Purification and Characterization of Recombinant Single-Chain Urokinase Produced in *Escherichia coli*

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ABSTRACT: Recombinant single-chain urokinase (rUK_1) has been purified from *Escherichia coli*. The purification utilizes a refractile body purification, followed by batch DE-52 cellulose extraction, hydroxylapatite chromatography, and S-200 chromatography. Two-chain rUK (rUK_2) is separated from rUK_1 on benzamidine–Sepharose. The purification eliminates proteases early in the procedure so the rUK_1 will not be cleaved to rUK_2. The rUK_1 has been characterized by amino-terminal analysis as well as carboxy-terminal analysis after cleavage by plasmin.

Urokinase (EC 3.4.21.31) is a serine protease which activates plasminogen to plasmin. The protein is synthesized in vivo as a single polypeptide chain (UK_1)[1] (Bernik, 1973). Although UK_1 has a low reactivity to synthetic chromogenic substrates or active-site titrants, it has recently been shown to have potent plasminogen activating properties due to its unusually low K_m of 0.3 μM for plasminogen binding (K_{cat} = 0.035 s^{-1}) (Lijnen et al., 1986; Collen et al., 1986). This one-chain form can be purified in trace amounts from urine and plasma and in larger amounts from fetal kidney cells, normal human foreskin fibroblasts, or certain tumor cells (Husain et al., 1981, 1983; Wun et al., 1982; Neilsen et al., 1982; Sumi et al., 1982; Eaton et al., 1984). UK_1 is cleaved between lysine-158 and isoleucine-159 to form a two-chain enzyme (UK_2). The two chains are believed to be held together by a single disulfide bond between cysteine-148 and cysteine-279. This form has a much higher reactivity to chromogenic substrates than UK_1, but its K_m for plasminogen binding is increased to 25 μM (K_{cat} = 2.0 s^{-1}) (Lijnen et al., 1986; Collen et al., 1986). A low molecular weight form of UK_2 is formed by cleavage after Lys-135. This causes the release of the first 135 amino-terminal amino acids from the UK_2 enzyme (see Figure 1).

Current therapies utilize UK_2 or streptokinase for dissolution of blood clots which occur in the clinical situations of myocardial infarction, deep vein thrombosis, and pulmonary embolism. Both therapies suffer from systemic activation of plasma proteases because of the lack of fibrin specificity (Verstraete, 1980). Systemic activation causes degradation of fibrinogen and leads to the decrease of circulating plasminogen, α_2-antiplasmin, and some clotting factors (Verstraete, 1980). Because of the higher fibrin specificity of tissue plasminogen activator (tPA), systemic activation is less of a problem with this experimental mode of therapy (Collen et al., 1984c). Both Sumi et al. (1983) and Husain et al. (1981) have reported that the single-chain form of UK has a higher specific thrombolytic activity and a better selectivity for fibrin than two-chain UK_2 when tested in vitro.

We have recently expressed the gene for human UK in *Escherichia coli*, in both the high and low molecular weight active forms (Holmes et al., 1985; Winkler et al., 1985). We describe here a purification procedure which eliminates *E. coli* proteases early in the preparation so that the final product is the single-chain form of urokinase (rUK_1). The purified rUK_1 can be activated with plasmin to yield rUK_2 which shows full reactivity to chromogenic substrates.

Materials and Methods

Purification of rUK_1 from E. coli. DNA coding for the amino acid sequence of UK was inserted in the plasmid pBR322 behind the *trp* promoter. The resulting plasmid was transfected into *E. coli* as described previously (Holmes et al., 1985). The bacteria were grown in a 10-L fermenter with a synthetic glucose medium containing 20% yeast extract. The production of rUK_1, under control of the *trp* promoter, was induced with indoleacetic acid when the cell density reached an OD_{550} of 20, and the cells were harvested 6 h later. The cells were stored at −20 °C for up to a week prior to use.

One kilogram of cell paste, harvested from two 10-L fermenters, was homogenized at 4 °C in 10 L of 0.05 M Tris, pH 7.2, containing 0.02 M EDTA, 0.5 g/L lysozyme (Sigma), and 0.01 g/L each of ribonuclease (Sigma) and deoxyribonuclease (Sigma). The solution was passed 3 times through a Menton Gaulin mill at 4500 psi and centrifuged for 30 min at 4700*g* at 5 °C. The resulting pellet, which contains the rUK_1 as monitored by SDS–PAGE and Western blotting (Burnette, 1981), was resuspended by homogenization in 2.5 L of 0.05 M Tris and 0.02 M EDTA, pH 7.2. This suspension was layered over 7.5 L of 50% glycerol and centrifuged again for 30 min at 4700*g*.

