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CHARGE HETEROGENEITY OF RECOMBINANT PRO-UROKINASE AND URINARY UROKINASE, AS REVEALED BY ISOELECTRIC FOCUSING IN IMMOBILIZED pH GRADIENTS

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SUMMARY

When analysing homogeneous preparations of recombinant pro-urokinase and urinary urokinase by isoelectric focusing (IEF) in immobilized pH gradients, an extreme charge heterogeneity was detected (at least ten major and ten minor bands in the pH range 7–10). This extensive polydispersity was not caused by different degrees of glycosylation, or by IEF artefacts, such as binding to carrier ampholytes or carbamylation by urea. A great part of this heterogeneity could be traced back to the existence of a multitude of protein molecules containing Cys residues at different oxidation levels (–SH, S–S–, even cysteic acid). Owing to the very large number of Cys residues in pro-urokinase (24 out of a total of 411 amino acids) and to the relatively high p*I* of its native forms (p*I* 9.5 9.8; the native form is believed to contain all Cys residues as –S–S bridges), the presence of SH or cysteic acid residues would increase the negative surface charge, as even SH groups would be extensively ionized. In pro-urokinase, part of the heterogeneity was also due to spontaneous degradation to urokinase and possibly also to cleavage into lower-molecular-mass fragments. When all these causes of heterogeneity were removed, the p*I* spectrum was reduced to only four, about equally intense, bands. The cause of this residual heterogeneity is unknown.

INTRODUCTION

Urokinase plasminogen activator (u-PA) is the factor responsible for the fibrinolytic activity of human urine. The enzyme is a serine protease that can convert, via a specific proteolytic clip, the zymogen plasminogen into the active form, plasmin, which in turn degrades the fibrin clots. For this property u-PA is widely used as

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fibrinolytic agent in thrombolytic therapy.

u-PA is a glycoprotein[1], present in human urine in two active forms: high-molecular-weight urokinase (HMW u-PA), consisting of two polypeptide chains, A and B, of 20000 and 30000 Da, respectively, connected by a disulphide bridge (also called two-chain urokinase or tcu-PA), and low-molecular-weight urokinase (LMW u-PA), of *ca.* 33000 Da, which contains the entire B chain and a short fragment (about 20 amino acids) of the A-chain. LMW u-PA is generally considered to be a degradation product, which retains catalytic activity, generated by the loss of an 18000 Da amino terminal fragments (ATF) from the A-chain of HMW u-PA. In urine the proenzyme is also present in the form of a single-chain urokinase (pro-urokinase or scu-PA[2]), which can be activated by plasmin to tcu-PA by proteolytic cleavage at the Lys-158 residue[3]. scu-PA is the most abundant form found in the culture media of different cell lines[2,4–8], and it is probably the only u-PA secreted by cells *in vivo*[9].

Although the primary structure of u-PA has been completely elucidated, some important biochemical features of the enzyme, such as glycosylation, amidation, phosphorylation and in general post-translational modifications, still require a more extensive investigation. For instance, although u-PA has been purified to homogeneity and shown to behave as a single band by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), some heterogeneity has been observed by isoelectric focusing (IEF), with a variable number of bands in the pH range 8–9.6[10–13]. Similar results have also been obtained for other, related proteins, such as plasminogen[14], but to date not general explanation has been proposed.

In this work, we investigated the behaviour in IEF (both conventional and in immobilized pH gradients) of u-PA in the glycosylated and neuraminidase-treated forms and extended our study to scu-PA and to molecular fragments of u-PA. It will be shown that the charge heterogeneity of u-PA is independent of the source of u-PA (be it urinary, from cell culture supernatant or from recombinant-DNA sources).

EXPERIMENTAL

Immobiline, Repel- and Bind-Silane, Ampholines, Gel Bond PAG, the Multiphor 2 chamber, the Multitemp thermostat and the Macrodrive power supply were from LKB (Bromma, Sweden) and Pharmalytes, activated Sepharose and the electrophoresis calibration kit for low-molecular-mass (M_r) proteins were purchased from Pharmacia (Uppsala, Sweden). Acrylamide, N,N'-methylenebisacrylamide (Bis), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate, Affi-Gel 10, sodium dodecylsulphate (SDS) and Coomassie Brilliant Blue R-250 were from Bio-Rad Labs. (Richmond, CA, U.S.A.). Dithiothreitol (DTT) and urea were from Merck (Darmstadt, F.R.G.) and 5,5'-dithiobis-2-nitrobenzoate (DTNB) was purchased from Serva (Heidelberg, F.R.G.). Iodoacetamide was from BDH (Poole, U.K.). Neuraminidase (*Clostridium perfringens*) was from Boehringer (Mannheim, F.R.G.). Commercially available, therapeutic-grade urinary u-PA was isolated from urine by Lepetit (Gerenzano, Italy). Human scu-PA was extracted from recombinant LB6 cell supernatant[15] by immunoaffinity and ion-exchange chromatography, essentially as described for A431 scu-PA[6]. LMW u-PA and ATF were purified from autocatalytically degraded u-PA by chromatography on Sephadex G-100, as described previously[16].

