Massey, V., & Edmondson, D. E. (1970) *J. Biol. Chem. 245*, 6595–6598.
Massey, V., Brumby, P. E., Komai, H., & Palmer, G. (1969) *J. Biol. Chem. 244*, 1682–1691.
Massey, V., Ghisla, S., & Yagi, K. (1986) *Biochemistry 25*, 8095–8102.
Massey, V., Schopfer, L. M., Nishino, T., & Nishino, T. (1989) *J. Biol. Chem. 264*, 10567–10573.
Moore, W. M., McDaniels, J. C., & Hen, J. A. (1977) *Photochem. Photobiol. 25*, 505–512.
Nishino, T., Nishino, T., & Tsushima, K. (1981) *FEBS Lett. 131*, 369–372.
Pouchert, C. J., & Campbell, J. R. (1974) *Aldrich Library of NMR Spectra*, Vol. 8, 93D, Aldrich Chemical Co., St. Louis.
Reiser, A., & Wagner, H. M. (1971) in *The Chemistry of the Azido Group* (Patai, S., Ed.) pp 441–501, Wiley, New York.
Saito, T., & Massey, V. (1991) *Flavins and Flavoproteins, 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 673–676, Walter de Gruyter, Berlin and New York.
Saito, T., Nishino, T., & Tsushima, K. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 413–416, Walter de Gruyter, Berlin and New York.
Saito, T., Nishino, T., & Massey, V. (1989) *J. Biol. Chem. 264*, 15930–15935.
Weber, G. (1961) in *Biochemist's Handbook* (Long, C., Ed.) pp 83–85, Spon, London.

Fragment E-2 from Fibrin Substantially Enhances Pro-urokinase-Induced Glu-Plasminogen Activation. A Kinetic Study Using the Plasmin-Resistant Mutant Pro-urokinase Ala-158-rpro-UK†

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ABSTRACT: In a previous study, it was shown that fibrin fragment E-2 selectively promotes the activation of plasminogen by pro-urokinase (pro-UK) [Liu, J., & Gurewich, V. (1991) *J. Clin. Invest. 88*, 2012–2017]. In this study, the kinetics of this promotion by fragment E-2 was studied. Alanine-158-rpro-UK (A-pro-UK), a recombinant plasmin-resistant mutant, was used in order to avoid interference by UK generation during the reaction. In some experiments, pro-UK was substituted in order to validate the mutant as a surrogate. In the presence of a range of concentrations (0–20 μM) of fragment E-2, a linear promotion of the catalytic efficiency of A-pro-UK against native Glu-plasminogen was seen which was 245.5-fold at the highest concentration of fragment E-2 and 450-fold at the highest ratio of E-2/plasminogen used. The promotion was largely a function of an increase in k_{cat}, since fragment E-2 induced a <10-fold reduction in K_M (8.50–1.40 μM). In contrast to this ligand, ϵ-aminocaproic acid (EACA) induced a biphasic promotion of the activation of Glu-plasminogen which was only 18-fold at maximum. Fragment E-2 did not promote the activation of Lys-plasminogen, but the catalytic efficiency of A-pro-UK was 19.7-fold greater against the open Lys-form than against the closed Glu- form of plasminogen. Fragment E-2 had no effect on the amidolytic activity of A-pro-UK or pro-UK, suggesting that the promotion of their activities was indirect and related to a fragment E-2-induced conformational change in Glu-plasminogen. Since EACA or Lys-plasminogen had relatively little effect on plasminogen activation by A-pro-UK, the fragment E-2-induced conformational change is believed to be novel and different from the well-established open Lys- form of plasminogen. Fragment E-2 caused little (2-fold) promotion of UK-induced plasminogen activation. The observed fibrin fragment E-2 effect on the activity of pro-UK may explain its fibrin specificity and illustrates a mechanism of fibrin-dependent plasminogen activation which is unrelated to fibrin affinity of the activator.

