Locating the unpaired cysteine of tissue-type plasminogen activator

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Variants of tissue-type plasminogen activator (t-PA) were constructed with selected cysteines replaced by alanine to evaluate the role of an unpaired cysteine, which has been presumed to be in the growth factor module. C75A, C83A, C84A and C83-84AA variants of t-PA were expressed transiently in human embryonic kidney cells. The biochemical properties of these variants provided experimental evidence to identify the unpaired cysteine in t-PA. Assays of amidolytic activity, plasminogen activation (in the presence or absence of fibrinogen or fibrin), plasma clot lysis, fibrin binding, clearance in mice, and interaction with a panel of monoclonal antibodies were performed as the basis for comparing these variants with wild-type t-PA. In all assays, C83A t-PA was biochemically equivalent to wild-type t-PA. C75A t-PA, C84A t-PA and C83-84AA t-PA variants exhibited reduced activities in a variety of functional assays. These variants displayed two- to threefold lower activity in fibrinogen or fibrin stimulated plasminogen activation, and fivefold reduced plasma clot lysis activity compared with that of wild-type t-PA. The affinity of C75A t-PA and C84A t-PA for fibrin was decreased more than two orders of magnitude compared with C83A t-PA or wild-type t-PA. Plasma clearance of C75A t-PA and C84A t-PA was reduced 2-fold in mice. The C75A, C84A and C83-84AA variants displayed significantly decreased reactivity with anti-tPA monoclonal antibodies specific for finger/growth factor domain epitopes. These data are consistent with a disulfide linkage of Cys75 with Cys84 and that Cys83 exists as an unpaired sulfhydryl. The significance of the unpaired cysteine is as yet undetermined since C83A t-PA and wild-type t-PA are functionally equivalent.

Keywords: free sulfhydryl group/site-specific mutants/tissue-type plasminogen activator/unpaired cysteine

Introduction

Tissue-type plasminogen activator (t-PA) converts plasminogen to plasmin by a single proteolytic cleavage. t-PA is a multidomain glycoprotein containing a region homologous to a fibronectin type I module (F, finger), a growth factor-like domain (GF), two kringle domains (K1 and K2) and a trypsin-like serine protease domain (P) (Banyai et al., 1983; Pennica et al., 1983; Ny et al., 1984; Pathy, 1985). There are 35 cysteine residues in t-PA, which form 17 disulfide bonds with one unpaired cysteine. The disulfide pairings, shown in Figure 1, were assigned based upon sequence homology with other proteins having known disulfide bonds (Banyai et al., 1983; Pennica et al., 1983; Ny et al., 1984; Pathy, 1985). Human t-PA possesses adjacent cysteines at positions 83 and 84 within the GF domain. Based upon homology with epidermal growth factor (EGF), Banyai et al. (1983) assigned a disulfide bond linking Cys75 and Cys84, leaving cysteine at position 83 unpaired. Interestingly, non-human forms of t-PA do not have cysteine at position 83, as Figure 2 illustrates (Ny et al., 1988; Rickles et al., 1988; Gardell et al., 1989; Feng et al., 1990; Krätzschmar et al., 1991), which provides indirect support for a disulfide bond between cysteines at 75 and 84. However, no experimental evidence has been presented that (i) identifies the free sulfhydryl residue in human t-PA, (ii) assigns the disulfide linkages of t-PA or (iii) indicates the functional significance of any of the unpaired cysteine in human t-PA. Several researchers have used sulfhydryl-specific reagents to label t-PA, assuming the free sulfhydryl to be at position 83 (Loscalzo, 1988; Nienaber et al., 1992; Stamler et al., 1992). However, classical chemical means have not successfully determined the position of the unpaired cysteine in human t-PA.

