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Effector-Assisted Refolding of Recombinant Tissue-Plasminogen Activator Produced in *Escherichia coli*

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ABSTRACT

Recombinant tissue-plasminogen activator (r-tPA), expressed in *Escherichia coli* cells in an aggregated form, was solubilized with a strong chaotrope in the absence of any reducing agent. The solubilized molecule was reactivated by a procedure that was developed to mimic the physiological conditions optimal for the functional folding and activity of the native protein. The use of partially purified fibrinogen, as a source of fibrin (the effector), is shown to facilitate the reactivation process and increase its yield by at least a factor of two. The yield of the process is also shown to be particularly dependent on the recombinant protein concentration. At a concentration level of 3–3.7 mg r-tPA/L in the reactivation mixture, up to a 90% yield of activity was obtained.

Purification of the activated form of r-tPA was achieved with a two-step column-chromatography scheme. This included a gel filtration step on a Sephadex G-50 column followed by an affinity chromatography step on a lysine-sepharose column. The product was composed of roughly equal amounts of one-chain and two-chain t-PA.

The feasibility of using a two water-soluble polymeric phase system, with a centrifugal partition chromatograph (CPC), in scaling up the reactivation process or the purification step was also evaluated.

Index Entries: Fibrinogen/fibrin-assisted refolding; recombinant t-PA; optimization.

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INTRODUCTION

Expression of eukaryotic proteins in recombinant mammalian cell lines yields products that are in principle identical to the naturally occurring protein. However, production costs in mammalian cell culture continue to be very high, and therefore, much interest persists in developing the expression of complex proteins in lower-cost bacterial systems such as *E. coli*. Depending on the mol wt of the protein and the genetic manipulation technique employed, expression levels in *E. coli* have exceeded 20% of the total cell protein (*1–3*). However, many of these recombinant proteins are expressed in the cytoplasm of the *E. coli* cells in an insoluble (inclusion bodies) and inactive form. Solubilization of the recombinant protein from inclusion bodies is usually achieved by the use of denaturants or strong chaotropic agents in combination with reducing agents. Obtaining the recombinant protein in its biologically active form from chaotropic solutions is particularly difficult with large proteins containing multiple sets of disulfide bonds (*3,4*).

Some naturally occurring high mol wt proteins such as serum albumin have been refolded successfully from a concentrated solution at 2mg/mL. This was done (*5*) with albumin derived from human plasma, which had been purified, unfolded, and then refolded. However, when such high-mol-wt proteins are expressed in the *E. coli* system, complete refolding of the molecule to its native and functional structure has not been achieved in this range of protein concentrations (*6*). In most cases, it is achieved in low yields with protein concentrations in the vicinity of μg/mL.

We report here our studies carried out with a relatively high-mol-wt protein, r-tPA, derived from expression in the *E. coli* system. Tissue-plasminogen activator is a serine protease which has an important function in the fibrinolytic system (*7*). It catalyzes the conversion of plasminogen to plasmin in the presence of a fibrin clot. It is a single-chain polypeptide of 530 amino acids, 69 kDa, and contains 17 disulfide bridges and three free cysteines. The enzyme is comprised of several domains, namely: a "finger," a growth factor-like domain, two "kringles," and a protease. Of particular interest are the finger and second kringle domains, which are involved in fibrin binding (*8*). A few attempts are described in the literature, to obtain fully active r-tPA from expressions in inclusion bodies of *E. coli* cells (*9–11*). The reports all describe a similar procedure for the isolation of r-tPA inclusion bodies and solubilization of the protein in 7*M* Gu. HCl under reducing conditions such as 0.15–4*M* dithiothreitol (DTT) (*11*) or 50 m*M* β-mercaptoethanol (*9,10*). This is followed by a step to reactivate (or refold) the fully reduced molecule. Such has been attempted by elimination of the reducing agent (particularly by dialysis) followed by diluting the resultant solution into an alkaline buffer [pH 10.5 (*11*) or pH 8.75 (*9,10*)]. The redox buffers contained reduced (GSH) and oxidized (GSSG) glutathione as well as a relatively weak denaturant (2.5*M* urea).

The reports claim a reactivation of the r-tPA to full activity but with a limited maximum yield. No explanations are given for these limitations, and we will discuss them in the last part of this report.

In this study, we describe and assess the feasibility of affecting the yield of refolding large proteins, such as r-tPA, by performing the process under conditions that mimic the physiological conditions favoring its activity. Also, we describe our results in the application of the two-phase affinity partitioning technique (*12,13*) to attempt scaling-up the reactivation process and/or the purification step.

MATERIALS AND METHODS

Materials and Reagents

For cell lysis and solubilization of r-tPA, *E. coli* cell paste, ECP786, containing t-PA was obtained from our in-house Biological Process Sciences Department. All chemicals used for cell lysis and solubilization of recombinant protein were obtained from Sigma (St. Louis, MO), except for Octyl-β-D-thioglucopyranoside (β-DPG), which was obtained from Calbiochem (CA). Homogenizations of cell paste suspensions were carried out using Tissuemizer® from Tekmar (OH). Dispersion of the cell lysate was carried out using a Branson (CA) sonicator, model 350. All centrifugations were carried out using preparative centrifuge, Beckman (CA) model J2-21M.

For total protein determination, electrophoresis, and Western blots, bovine serum albumin (BSA), standard II, from Bio-Rad (CA) was used as a standard for protein determinations. A mixture of protein markers, low-mol-wt Rainbow, no. RPN756, from Amersham (UK) was used as a standard for mol wt in the SDS-PAGE and blot. Samples that contained water-soluble polymers, particularly polyethylene glycol 8000 and its derivatives, were processed prior to electrophoresis on either an S-sepharose column or a Q-sepharose column, both from Pharmacia (Uppsala, Sweden). Goat-antihuman melanoma t-PA, no 387, from American Diagnostica (NY) was used as the first t-PA antibody in the development of the blots. All of the other specialty chemicals were obtained from Bio-Rad. A Speed-Vac Concentrator, model SVC100H from Savant (NY) was used in the preparation of samples for protein determination and electrophoresis. A Mini-Protean II system from Bio-Rad was used to perform the electrophoresis and Western blots.

For enzyme reactivation, L-arginine (free base), p-amino-benzamidine (PABA); 1,1'-carbonyl-diimidazole (CDI); dextran 500 (DEX500); fibrinogen; glycyl-glycyl-L-arginine (GGR); and polyethylene glycol 8000 (PEG8000) were obtained from Sigma. Reduced (GSH) and oxidized (GSSG) glutathione were from Calbiochem. Dioxane and sodium tetraborate were from Aldrich (WI) and arginine-semicarbazone (ArgSC) from SKB (PA).