The rUK_1, which again is found in the pellet, was dissolved with stirring for 6–18 h in 6.0 M guanidine hydrochloride at 4 °C. Insoluble material was removed by centrifugation for 30 min at 4700*g*. The supernatant was diluted to 30 L for refolding. The final concentration of salts in the pH 9.0 refolding buffer was 0.05 M Tris, 1.0 M guanidine hydrochloride, 0.2 M arginine, 0.005 M EDTA, 0.005% Tween 80, 1.25 mM reduced glutathione, and 0.25 mM oxidized glutathione. The volume of 30 L was calculated to give an OD_{280} < 1. The solution was allowed to stand 24 h at 4 °C to obtain maximal yields of activity as measured by the chromogenic substrate, S-2444, after cleavage of rUK_1 by plasmin. Refolding reagents were removed by dialysis at 4 °C against two

[1] Abbreviations: CU, casein units; DFP, diisopropyl fluorophosphate; DNA, deoxyribonucleic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; kDa, kilodalton(s); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; PU, Plough units; RNA, ribonucleic acid; rUK_1, recombinant single-chain urokinase; rUK_2, recombinant two-chain urokinase; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; tPA, tissue plasminogen activator; Tris, tris(hydroxymethyl)aminomethane; UK_1, single-chain urokinase; UK_2, two-chain urokinase.

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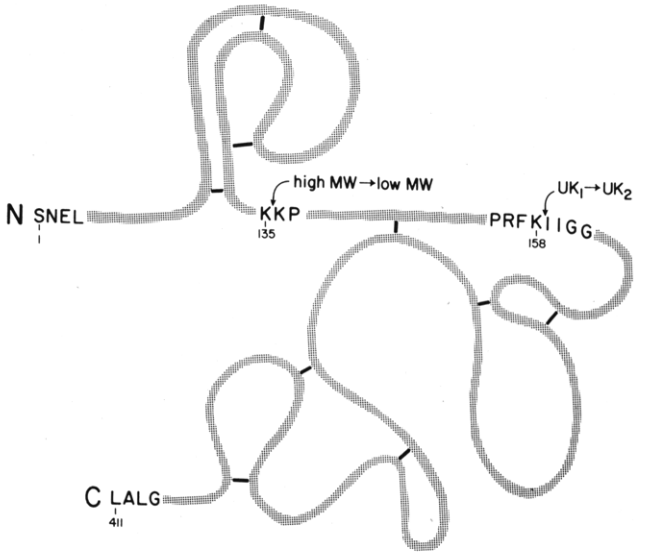

FIGURE 1: Schematic drawing of UK, showing the site of cleavage for the conversion of UK_1 to UK_2. The cleavage point for the conversion of high molecular weight UK to low molecular weight UK is also indicated.

changes of 300 L each of 0.05 M sodium phosphate, pH 6.8, containing 0.005% Tween 80. The dialysis was completed in 6 h to reduce the amount of cleavage after lysine-158, which yields UK_2. At this point in the purification, cleavage after lysine-158 seems to occur quite readily. All subsequent purification steps were carried out at 4 °C.

The dialyzed solution was batch extracted with 400 mL of DE-52 cellulose (Whatman) equilibrated in the dialysis buffer. The slurry was filtered with a Büchner funnel. The supernatant, containing unadsorbed rUK_1, was loaded immediately onto a 250 mL (7.5 × 5 cm) hydroxylapatite (Bio-Rad) column previously equilibrated with the dialysis buffer. The column was washed with 0.125 M sodium phosphate, pH 6.8, containing 0.005% Tween 80. rUK_1 was eluted with 0.4 M sodium phosphate, pH 6.8, containing 1.0 M guanidine hydrochloride and 0.005% Tween 80 (Figure 2).