Unpaired cysteines in extracellular proteins are relatively unusual (Carter and Ho, 1994). In addition to the unpaired cysteine of t-PA, examples of free thiols in circulating plasma proteins include Cys125 of interleukin-2 (IL-2) (Robb et al., 1984), Cys17 of human granulocyte-colony stimulating factor (G-CSF) (Lu et al., 1989; Arakawa et al., 1993), Cys34 of human serum albumin (HSA) (Peters, 1985; He and Carter, 1992; Carter and Ho, 1994), Cys4057 in apolipoprotein(a) [apo(a)] and an unidentified cysteine in apolipoprotein B-100 (apoB-100) (Koschinsky et al., 1993). The identities of the unpaired cysteine residues were established for these proteins, either by chemical modification with sulfhydryl-specific reagents or by site-directed mutagenesis. Cys125 of IL-2 was demonstrated to be unpaired by chemical methods using radiolabeled iodoacetic acid. Peptide mapping and sequencing indicated the location of the unpaired cysteine (Robb et al., 1984). Mutagenesis studies confirmed the results of the chemical identification and examined the functional significance of the unpaired cysteine and the single disulfide bond in IL-2 (Wang et al., 1984). For the much larger protein apo(a), the cDNA sequence indicated the presence of a free sulfhydryl at Cys4057, which was believed to be unpaired on the basis of sequence homology (McLean et al., 1987). The identity and functional role of an unpaired cysteine in apo(a) were evaluated utilizing mutational analysis. The formation of a covalent complex with apoB-100 did not occur for the C4057S apo(a) mutant, confirming Cys4057 as the unpaired cysteine and the site of intermolecular disulfide formation with apoB-100 (Koschinsky et al., 1993).

In this paper, we describe a site-directed mutagenesis approach to identify the unpaired cysteine of t-PA. C75A, C83A, C84A and C83-84AA variants of t-PA were constructed, expressed and evaluated for amidolytic activity and
plasinogen activation. Based on the sequences of t-PA from other species, C83R t-PA was also constructed. The cysteine substitution variants were characterized with respect to fibrin binding, interaction with anti-t-PA monoclonal antibodies and binding of t-PA variants to hepatic receptors (as indicated by in vivo plasma clearance). Molecular modeling of the t-PA growth factor domain, derived from the murine epidermal growth factor structure (Montelione et al., 1987), was used to evaluate the feasibility of alternative disulfide linkages, such as Cys75-Cys83 versus Cys75-Cys84. The experimental evidence presented in this paper indicates that Cys83 is the unpaired cysteine in human t-PA.

Materials and methods

Materials
The restriction endonucleases KpnI and HindIII (and NEBuffer for KpnI) were purchased from New England Biolabs (Beverly, MA). [α-35S]d-ATP was from Amersham (Arlington Heights, IL). Human plasmin, H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide dihydrochloride (S-2288) and H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride (S-2251) were obtained from Kabi Pharmacia Hepar (Franklin, OH). H-D-tyrosyl-L-prolyl-L-arginyl chloromethyl ketone (YPRck) was purchased from Bachem (Philadelphia, PA). Human fibrinogen and human thrombin were products of Calbiochem (La Jolla, CA). Cyanogen bromide-activated Sepharose 4B resin and PD-10 columns were purchased from Pharmacia LKB (Piscataway, NJ). Human plasma was obtained from Peninsula Memorial Blood Bank (Burlingame, CA). Glu-plasminogen was purified from human plasma by a modification of the Deuts and Mertz procedure (Deutsch and Mertz, 1970; Bennett et al., 1991).

Mutagenesis and dsDNA preparation
Oligonucleotide-directed mutants were constructed using the method of Kunkel (Kunkel, 1985; Kunkel et al., 1987) with the Muta-Gen M13 in vitro mutagenesis kit from Bio-Rad (Richmond, CA). Mutagenic oligonucleotides were synthesized by the Genentech DNA synthesis laboratory as described previously (Bennett et al., 1991). Double-strand DNA samples were prepared by Qiagen midi preparation procedures (Qiagen, Chatsworth, CA) from Escherichia coli tonAmm294 cells (Crowe, 1992). Successful mutagenesis was confirmed by directly sequencing double-strand DNA. Oligonucleotide primers were synthesized as described previously (Bennett et al., 1991). DNA strands were separated prior to sequencing with sodium hydroxide according to a published procedure (Sambrook et al., 1989). Sequencing was accomplished by the dideoxy method using United States Biochemical (Cleveland, OH) Sequenase reagents and protocols (Sanger et al., 1977; Bennett et al., 1991). DNA was labeled with [α-35S]d-ATP.

Transient expression of t-PA
Human embryonic kidney 293 cells were employed for transient expression of the t-PA molecules as described previously (Gorman et al., 1990; Bennett et al., 1991). Expression levels were typically in the range 1–5 µg/ml as determined using a dual monoclonal enzyme-linked immunosorbent assay (ELISA). The monoclonal antibodies used in the ELISA, Mab 672 and 621, were specific for antigenic determinants in kringle 2 and protease, respectively (Nguyen et al., 1993).