For preparation of PEG derivatives, molecular sieves, 4A, 8–12 mesh were obtained from Aldrich. An evaporator, Rotavapor model RE-120, was from Brinkmann (NY). Dialysis tubing, Spectrapor, with 5000 MWCO was obtained from Thomas Scientific (PA). The PEG derivatives were prepared essentially according to Beauchamp, et al. (*14*). Per each 20 g of PEG8000 (i.e., 2.5 mmol hydroxyl groups), 50 mL of dried dioxane was added, and the suspension was mixed at 37°C until all the PEG dissolved. To this solution, 4.055 g (25 mmol) CDI was added, and the solution was mixed for 2 h at 37°C. Afterward, dioxane was removed with the Rotavapor until a powder was obtained. To this powder (i.e., CDI-activated PEG), 25 mmol of a ligand (either arginine, lysine, PABA, GGR, or ArgSC) solubilized in a 100-mL solution of 0.05*M* borate buffer, pH 8.5, was added. The solution was stirred for 96 h at 4°C. The unreacted ligand was removed by dialysis using Spectrapor tubing. The PEG derivative thus obtained was dried using the Rotavapor.

For r-tPA activity assay, the Kabi AB substrate, S-2251, was obtained from Helena Labs (TX).Glu-plasminogen was obtained from American Diagnostica. A t-PA house standard (YL-14856-127) that had been calibrated against a World Health Organization (WHO) standard was obtained from SKB Pharmaceutics. The rest of the materials used were obtained from Sigma. For r-tPA protein determination, a laser densitometer, Ultroscan model 2202 from LKB (Bromma, Sweden), was used to scan SDS-PAGE gels. For r-tPA purification, CPC, model LLN from Sanki (Kyoto, Japan), was used for purifications in a two-phase partition method. Sephadex G-50 and lysine-sepharose, both from Pharmacia, were used for the chromatographic purification steps.

Cloning and Expression of r-tPA

The Bgl II-SacI fragment containing the entire t-PA coding sequence was isolated from pRDZBtPA.JT (*15*). It was inserted into the expression vector pOTS (*16*) between filled in BamH I and SAC I sites. The vector pOTS-tPA thus created had the ATG initiation codon (provided by the expression vector) placed immediately adjacent to the second codon of the t-PA coding sequence. The plasmid was transformed into *E. coli* strains AR58 and AR120 (*17*), and cultures were grown at 32 and 37°C, respectively. Expression of t-PA was induced in these cultures through the use of thermal induction (in AR58) or nalidixic acid induction (AR120). The production of t-PA was followed by SDS-PAGE gels and Western blots, as described later.

Cell Lysis and Solubilization of r-tPA

Batches of 10 g (or multiples) of cell paste were lysed and the r-tPA solubilized according to the procedure outlined in (Fig. 1). All steps were carried out at 4°C. Each 10 g of cell paste was suspended in a 100-mL solu-

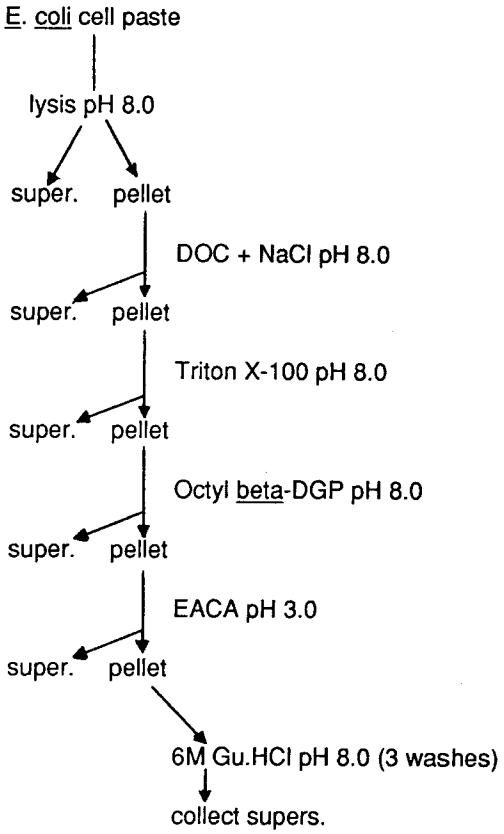

Fig. 1. Isolation of r-tPA inclusion bodies and solubilication of the recombinant protein—schematic description.

tion of cold lysis buffer (0.1*M* Tris, 5 m*M* EDTA, 0.1*M* NaCl, 5% Glycerol—buffer A) and homogenized with the Tissuemizer.® The homogenate obtained was then sonicated (in pulse mode of 10 bursts of 30 s each) and centrifuged for 20 min at 20,000*g* (ca. 12,000 rpm). The supernatant was discarded and the pellet resuspended in a 100-mL solution of 0.05% DOC, 1*M* NaCl, in buffer A. This suspension was then centrifuged as above and the pellet resuspended in 1% Triton X-100 (in buffer A). This step was repeated twice. The pellet obtained was then resuspended in a solution of 1% β-DPG in buffer A and divided (equal vol) into several bottles and centrifuged. The pellets obtained were then resuspended each in a different type of solution and pH. This step was chosen to identify a final solubilization step for the r-tPA, which resulted in good yields in both the solubilization step and later on in the reactivation step.

Protein Determination

Total protein content was determined essentially according to the Bradford assay procedure (*18*), using BSA as a reference. However, to

avoid interference by reagents present in the samples, each sample was treated as follows: A cold solution of 25% trichloroacetic acid (TCA) was added. Following a vigorous mixing step, the samples were kept on ice for at least 30 min. The samples were then centrifuged, and the precipitates obtained were washed with cold acetone. The acetone supernatant was discarded, and the remaining precipitates were dried using the Speed-Vac Concentrator. Finally, the precipitates were solubilized in 10 m*M* Acetic acid. The solutions so obtained were used for the assay.

Electrophoresis and Western Blotting

Samples for SDS-PAGE and blots were prepared essentially according to the procedure described under protein determination. However, in this case, the precipitates obtained after the acetone wash were solubilized with the electrophoresis buffer [0.0625*M* Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.0005% bromophenol blue, in the absence (native) or presence (reduced) of 50 m*M* DTT] instead of the acetic acid.

The 12.5% SDS-PAGE gels were prepared and used essentially according to Laemmli's procedure (*19*). Western blots for t-PA, were carried out by electrotransfer of protein from SDS-PAGE gels onto nitrocellulose papers. Development of the blots was obtained by soaking the nitrocellulose paper, in a sequential manner, in solutions of: goat anti-human t-PA, antigoat IgG-horseradish peroxidase, and horseradish color development reagent in the presence of H_2O_2.

Reactivation

Two approaches were evaluated. The first was "reactivation in the absence of polymers," and the second was "reactivation in the presence of polymers." In the first approach, the solution containing the solubilized recombinant protein was diluted simply into a buffered solution. The increase in activity of r-tPA was measured with respect to the composition of the buffered solution and other physicochemical conditions.