Pro-urokinase (pro-UK)[1] or single chain urokinase-type plasminogen activator is a precursor of two-chain urokinase (UK) which, unlike the latter, induces fibrin-specific clot lysis in a plasma milieu (Gurewich et al., 1984; Zamarron et al., 1984). In plasma, pro-UK is inert and stable, but when a fibrin clot is introduced, plasminogen activation is triggered (Pannell & Gurewich, 1986). Since pro-UK has no significant fibrin affinity, the mechanism by which its fibrin specificity may be explained has remained elusive.

The fibrin dependence of pro-UK has been postulated to be due either to neutralization by fibrin of a competitive in-

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[1] Abbreviations: pro-UK, pro-urokinase or single-chain urokinase type plasminogen activator; UK, two-chain urokinase; fragment E-2, a purified derivative of fibrin; A-pro-UK, alanine-158 mutant recombinant pro-urokinase made by site-directed mutagenesis (Lys-158 → Ala-158); EACA, ϵ-aminocaproic acid; SK, streptokinase; u-PA, urokinase-type plasminogen activator; Plg, plasminogen; Pln, plasmin; pNA, *p*-nitroalanine; F, promotional factor; CNBr-2, cyanogen bromide-2 fragments derived from fibrinogen; t-PA, tissue-plasminogen activator.

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hibitor in plasma (Lijnen et al., 1986) or to a particular fibrin-mediated conformational change in Glu-plasminogen (Pannell & Gurewich, 1986). Later, certain carboxy-terminal lysines in partially degraded fibrin were implicated in this mechanism since clot lysis by pro-UK, but not by other activators, was accelerated by plasmin pretreatment of the fibrin clot, and this effect was nullified by carboxypeptidase B (Pannell et al., 1988). Carboxy-terminal lysines have been shown to be responsible for high-affinity binding to plasminogen (Christensen, 1985), and recent evidence was obtained that the carboxy-terminal lysines in fibrin fragment E are involved in the promotion of pro-UK-induced plasminogen activation (Liu & Gurewich, 1991). Fragment E has a carboxy-terminal lysine on each of its three chains and has an especially high affinity for plasminogen (Varadi & Patthy, 1984).

In the present study, the kinetics of this promotion of pro-UK activity by purified fibrin fragment E-2 against Glu-plasminogen, the native form, were analyzed. The findings were compared with those against Lys-plasminogen, the plasmin-cleaved form lacking N-terminal residues Glu-1–Lys-76 and with promotion by ϵ-aminocaproic acid (EACA). The plasmin-resistant mutant rpro-UK was used as a surrogate in order to avoid interference by UK generation during the reaction.

Materials and Methods

A plasmin-resistant mutant rpro-UK constructed by site-directed mutagenesis of Lys-158 to Ala-158 (A-pro-UK) was a gift from Collaborative Research Incorporated (Bedford, MA). Its concentration was determined from the absorbance (280 nm) divided by the extinction coefficient ($E^{1\%}_{280nm}$ = 13.6)(White & Barlow, 1970) of UK. Urokinase (55 kDa) was purchased from Green Cross (Osaka, Japan), and its concentration was standardized against the UK International Reference (NIBSC, London, UK). Native Glu-plasminogen was prepared from DFP-treated human bank plasma (Castellino & Powell, 1981). Lys-plasminogen was obtained by plasmin treatment of purified Glu-plasminogen (Lucas et al., 1983a). ϵ-Aminocaproic acid (EACA) was obtained from Sigma, and synthetic substrate for plasmin (S2251) was obtained from Kabi (Franklin, OH).

Fragment E-2 was prepared by a modification of the method of Varadi and Patthy (1983, 1984) as previously described (Liu & Gurewich, 1991).

The preparation was tested for plasmin activity with S2251, and trace activity was found. To remove this, the preparation was treated with diisopropyl fluorophosphate (5 mM) for 1 h (37 °C) and then dialyzed exhaustively against buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.8).