The elution pool from the hydroxylapatite column was concentrated to approximately 30 mL by using a YM10 Amicon filter and was loaded onto a 2.5 × 100 cm Sephacryl S-200 sizing column equilibrated with 0.05 M sodium phosphate, pH 6.8, containing 1.0 M guanidine hydrochloride and 0.005% Tween 80. The peak containing rUK_1 (see Figure 3) was pooled and dialyzed against 100 volumes of 0.05 M sodium phosphate, pH 7.3, containing 0.15 M sodium chloride and 0.005% Tween 80.

Any rUK_2 generated during the purification by the action of *E. coli* proteases was removed from the rUK_1 by passing the sample over a benzamidine–Sepharose column. UK_2 was observed to have a significantly higher affinity for benzamidine–Sepharose than the one-chain form. The column size was therefore determined by the binding capacity of the resin and the amount of rUK_2 in the sample, as measured by the S-2444 chromogenic assay.

Storage of rUK_1. For long-term storage, the purified rUK_1 was stored at −70 °C to prevent conversion to its active form by trace amounts of *E. coli* proteases.

Assay of UK_2, rUK_2, and rUK_1 Activity. UK_2, rUK_2, and rUK_1 were assayed on fibrin plates by using a procedure similar to that of Ploug and Kjeldgaard (1957). There appeared to be no delay in the appearance of lysis zones for rUK_1 as compared to UK_2 or rUK_2. Both rUK_1 and rUK_2 were also assayed by using the direct chromogenic substrate S-2444

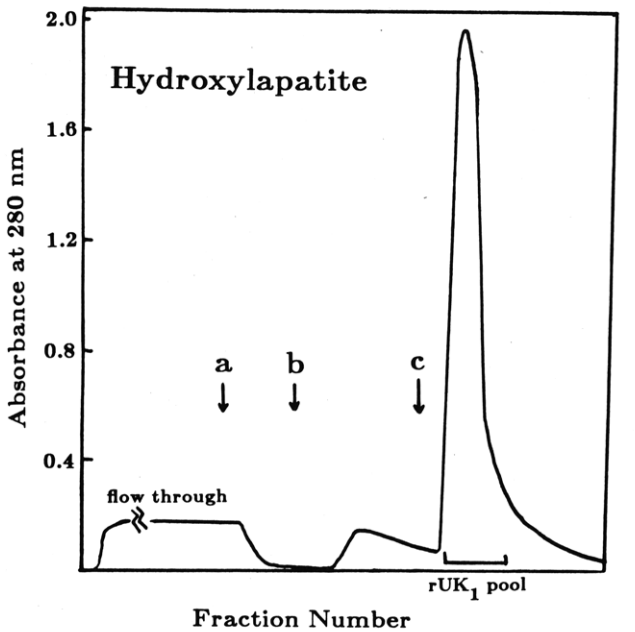

FIGURE 2: Elution profile of hydroxylapatite chromatography. At arrow a, the column was washed with 0.05 M sodium phosphate, pH 6.8, containing 0.005% Tween 80; at arrow b, further contaminating proteins were washed off the column with 0.125 M sodium phosphate, pH 6.8, containing 0.005% Tween 80; at arrow c, the rUK_1 was eluted with 0.4 M sodium phosphate containing 1 M guanidine hydrochloride and 0.005% Tween 80.

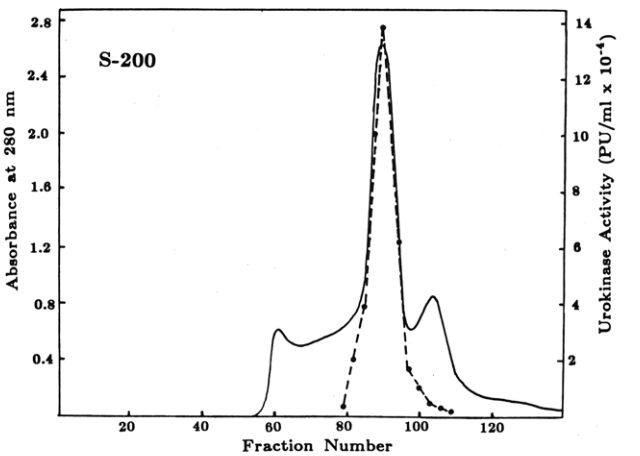

FIGURE 3: Elution profile of chromatography on S-200. (—) Absorbance at 280 nm; (---) activity of rUK as measured by using S-2444 substrate after cleavage of rUK_1 to rUK_2 using plasmin.