In the second approach, the solubilized r-tPA was diluted into a solution containing either one or two water-soluble polymers. In any case, one of the polymers was PEG, which had been conjugated with a biospecific ligand (*see above*). Two parameters were measured: an increase in activity of the r-tPA and the extent of purification of active enzyme caused by its preferential partition into one of the polymeric phases.

Determination of t-PA Activity

The t-PA activity was determined with the chromogenic substrate S-2251 and the procedure described by Conners et al. (*20*). Purified CHO-tPA, calibrated against WHO International Standard (*21*) was used as a reference.

Determination of Amount of t-PA

The t-PA was quantitated by scanning coomassie-stained SDS-PAGE gels of the sample with a laser densitometer. The t-PA–related bands on gels were identified by appropriate Western blots. From the amount of total protein in a sample (determined as previously described) and the percentage of t-PA–related bands on gels, the amount of r-tPA was calculated. For accuracy, total t-PA–related protein was also calculated with respect to calibration gels, which were prepared with protein standard and pure CHO-tPA. Results from both ways were identical.

Determination of t-PA Specific Activity

Specific activity was calculated from the ratio between the activity of a sample on S-2251 and the amount of total r-tPA protein in the sample (determined as described above).

Purification

The isolation of active r-tPA from its reactivation mixture was carried out in two different ways. In the first, the CPC was used to apply the two-phase partition method (22). This involved using two water-soluble polymers: PEG8000 and DEX500. For each experiment, a solution containing 3.5% (w/w) PEG8000 and 5.0% (w/w) Dex500 was made in a buffer of defined pH value. The solution was mixed well and poured into a separatory funnel. On standing overnight to allow equilibration, the mixture separated into two distinct and clear phases. The two phases thus obtained were then isolated and used as the two phases for the experiments. The CPC was prepared for each experiment in the following manner: First, the phase designated to be the stationary phase was pumped into the CPC at maximal flow rate until its emergence from the outlet of the instrument. The emergence was followed either by the change in absorption at 280 nm or by chemical means (e.g., addition of methanol to effluent will cause a white precipitate if DEX is present). Second, the phase designated to be the mobile phase was pumped into the CPC at a flow rate of 0.5–1.0 mL/min until its emergence from the instrument was noted by the change in absorption at 280 nm and/or the appearance of two distinct phases in the effluent.

When the above steps were completed, the sample was injected, and the mobile phase was pumped through the system until the absorbance at 280 nm reached the baseline. At this stage, the direction of flow through the CPC was reversed, and the stationary phase was pumped through the system until absorbance at 280 nm reached the baseline. The tubes containing proteins (according to absorption at 280 nm) were further processed to remove the phase-defining polymers and thus enable SDS-PAGE analyses. Either S-sepharose (for nonsubstituted PEG) or Q-sepha-

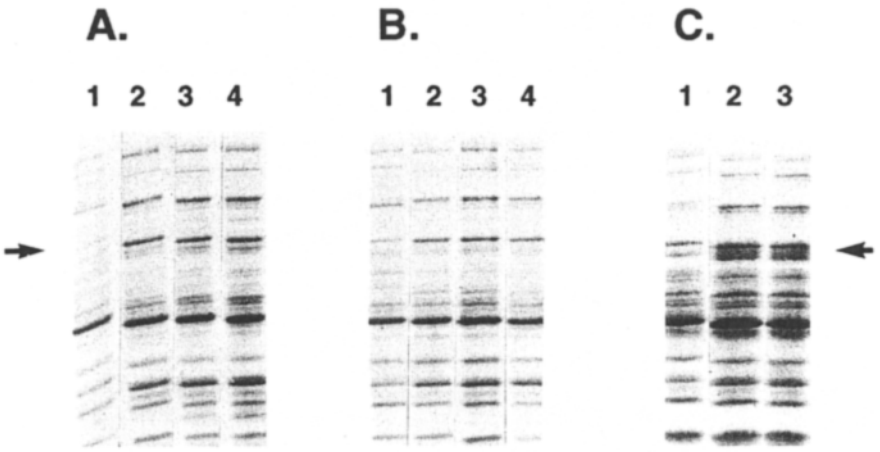

Fig. 2. Coomassie-stained gel of *E. coli* pellets obtained from shake flask cultures. The AR58 cultures containing either pOTS-tPA or pOTS expression vectors (2A and B respectively) were thermally induced and samples withdrawn just prior to induction (lane 1) and 1, 2, and 3 h postinduction (lanes 2, 3, and 4, respectively). An AR120 culture containing pOTS-tPA was chemically induced with nalidixic acid (2C) with samples taken for analysis just prior to induction (lane 1) and 3 and 5 h postinduction (lanes 2 and 3, respectively). Arrows indicate the position at which tPA migrates.

rose (for substituted PEG) was used for this purpose. The second approach to purify the r-tPA from its reactivation mixture involved the use of a two-step column chromatography scheme employing Sephadex G-50 and lysine-sepharose.

RESULTS

Cloning and Expression of r-tPA

Analysis of the *E. coli* pellets after 3 h from the induction, on SDS-PAGE gels, showed (Fig. 2) the presence of a new protein at the expected mol wt of full-length t-PA (65 kDa) (Fig. 2C). This protein was not present in an identical culture that contained only the parent vector pOTS and had been similarly induced. Western blot analysis confirmed the identity of the protein as full-length t-PA (Fig. 3). Also, as shown in Figs. 2 and 3, the chemical induction of *E. coli* cells revealed to be more efficient than the common thermal mode and produced a more homogenous, less degraded product. In the shaker flask system, expression levels reached as much as 5% of the total cell protein. In the larger-scale fermentor, this level was lower, as described later.

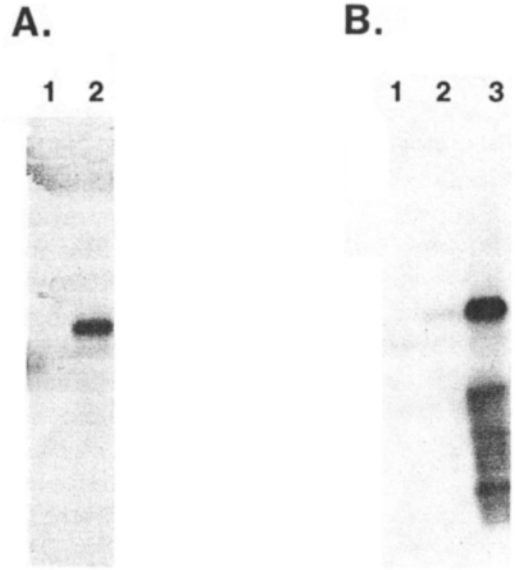

Fig. 3. Western blot analysis of cell pellets from shake flask cultures of AR120 (3A) and AR58 (2B) containing either the pOTS-tPA or pOTS vectors. Expression of tPA before and 5 h after chemical induction is shown in panel A, lanes 1 and 2, respectively. Panel B (lanes 1 and 2) shows pre- and 3 h post-thermal induction samples (with equal vol loaded to samples in panel A). In lane 3 is a 10-fold overloading of the sample in panel 2.

