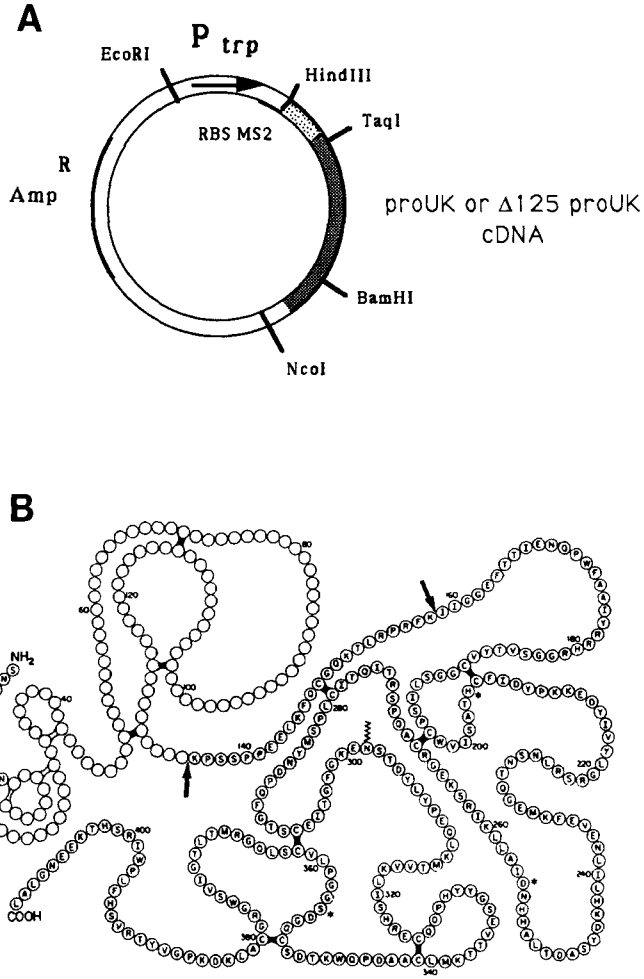

Fig. 1. *Expression plasmid and schematic structure of proUK and Δ125-proUK.* (A) Circular map of pFC16; the tryptophan promoter (P_{trp}), the MS-2 Shine-Dalgarno ribosome-binding site (RBS) and the cDNA sequence encoding mature proUK are shown. A similar plasmid, named pFC45, was utilized for the Δ125-proUK expression as described in Experimental Procedures. (B) Schematic representation of the 411-amino-acid sequence of human proUK. Amino acids are given in single-letter notation. Solid black bars indicate potential disulfide bridges based on homology with other serine proteases. Arrows indicate the position of plasmin-sensitive sites. Open circles indicate the sequence (positions 11 – 135), which is missing in the Δ125-proUK mutant

Table 1. *Purification of proUK and Δ125-proUK*
Bacteria were harvested by centrifugation after high-biomass fermentation (A_{600} = 120–140) to give 250–300 g wet cell paste/l fermentation. Refolding was carried out after cell disruption and washing of the insoluble pellets containing approximately 3 g protein/l fermentation (roughly correspondent to 6% total cell protein). Values are referred to 1 l fermentation broth. Amidolytic activity was assayed using S-2444 chromogenic substrate. Protein was estimated by the Bradford method [11], with bovine serum albumin as standard

Protein purified	Purification step	Total protein	Total activity	Specific activity	Recovery
		mg	IU × 10^{-6}	IU/mg protein	%
proUK	refolded solution	2813	42.2	15000	100
	Sepharose-S	334	27.4	82000	65
	hydroxyapatite	131	17.0	130000	40
	Sephacryl S-200	80	13.0	160000	31
Δ125-proUK	refolded solution	2440	111.8	45750	100
	Sepharose-S	430	83.0	194500	74
	Sephacryl S-100	280	70.0	249000	63

pH 7.6, and loaded directly on to an agarose-coated hydroxyapatite (IBF) column (25 ml) equilibrated with the same buffer. ProUK was eluted with 0.5 M NaCl 20 mM phosphate buffer. When appropriate, the volume was reduced by ultrafiltration before gel-filtration chromatography on Sephacryl S-200 (Pharmacia); column equilibration and elution were carried out in 50 mM ammonium bicarbonate and the eluted fraction containing proUK was directly lyophilized to obtain the final solid preparation.