Conventional isoelectric focusing (IEF)

IEF in carrier ampholyte buffers (CA) was performed in 0.5-mm thick gels, supported by a gel Bond PAG plastic film[17]. The gel had a 4%T, 4%C composition (%T = total amount of monomers; %C = grams of Bis per 100 grams of total monomers). The gels were polymerized as "empty" matrices, in the absence of CA; after two washing steps in distilled water (30 min each) they were dried and reswollen in a mixture of 2% CA in the pH range 5–10 and 8 *M* urea. In some experiments, this mixture also contained 5 m*M* DTT. The sample size was 100 μg for Coomassie Brilliant Blue and 2 μg for silver staining (in both instances in a 30-μl volume). The running conditions were 5 W limiting, with a final voltage of 1500 V, for a total of 5 h, at 10°C. Staining was with Coomassie Brilliant Blue–Cu^{2+} according to Righetti and Drysdale[18] and with silver according to Merril *et al.*[19].

Immobilized pH gradients (IPG)

IEF in IPG was performed in 4%T, 4%C, 0.5-mm thick matrices, according to Bjellqvist *et al.*[20]. After standard polymerization (1 h at 50°C)[21], the gels were washed twice in distilled water (20 min each) and then for an additional 20 min in 1% glycerol. After drying, they were reswollen in 8 *M* urea and 1% CA in the pH range 3.5–10 (in some experiments, 5 m*M* DTT was added to the reswelling mixture). Most IPG experiments were performed in the pH range 5.0–10.0; in some pH 5.0–10.5 was utilized. The recipe for the IPG pH 5–10 range was as follows: acidic dense solution (3.8 ml final volume), 143 μl of p*K* 3.6, 117 μl of p*K* 4.6, 75 μl of p*K* 6.2, 69 μl of p*K* 7.0, 57 μl of p*K* 8.5 and 32 μl of p*K* 9.3 Immobilines; basic, light solution (3.8 ml final volume), 5 μl of p*K* 3.6, 15 μl of p*K* 4.6, 8.5 μl of p*K* 6.2, 106 μl of p*K* 7.0, 79 μl of p*K* 8.5 and 69 μl of p*K* 9.3 Immobilines (for IPG recipes, see also ref. 22). The above recipe was also used for the IPG pH 5–10.5 range, except that the basic solution was titrated to pH 10.5 with a p*K* 10.3 Immobiline. The samples were applied in pockets, precast at the anodic side (same amounts and volumes as described under conventional IEF). Electrophoresis was performed for the first 4 h at 400 V, followed by overnight at 2000 V at 10°C (the initial low-voltage period is necessary to prevent sample denaturation before entry into the gel[23]).

IPG in alkaline pH ranges

For IPG in strongly alkaline ranges (pH 10–11) the gel must be supported on silanized glass plates (with Bind-Silane)[24] and must contain a pH 8.0 plateau for sample loading. For the recipe and gel handling, see ref. 25. During electrophoresis, adsorption of carbon dioxide is minimized by alkali traps (filter-paper strips impregnated with 1 *M* sodium hydroxide solution, deposited on the empty surface of the cooling block of the Multiphor II chamber) and by covering the gel surface (except in the region of the pockets for sample application) with a plastic foil (Gel Bond PAG, hydrophobic surface facing the gel). Staining is carried out with Coomassie Brilliant Blue, as very dark background results with silver staining.

SDS-PAGE

SDS-PAGE was carried out in the discontinuous system according to Laemmli[26]. The stacking gel was 5%T–4%C–125 m*M* Tris–HCl (pH 6.8)–0.1% SDS and the running gel was a 10–15%T gradient–4%C–374 m*M* Tris–HCl (pH 8.8)–0.1% SDS.

The sample and marker proteins were denatured in 50 m*M* Tris–HCl (pH 6.8), containing 2% SDS–1% DTT–6 *M* urea–30% glycerol. The denaturating solution can be kept frozen for several months, but DTT is always added at the last moment, just prior to use. Electrophoresis is carried out at a constant 300 V, until the Bromophenol Blue front begins to leach out at the anode.

SH group alkylation

Alkylation of SH groups is performed according to Crestfield *et al.*[27] at room temperature. A 500-μg amount sample is dissolved in 500 μl of 8 *M* urea–1 *M* Tris–10 m*M* EDTA and titrated to pH 8.5 with acetic acid under nitrogen and with continuous stirring. After 15 min, the solution is made 10 m*M* in DTT. After 2 h, monoiodoacetamide is added and the reaction is allowed to continue in the dark for 20 min. The reaction is terminated with an excess of 2-mercaptoethanol.