Cell Lysis and Solubilization of r-tPA

A 30-g batch of *E. coli* cell paste (AR120) was lysed, and inclusion bodies were isolated (Fig. 1). Final solubilization of r-tPA was carried out in two steps. In the first step, the suspension obtained with β-DGP was divided into six equal vol. These six portions were centrifuged, and the pellet in each was resuspended in one of the following solutions (5 mL each): 0.1*M* phosphoric acid/glycine pH 3.0, 0.5*M* L-arginine pH 3.0, 0.5*M* ε-aminocaproic acid (EACA) pH 3.0, 0.1*M* Tris, 6*M* Gu.HCl, 1 m*M* EDTA pH 8.0, 0.1*M* Tris, 0.5*M* arginine, 1 m*M* EDTA pH 8.0, and 0.1*M* Tris, 0.5*M* EACA, 1 m*M* EDTA pH 8.0. All these suspensions were centrifuged, and the supernatants were examined for presence of r-tPA. The r-tPA was solubilized completely only by the 6*M* Gu.HCl solution. This source of the solubilized r-tPA will be denoted as source I.

In the second step, the pellets obtained from the six types of suspensions described above were all resuspended in the 6*M* Gu.HCl solution and agitated overnight at 4°C. The resulting suspensions were centrifuged and pellets separated from supernatants. This procedure of resuspension of the pellets in 6*M* Gu. HCl, and separation between pellets and

supernatants was repeated two more times. This yielded three pairs of pellet and supernatant from each of the six pellets described in the preceding step. Of all the pellets studied, only one that had been previously washed with EACA at pH 3 yielded soluble r-tPA in this second-step scheme. Most of the r-tPA was found in the second and third 6*M* Gu.HCl washes (supernatants). This material upon pooling was designated as source II.

In source I, the soluble r-tPA was 2.8% of the total protein, and in source II, it constituted 10–12% of the total protein, i.e., a three- to four fold purification was achieved by prewashing a pellet with the acidic solution of the EACA. As will be seen later on, this difference significantly affected the yield of the reactivation. Overall, 10 g of *E. coli* cell paste (harvested from a 2-L broth) yielded a total of 1.5 mg of soluble r-tPA.

Reactivation

As described above, two sources of soluble r-tPA were obtained and denoted sources I and II. Since preliminary experiments indicated that r-tPA derived from source II showed more reactivation than that from source I, our study was carried out mostly with the former, unless otherwise mentioned.

In the Absence of Polymers

Reactivation was carried out by diluting the r-tPA solution into an alkaline, redox buffer, which contained GSH and GSSG in a fixed molar ratio and concentration. The mixtures incubated for at least 24h. The yield of the process, represented as percent reactivation, is calculated from the ratio between the specific activity of r-tPA as obtained from the reactivation mixture and the specific activity of wild-type standard t-PA. The specific activity of the latter was taken as 500 IU/μg, as determined with respect to the WHO International Standard (*21*).

To determine optimal conditions for obtaining maximum yield of the reactivation process, a broad range of parameters was examined. Among these were: temperature, pH, molar ratio and concentration of GSH and GSSG, total protein and target protein concentrations, concentration of denaturants derived from the source solution, and presence of amino acids. In addition, we studied the use of biospecific effector substances that could promote refolding of the target protein. Our study here focused on partially purified fibrinogen (denoted as fibrinogen/fibrin).

Preliminary results (unpublished data) pointed out two things: First, no reactivation of fibrinolytic activity was obtained in the absence of the redox components (GSH and GSSG). Second, the absence of free arginine in the reactivation mixture resulted in yields that were only 10% of those achieved when arginine was present at a final concentration of 0.25*M*. These outcomes persisted regardless of the value of other parameters affecting the process, such as temperature, pH, and so on.

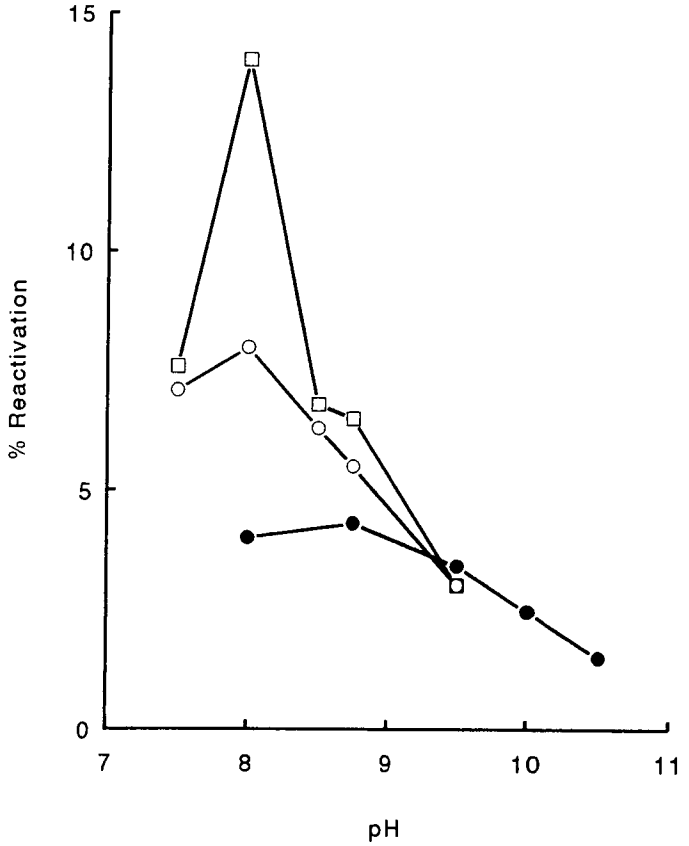

Fig. 4. Effect of temperature, pH, and effector on reactivation process. Experiments were carried out with r-tPA from source II containing 180 μg/mL total protein, 11 μg/mL r-tPA, GSH:GSSG=2.0:0.4 m*M*. 0.25 arginine, and in absence of Tween-80. All experimental systems were carried out in duplicates. In general, experiments at pH 8.0 and 8.75 were each carried out in 15 and 5 replicates, respectively. All other experiments were performed each in three replicates. (●), 4°C no fibrinogen/fibrin; (○), rt no fibrinogen/fibrin; and (□), rt with fibrinogen/fibrin.