Purification of Δ125-proUK

The purification scheme was essentially as described above, with the following modifications. Denaturation was in 6 M guanidine hydrochloride 10 mM Tris, pH 8.5, and refolding in 2.5 M urea, 10 mM Tris and 5 mM EDTA, pH 8, containing 3 mM 2-mercaptoethanol. Hydroxyapatite chromatography was omitted, and the final gel filtration was performed on Sephacryl S-100 column.

Plasmin-activation experiments

1 μM proUK or Δ125-proUK were incubated at 37 °C in 0.05 M Tris, 38 mM NaCl, 0.01% Tween 80, pH 7.5, with 0.5 CU/l human plasmin (KabiVitrum), in a total volume of 0.05 ml. At time intervals, proteolytic reaction was blocked by adding 0.5 ml 0.05 M Tris, 38 mM NaCl, 0.01% Tween 80, pH 8.8, containing 100 KIU/ml aprotinin. Amidolytic activities were then measured with chromogenic substrate S-2444, as described.

Complete cleavage of proUK and Δ125-proUK to the corresponding two-chain forms by plasmin was carried out by incubating 1 mg/ml protein with 0.02 mg/ml porcine plasmin (Sigma) in 0.05 M Tris, 38 mM NaCl and 0.01% Tween 80, pH 7.5, at 37 °C for 30 min. Control samples were incubated without plasmin. Samples were assayed by SDS/PAGE under reducing and non-reducing conditions.

Fibrin clot-lysis experiment

Fibrinolytic activity was evaluated by an *in vitro* clot-lysis test [14]. ^{125}I-labeled clots were prepared from citrated normal human plasma mixed with ^{125}I-human fibrinogen (Amersham), 25 mM $CaCl_2$ and 2 NIH unit/ml thrombin (Fibrindex, Ortho). Clots were formed in 4-mm-internal-diameter silicon tubes, incubated for 25 min at 37 °C, and washed in NaCl/P_i. Clots were extruded from silicon tubes cut into 1.5-mm pieces, suspended in 2.5 ml citrated human pooled plasma with added activators, and incubated at 37 °C. Controls had no added activators. Lysis was monitored by taking 0.1-ml aliquots from the supernatants at various time intervals and measuring the radioactivity released from the clots. Results were expressed as a percentage of lysis of the clot relative to initial radioactive content.

RESULTS

Expression of proUK and Δ125-proUK

Recombinant proUK and Δ125-proUK have been expressed in an *E. coli* type-B strain using the tryptophan promoter and the Shine-Dalgarno MS-2 ribosome-binding site [15]. The final expression plasmid, pFC16 is shown in Fig. 1, and its construction is detailed in Experimental Procedures. For expression of Δ125-proUK, an isogenic plasmid, pFC45, was utilized.

The gene coding for Δ125-proUK was obtained by site-directed mutagenesis. The deletion involves a DNA sequence coding for EGF-like and kringle domains, from amino acid residues 11–135 (Fig. 1). The deleted sequence did not include the first 10 amino acids, since we have verified that this sequence is important for achieving high expression levels. Indeed, in previous experiments where the first 135 amino acids were completely deleted, no expression of the resulting mutant was obtained. It is well known that the efficiency of initiation of protein synthesis depends to a great extent on the nucleotide sequence proximal to the ribosome-binding site [16].

Renaturation and purification

Despite the structural complexity of proUK, we have developed a simple renaturation process that allows us to produce this protein on a large scale following high biomass growth of the recombinant *E. coli* type-B strain.