Enzymatic activity assays
Conditioned media from transient expression of wild-type t-PA and t-PA variants were assayed for amidolytic activity (in both one- and two-chain form), plasinogen activating activity (unstimulated, fibrinogen and fibrin stimulated) and plasma clot lysis activity (one-chain form) as published previously (Jones and Meunier, 1990; Bennett et al., 1993). The data are reported as values normalized to activities obtained with wild-type t-PA.

Fibrin binding assays
YPRck was labeled with iodine-125 and covalently bound in the active site of t-PA molecules as described previously (Keyt et al., 1992). Fibrin binding of [125I]YPRck-labeled variants was evaluated at various fibrinogen concentrations using previously published methods (Bennett et al., 1991).

Pharmacokinetic analysis
Clearance of [125I]YPRck-labeled wild-type and variant forms of t-PA was determined in mice according to procedures established for rats (Paoni et al., 1993). Four-week-old ICR mice (Simonsen Labs, Gilroy, CA) weighing ~20 g each were chosen for the pharmacokinetic experiments. Wild-type t-PA or variants of t-PA (~80 ng of t-PA labeled with 0.1 µCi of [125I]YPRck) were administered to groups of four mice by intravenous injection in the tail. Blood samples (70 µl each) were obtained at various times by orbital bleeds and precipitated with trichloroacetic acid (TCA) to a final concentration of 10%. The TCA-precipitable radioactivity was quantitated using an LKB gamma scintillation counter. Clearance from blood was calculated from the trapezoidal area under the curve from zero to 40 min.

Immunochromatography
Monoclonal antibodies, raised against recombinant human t-PA (Activase), were used to evaluate the immunochromatographic reactivity of cysteine substitution variants of t-PA. Antigen specificity of the monoclonal antibodies was initially characterized by the interaction of the antibodies with domain deletion variants of t-PA (Sinicropi et al., 1989). A higher resolution epitone map of t-PA was obtained by comparing the immunochromatographic reactivity of 35 monoclonal antibodies with a series of alanine scan variants of t-PA (Nguyen et al., 1993). The methods utilized in these reports were modified as follows for the evaluation of cysteine to alanine t-PA variants. Microtiter plates (96-well, polycarbonate, from Dynatech) were coated with 100 µl per well of affinity-purified goat polyclonal anti-tPA at 0.625 µg/ml in 0.05 M sodium carbonate (pH 9.6) for 16 h at 4°C. The plates were washed with PBS containing 0.01% Tween 20. The plates were incubated with bovine serum albumin at 5 mg/ml in PBS for 1 h at 25°C, then emptied and washed with PBS containing Tween 20. Aliquots of samples containing t-PA or t-PA variants, in conditioned medium (100 µl) from transiently transfected 293 cells, were diluted to 10 ng/ml (by the ELISA described above), added to the wells of the microtiter plate and incubated for 2 h at 25°C with gentle agitation. The microtiter plates were emptied and washed with PBS containing Tween 20. Various anti-t-PA monoclonal antibodies (100 µl) were added to microtiter plate wells at specified concentrations for each monoclonal antibody. Anti-t-PA monoclonal antibodies were titrated, in an ELISA format using purified t-PA at 10 ng/ml, to determine the concentration of each antibody that yielded absorbance in the range 1.0–1.2 at 492 nm. The absorbance values for each t-PA variant were normalized to the values obtained with wild-type t-PA for each antibody to give the relative binding data reported.
loop residues (residues Cys51 through to Cys56 using t-PA numbering) were flexible while the remainder of the molecule was constrained. In a final series of energy minimizations, the side-chain atoms of the entire GF domain and all atoms in the loop were allowed to adopt new conformations with respect to the original EGF structure. Other than the loop from Cys51 to Cys56, the backbone conformations of EGF and the GF model are identical. The C84A model of t-PA growth factor domain was made by first substituting alanine for Cys84 and subsequently forming a new disulfide bond between Cys75 and Cys83. The new disulfide was optimized by energy minimization allowing only the Cys75 and Cys83 side chain atoms to move.