As shown in Fig. 4, reactivation of the protein at a concentration of 11 μg/mL reached a maximum at pH 8.0, particularly at room temperature. A similar effect of the pH on the yield of the reactivation was observed also (Fig. 5) with lower protein concentration. Although the results reported herein correspond to 24-h reactivation, no change in yield was seen even after 48 h. The presence of fibrinogen/fibrin during the reactivation process contributed to an additional increase in the maximum yield of activity (Fig. 4). This increase was particularly significant at pH 8.0—at room temperature, about twofold.

The yield of the reactivation process was inversely dependent on the total protein and r-tPA concentrations, as seen in Fig. 6. However, the

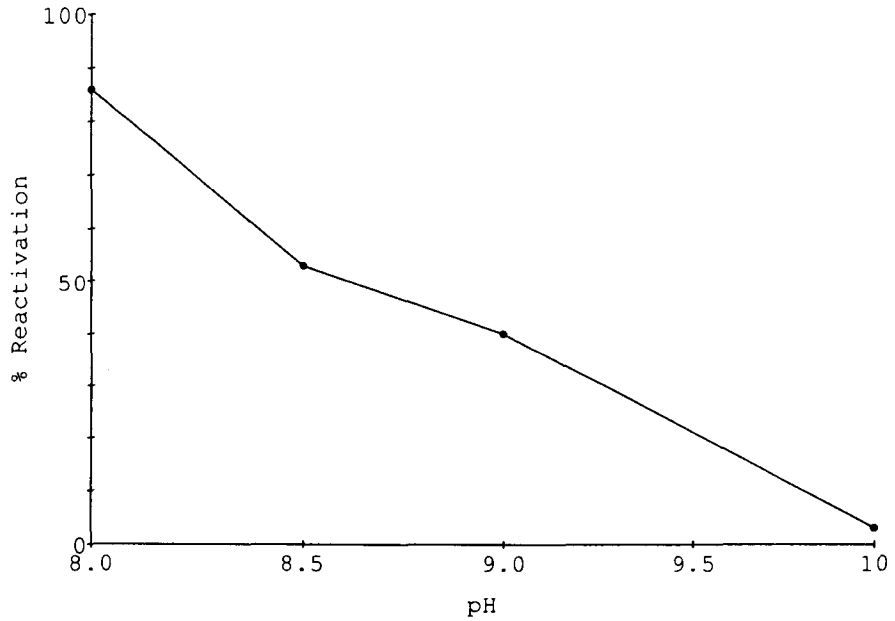

Fig. 5. Effect of pH on the yield of the reactivation process. All experiments were carried out at room temperature in the presence of fibrinogen/fibrin (0.2 mg/mL), 0.01% Tween-80, GSH:GSSG=1:0.5 m*M*. r-tPA from source II contained 21.8 μg/mL total protein, and 2.63 μg/mL r-tPA.

contribution of biospecific effector to the yield of activity remained quite constant, regardless of the protein concentration (apparently a result of its molar excess). Reactivation yield increased twofold with the presence of effector. Under these conditions, nearly full reactivation was achieved when the r-tPA concentration was in the range of 2.5–4 μg/mL.

In the Presence of Polymers

To study the feasibility of using a two aqueous polymeric phase system, such as PEG8000 and DEX500, in scaling up the reactivation process, the effect of each polymer individually on the yield of the reactivation was first examined. As shown in Table 1, when PEG-based biosorbents were added to the reactivation mixture, the yield of the process fell dramatically. Table 1 presents an example of the results obtained at 4°C. Similar results were obtained (data not shown) at room temperature, independent on the pH in the range of values between 8.0 and 10.5. The total yield of the reactivation was very low, particularly because the process was accompanied with precipitation of r-tPA. This effect of PEG persisted even when the reactivation was carried out in the presence of DEX500, in a two-phase system consisting of 3.5% (w/w) PEG8000 and 5% (w/w) DEX500.

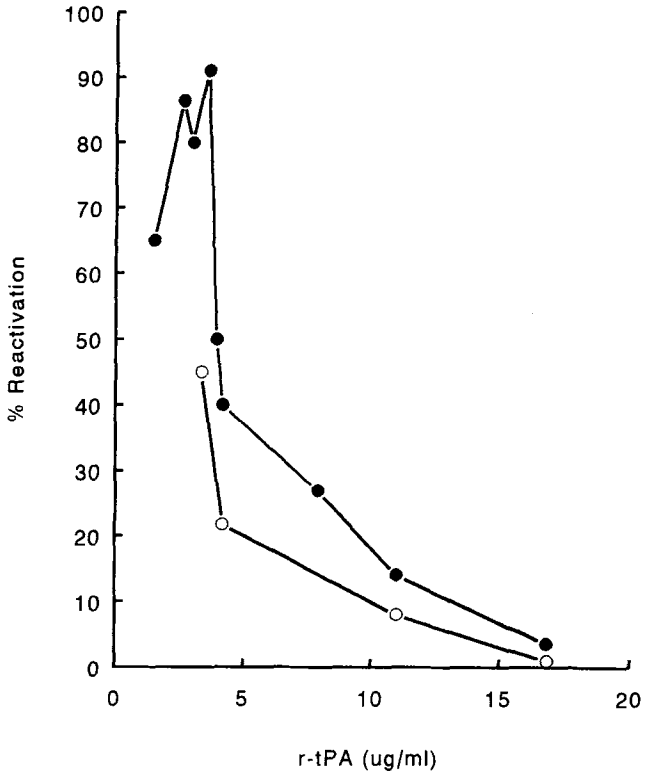

Fig. 6. Effect of r-tPA concentration on the yield of the reactivation process. Reactivation was carried out under the conditions described in Table 1 (in presence of 0.6*M* GuHCl). (●) with fibrinogen/fibrin, (○) without.

Purification

Two approaches were used to purify the r-tPA from its reactivation mixture. The first, an application of two aqueous polymeric phase system. The second, a column-chromatography-based purification scheme.

Purification by Two-Phase System

Several two-phase systems were investigated, differing in their bulk physico-chemical properties or in the presence or absence of an immobilized ligand on one of the polymers. PEG8000 and DEX500 were used to define the aqueous phases. Each two-phase system was prepared as described under Methods. Either nonsubstituted PEG or PEG-ligand was used. The characteristic total composition of each system was 3.5% (w/w) PEG, and 5% (w/w) dextran. The r-tPA reactivated according to the optimal procedure developed in this study (*see* details in Table 2) was used for the purification studies.