Titration of free SH groups with DTNB

A 10 m*M* solution of DTNB in 300 m*M* Tris–HCl (pH 8) was prepared in the dark just prior to use. For the assay, 100 μl of DTNB solution and 100 μl of sample solution are added to 2.8 ml of the above buffer. The sample is composed from the major isoelectric bands of u-PA, isolated from excized gel portions in an IPG gel, in amounts calculated to contain up to 10 nmol of cysteine residues (see Fig. 7). The absorbance at 410 nm is measured immediately against blank tubes. The calibration graph is constructed with standards of glutathione (0–10 nmol). The molar absorption coefficient of reduced DTNB is taken as 13600, according to Ellman[28].

RESULTS

Size homogeneity of different urokinases

When recombinant scu-PA and tcu-PA were analysed by SDS-PAGE they were found to be essentially homogeneous (Fig. 1A). The same gel also shows the position of LMW u-PA (track C) and the pattern given by ATF (track D). When the same experiment was repeated in the presence of an excess of 2-mercaptoethanol, scu-PA still exhibited a single band while tcu-PA was split into two bands, corresponding to the B-chain (30000 Da) and to the A-chain (20000 Da), respectively. The present data clearly indicate that all species isolated are free from contaminant polypeptides of different molecular mass.

Charge heterogeneity of different urokinases

However, when the above tcu-PA, purified from human urines, was analysed by IEF in IPG in the pH range 5–10, it was found to be extensively heterogeneous, exhibiting at least ten major and ten minor isoelectric bands, with p*I* values covering the pH range 7–10 (Fig. 2). This most pronounced polydispersity prompted us to study its possible causes. It is in general believed that one of the most frequent causes of charge heterogeneity is post-synthetic glycosylation of the polypeptide chains, producing a series of charge variants due to the different content of sialic acid or other charged sugars. To verify this, scu-PA was subjected to treatment with neuraminidase, and then analysed by conventional IEF. As seen in Fig. 3, the train of bands in the control (at least six major zones) is shifted to higher p*I* values, indeed suggesting

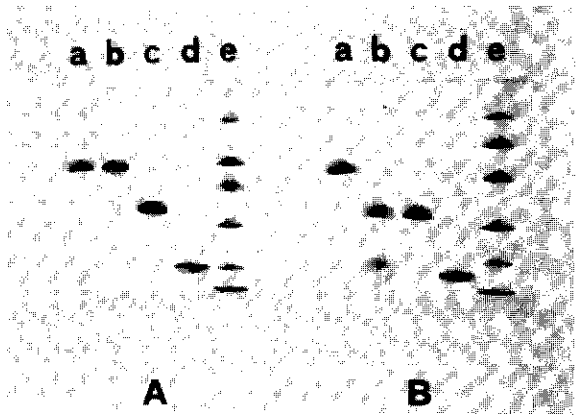

Fig. 1. Analysis of different urokinase preparations by SDS-PAGE was performed in the discontinuous system according to Laemmli[26] in a 10–15%T porosity gradient under (A) non-reducing and (B) reducing conditions. Lanes: (a) recombinant single-chain urokinase plasminogen activator (scu-PA); (b) two-chain u-PA (tcu-PA); (c) low-molecular-mass urokinase (LMW-UK); (d) amino-terminal fragment (ATF) of urokinase; (e) molecular-mass standards (Pharmacia), from top to bottom phosphorylase B (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100) and β-lactalbumin (M_r 14 400). SDS-PAGE was performed in Phast-gel apparatus (Pharmacia). 2-Mercaptoethanol was used as reducing agent. Staining: Coomassie Brilliant Blue R-250.

the presence of sialic acid, but the heterogeneity is not reduced, although the relative band intensity seems to be altered in favour of the most alkaline components.

In a search for other possible sources of heterogeneity, we hypothesized that some of it could be produced by the presence of 8 *M* urea in the gel, as electrophoresis occurs at alkaline pH values, which could favour degradation of urea to cyanate and the subsequent carbamylation of deprotonated amino groups in proteins. However, when electrophoresis was repeated in the presence of 50% dimethyl sulphoxide, as a substitute for urea, essentially the same pattern was obtained (not shown), thus ruling out modifications due to the presence of urea.

Another possible cause of heterogeneity could be potential binding to the carrier ampholytes, admixed with the IPG gel. We therefore repeated the experiment in an IPG pH 5–10 gel in the absence of CA chemicals. As shown in Fig. 4, urokinase exhibits essentially the same polydisperse spectrum, although the bands are more diffuse, possibly owing to adsorption on the IPG matrix. This also rules out potential artefacts due to binding to the CA buffers.