After mechanical breakage of the bacterial cells the insoluble inclusion bodies were separated by centrifugation. Approximately 3 g protein/l fermentation mixture (roughly corresponding to 6% total cell protein) were recovered as an insoluble pellet. In the pellet, inactive proUK and Δ125-proUK were estimated by SDS/PAGE to represent 60–80% of the protein content (data not shown). The partially purified, insoluble pellets were solubilized in 6 M guanidine/HCl. Addition of a reducing agent like 2-mercaptoethanol was neces-

sary for total solubilization of proUK, indicating that these aggregates are partially linked by disulfide bonds; these are probably formed upon air oxidation after the cell breakage [17].

The subsequent renaturation of proUK and Δ125-proUK was achieved by a 25-fold dilution of the guanidine/HCl solution in refolding buffer (2.5 M urea, 10 mM Tris and 5 mM EDTA, pH 8.8, containing 3 mM 2-mercaptoethanol); this concentration of urea is necessary to prevent reaggregation of the polypeptide chains. The optimal protein concentration in the refolding solution was about 200 μg/ml. The renaturation was allowed to proceed for about 24 h at 15 °C.

The recovery of amidolytic activity after plasmin activation was used to probe the correct folding of the polypeptide chain (see Experimental Procedures).

It has been frequently reported that a redox couple like a mixture of oxidized and reduced glutathione should improve the refolding of reduced proteins [18], but no such improvement was observed in this case when 2-mercaptoethanol was replaced by different ratios of oxidized/reduced glutathione or other thiol/disulfide mixtures. The added 2-mercaptoethanol, however, is slowly oxidized during the refolding even in presence of EDTA. It is likely, therefore, that sufficient amounts of hydroxyethyldisulfide are generated in this way to promote the oxidative renaturation of proUK.

The renaturated proUK and Δ125-proUK were submitted to purification as described in Table 1. For Δ125-proUK, a two-step purification process, including adsorption on to Sepharose-S and gel filtration through Sephacryl S-100, was sufficient to obtain a final product more than 95% pure, as determined by SDS/PAGE and reversed-phase HPLC. In the case of proUK, an additional chromatographic step through hydroxyapatite was necessary to reach the same purity (Fig. 2).

During purification, we did not encounter the difficult problem of cleavage of the renaturated proteins in the two-chain form by *E. coli* proteases as previously reported [19]. Contamination by two-chain forms did not exceed 2%, and this level of contamination could be easily reduced to less than 0.5% by application of the products to a small benzamidine-Sepharose column [20].