Table 1
Reactivation of r-tPA in Presence of PEG and its Derivatives[a]

PEG type[a]	Arg. (*M*)	GSH:GSSG (mola ratio)	r-tPA (IU/system)	r-tPA (IU/μg)	Soluble r-tPA (%)	Soluble r-tPA (IU/μg)
P-ARG	0.25	2:1	37.8	3.5	11.0	31.2
"	—	2:1	0.0	—	—	—
"	0.25	5:1	49.5	4.5	13.4	33.5
"	—	5:1	0.0	—	—	—
"	0.25	10:1	42.0	3.8	2.2	176.2
"	—	10:1	0.0	—	—	—
P-ARGsc	0.25	2:1	53.2	4.8	6.6	73.2
"	—	2:1	0.0	—	—	—
"	0.25	5:1	45.9	4.2	4.3	97.5
"	—	5:1	0.0	—	—	—
"	0.25	10:1	44.6	4.1	14.2	28.6
"	—	10:1	0.0	—	—	—
P-GGR	0.25	2:1	52.5	4.8	11.5	41.3
"	—	2:1	0.0	—	—	—
"	0.25	5:1	57.6	5.3	9.3	56.5
"	—	5:1	0.0	—	—	—
"	0.25	10:1	43.2	4.0	15.9	24.7
"	—	10:1	0.0	—	—	—
P-PABA	0.25	2:1	72.9	6.6	9.8	67.5
"	—	2:1	0.0	—	—	—
"	0.25	5:1	68.5	6.2	5.5	113.5
"	—	5:1	0.0	—	—	—
"	0.25	10:1	60.8	5.5	5.6	98.0
"	—	10:1	0.0	—	—	—

[a]r-tPA at 11 μg/mL; PEG at 5% (w/w); all experiments were carried out at pH 9.5, 4°C.
[b]P-AGR = PEG-arginine; P-ARGsc = PEG-argininal semi-carbazide; P-GGR = PEG-glycyl-glycyl-arginine; and P-PABA = PEG-p-amino-benzamidine.

When two-phase systems of nonsubstituted PEG (upper phase) and DEX (lower phase) of fixed composition were used at the pH values of 3.0, 6.0, 7.5, and 8.5, similar chromatograms were obtained. A partition coefficient of 1.1 for r-tPA was seen at the pH values tested. Active r-tPA was recovered only in the PEG phase emerging next to the void vol of the system. Recoveries were modest with 15, 50, 30, and 50% at the pH values of 3.0, 6.0, 7.5, and 8.5, respectively. At the two higher pH values, we obtained a twofold purification of the enzyme (40 IU/μg total protein to 82 IU/μg.

When PEG-ligand conjugates were used (e.g., PEG-arginine, PEG-lysine, and PEG-PABA) as a stationary phase, the experiments were limited to pH 7.5. Enzyme purification results (data not shown) were similar to those seen with systems containing nonderivatized PEG. Recoveries of

Table 2
Reactivation of r-tPA (Source II)[a]

Gu.HCl (*M*)	Total protein[b] (μg/mL)	r-tPA (μg/mL)	r-tPA (IU/mL)	r-tPA (IU/μg)	Reactivation (%)
0.60	21.8	2.63	1135.6	431.0	86.4
0.60	70.3	3.00	1179.0	401.0	80.0
0.60	123.7	3.60	1630.0	455.3	91.0
0.60	32.7	3.95	987.5	250.0	50.0
0.60	35.0	4.20	847.3	200.0	40.0
0.60	73.8	4.20	365.0	86.9	17.4
0.15	73.8	4.20	121.0	28.8	5.8
0.60	65.4	7.90	1066.5	135.0	27.0
0.60	147.5	8.40	380.0	45.2	9.0
0.30	147.5	8.40	192.0	22.9	4.6
0.60	295.1	16.80	299.5	17.1	3.6

[a]At room temperature, pH 8.0, in the presence of fibrinogen/fibrin (0.2 mg/mL), 0.25*M* arginine, 0.01% tween-80, GSH:GSSG=1:0.5m*M*.
[b]Total protein in the crude sample from source II, not the final value for the total protein in the reactivation mixture.

active r-tPA were 31, 50, and 25% when PEG-Arg, -Lys, and -PABA were used, respectively.

Purification by Column Chromatography

Purification of r-tPA from its reactivation mixture was carried out in two steps at 4°C. First, the reactivation mixture was filtered through a column of Sephadex G-50 (2.5×16.3 cm for a 5-mL load or 4.5×18 cm for a 25-mL load) preequilibrated at pH 7.6 (0.05*M* Tris, 0.5*M* NaCl, 0.01% Tween-80). As expected, the r-tPA eluted in the void vol (early peak), leaving behind the Gu.HCl and arginine (late peak). The fractions of r-tPA thus obtained were combined and loaded on a lysine-sepharose column (1×2.5 cm) preequilibrated at pH 7.6 (same buffer as in the former step). After sample loading, the column was washed with the same buffer until the $A280_{nm}$ of the effluent returned to baseline. The r-tPA was recovered by washing the column with the same buffer containing 0.25*M* arginine.

Having started typically with specific activity at about 40 IU/μg protein in the refolding mixture, the product from lysine-sepharose had values of 450 IU/μg—indicating greater than 11-fold purification. Owing to low sample concentrations in eluates obtained from the lysine-sepharose column, the coomassie-stained bands were faint (Fig. 7B). But one band corresponding to a 65-kDa protein (Fig. 7B, Lane 4) was seen. A Western blot of the same gel (Fig. 8) showed that the r-tPA product was comprised of roughly equal amounts of one-chain (MW_{app}=65 kDa) and two-chain (MW_{app}=34 and 32 kDa fragments) forms. These are commonly found in preparations done in absence of protease inhibitors and are