In search for other possible causes of such extensive heterogeneity, we noticed that pro-urokinase contains a very large number of Cys residues (a total of 24, all of them believed to be in the form of –S–S– bridges) in the native molecule. If the fully oxidized molecule were in equilibrium with partially reduced species (containing SH groups), this could greatly contribute to the charge heterogeneity, as these groups, at the pH prevailing during IEF, would be extensively ionized to $-S^-$, thus automatically generating lower -p*I* components. Evidence for such a mechanism comes from Fig. 5; here, the same IPG gel was divided into two sections and reswollen either in the presence (left) or in the absence (right) of DTT. It is seen that "native" urokinase

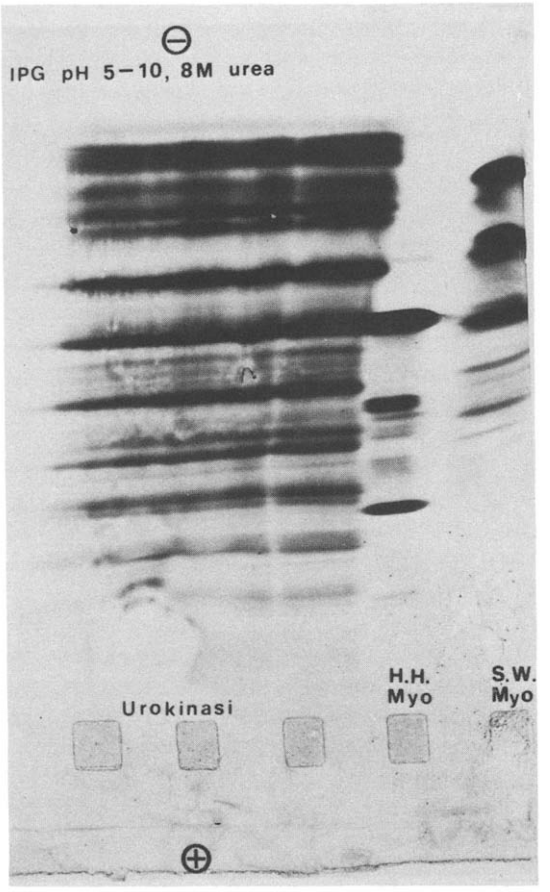

Fig. 2. Analysis of urokinase by IEF in IPG. A 4%T–4%C polyacrylamide gel was prepared, containing a pH 5–10 immobilized gradient. The gel was washed, dried and reswollen in 8 *M* urea–1% CA in the same pH 5–10 range. Samples were applied in pockets, precast at the anodic side. Electrophoresis: overnight at 2000 V, 10°C. The three urokinase lanes contained a total of 100 μg of protein and the two pH indicators were 20 μg each. HH Myo and SW Myo: horse heart and sperm whale myoglobins, respectively. Staining: Coomassie Brilliant Blue R-250 in the presence of copper sulphate.

consists of a major group of tightly packed species with high p*I* values (pH range 9.5–9.8) followed by a number of minor bands with lower p*I* values (pH 7–9). In the presence of DTT (left panel) (added also to the sample zone) the high-p*I* components almost disappear and the lower-p*I* components are strongly reinforced. Note that the major lower-p*I* components of reduced urokinase coincide, in isoelectric points, with the minor lower-p*I* species present in "native" urokinase, suggesting a precursor–product relationship. In order to prove this hypothesis, the IPG gel was used in a small-scale preparative version, by applying a single sample to an anodic trench. A lateral gel segment was stained, for detecting the band position, while leaving the remainder of the gel under voltage. By aligning the stained with the unstained gel

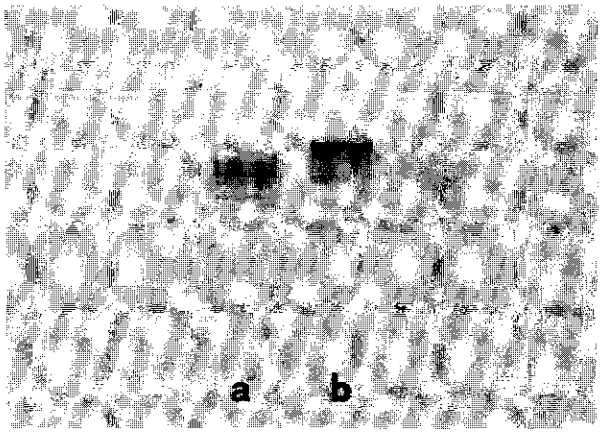

Fig. 3. Conventional IEF (in CA buffers) in the pH range 7–10 of recombinant scu-PA, (a) before and (b) after treatment with neuraminidase (from *Clostridium perfringens*). IEF was in a 1-mm thick, 6%T polyacrylamide gel, containing 6 *M* urea and 0.5% Nonidet P-40. scu-PA was incubated with neuraminidase in 50 m*M* acetate buffer (pH 5), containing 15 m*M* sodium chloride–0.01% bovine serum albumin–10 m*M* benzamidine, for 18 h at 37°C. Electrophoresis: 5 h at 1500 V, 10°C. Staining as in Fig. 2.