Results
Wild-type and variant forms of t-PA were expressed by transient transfection of human fibroblasts and the conditioned media was quantified by a dual monoclonal antibody-based ELISA. The monoclonal antibodies used in the ELISA, Mab 672 and Mab 621, were specific for antigenic determinants in the kringle 2 and protease domains, respectively, as previously determined with domain deletion variants of t-PA (Sinicropi et al., 1989). As such, the concentration of variant t-PA protein in transected 293 cell conditioned media was accurately determined by this ELISA, and varied from 1 to 5 μg/ml.

Enzymatic activities of the t-PA mutants
Amidolytic activity, plasminogen activation (in the presence or absence of fibrin) and plasma clot lysis activity data for the C75A, C83A, C83R, C84A and CC83-84AA mutants are presented in Table I. These data indicate that C83A, C83R and wild-type tPA exhibit similar properties in all assays. The data also show that all of the mutants are similar to wild-type tPA with respect to one- and two-chain amidolytic activities. Non-stimulated plasminogen activation for C83A, C83R and C84A mutants is normal, whereas the activities of C75A and CC83-84AA mutants are moderately to severely decreased (30-50% loss of wild-type activity, respectively). In the plasminogen activation assays involving a stimulator, C75A, C84A and CC83-84AA mutants are two- to threefold less active than wild-type. The same mutants are more severely impaired (decreased 5-fold) in the plasma clot lysis system. As a whole, the data indicate that each of the mutants is a fully functional serine protease and that C83A and C83R are functionally similar to wild-type t-PA. However, the C75A, C84A and CC83-84AA variants are impaired with respect to their ability to be stimulated by fibrin, particularly in a plasma clot lysis system.

Fibrin binding
The importance of the amino-terminal domains in t-PA for fibrin binding has been established (Banyai et al., 1983; van Zonneveld et al., 1986a,b; Verheijen et al., 1986; Bennett et al., 1991). Structural alterations in these regions of the molecule are likely to result in reduced fibrin affinity. As displayed by the binding curves in Figure 3, the C83A variant is similar to wild-type t-PA in fibrin affinity, while the affinities of C75A and C84A are reduced by approximately two orders of magnitude. Significantly decreased fibrin affinity observed for both C75A and C84A mutants is consistent with the data for plasminogen activation in the presence of fibrin or fibrinogen (especially plasma clot lysis). It is also consistent with data for t-PA variants in which presumed disulfide pairs were eliminated from the amino-terminal region of the
Table I. Enzymatic activities of t-PA variants

<table>
<thead>
<tr>
<th>Assay*</th>
<th>Activity of variant normalized to that of wild-type t-PA</th>
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<tr>
<td></td>
<td>C83A</td>
</tr>
<tr>
<td>1-ch 2288</td>
<td>0.94</td>
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<tr>
<td>2-ch 2288</td>
<td>1.08</td>
</tr>
<tr>
<td>1-ch non-stim 2251</td>
<td>1.21</td>
</tr>
<tr>
<td>2-ch non-stim 2251</td>
<td>1.09</td>
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<tr>
<td>1-ch fg stim 2251</td>
<td>1.02</td>
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<tr>
<td>2-ch fg stim 2251</td>
<td>1.03</td>
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<tr>
<td>1-ch fn stim 2251</td>
<td>0.95</td>
</tr>
<tr>
<td>2-ch fn stim 2251</td>
<td>1.15</td>
</tr>
<tr>
<td>Plasma clot lysis</td>
<td>1.34</td>
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*Assays: 1-ch and 2-ch refer to results obtained with t-PA or t-PA variants assayed in the predominantly single-chain or in the two-chain form, respectively. 2288 and 2251 identify the chromogenic substrates for t-PA and plasmin, respectively. The co-factor status in the plasminogen activation assay is either ‘non-stim’ (absence of fibrin co-factor), ‘fg stim’ (presence of fibrinogen) or ‘fn stim’ (presence of fibrin).

molecule. The putative disulfide bonds were individually eliminated by substitution of disulfide-linked cysteine pairs to alamines. The t-PA variants C6A, C36A; C34A, C43A; C51A, C62A; C56A, C73A and C75A, C84A were expressed transiently in human kidney 293 cells. Assay of their fibrin-binding properties indicated that each of these mutants was severely compromised with respect to fibrin binding (data not shown). Mutations of the GF domain cysteines (51, 62; 56, 73 and 75, 84), particularly the C75A, C84A mutation, were associated with the greatest effect on fibrin binding. Variants with disulfide deletions in the growth factor domain exhibited decreased fibrin affinity by two to three orders of magnitude. Similar effects of the single mutations, C75A and C84A, compared with the putative disulfide deletions, suggest that the single mutations disrupt the 75–84 disulfide bond, altering the structure of t-PA in the F/GF region of the molecule. The lack of an effect for the C83A mutation is consistent with Cys83 being unpaired and not required for the structural integrity of the amino-terminal region of the protein.