The concentration of fragment E-2 was determined from the absorbance (280 nm) divided by the extinction coefficient of fragment E-2 ($E^{1\%}_{280nm}$ = 10)(Panyim & Chalkley, 1969). It was additionally measured using a Bio-Rad Protein Assay Kit. The final preparation was examined by gradient (5–15%) SDS–PAGE.

Characterization of A-pro-UK—A Comparative Study with pro-UK. The amidolytic activities of A-pro-UK and pro-UK were measured with synthetic substrate S2444 as previously described (Pannell & Gurewich, 1987). The plasminogen activating activities were measured in reaction mixtures containing 0.48 μM Glu-plasminogen and 1.0 nM A-pro-UK or pro-UK in the presence and absence of fragment E-2 (20 μM) incubated at room temperature over time. At 10-min intervals, 50-μL aliquots were removed and assayed for plasmin generation by addition to 50 μL of a solution containing S2251 (3 mM) and amiloride (0.5 mM) and 0.15 M NaCl, 0.1% BSA, 0.01% Tween 80, and 0.05 M sodium phosphate (pH 7.8). Two-chain urokinase generated during the reaction was monitored by including ^{125}I-pro-UK/^{125}I-A-pro-UK in the reaction mixtures. The sensitivities to plasmin and thrombin were studied as previously described (Gurewich et al., 1988).

Continuous Assay for Plasmin Generation by A-pro-UK or UK in the Presence of Fragment E-2 or EACA. Time–absorbance curves of plasminogen activation were obtained by measuring the OD increase of the reaction mixture with time at the selected wavelength 410 nm and the reference wavelength 490 nm (410/490 nm) on a microtiter plate reader (Dynatech MR 5000). The reaction mixture contained S2251 (1.5 mM), Glu-plasminogen or Lys-plasminogen (4 μM and 8 μM), and UK (1.0 nM) or A-pro-UK (1.0 nM), in the presence of fragment E-2 (0–20 μM) or EACA (0–20 mM). The reactants were made up in 0.05 M sodium phosphate, 0.15 M NaCl, 0.2% BSA, 0.01% Tween-80, pH 7.8, and incubated at room temperature. A fixed Cl^- concentration was used in all the reactions since this has been shown to affect plasminogen activation (Urano et al., 1987a,b).

Methodology Used for the Kinetic Analysis. Plasminogen activation by UK or A-pro-UK coupling with measuring plasmin generation with chromogenic substrate S-2251 can be described by the following two reactions:

$$\text{plasminogen} \xrightarrow{\text{u-PA}} \text{plasmin}$$

$$\text{S2251} \xrightarrow{\text{plasmin}} \text{peptide} + \text{pNA}\ (\Delta A_{410/490nm})$$

The time–absorbance curves of these reactions were plotted from the assay described above. The value for K_M was then determined directly by curve fitting using the integrated rate equation (eq 1), adapted from Chibber et al. (1985), where

$$A(t)_{i\,410/490nm} = \frac{\epsilon D}{2}\,\frac{k_{cat,uPA}[uPA][Plg]_i}{K_{M,uPA} + [Plg]_i}t^2 + B \qquad (1)$$

ϵ is the molar extinction coefficient for pNA in a 100-μL well, A is absorbance, the subscript i represents the concentration used, and B, is the value for the background ($B = A_{t=0}$). Curve fitting was performed on early time points, before depletion of substrates became significant, using the Enzfitter program (Elsevier-Biosoft, Cambridge, UK). During this time D is constant:

$$D = \frac{k_{cat,pln}[S2251]}{K_{M,pln} + [S2251]}$$

The K_M of u-PA can be determined from the following experiments and calculations:

$$H_i = \frac{\epsilon D}{2}\,\frac{k_{cat,uPA}[uPA][Plg]_i}{K_{M,uPA} + [Plg]_i} \qquad (2)$$

so that

$$A(t)_{i\,410/490nm} = H_i t^2 + B \qquad (3)$$

H_i was directly obtained by nonlinear regression analysis using the Enzfitter program.