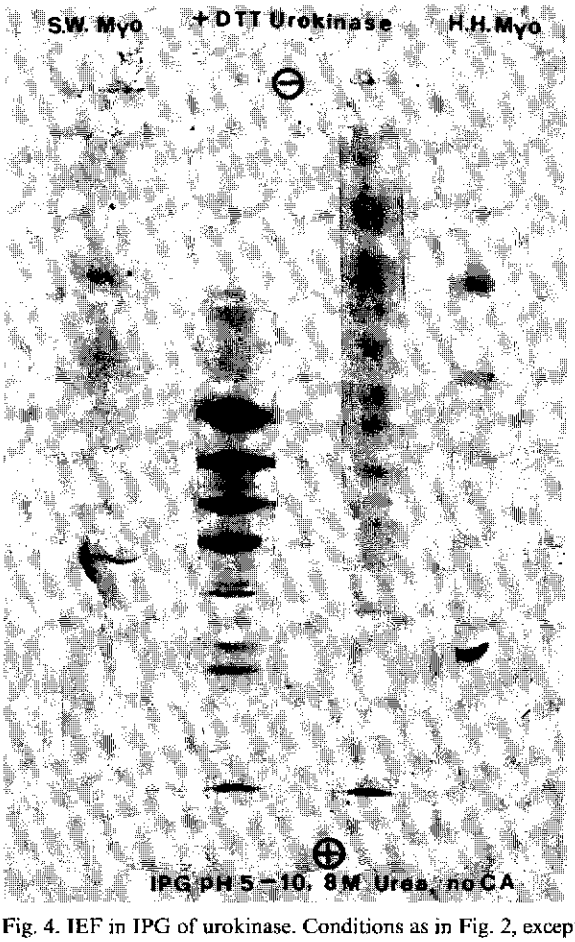

Fig. 4. IEF in IPG of urokinase. Conditions as in Fig. 2, except that the gel contained no carrier ampholytes. Of the two urokinase tracks, that on the right is in the absence and that on the left in the presence of

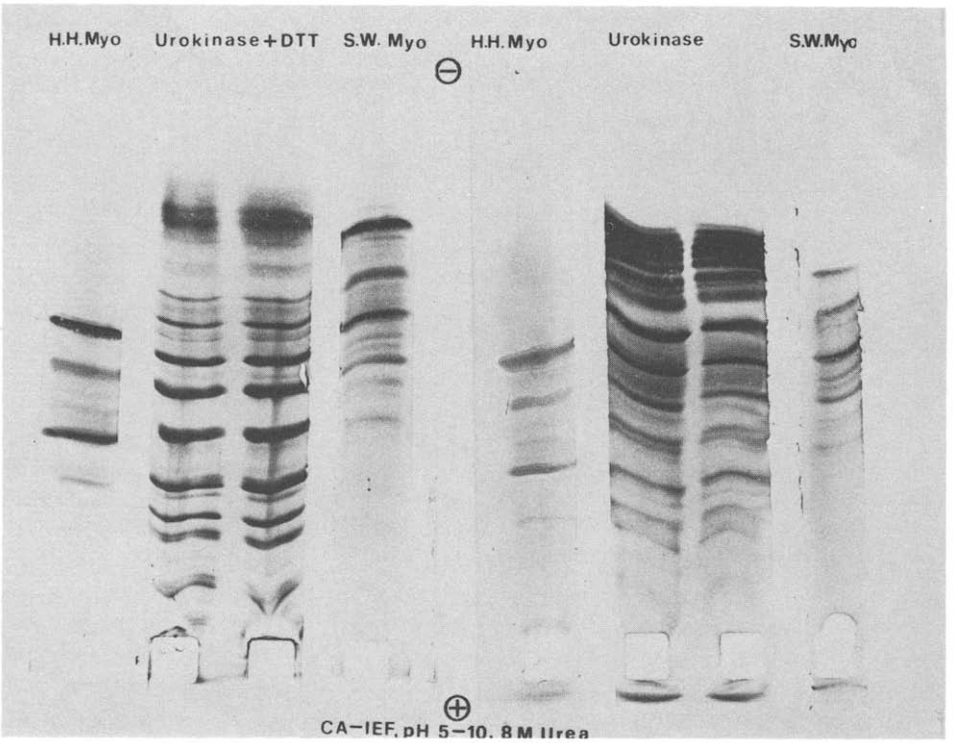

Fig. 5. IEF in IPG of urokinase in the presence (left) and in the absence (right) of reducing agents (DTT). Other conditions as in Fig. 2. Two gels were prepared; the left one was reswollen in 1% CA-8 *M* urea–5 m*M* DTT, while the right one contained the same reswelling mixture without DTT. Note the disappearance of the very alkaline urokinase bands in the DTT gel.

portions, a group of six bands (see Fig. 7) were excized, eluted and assayed for free SH groups.