**Pharmacokinetic analysis**

Mutations of t-PA with decreased fibrin binding have been associated, in some cases, with reduced plasma clearance.
Fig. 6. Computer-generated stereoscopic diagram of t-PA growth factor based on the NMR-derived structure of murine epidermal growth factor (Montelione et al., 1987). Oval ribbon diagrams of t-PA growth factor models are displayed with the expected disulfide bonds indicated as yellow ‘ball-and-stick’ connections between the appropriate carbon and sulfur atoms. The disulfides Cys51-Cys62, Cys56-Cys73 and Cys75-Cys84 appear at the top, center and bottom of the GF model, respectively. In the model of wild-type growth factor structure, the unpaired sulfhydryl is indicated as a short yellow ‘ball-and-stick’ at position 83. With respect to the variant C84A t-PA, a hypothetical disulfide bond connecting Cys74 and Cys83 is shown in red, near the yellow disulfide connecting Cys75 and Cys84. The amino and carboxy termini of the growth factor domain are indicated with N and C.

(Ahern et al., 1990; Yahara et al., 1992). Cysteine-to-alanine mutants in this study with decreased fibrin binding, C75A t-PA and C84A t-PA, also exhibit decreased plasma clearance (Figure 4). C83A t-PA, which exhibited normal fibrin binding, demonstrates the same rate of plasma clearance in mice as wild-type t-PA (28.7 ± 0.9 and 28.1 ± 5.6 ml/min.kg, respectively), while C75A t-PA and C84A t-PA are cleared approximately twofold more slowly (14.2 ± 0.7 and 13.4 ± 1.5 ml/min.kg, respectively). These data indicate that the hepatic receptors that mediate the rapid plasma clearance of t-PA have reduced in vivo binding of the C75A and C84A mutants of t-PA.

Immunological reactivity of cysteine substitution variants
To detect structural disruptions due to misfolding of the mutant t-PA molecules, cysteine substitution variants were screened for differential immunoreactivity (with respect to wild-type t-PA) using a series of 35 monoclonal antibodies (Mabs) raised against t-PA. The antigenic specificities of these antibodies were initially determined using domain deletion variants of t-PA (Sinicropi et al., 1989). The reactivities of C75A t-PA, C83A t-PA, C84A t-PA and CC83-84AA t-PA were evaluated with the panel of monoclonal antibodies. These results indicate that C83A t-PA was only minimally disrupted with respect to the reactivity of the entire panel of monoclonal antibodies. A selected set of results, shown in Figure 5, were obtained with five Mabs having anti-finger and/or growth factor specificity (anti-F/GF) and two representative Mabs directed against each of the other domains (anti-K1, anti-K2 or anti-P). Three antibodies (363, 740 and 742) with anti-F/GF specificity display a set of shared antigenic determinants as indicated by similar reactivity with charged-to-alanine mutants (Nguyen et al., 1993). These Mabs demonstrated similar reactivity with the t-PA cysteine-to-alanine mutants of t-PA (especially Mabs 740 and 742), although Mab 363 displayed greater reactivity for all cysteine mutants (Figure 5). Antibodies 740 and 742 exhibited a complete loss of reactivity with cysteine variants C75A, C84A and CC83-84AA. The binding of C83A t-PA was decreased approximately twofold with Mabs 740 and 742. In contrast, the C83A mutant appeared equivalent to wild-type t-PA with respect to Mab 363 binding, while C75A, C84A and CC83-84AA mutants were 7 to 10-fold decreased relative to wild-type t-PA. An additional pair of F/GF specific antibodies (Mabs 369 and 371) displayed decreased reactivity with all the cysteine mutants of t-PA; especially CC83-84AA t-PA. C75A, C83A and C84A mutants exhibited reduced binding to Mabs 369 and 371.