(Hayashi & Yameda, 1981) (Helena Laboratories, Beaumont, TX). The rUK_1 (0.5–50 PU) was completely activated by incubation with 0.005 CU of plasmin at 37 °C for 15 min prior to assaying. All assays were compared to the UK_2 standard (Calbiochem) to obtain absolute activities.

SDS–Polyacrylamide Gel Electrophoresis (PAGE). Samples containing rUK_2 or rUK_1 were concentrated by centrifugation under vacuum and were then dissolved in 2% sodium dodecyl sulfate (SDS)/10% glycerol; 10 mM dithiothreitol (DTT) was added to reduce the protein disulfides where indicated. Discontinuous SDS electrophoresis, using 10% polyacrylamide resolving gels, was performed according to the procedure of Laemmli (1970).

NH_2-Terminal Analysis. NH_2-terminal analysis was performed on a Beckman 890C sequenator with on-line HPLC conversion and detection (Rodriguez et al., 1984).

Carboxy-Terminal Analysis. rUK_1 was cleaved by plasmin in 50 mM sodium phosphate, pH 8.0, containing 0.15 M NaCl

Table I: Purification of rUK_1

	volume	act. (PU × 10^6)	sp act. (PU/mg)	x-fold purification	cumulative purification	cumulative yield (%)
cell paste (1 kg)	30 L	42.8[a,b]	240[a,b]			
refolded refractile bodies	30 L	30.0[b]	2 200[b]	9.2	9.2	70
DE-52	30 L	28.7[c]	16 900[c]	7.7	70.8	67
hydroxylapatite	300 mL	22.7[c]	33 800[c]	2.0	142	53
S-200	200 mL	17.1[c]	84 500[c]	2.5	354	40
benzamidine–Sepharose	200 mL	13.7[c]	89 000[c]	1.05	372	32

[a] Cell paste was submitted to refolding conditions to determine initial activity. [b] Based on the fibrin plate assay. [c] Based on the S-2444 direct chromogenic assay after plasmin activation.

and 0.005% Tween 80. The ratio of plasmin to rUK_1 was 1:10 by mass. The carboxy-terminal amino acid of the A chain, generated by the activation cleavage by plasmin, was detected after a 90-min digestion with carboxypeptidase B at room temperature in 0.1 M *N*-ethylmorpholine buffer at pH 8.0. The ratio of carboxypeptidase B to rUK was 1:40 by mass. Norleucine was added as an internal standard. Intact peptides were removed from the free amino acids in the samples by passage over a Sep-Pak C-18 (Waters) cartridge equilibrated to 0.1% trifluoroacetic acid (TFA)/15% acetonitrile. Amino acid analysis was performed on a Beckman 6300 high-performance analyzer with a Hewlett Packard integrator.

Results

rUK_1, expressed in *E. coli*, exists as amorphous insoluble aggregates, most likely due to imperfectly aligned disulfides and improper folding of the protein. Nonreduced SDS–PAGE shows the protein to be disulfide-bonded polymers. The protein can be solubilized with 6.0 M guanidine hydrochloride. However, it must be refolded before active monomer can be obtained.

Various ratios of reduced to oxidized glutathione (Ahmed et al., 1975) from 1:1 to 20:1 were tested in the refolding buffer, and the ratio of 5:1 was found to give maximal activity. Also, the concentrations of guanidine hydrochloride and total protein have been optimized, as well as the pH, to give high yields of activity. Estimating the level of rUK_1 expression from SDS–PAGE, we calculate approximately 10% of the expressed rUK_1 has been refolded. However, using radioimmunoassay, we could not precisely determine the fraction of expressed rUK_1 which was folded to give active plasminogen activator because the antibodies raised against natural UK_2 are sensitive to the conformation of the native protein.

The rUK_1 in the refolding buffer does not appear to be digested by *E. coli* proteases, which were found to be inhibited by the reagents 1.0 M guanidine hydrochloride, 0.2 M arginine, and 5 mM EDTA. the rUK_1 is very dilute at this stage, and this may also be advantageous in preventing proteolysis. rUK_1 has been left for up to a week in this buffer without significant cleavage to rUK_2.