As shown in Fig. 6, on a molar basis, the lower-p*I* components contain progressively higher amounts of titratable SH groups than the native high-p*I* components, in which less than 1 mol of SH per mole of protein can be evidenced. However, even the low-p*I* group (*ca.* pH 7.5) is far from exhibiting the full number of 24 SH groups, which should be present in a completely reduced pro-urokinase (see the Discussion for this phenomenon). It therefore appears that preparations of urokinase are indeed a mixture of molecules in equilibrium, with the high-p*I* components representing "native" species and the lower-p*I* species typifying conversion products with progressively broken S–S - bridges. As this pattern is exhibited by all preparations that we analysed (scu-PA and tcu-PA), it appears to be a general phenomenon. It remains to be seen how this happens.

Some evidence for the genesis of these bands comes from Fig. 7. Here, an IPG gel containing "native" molecules (in the absence of DTT) is cut again into six major zones, as described above. These gel segments (labelled 1–6) are immediately reloaded on to two IPG gels, one in the absence and the other in the presence of DTT

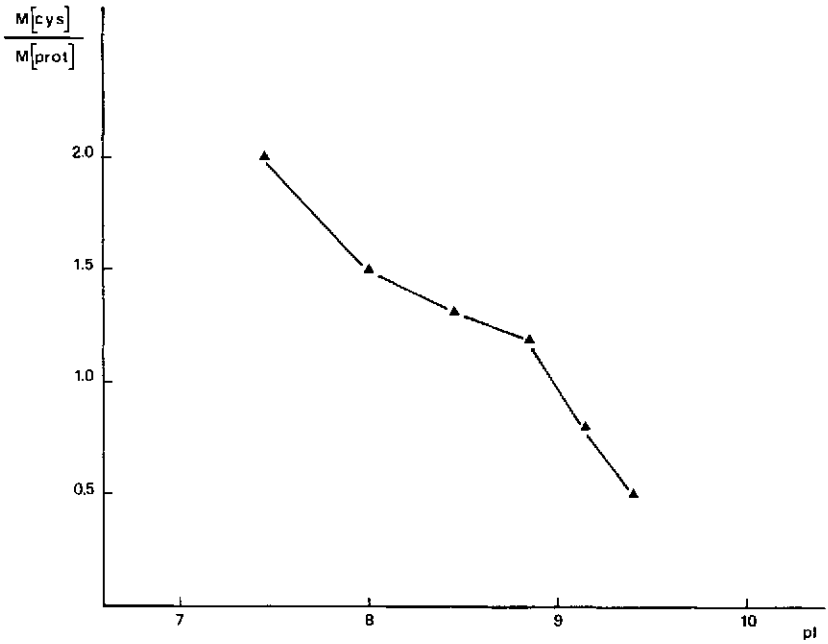

Fig. 6. Titration of free SH groups in urokinase bands of different isoelectric points. A preparative gel was run and six urokinase zones with p*I* values ranging from 7.5 to 9.5 were excized and eluted from the polyacrylamide gel (the IPG pattern with the six labelled zones is shown on the extreme right of Fig. 7). The supernatant was assayed for protein and for free SH groups, as described under Experimental, and the results were expressed in mol Cys/mol protein as a function of the observed p*I* values in the IPG gel.

at the anodic side, and re-focused as such. It is seen that, whereas the bands in the DTT gel give the usual spectrum of reduced, lower-p*I* components (four major plus a number of minor zones; *cf.*, Fig. 5), surprisingly, in the native gel (in the absence of DTT) all the components run again to the original position of the six groups of zones, while simultaneously forming the entire spectrum of lower-p*I* components, typical of the DTT gel (left panel). The group of bands labelled No. 6 appears to be the final conversion product, as its position does not change upon re-running. Hence it appears that the lower-p*I* components are formed spontaneously, possibly even in the test-tubes, on storage, and definitely during IPG (at least 30% conversion during an overnight run).

The situation is further complicated by the fact that, *e.g.*, in the case of pro-urokinase, the molecule could be spontaneously activated by proteolytic cleavage to form urokinase. We therefore analysed, side-by-side, pro-urokinase and urokinase in an IPG pH 5–10 gel in the absence of DTT. As shown in Fig. 8, pro-urokinase indeed gives a major, strongly alkaline band (p*I* 9.8), followed by a multitude of lower-p*I* components (rendered visible only by silver-staining this portion of the gel). It would appear that all these minor components are shared by preparations of urokinase, which, in fact, lacks only the strongly alkaline species. At this point, we took all the possible interconversion products and analysed them in the same gel. As shown in