Anti-K1, anti-K2 and anti-protease specific t-PA antibodies were also used to evaluate the overall structural integrity of cysteine mutants of t-PA. Included in Figure 5 are the binding data for representative monoclonal antibodies specific to K1 (Mabs 668 and 387), K2 (Mabs 672 and 383) and the protease domain (Mabs 362 and 728) of t-PA (data not shown for 24 additional Mabs against non-F/GF epitopes). The results of these studies indicate that antibodies to non-F/GF regions of t-PA do not distinguish any significant immunoreactive difference between wild-type t-PA and the C75A, C83A, C84A and CC83-84AA mutants of t-PA. The structural disruptions due to cysteine-to-alanine mutations appear to be restricted to the F/GF region of the molecule. These data suggest that the antigenic structure of C83A t-PA is similar to wild-type
t-PA as detected by antibodies specific to the F/GF region. In addition, C75A t-PA and C84A t-PA are recognized as significantly different from wild-type t-PA by three F/GF specific antibodies. Furthermore, CC83–84AA t-PA is immunochemically distinct from wild-type t-PA, as indicated by low reactivity with all five F/GF specific monoclonal antibodies.

**Molecular modeling of the GF domain of t-PA**

The GF domain of t-PA was modeled using the structure of murine EGF determined by Montelione et al. (1987). Figure 6 shows two models of the GF domain that are superimposed for comparison. One model is the wild-type GF which possesses three disulfides including Cys75 and Cys84. To examine the possibility of alternate disulfide pairing in the GF domain, another model was constructed of a variant containing the C84A mutation and a disulfide bond between Cys75–Cys83. Other than the alternative disulfide linkages and the C84A mutation, the models are identical. These models illustrate that a 75–83 disulfide bond is not likely to form without significant perturbation of the GF structure. The modeled 75–83 disulfide was considerably different from the presumed "normal" 75–84 disulfide bond. The 75–84 disulfide possesses a bond length of 1.99 Å and a χ3 side chain dihedral angle of 90.8°, which are consistent with expected disulfide bond values (Richardson, 1981; Thornton, 1981; Katz and Kossiakoff, 1986). The values for the length and χ3 angle of the 75–84 disulfide bond (2.16 Å and -106.1°, respectively) are not within the expected ranges. The χ3 angle for this bond has an opposite twist compared with the 75–84 bond. Additionally, the χ1' and χ2' side chain dihedral angles for the 75–83 disulfide indicate a strained system which was created in an attempt to accommodate the bond length and χ3 requirements for disulfide formation. The strained nature of the 75–83 disulfide as compared to the normal disulfide structure represented by the 75–84 bond is in agreement with the experimental data. The modeled GF domain provides a reasonable explanation for the observed differences among the variants of t-PA and further supports the conclusion that Cys83 is unpaired in t-PA. These data suggest that t-PA GF domain contains a Cys75–Cys84 bond which is necessary for t-PA structure and function.

**Discussion**

In addition to its substrate, plasminogen, t-PA interacts with a variety of other molecules. Fibrinogen stimulates plasminogen activation by t-PA (Hoylaerts et al., 1982; Bennett et al., 1991). t-PA also binds to fibrin (Banyai et al., 1983; van Zonneveld et al., 1986a,b; Bennett et al., 1991). Plasminogen activator inhibitor-1 (PAI-1) rapidly inhibits circulating t-PA (Wiman et al., 1984; Vaughan et al., 1989). t-PA is cleared from circulation via one or more hepatic receptors (Nilsson et al., 1985; Otter et al., 1991; Bu et al., 1994). Lysine and lysine analogs also interact with t-PA via a site on the K2 domain (Rijken et al., 1982; Cleary et al., 1989; Bennett et al., 1991). The numerous intermolecular interactions of t-PA provide the basis for a variety of functional assays which can be used to evaluate the effects of discrete mutations. For example, residues in the 296–299 region of the molecule affect both fibrin specificity of t-PA and the rate of inhibition by PAI-1 (Madison et al., 1989, 1990; Bennett et al., 1991; Eastman et al., 1992; Paoni et al., 1992, 1993). Residues 67–69 in the growth factor domain have been implicated in clearance of t-PA (Anderson et al., 1988; Browne et al., 1990; Bassel-Duby et al., 1992). Certain residues in the finger domain were demonstrated to be important for fibrin binding; others for clearance (Ahern et al., 1990). In the current study, cysteine residues in the GF domain of t-PA have been mutated to alanine. The properties of these variants were used to identify the unpaired cysteine in t-PA and to determine what effect, if any, its replacement would have on the properties of the molecule. The results of this study demonstrate that removal of the Cys83 sulfhydryl has no effect on the enzymatic activity of t-PA. C83A t-PA also binds fibrin like wild-type t-PA and is cleared in mice at the same rate as wild-type. Mutation of the adjacent cysteine, Cys84, to alanine yielded a t-PA variant with reduced clearance and greatly decreased fibrin binding, fibrin stimulation and plasma clot lysis. These results are consistent with Cys83 being unpaired and Cys84 being involved in a disulfide bond with Cys75.