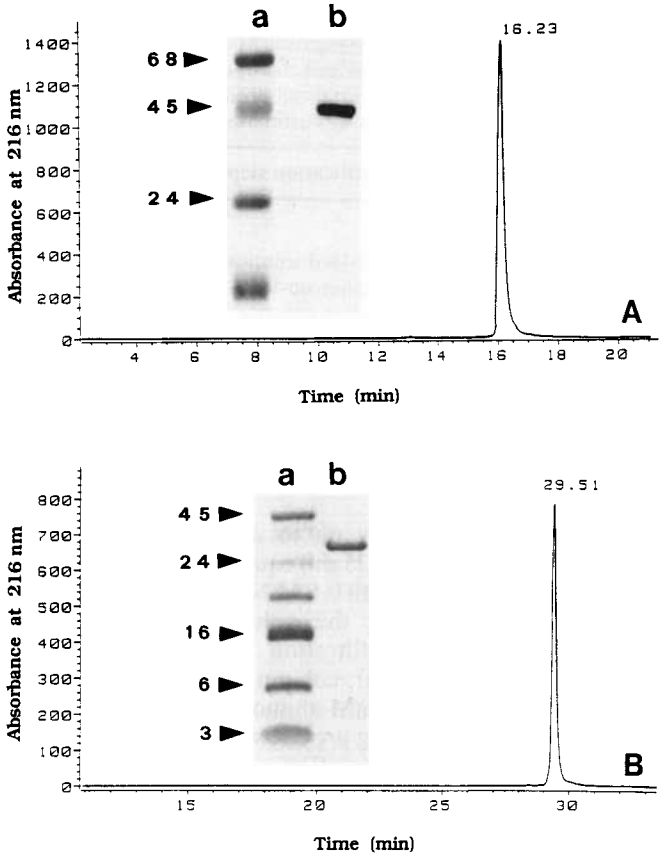

Fig. 2. *HPLC and SDS/PAGE analysis of purified recombinant proUK and Δ125-proUK.* (A) Reversed-phase HPLC of purified recombinant proUK applied to a Aquapore butyl 2.1 × 30 mm column and eluted at 40 °C with a linear gradient 15–61% acetonitrile in water with 0.08% trifluoroacetic acid. Detection was by absorbance at 216 nm. Inset: Coomassie blue staining of SDS/12.5% polyacrylamide gel electrophoresis of relative molecular mass markers (a) and purified recombinant proUK (b) under reducing conditions, showing no contamination by two-chain form. Molecular mass standards: bovine serum albumin (68 kDa), ovalbumin (45 kDa) and chymotrypsinogen (24 kDa). (B) Reversed phase HPLC of purified recombinant Δ125-proUK applied to a Vydac C_4 column and eluted at 40 °C with a linear gradient of 10–70% acetonitrile in water with 0.08% trifluoroacetic acid. Detection was by absorbance at 216 nm. Inset: Coomassie blue staining of SDS/15% polyacrylamide gel electrophoresis of relative molecular mass markers (a) and purified recombinant Δ125-proUK (b) showing no contamination by two-chain forms. Molecular mass standards: ovalbumin (45 kDa), chymotrypsinogen (24 kDa), lysozyme (16 kDa), bovine trypsin inhibitor (6 kDa) and insulin A and B chains (3 kDa)

Refolding kinetics

To monitor the kinetics of renaturation of proUK and Δ125-proUK, aliquots were removed from the refolding solution at various time intervals. The amidolytic activity was measured after sufficient dilution of the samples to avoid interference with the assay by the refolding buffer.

Following dilution of the denaturated proUK into the refolding buffer, a time-dependent increase of the latent amidolytic activity of proUK is observed.

After 24 h of incubation, the curve reaches a plateau that corresponds to an activity of about 3000 IU/ml and a specific activity of 15000 IU/mg protein. From the content of proUK in the starting pellet, we estimate an average refolding efficiency of about 15%. The kinetics of refolding were apparently of first order; by linear regression analysis of the first-order plot, a rate constant of 0.25 h^{-1} was obtained. Interestingly, when samples of purified recombinant proUK were subjected again to the same denaturation/renaturation treatment, a much higher refolding efficiency of about 80% was obtained. Moreover, when compared on a normalized scale, the refolding kinetics of the purified protein was equivalent to that found for the protein from the inclusion bodies (Fig. 3).

Refolding of Δ125-proUK pellet gave a final amidolytic activity of about 10000 IU/ml corresponding to a specific activity of 50000 IU/mg protein and a refolding efficiency of around 30%. Moreover, from a calculated first-order rate constant of 0.57 h^{-1}, it appears that this mutant refolds faster than intact proUK. Thus the removal of two domains in the protein improves significantly the rate and the efficiency of refolding. This result is consistent with the observation that individual domains refold more rapidly than when part of a multidomain polypeptide, presumably because the domains interfere with each other during folding [21].