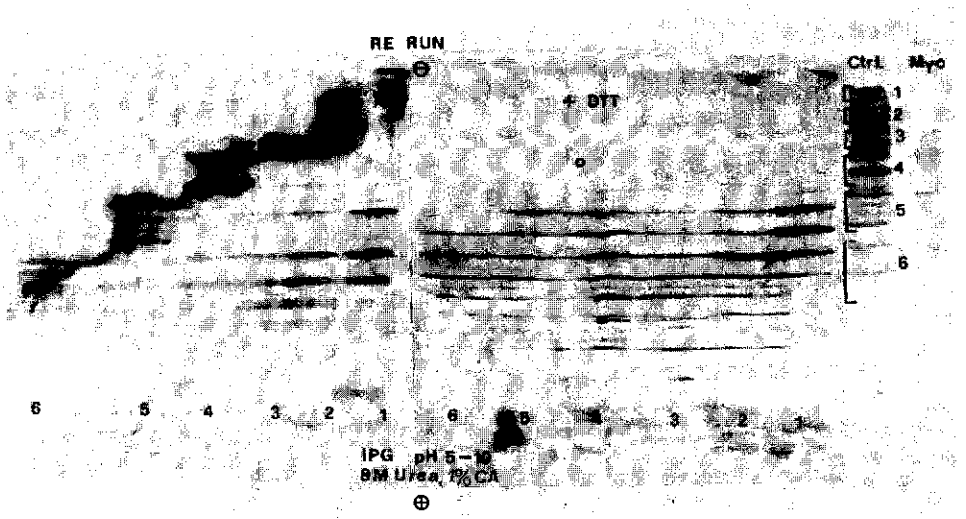

Fig. 7. IEF in IPG of urokinase. Gel and running conditions as in Fig. 2. After electrophoresis, a lateral segment was cut and stained in Coomassie Brilliant Blue, while leaving the remaining of the gel under voltage. Six major zones of urokinase were selected (see the right lane marked Ctrl., control), the bands excized and applied directly at the anodic side, on the gel surface, of two different gels, both reswollen in 8 *M* urea and 1% CA, one in the presence and the other in the absence (left portion) of DTT. These two gels were subjected to a second overnight focusing, run at 2000 V. Note that the "native" bands rerun in the left gel focus again at their original p*I* position, but forming a substantial amount of lower-p*I* components (*ca.* 30% during an 18-h run). Myo: horse heart myoglobin marker.

Fig. 9, pro-urokinase gives, as expected, a major alkaline zone, followed by a fine spectrum of lower-p*I* components. Urokinase, run in DTT, reproduces this spectrum of low-p*I* species, which is essentially identical, in position and intensity, with the bands produced by LMW u-PA. In contrast, ATF gives only two or three very faint bands, coincident with some minor bands in the LMW u-PA track. As the amount of ATF loaded is the same as that of LMW u-PA, there is clearly something missing. As the ATF fragment, on the basis of its amino acid composition, should have a very high p*I*, we repeated the run in an IPG in the pH 10–11 range. Now ATF was found to give two intense bands, the major one with a p*I* of *ca.* 10.4 and the minor one of *ca.* 10.2; however, owing to the very poor stainability of these alkaline bands, they could be revealed only by visual inspection and could not be reproduced photographically.

DISCUSSION

These studies reveal an often overlooked source of charge heterogeneity for protein molecules, *viz.*, coexistence among species in the SH, –S–S– and SO_3^{2-} states. This equilibrium could remain undetected in most instances, as two thirds of the known proteins have isoelectric points falling in the acidic portion of the pH scale. Given the mildly alkaline p*K* of the SH group of Cys (p*K* = 8.3), the presence of an SH rather than an –S–S– group would remain unnoticed in acidic proteins, as neither

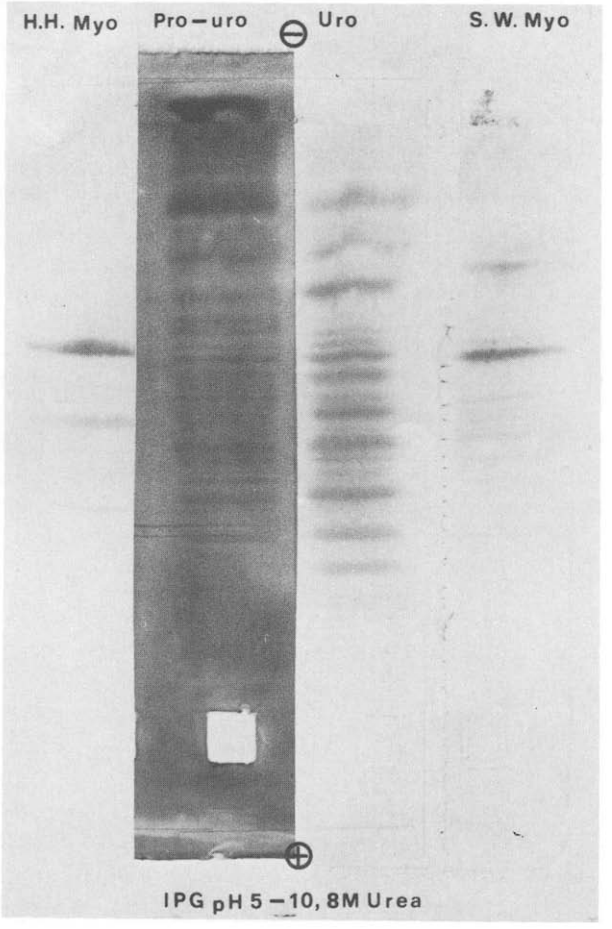

Fig. 8. IEF in IPG of pro-urokinase (pro-uro) and of urokinase (uro). Conditions as in Fig. 2. Owing to the small amount present, the pro-uro track was stained with silver, while the others were dyed with Coomassie Brilliant Blue. Note the strongly alkaline major band in the pro-uro preparation.