Cysteine mutations were chosen on the basis of disulfide pair assignments made by sequence homologies between t-PA and other proteins with known disulfide bond patterns (Banyai et al., 1983; Pennica et al., 1983; Ny et al., 1984; Patthy, 1985). In view of the sequence homology, it has been suggested that Cys83 is the unpaired cysteine of the 35 such residues in t-PA (Banyai et al., 1983). Experimental evidence provided by a number of researchers has indicated that a single titratable cysteine is present in t-PA (Loscalzo, 1988; Nienaber et al., 1992; Stamler et al., 1992). These researchers have reported 0.8–1.0 mol of reactive cysteine per mole of t-PA. However, no experimental evidence has as yet identified the site of label incorporation. Typically, identification of an unpaired cysteine would involve reaction of the protein with a sulfhydryl-specific label and subsequent isolation of the labeled residues. By various chemical approaches, identifying the unpaired cysteine of t-PA has proven fruitless. The difficulties include enzymatic digestion of native t-PA and the presence of consecutive cysteines at positions 83 and 84. t-PA is relatively resistant to protease digestion unless denatured, reduced and carboxymethylated (Ling et al., 1991), hence the assignment of disulfide bonds presents an extremely challenging problem. The current investigation utilized the approach of replacing cysteines with alanines and studying the properties of the variant t-PA molecules produced by those mutations.

As described above, the C83A mutation had little or no effect on the properties of t-PA and the data are consistent with Cys83 being unpaired with an unknown function. The effects of the C75A C84A and C83–84AA mutations on the properties of t-PA are consistent with a 75–84 disulfide bond in the wild-type molecule. Such mutations would eliminate the 75–84 disulfide bond, and the decreased activity of the corresponding t-PA variants supports that hypothesis. The monoclonal antibody binding data for those mutants are indicative of structural defects; likewise, their decreased fibrin affinity is consistent with data obtained for double cysteine mutations which delete individual disulfide bonds in the finger and GF domains. C6A, C36A, C34A, C51A, C62A, C56A, C73A and C75A, C84A variants of t-PA were expressed and evaluated with respect to fibrin binding. All of the disulfide deletion mutants exhibited significantly decreased fibrin affinity. These observations of the properties of C75A, C83A, C84A and CC83–84AA t-PA variants, and also the sequence homology information, are indicative of the existence of a Cys75–Cys84 bond.

C84S, another t-PA mutant which disrupts the 75–84 disulfide bond, was reported by Adachi et al. (1992). The C84S molecule, designated E6010, possesses significantly less clot...
lysis activity \textit{in vitro} and is cleared more slowly \textit{in vivo} than wild-type t-PA. The data for C84S and C84A mutants of t-PA are consistent with molecules that are cleared more slowly owing to the disruption of a key disulfide bond and partial unfolding of the GF domain. According to Adachi \textit{et al.} (1992), the molecule shows promise as a thrombolytic agent despite a reduction in clot lysis activity \textit{in vitro}. Ahern \textit{et al.} (1990) suggested that a t-PA variant with reduced clearance could be an effective thrombolytic drug, although \textit{in vitro} activity was lower than that of the wild-type enzyme.