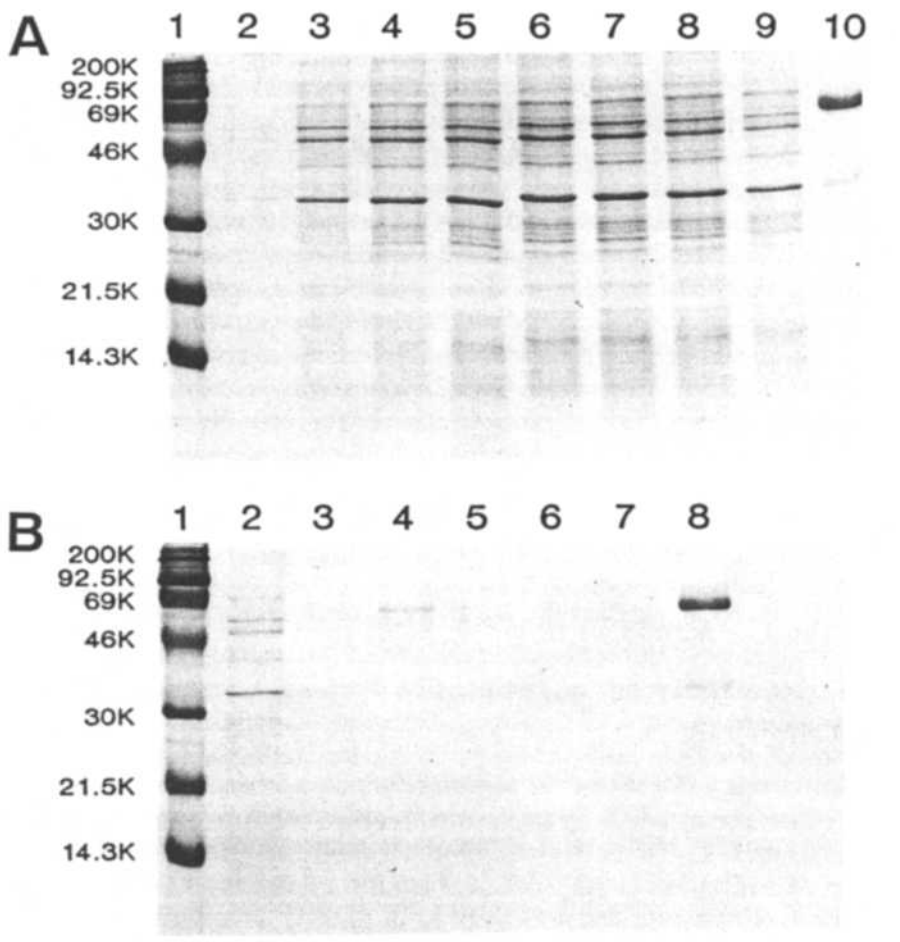

Fig. 7. SDS-PAGE gel of fractions eluted from the lysine-sepharose column. Chromatography was carried out at 4°C and pH 7.6. The 400 μL of each fraction was prepared for electrophoresis (as described under Methods), then solubilized in 25 μL electrophoresis buffer, of which 7.5 μL were loaded in each lane. **(A)** One mL fraction. Lane 1: LMW markers; Lanes 2–9: fractions of the unbound proteins; Lane 10: tPA standard. **(B)** Two mL fractions. Lane 1: LMW markers; Lane 2: last fraction of unbound protein eluted from column; Lanes 3–7: fractions of r-tPA activity eluted with 0.25*M* arginine/buffer; Lane 8: CHO-tPA standard, 7.5 μg.

extensively described elsewhere (23). No other degradation fragments were detected. Specific activity of this purified r-tPA was identical to that predicted from the reactivation step (Table 2) where the specific activity was computed on the basis of r-tPA protein rather than total protein in the mixture.

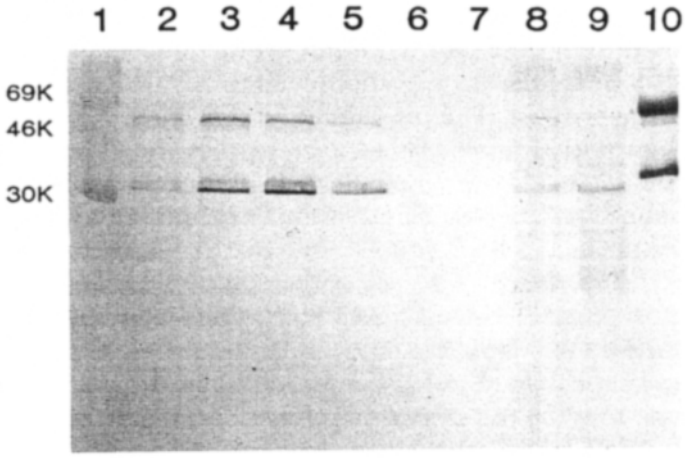

Fig. 8. Western blot of fractions eluted from the lysine-sepharose column. Lane 1: LMW markers. Lanes 2–5: fractions containing r-tPA activity (in order of elution) eluted in the presence of 0.25*M* arginine. Lanes 6 and 7: fibrinogen in the concentration used for reactivation. Lanes 8 and 9: t-PA recovered in the flowthrough fraction. Lane 10: standard CHO t-PA.

DISCUSSION

Like many other eukaryotic proteins overexpressed in *E. coli* cells, r-tPA was obtained in an insoluble form (possibly inclusion bodies). Our expression level was, on the average, 0.8 mg/L of broth or 0.15 g/g cell paste.

As shown in this study, solubilization of the protein could be achieved with a strong denaturant, 6*M* Gu. HCl, but without the use of any reducing agent. The addition of reducing agents has been considered necessary by other workers [Rudolph (*11*); Sarmientos et al. (*9,10*)], for *both* the solubilization step and the subsequent refolding or reactivation step. Our interest in omitting the reducing agent during solubilization was prompted by the hypothesis:

1. That even a complex molecule such as r-tPA (containing 17 disulfides) when expressed in *E. coli* is mostly folded correctly
2. That owing to the intracellular redox potential, some of the disulfides may not be formed until cell disruption and/or solubilization of the insoluble protein body allows oxidation to take place and
3. That full recovery of activity could be attained after solubilization (using chaotropes) by controlled oxidation of the few remaining cysteines.

From our experience with other recombinant eukaryotic proteins as well as from studying the work of other groups, it seems that use of high concentrations of thiols during solubilization results in complete reduction of disulfide bridges. The probability of refolding or reactivating such a completely unfolded molecule is expected to be much lower than that of a partially folded species, e.g., 12 of 17 disulfide groups already formed.

To enhance the process of refolding a *rec*-protein, it is desirable to obtain the molecule in high purity. Toward this end, it was found that washing the *E. coli* pellet with an acidic solution of EACA (Fig. 1) was useful. This increased the purity of the protein that could be solubilized by threefold and facilitated reactivation of the r-tPA.

The chaotrope-solubilized r-tPA so obtained was not fully active. It showed some amidolytic activity (as judged by assays with oligopeptide nitroanilides, data not shown) but completely lacked the ability to activate plasminogen in the presence of fibrin. To infuse the molecule with the required fibrinolytic function (i.e., full reactivation), we investigated several routes. As shown in this report, best results were obtained when the reactivation was carried out under conditions that favored the native folded state and catalytic activity. To this end, reactivation was carried out at pH 8.0 and in the presence of fibrinogen/fibrin. L-arginine was also observed to enhance the refolding process. In contrast to the recent report of Sarmientos et al. (*10*) but in agreement with a previous report of Rudolph et al. (*11*), our use of lysine instead of arginine to assist reactivation resulted in very low yields. These yields were even below those obtained at 4° C (Fig. 4).

The use of fibrinogen/fibrin to assist the reactivation of t-PA was based on the concept (*24,25*) that the native structure of many proteins can be stabilized by the presence of their substrate, inhibitor, and/or cofactor (i.e., a specific ligand). Unfortunately, little is yet known about how such ligands facilitate the process of refolding/reactivation a catalytic protein. There have been reported only a few studies (*26–28*) where experimentally denatured naturally occurring proteins have been refolded with the aid of specific ligands. All of the reports indicated that such ligands had a profound effect on the yield of active proteins.