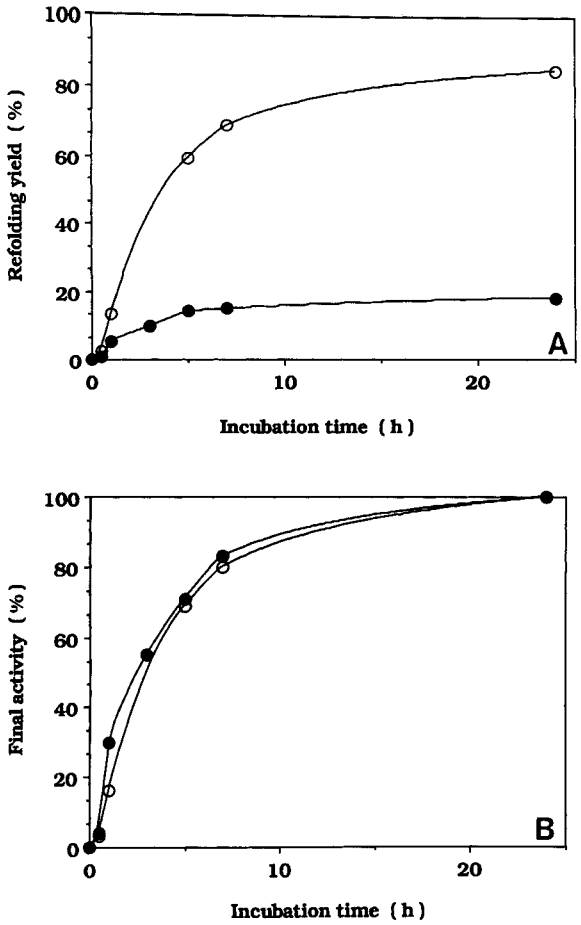

Fig. 3. *Refolding kinetics of purified recombinant proUK and insoluble proUK from bacterial pellets.* (A) 6 M guanidine hydrochloride/50 mM 2-mercaptoethanol extracts were diluted to 0.17 mg pure proUK/ml (○) or 0.20 mg insoluble pellet protein/ml (●) in a refolding buffer containing 2.5 M urea/10 mM Tris. The extent of reactivation was monitored by measuring amidolytic activity at various time intervals in the range 0.5–24 h. For comparison, experimental data were reported as a percentage of the theoretical yield of refolding. Final activities measured at 24 h were 20875 IU/ml for pure proUK and 3170 IU/ml for the insoluble pellet, corresponding, respectively, to refolding efficiencies of 84% and 18%. (B) Same data of (B) when plotted as a percentage of final activity versus incubation time show same refolding kinetics for pure proUK and insoluble pellet, with a half-reaction time of about 3 h