When the refolding reagents are dialyzed out of the rUK_1 solution, some proteolysis starts immediately. Therefore, the dialysis time must not exceed 6 h at 4 °C. We have tried adding low molecular weight protease inhibitors [such as phenylmethanesulfonyl fluoride (PMSF) or diisopropyl fluorophosphate (DFP) (Prouty & Goldberg, 1972)] to the sample and dialysis buffers. These not only were unsuccessful at blocking proteolysis but also high concentrations seem to inactivate rUK_1 activity (measured after dialysis to remove inhibitors and subsequent cleavage to rUK_2 by plasmin). In separate experiments, we also tried inhibiting proteolysis by the addition of aprotinin, pancreatic trypsin inhibitor, or soybean trypsin inhibitor to the rUK_1 solutions during the purification. Of these, the only inhibitor which was effective in blocking the cleavage of rUK_1 to rUK_2 was aprotinin. However, even this inhibitor was not very effective and required 0.5 g/L to achieve the necessary inhibition.

The batch DE-52 adsorption step seems to remove most of the *E. coli* proteolytic activity and moreover purifies the rUK_1 7.6-fold with a 95% recovery (Table I). The adsorbed proteolytic enzymes can be eluted from the cellulose with 1.0 M sodium chloride, and the eluted pool has been found to be very effective in cleaving rUK_1 to rUK_2.

The rUK_1 eluted from the hydroxylapatite column is very concentrated and as thus was observed to be susceptible to proteolysis by proteases which coelute with it. However, the rUK_1 appears to be stable when bound to the hydroxylapatite column. If the rUK_1 is eluted with 0.4 M phosphate without 1.0 M guanidine hydrochloride, it is quite susceptible to proteolysis and after concentration by ultrafiltration will be mostly cleaved to rUK_2. When 1.0 M guanidine is included in the elution buffer of the hydroxylapatite column and the Sephacryl S-200 column, the proteolysis is inhibited. The Sephacryl S-200 column gives a 2–3-fold purification and a 75% recovery of the rUK_1 (Table I, Figure 3).

The preparation was always found to contain low amounts of rUK_2. Separation of the rUK_2 from rUK_1 was accomplished by chromatography on benzamidine–Sepharose. We have shown previously (Winkler et al., 1985) that high molecular weight UK_2 has an affinity for benzamidine–Sepharose mediated not only through the active site but also through the amino-terminal "kringle" region. Thus, both rUK_2 and rUK_1 bind to benzamidine–Sepharose. However, since the active site of rUK_1 is different from that of rUK_2, it has a much lower affinity for the benzamidine resin than rUK_2. A 3-mL benzamidine–Sepharose column will bind any rUK_2 (up to 10 mg) and possibly some remaining *E. coli* proteases whereas most of the rUK_1 will flow through.

Analysis of the purified rUK_1 by SDS–PAGE is shown in Figure 4. The product is 90–95% pure after the three-column purification procedure. The rUK_1 migrates at M_r 42 500 on nonreduced SDS–PAGE and at M_r 52 000 on reduced SDS–PAGE. When the nonreduced gel is soaked in 0.1% Triton X-100 and then overlayed with a fibrin–agarose sheet (zymography) (Granelli-Piperno & Reich, 1978), plasminogen activating activity can been seen mainly in the M_r 42 500 rUK_1 band. However, a small amount of activity is also seen at M_r 38 000, suggesting that a small amount of cleavage may have occurred in the amino terminus of the rUK_1. Contaminants visualized on SDS–PAGE at M_r 35 000 and below do not show any corresponding plasminogen activating activity.

The activity, as measured by the chromogenic substrate S-2444, increases 50-fold after activation by plasmin, indicating that the rUK_1 is at least 98% in the one-chain form. The specific activity of the purified rUK_1 is 89 000 PU/mg after activation by plasmin as measured by S-2444 (Table II). This compares favorably with the specific activity of rUK_2 of 102 000 PU/mg as measured by S-2444 (Winkler et al., 1985).