The use of this concept in the refolding of recombinant proteins produced in *E.coli* has been done to date using only small molecules such as amino acids (*9–11*). The use of larger structural agents to assist the in vitro refolding process has not yet been reported. In this study, we showed (*see* particularly Fig. 6) that in addition to arginine, which had been reported elsewhere to stabilize r-tPA (*11*), fibrin was necessary for efficient refolding of this complex molecule.

Until recently, most of the attempts to obtain active r-tPA from *E. coli* in the absence of a reducing agent in the solubilization step resulted in very low, close to negligible yield. Pennica et al. (*29*) and Harris et al. (*30*) reported total yields of 0.2%. Other reports (*see also* Table 3) showed that

Table 3
Reactivation of r-tPA Expressed in *E coli:*
A Comparative Summary[a]

System #	Expression level (mg/g cell)	Expression level (mg/L)	r-tPA in reactivation mixture (mg/L)	Total yield %	Ref.
1	0.004	0.04	0.04–1.60	0.02	30
2	0.032	0.64	same as #1	same as #1	30
3	NA	NA	0.20–2.00	12	11
4	NA	>10	>10	20-50[b]	9,10
5	0.15	0.80	2.50–3.70	90	this report

[a]Pennica et al. (*29*) reported on obtaining active r-tPA in the range of concentration of 50–80 μg/L culture. The authors did not report by any mean on their expression level the concentration of the total r-tPA, which was solubilized and the total yield of their recovery of activity.

[b]This is the result from the laboratory scale protocol. In the larger scale, the total yield was only 10%.

when a reducing agent was incorporated in the solubilization step prior to the reactivation of the protein, the yield of the reactivation increased dramatically. Rudolph et al. (*11*) obtained a final yield of 12% (fully active r-tPA) from a reactivation mixture that contained 0.2–2 μg r-tPA/mL. Sarmientos et al. (*9,10*) did not explicitly show the total yield of their reactivation process. However, they did recover fully active r-tPA in a concentration of 2–5 μg active r-tPA/mL in their reactivation mixture. We have estimated from their data that the total yield from reactivation was 20–50% at bench-scale and about 10% at a larger scale.

Our results here show that it is possible to achieve high total yields in the reactivation of a relatively large protein such as r-tPA expressed in an intracellularly insoluble form. This was attained without using reducing agents during the process to solubilize the protein. We also found that the optimal protein concentration during reactivation process fell in a narrow range. Total yields of about 90% were achieved with the r-tPA concentration of the reactivation mixture in the range of 2.6–3.7 mg/L. When these results are compared to that reported by others (Table 3), the importance of the concentration of r-tPA in the refolding mixture becomes evident. It appears that r-tPA concentrations below 2 mg/L or above 10 mg/L are less than optimal.

The optimal range of r-tPA concentrations in the reactivation mixture, as observed in this study, is comparable to the range of concentrations obtained when the protein is expressed in recombinant mammalian cells and secreted into the media in its active form (*31*). Some reports have described yields of as much as 55 mg/L of t-PA when expressed in mammalian cells (*32*). This emphasizes the need to scale up the reactivation process in a way that will make *E. coli* a more economically advantageous

source for large proteins. However, as we have shown quantitatively and as a few reports (*1,2*) have discussed only qualitatively, the reactivation of an unfolded molecule, particularly one containing many pairs of cysteines, is very adversely affected by intermolecular protein–protein interactions. These adverse interactions increase with increasing protein concentration. For this reason, dilution of the protein solution is required to achieve proper refolding.

Despite the preceding conclusions, we saw the need to overcome this requirement for dilution. The prospect of involving gargantuan holding tanks to achieve refolding of proteins in dilute solutions at manufacturing scale is not attractive (nor practical). This prompted us to study the t-PA reactivation process in a two-phase partition system. We hypothesized that partially unfolded r-tPA might be refolded in one aqueous phase and that the fully functional enzyme could partition to the second phase, which is comprised of polymers bearing a biospecific ligand. By performing the process in the CPC, we hoped to develop a continuous refolding process accompanied by an efficient separation of active from inactive enzyme.

As shown in the results, when PEG and DEX were used to define the two phases, significant problems were encountered as a result of precipitation of the enzyme. Precipitation was also encountered when fully active r-tPA was loaded onto the system for purposes of purification. While Cleland and Wang (*33*) reported recently that PEG could enhance protein folding recovery of bovine carbonic anhydrase, we feel that r-tPA, owing to its greater complexity (anhydrase lacks disulfide bonds) and mol wt (anhydrase is only 30 kDa), poses a different problem. The effect of PEG on protein folding is likely to vary with molecular species. Results (data not shown) were also discouraging when AquaphasePPT (AqPPT, an hydroxypropyl derivative of starch developed by Perstorp Biolytica, Lund, Sweden) was used instead of PEG. The high viscosity of AqPPT and the very low recoveries of r-tPA activity both interfered with the maintenance of the protein in its soluble and active form.

Thus, for now, dilution still appears to be the most effective way to achieve reactivation of partially folded r-tPA that has been solubilized with a chaotropic agent. Although it poses disadvantages for scaling up the refolding step, others have suggested (*34*) that the dilution process can be scaled up by using a controlled ultrafiltration unit. Such an approach has been found to even increase the efficiency of the refolding step.

The results of this study suggest that an effective approach to scale up reactivation of r-tPA should include the use of fibrin-sepharose to facilitate protein refolding. Purification of wt t-PA (from mammalian cells) has been extensively studied and reported in the literature (*35–37*). In this work, purification of r-tPA (in a nonglycosylated form) from its reactivation mixture was approached via the two-phase partition technique (using the CPC) to achieve a one-step purification. As shown, a substantial purification was achieved but with limited efficiency. Purification of proteins

from a complex mixture, such as *E. coli* lysate, using a two-phase partitioning system seems to be an efficient "upstream" technique when applied in a one-step batch mode. However, the CPC as designed may be useful as a "downstream" step but is limited in its scalability. There are two reasons for this: It is limited in load vol. Target proteins derived from large vol soltuions (either conditioned media or reactivation mixture) cannot be loaded to high capacity as in adsorption-type column chromatography. Second, the requirement for an additional step to remove the phase-defining polymers from the target protein makes the CPC process less attractive than conventional and readily scalable chromatographic columns.

As shown in this study, an appropriate affinity resin packed in a column and operated in the common continuous form may lead to a scalable and efficient mode of purification of r-tPA. If a much more biospecific ligand is to be used, such as a MAb, the purification should be achieved even more efficiently.

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