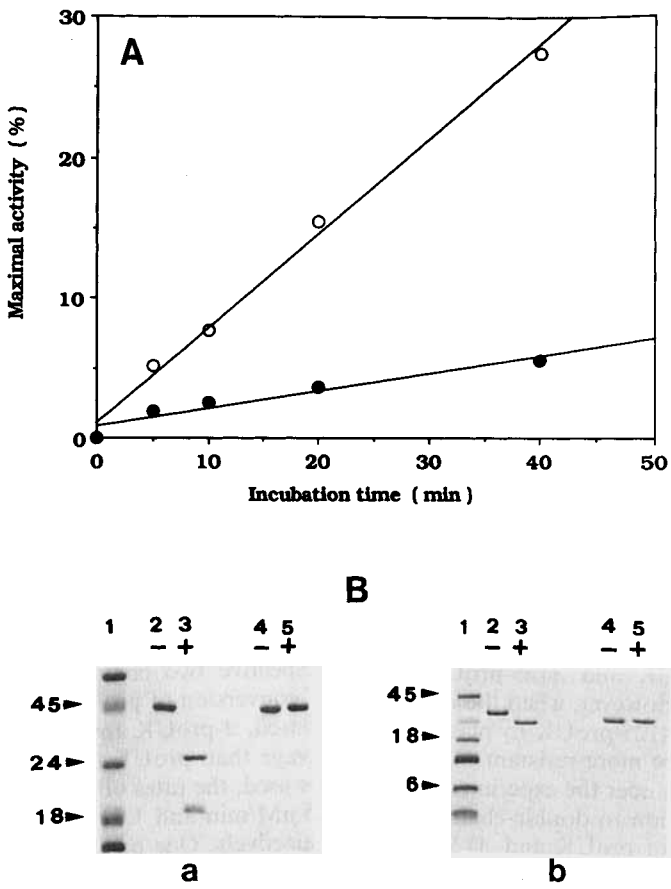

Fig. 4. *Plasmin activation of recombinant proUK and Δ125-proUK.* (A) Time course of activation of 1 μM recombinant proUK (○) and Δ125-proUK (●) incubated at 37 °C with 0.5 CU/l plasmin. Activation was monitored by assaying the amidolytic activity with chromogen substrate S-2444. Amidolytic activity is expressed as a percentage of maximal activity. Each point is the average of two replications. Mean conversion rates were 7.5 nM · min^{-1} and 1.9 nM · min^{-1}, respectively, for proUK and Δ125-proUK. (B) Effect of plasmin treatment on purified recombinant proUK (a) and on purified recombinant Δ125-proUK (b). Samples (1 mg/ml) were incubated for 30 min at 37 °C with porcine plasmin (0.02 mg/ml) in 38 mM NaCl, 0.05 M Tris and 0.01% Tween 80, pH 7.5. Samples with (+) or without (−) plasmin treatment were analysed by SDS/PAGE under reducing (lanes 2 and 3) and non-reducing conditions (lanes 4 and 5). Lane 1 represents a mixture of molecular mass markers: ovalbumin (45 kDa), chymotrypsinogen (24 kDa), lactoglobulin (18 kDa) and bovine trypsin inhibitor (6 kDa). A disulfide bridge links the plasmin-derived two-chain forms, so no molecular mass change was observed under non-reducing conditions (lanes 5). Cleavage into two-chain derivatives was observed only after reduction into the two expected polypeptides of lower molecular mass (lanes 3). Note that for Δ125-proUK (b), a band corresponding to the light chain was barely visible only on the original gel (at about 4 kDa), due to its low mass and a tendency to be washed away during the destaining procedure

Biochemical characteristics of proUK and Δ125-proUK

Specific activities of final preparations, measured as amidolytic activity, were 160000 IU/ml and 250000 IU/mg protein for proUK and Δ125-proUK respectively. No free thiols were detected in either preparation upon thiol group titration under denaturing conditions [22]. No aggregates were observed by SDS/PAGE under non-reducing conditions, indicating that all the cysteines of the renaturated proteins had been reoxidized into internal disulfides. N-terminal sequence analysis of both preparations gave the expected sequences, with less than 5% of initial methionine, indicating an essentially complete intracellular cleavage of the initial methionyl residue.

Peptide mapping experiments following digestion with trypsin in non reducing conditions, where the recombinant proUK was compared to a preparation of glycosylated proUK from mammalian cells (specific activity 124000 IU/mg protein, obtained from Sandoz, Wien) gave equivalent profiles suggesting that the arrangement of disulfide bridges in recombinant proUK is equivalent to the non-recombinant protein (data not shown).

Like natural proUK, our preparations of recombinant proUK and Δ125-proUK are practically devoid of intrinsic amidolytic activity. Only after plasmin treatment are both proUK and Δ125-proUK converted to the active double-chain form, as shown by SDS/PAGE analysis. Despite the deletion of 30% of total sequence, Δ125-proUK is still a proenzyme.

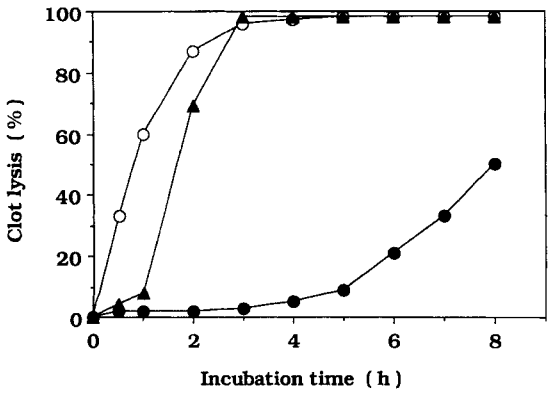

Fig. 5. *Clot-lysis activity of recombinant proUK and Δ125-proUK.* Lytic activities of 100 IU/ml urokinase (○), recombinant proUK (▲) and recombinant Δ125-proUK (●) were estimated by measuring the time course of release of radioactivity from ^{125}I-labeled human plasma clots incubated at 37 °C in human plasma. Less than 5% lysis was observed in control incubations

Plasmin causes a time-dependent conversion of both proUK and Δ125-proUK to the respective two chain-forms. However, when the initial rates of conversion of proUK and Δ125-proUK by plasmin are compared, Δ-proUK appears to be more resistant to plasmin cleavage than proUK (Fig. 4). Under the experimental conditions used, the rates of conversion to double-chain form are 7.5 nM/min and 1.9 nM/min for proUK and Δ125-proUK, respectively. One possible explanation for this difference is that a secondary site of interaction with plasmin is located in the EGF and/or kringle domain of proUK. Δ125-proUK lacking this site is relatively more resistant to plasmin cleavage than proUK.