The properties of C83A t-PA indicate that the unpaired cysteine of t-PA may not contribute to t-PA function in spite of its apparent solvent accessibility to sulfhydryl-specific reagents. For some plasma proteins the functions of free thiols are known, whereas for other proteins, including t-PA, the free thiol groups have no known function. An example of a functionally characterized unpaired thiol is Cys34 in HSA, which is essential for complex formation with cysteine or glutathione, ligation of metal ions and metal-containing complexes and dimerization of the molecule as it ages (Carter and Ho, 1994). Human plasma fibronectin contains at least one unpaired cysteine which is fully exposed by partial denaturation (Wagner and Hynes, 1979). The free sulfhydryl group has been implicated in the formation of covalent multimers of fibronectin (Mosher and Johnson, 1983). Cys4057 of apo(a) is necessary for the formation of an intermolecular disulfide bond with apoB-100, a covalent interaction in the formation of the lipoprotein(a) particle (Koschinsky \textit{et al.}, 1993). In contrast, the function of Cys17 of G-CSF is not known, although it is associated with decreased protein stability (Arakawa \textit{et al.}, 1993). Interestingly, IL-12 has an unpaired cysteine (Cys252) that has been demonstrated to be complexed with cysteine or thioisoglycic acid (Tangarone \textit{et al.}, 1995).

With respect to the current study, Cys125 of IL-2 is an interesting and pertinent example of an unpaired cysteine in an extracellular protein. This free sulfhydryl group was identified by using chemical modification (Robb \textit{et al.}, 1984) and mutagenesis approaches (Wang \textit{et al.}, 1984), has no known role, and participates in alternative disulfide pairs when denatured (Browning \textit{et al.}, 1986) or in certain IL-2 mutants (Rong \textit{et al.}, 1992). C125S IL-2 possesses full biological activity (Wang \textit{et al.}, 1984; Liang \textit{et al.}, 1986; Rong \textit{et al.}, 1992), much like the C83A variant of t-PA. Native IL-2 contains a disulfide bond between Cys58 and Cys105 (Robb \textit{et al.}, 1984). In the C85S variant of IL-2, a 105-125 disulfide bond is formed. However, the biological activity of this form of IL-2 is greatly reduced (Wang \textit{et al.}, 1984; Liang \textit{et al.}, 1986; Rong \textit{et al.}, 1992). Investigation of disulfide pairing for IL-2 under denaturing conditions also demonstrated the formation of a 105-125 disulfide bond and that the conformation of the IL-2 in the presence of such a disulfide bond is significantly different than that of native IL-2 (Browning \textit{et al.}, 1986).

In the light of the report of alternate disulfide pairing in mutant IL-2 and other molecules, the possibility for t-PA, that either Cys83 or Cys84 could form a disulfide bond with Cys75 was considered and addressed by molecular modeling. This analysis indicated that a 75-83 disulfide bond would be very different from a 75-84 disulfide bond if each formed in the context of the GF domain structure (Figure 6). A 75-83 bond would be strained unless the GF structure was altered to accommodate a more favorable disulfide. According to the work of Katz and Kossiakoff (1986), a strained system does not preclude disulfide bond formation. However, in the case of t-PA GF domain, the experimental data indicate that (i) a 75-83 bond does not form, or (ii) it forms in an altered GF domain structure and the functional integrity of the domain is compromised in the presence of a C84A mutation. Combined with the experimental data, differences between the predicted 75-84 and the 75-83 disulfide bonds provide a strong indication that a 75-83 bond does not occur, or that the structure of the GF domain is significantly altered if such a bond does occur. Although the structure of a recombinant finger/growth factor domain pair has been reported (Smith \textit{et al.}, 1994), it does not address this issue since the cysteine at position 83 was mutated to serine to simplify the expression of the polypeptide.

C83A t-PA is essentially identical with wild-type t-PA in all of the data described above, suggesting that 83 is the unpaired cysteine of t-PA. These data also suggest that Cys83 is not important for t-PA function. Stamler \textit{et al.} (1992) have proposed that the unpaired sulfhydryl group of t-PA may be nitrosylated. Since the homologous plasminogen activators in mouse, rat, hamster and bat do not contain cysteines at analogous positions in their sequences (Ny \textit{et al.}, 1984; Rickles \textit{et al.}, 1988; Gardell \textit{et al.}, 1989; Feng \textit{et al.}, 1990; Kratzschmar \textit{et al.}, 1991), nitrosylation and its possible function would be unique to human t-PA. Referring to Figure 2, the bat molecule has a glutamine at position 83 (with respect to the human sequence) whereas the other species of t-PA have an arginine at that position. t-PA molecules from other species do not contain an unpaired cysteine and a function of the enzyme that is specific to humans is not currently known. However, this does not eliminate the possibility that Cys83 of t-PA may be involved in an as yet unknown covalent intermolecular interaction. With regard to the current data, the conclusion must be that Cys83 is the unpaired cysteine in t-PA and that it has no currently defined structural or functional role.

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