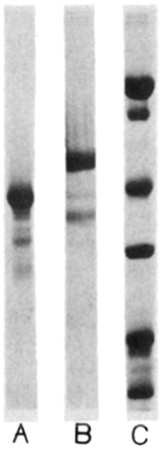

FIGURE 4: SDS–PAGE of rUK_1. Lane A, rUK_1; lane B, rUK_1 in the presence of 10 mM DTT; lane C, molecular weight standards in the presence of 10 mM DTT (from top to bottom): phosphorylase *b*, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

Table II: Specific Activity of Recombinant and Natural Urokinase

	rUK_1	$rUK_1 \rightarrow rUK_2$	rUK_2[a]	UK_2[a]
S-2444 (PU/mg)	1800	89000	102000	92700
fibrin plate (PU/mg)	92000	ND[b]	121000	126200

[a] From Winkler et al. (1985). [b] ND, not determined.

The specific activity of the rUK_1 was also very similar to that of high molecular weight rUK_2 (Winkler et al., 1985) when measured on a fibrin plate (Table II).

Amino-terminal analysis shows just one amino terminus to be present in the rUK_1 (Ser-Asn-Glu-Leu...). This shows that the *E. coli* process the rUK_1 to remove the NH_2-terminal Met required for initiation of translation.

The amino acid sequence around the cleavage site of UK_1 is -Pro-Arg-Phe-Lys-Ile-Ile-. Cleavage after lysine-158 results in an amino-terminal isoleucine residue on the B chain. When natural high molecular weight UK_2 is purified, carboxy-terminal analysis of the A-chain sequence shows phenylalanine to be present (Gunzler et al., 1982). This carboxy-terminal phenylalanine indicates that lysine-158 has been released from natural high molecular weight UK_2. Carboxy-terminal analysis of natural low molecular weight UK_2 shows the additional release of phenylalanine-157, giving -Pro-Arg at the carboxy terminus and Ile-Ile- at the amino terminus, adjacent to the lysine-158 cleavage site (Steffens et al., 1982). Carboxy-terminal analysis of rUK_2 generated by the cleavage of rUK_1 with plasmin (Figure 5) shows only 35% of the protein has a carboxy-terminal arginine after 60 min of digestion with plasmin. Amino-terminal analysis of the plasmin-generated rUK_2 shows additionally some tendency for cleavage in the amino part of the protein. We have noted cleavage after lysine-46 which releases the amino-terminal domain of rUK_2.

Discussion

The presence of proteases in bacterial supernatants is a problem often encountered when purifying recombinant proteins. These proteases can cause unwanted processing of the recombinant protein or even complete degradation of the protein. UK_1 is quite easily cleaved from the one-chain form to UK_2 by trypsin, plasmin, or *E. coli* proteases. Many *E. coli* proteases are inhibited by protease inhibitors such as PMSF or DFP (Prouty & Goldberg, 1972). Others seem to be dependent on divalent cations and thus are sensitive to reagents

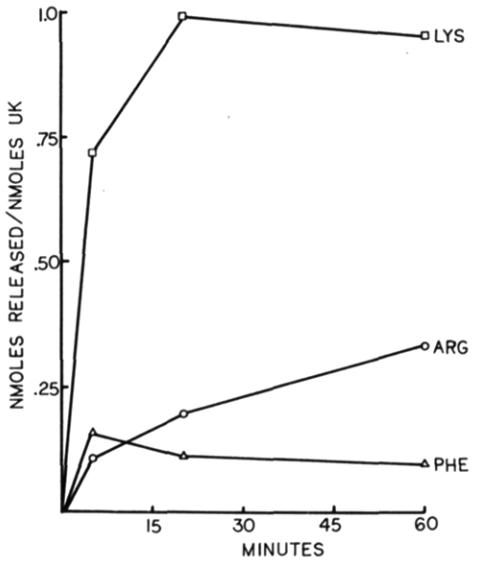

FIGURE 5: Carboxy-terminal amino acids released from rUK_1 which has been incubated with plasmin for varying lengths of time. The carboxy-terminal amino acids were released by digestion with carboxypeptidase B for 90 min at room temperature. Values have been corrected for release of the carboxy terminal amino acids of plasmin.

such as EDTA. However, even with these compounds present, there is significant conversion of rUK_1 to rUK_2 in *E. coli* cell lysates. Clearly, the best way to deal with these proteases is to eliminate them as early as possible in the preparation.