Fibrinolytic activity

The fibrinolytic potency of both proUK and Δ125-proUK were evaluated using a fibrin-clot lysis test (Fig. 5).

Complete lysis was obtained in 3 h with 100 IU/ml proUK. The shape of the curve was characterized by a lag phase in which less than 10% lysis occurred within the first hour. In the case of Δ125-proUK, this lag-phase was greatly increased, and less than 10% of the lysis was obtained within 4 h. When the same concentration of urokinase was assayed, this lag-phase was not observed and the enzyme displayed nearly immediate onset of lysis. As expected [23, 24], urokinase, whose activity is not specific for fibrin, caused an important degradation of coagulable fibrinogen present in the plasma milieu in which clots were immersed, but no significant fibrinolysis was observed when proUK and Δ125-proUK were used at the same concentration (data not shown).

The increase of the lag time displayed by Δ125-proUK in clot-lysis experiments could be related to the relative resistance of this protein to plasmin cleavage. This would confirm that, at least *in vitro*, the conversion of proUK into active two-chain urokinase by plasmin is a rate-determining step in lysis of the fibrin clot.

DISCUSSION

In this paper we describe the characteristics of an efficient renaturation procedure for recovery of active proUK from inclusion bodies of *E. coli*. The same procedure has been applied to a low-molecular-mass derivative of proUK which displays an interesting fibrinolytic behaviour.

ProUK is a well-known thrombolytic enzyme whose preparation from recombinant *E. coli* has been hampered by the initial difficulties of renaturing the polypeptide chain. On the other hand, the production of this enzyme from mammalian cells has also encountered some limitations in terms of yield and undesired conversion to the double-chain form. Our data demonstrate the possibility of efficiently renaturing even complex molecules like proUK, and consequently offer the opportunity of an alternative recombinant DNA process, exploitable on a large scale.

The efficiency of our refolding process is also demonstrated by the finding that when applied to purified recombinant proUK, almost quantitative yields of renaturation are obtained. These data suggest that the lower refolding yield obtained with inclusion bodies results either from the presence of interfering substances in the pellets, which favour the formation of inactive, uncorrectly folded proteins and/or aggregates, or from some unknown covalent modification of the recombinant protein. It has been proposed that polyanionic compounds, tightly bound to recombinant basic proteins, could affect their folding properties [25]. Attempts to purify proUK from the inclusion body proteins in denaturating conditions before the refolding step, however, have till now given only marginal improvements.

Using the same renaturation methodology, we have prepared a mutant of proUK, Δ125-proUK, which lacks the kringle domain and most of the EGF-like region. This molecule seems to have kept most of the biochemical features of the wild-type protein, with the exception of the very interesting finding that it is converted by plasmin to the double-chain form at a lower rate. In other words, this new molecule is not as a good substrate for plasmin as intact proUK, suggesting that a site of interaction between proUK and plasmin may be present in the deleted sequence.

A similar low-molecular-mass derivative of proUK, lacking the first 143 amino acids and expressed in mammalian cells, has been described; however its biochemical and fibrinolytic properties were found to be the same as intact proUK [26]. It must be considered that our mutant lacks glycoside chains, whose presence may cause different behaviour. Nevertheless, the difference in terms of plasmin activation that we have found between proUK and Δ125-proUK, both non-glycosylated, would exclude any effect of glycoside chains.

The observation that many plasma factors involved in the coagulation and fibrinolytic cascades do contain kringle structures, leads to the temptation to speculate that the kringle domain of proUK, whose function is still unknown to date, may be important for the interaction with plasmin. Further studies are in progress in our laboratory to demonstrate such hypothesis.