would contribute to the surface charge. However, in urokinase-like molecules, owing to the high isoelectric point of the "native" forms and to the presence of an unusually large number of Cys (24 out of a total of 411 amino acid residues), an equilibrium between SH and –S–S– states would be immediately visible by producing a series of charge-altered species. For example, the rupture of any single –S–S– bridge, with the formation of two SH groups, would produce species with a net increment of *ca.* two negative charges because at the high p*I* of the "native" protein molecules (p*I* 9.8), such groups would be extensively ionized. It is a fact that the high-p*I* species were found to have almost no titratable SH groups (less than 1 mol per mole of protein), in agreement with literature data, whereas the lower-p*I* components had a larger number of available SH groups. It could be asked, however, why this number (see Fig. 6) is much lower than the theoretically expected number of 24 SH groups per mole of

Fig. 9. IEF in IPG of pro-urokinase (pro-UK), urokinase (UK), low-molecular-weight urokinase (LMW) and amino-terminal-fragment (ATF). HH Myo: horse heart myoglobin. Conditions as in Fig. 2, except that all tracks were stained with silver. The gel was reswollen in 8 *M* urea–1% CA–5 m*M* DTT.

protein. The answer could be that at high pH values, in the presence of atmospheric oxygen and under an electric field (the anode is a strongly oxidizing compartment), oxidation of Cys could proceed to an irriversible state, *i.e.*, cysteic acid. From the point of view of charge, high-p*I* molecules carrying a free SH group or its fully oxidized cysteic acid derivative should be indistinguishable, as both would carry a net negative charge. However, titration with Ellman reagent would reveal only the former and ignore Cys residues transformed to cysteic acid. Indeed, this phenomenon was reported so long ago that is has probably been forgotten. Thus, as early as 1971, Jacobs[29,30] first reported the partial modification of Cys and Met to cysteic acid and methionine sulphoxide, upon prolonged IEF, in bovine ribonuclease (also an alkaline protein). These oxidation phenomena could be largely suppressed by removal of oxygen from the IEF column (at that time most IEF experiments were run in a vertical column in a sucrose density gradient) and by addition of antioxidants, such as thiodiglycol and ascorbic acid. With the advent of open-face IEF gels, both of these

remedies were abandoned as impractical (thiol groups are inhibitors of gel polymerization). However, with IPG technology, such remedies can easily be exploited again, as IPG gels are routinely washed and dried and can be reswollen in the solvent of choice (including any desired reducing agent).

Another possible source of heterogeneity comes from the nature of the protein itself. Pro-urokinase can be spontaneously cleaved with the formation of urokinase, which can further split into LMW u-PA and ATF. The former has a p*I* spectrum similar to that of urokinase, while the latter produces two major strongly alkaline bands ($I > 10$) and only a few, very faint zones in the pH 7–8 region. However, it is surprising that, under strongly reducing conditions (addition of DTT), one can dramatically reduce the enormous p*I* spectrum (ten major and at least 10 minor p*I* components) without obtaining a single p*I* band. The best that can be achieved, in fact, is the reduction of this multiple spectrum to a set of four bands of about equal intensity. Having ruled out different extents of glycosylation, the other possible cause of this residual heterogeneity would be the existence in the molecule of residues in the Asp–Asn or Glu–Gln states. Further work is needed to elucidate these more elusive aspects. It is nevertheless hoped that this work will be of help in understanding the enormous complexity of urokinase-type molecules, when analysed by surface-charge-dependent methods, and that it will offer some guidelines to scientists purifying recombinant-DNA products and having to meet the stringent requirements of FDA regulations.

ADDED NOTE

After this paper had been accepted, on continuing our studies on IPG matrices, we have now obtained proof that alkaline IPG ranges indeed have a strong oxidizing power. This residual oxidizing power is typical of IPG matrices, and remains even on prolonged washing of the gel and electrophoretic removal of any residual persulphate. Thus, considering that native urokinase molecules contain essentially only cystine residues, the only possibility left is that their further oxidation produces cysteic acid. This is in agreement with our hypothesis and with the experimental evidence given (Fig. 6) that the low-p*I* species exhibited only a modest increment in free, titratable SH groups.

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