Fortunately, when *E. coli* produce large amounts of rUK_1, they segregate the recombinant protein into large intracellular inclusions. These structures, which refract light under the microscope, are termed "refractile bodies" (Wetzel & Goeddel, 1983). The refractile bodies are released when the cells are broken, and the rUK_1 contained in them can be purified from a majority of troublesome *E. coli* proteases by a selective two-step centrifugation. The first centrifugation step separates the refractile bodies from soluble DNA, RNA, and free proteins, whereas the second centrifugation, through a 50% glycerol cushion, allows the relatively dense refractile bodies to be separated from lighter cellular debris such as membranes or cell wall fragments. Both of these steps are necessary to remove the bulk of the *E. coli* proteases which were found to cleave rUK_1 to rUK_2 during the rest of the purification procedure. If this purification is not undertaken, cleavage to rUK_2 is 50–90% complete after dialysis to remove the refolding reagents.

Throughout the purification procedure, steps were used which would minimize proteolysis. Columns which concentrated the sample were avoided where possible. When high sample concentrations were encountered, 1.0 M guanidine hydrochloride was added to inhibit proteolysis. Dialysis was not allowed to proceed for more than 6 h and was always carried out at 4 °C. Chromatographic steps, where possible, were chosen to favor nonadsorption of rUK_1 to facilitate speed of processing and unnecessary concentration. The DE-52 resin, which efficiently separates many *E. coli* proteases from the rUK_1, was used early in the scheme.

The specific activity of the purified rUK_1 was found to be 89000 PU/mg (Table II). This activity is somewhat lower than the 102000 PU/mg measured for rUK_2 (Winkler et al., 1985). Most of this difference can be accounted for by the 5–10% impurities found in the rUK_1. However, it may be that a small amount of imperfectly folded rUK is also in the preparation. The active site of rUK_1 is different from that of rUK_2, as is evidenced by the different binding affinities and

cleavage rates for plasminogen. Therefore, the benzamidine–Sepharose column can no longer be used to separate inactive rUK_1 from active rUK_1 as was possible with the purification of rUK_2 (Winkler et al., 1985). Rather, the column is only useful in separating rUK_1 from rUK_2. To improve the purification of rUK_1, it may be useful to include an affinity column or antibody column at the end of the purification scheme to select for active rUK_1.

The amino-terminal methionine, necessary for the initiation of translation, is completely removed from the rUK_1 by the *E. coli*. For several recombinant proteins, such as human growth hormone, *E. coli* does not cleave after this methionine (Olsen et al., 1981). It is not clear what factors are involved which determine the removal of this initiator methionine.

We have shown previously that rUK_2, which has been cleaved to rUK_2 by *E. coli* proteases during the purification, has been cleaved entirely between lysine-158 and isoleucine-159 (Winkler et al., 1985). Forty-five percent of the rUK_2 has had a second cleavage after arginine-156 which releases the Phe-Lys dipeptide (Winkler et al., 1985). In the present paper, we show that plasmin also activates rUK_1 by cleaving between lysine-158 and isoleucine-159, with little additional cleavage after arginine-156. The cleavage between lysine-158 and isoleucine-159 is the only cleavage necessary for increased activity toward chromogenic substrates. It is not clear which enzymes activate UK_2 in vivo, but it has been shown that lysine-158 is excised from the UK_1 during activation, or before release into the urine, since it is not present in high molecular weight UK_2 purified from urine (Gunzler et al., 1982). It has been shown that UK_1 is not activated in the urine in vivo but is stable as the single-chain enzyme (Pannell & Gurewich, 1983). Plasmin cannot be the sole protease which processes UK_2 in vivo since it is not capable of cleaving after phenylalanine-157 to release lysine-158 and only cleaves very slowly after arginine-156. Either two proteases, such as plasmin and a carboxypeptidase, or a completely different protease must release lysine-158 during activation.

Recent reports on in vivo studies using rUK_1 (Collen et al., 1984b) show this protein to behave similarly to UK_1 isolated from tissue culture sources (Gurewich et al., 1984). That is, it requires less rUK_1 than rUK_2 or UK_2 to dissolve an experimental thrombosis in rabbits, with systemic activation of plasma proteases only at high doses. The rUK_1 has a short half-life even though it is not glycosylated and has not been cleaved to the two-chain form (Collen et al., 1984a). Therefore, the protein must be cleared by mechanisms other than by formation of protease–inhibitor complexes of the two-chain form or through sugar receptors in the liver. The purification of large amounts of rUK_1 will allow further in vitro and in vivo evaluations of UK_1 as a potential thrombolytic agent.

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