The decreased plasmin sensitivity of Δ125-proUK has probably also important consequences on its thrombolytic properties. In *in vitro* clot-lysis experiments, this new mutant requires a much longer time to achieve a complete lysis of the blood clot. The observed initial lag phase, quite long compared to proUK, might be explained by its resistance to plasmin, which tends to keep it in the form of a proenzyme. The behaviour of this mutant further demonstrates, therefore, that conversion to the double-chain form is the main event of clot lysis induced by proUK.

We are very grateful to Umberto Breme, Gianpaolo Nitti and Irma Facchetti for carrying out HPLC analyses and peptide-mapping

experiments. We also greatly appreciate the critical reading of the manuscript and suggestions by Francesco Blasi, Thomas E. Creighton and Gennaro Marino.

REFERENCES

1. Gurevich, V., Pannel, R., Louie, S., Kelley, P., Suddith, R. L. & Greenlee, R. (1984) *J. Clin. Invest. 73*, 1731–1739.
2. Holmes, W. E., Pennica, D. M., Blaber, M., Rey, M. W., Guenzler, W. A., Steffens, G. J. & Heyneker, H. L. (1985) *Bio-technology 3*, 923–929.
3. Gunzler, W. A., Steffens, G. S., Oetting, F., Base, G. & Flohe, L. (1982) *Hoppe Seyler's Z. Physiol. Chem. 363*, 133–141.
4. Van De Werf, F., Vanhaecke, J., De Geest, H., Verstraete, M. & Collen, D. (1986) *Circulation 74*, 1066–1070.
5. Hiramatsu, R., Kasai, S., Amatsuji, Y., Hirose, M., Morita, M., Tanabe, Y., Kawabe, H., Arimura, H. & Yokoyama, K. (1989) *Fibrinolysis 3*, 147–151.
6. Anfinsen, C. B. (1973) *Science 181*, 223–230.
7. Pain, R. (1987) *Trends Biochem. Sci. 12*, 309–312.
8. Brandazza, A., Mazue, G. & Sarmientos, P. (1989) European patent application no. 0338409.
9. Brandazza, A., Orsini, G. & Sarmientos, P. (1989) European patent application no. 0365894.
10. Duester, G., Elford, R. M. & Holmes, W. M. (1982) *Cell 30*, 855–864.
11. Bradford, M. M. (1972) *Anal. Biochem. 72*, 248–254.
12. Schaegger, H. & Von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
13. Laemmli, U. K. (1970) *Nature 227*, 680–685.
14. Zamarron, C., Lijnen, H. R. & Collen, D. (1984) *Thromb. Res. 35*, 335–345.
15. Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Iserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raeymaekers, A., Van den Berghe, A., Volckaert, G. & Ysebaert, M. (1976) *Nature, 260*, 500–507.
16. Isacchi, A., Sarmientos, P., Lorenzetti, R. & Soria, M. (1989) *Gene 81*, 129–137.
17. Marston, F. A. O. (1986) *Biochem. J. 240*, 1–12.
18. Ahmed, A. K., Schaffer, S. W. & Wetlaufer, D. B. (1975) *J. Biol. Chem. 250*, 8477–8482.
19. Winkler, M. E. & Blaber, M. (1986) *Biochemistry 25*, 4041–4045.
20. Holmberg, L., Bladh, B. & Asted, B. (1976) *Biochim. Biophys. Acta 445*, 215–222.
21. Dautry-Varsat, A. & Garel, J. (1978) *Proc. Natl Acad. Sci. USA 75*, 5979–5982.
22. Riddles, P. W., Blakeley, R. L. & Zerner, B. (1983) *Methods Enyzmol. 91*, 49–60.
23. Stump, D. C., Lijnen, H. R. & Collen, D. (1986) *J. Biol. Chem. 261*, 17120–17126.
24. Pannell, R. & Gurevich, V. (1986) *Blood 67*, 1215–1223.
25. Darby, N. S. & Creighton, T. E. (1990) *Nature 344*, 715–716.
26. Lijnen, H. R., Nelles, L., Holmes, W. E. & Collen, D. (1988) *J. Biol. Chem. 263*, 5594–5598.