In the presence of fragment E-2 (0–20 μM) or EACA (0–20 mM), plasminogen forms complexes with ligands as a real substrate for u-PA. The concentration of new substrate can be calculated as follows:

$$K_d = \frac{(A - X)(B - X)}{X} \qquad (4)$$

where A is the concentration of plasminogen, and B is the

concentration of fragment E-2 or EACA. X is the concentration of the complex. The K_d for fragment E-2 (fibrin degradation products) of Glu-plasminogen or of Lys-plasminogen is about 0.3 μM (Tran-Thang et al., 1986; Bachmann & Tran-Thang, 1990). The K_d for EACA of Glu-plasminogen is about 0.01 mM and of Lys-plasminogen it is about 0.02 mM (Lucas et al., 1983a). According to eq 4

$$X = \frac{A + B + K_d - \sqrt{(A + B + K_d)^2 - 4AB}}{2} \quad (5)$$

In this experimental condition, $X_m \approx [\mathrm{Plg}]_m$, $X_n \approx [\mathrm{Plg}]_n$

If [u-PA] is fixed, using two different concentrations of substrate, plasminogen $[\mathrm{Plg}]_m$ or $[\mathrm{Plg}]_n$, different values for H_i can be obtained as follows:

$$H_m = \frac{\epsilon D}{2} \frac{k_{cat,uPA}[uPA][Plg]_m}{K_{M,uPA} + [Plg]_m} \quad (2a)$$

$$H_n = \frac{\epsilon D}{2} \frac{k_{cat,uPA}[uPA][Plg]_n}{K_{M,uPA} + [Plg]_n} \quad (2b)$$

If $[\mathrm{Plg}]_m = c$, $[\mathrm{Plg}]_n = nc$, and the following is defined:

$$\alpha = \frac{H_m}{Hn} = \frac{K_{M,uPA} + [Plg]_n}{K_{M,uPA} + [Plg]_m} \frac{[Plg]_m}{[Plg]_m} = \frac{K_{M,uPA} + nc}{K_{M,uPA} + c} \frac{c}{nc} \quad (6)$$

Therefore

$$n\alpha K_{M,uPA} + nc\alpha = K_{M,uPA} + nc$$

$$K_{M,uPA}(n\alpha - 1) = nc(1 = \alpha)$$

The following equation, obtained from the above calculations, was used to calculate the K_M:

$$K_{M,uPA} = \frac{(1 - \alpha)nc}{n\alpha - 1} = \frac{1 - \alpha}{\alpha - n^{-1}}c \quad (7)$$

In the presence of different concentrations of fragment E-2 or EACA, on the basis of the studies of Christensen (1978) with EACA which we have duplicated with fragment E-2, the values for D are constant within the range of concentrations of EACA, fragment E-2, and plasminogen which were used. Therefore, it was possible to compare the catalytic efficiencies of u-PA with or without fragment E-2 or EACA by the following procedure.

The kinetic parameters (K_M and k_{cat}) of A-pro-UK were postulated to change with the concentration of fragment E-2 or EACA whose presence is indicated by a prime ($'$). Therefore, the following equations were used:

$$H_i = \frac{\epsilon D}{2} \frac{k_{cat,uPA}[uPA][Plg]_i}{K_{M,uPA} + [Plg]_i} \quad (2)$$

$$H'_i = \frac{\epsilon D}{2} \frac{k'_{cat,uPA}[uPA][Plg]'_i}{K'_{M,uPA} + [Plg]'_i} \quad (2')$$

if $[\mathrm{Plg}]_i = c$, $[\mathrm{Plg}]_i' = c'$, $p = [\mathrm{Plg}]_i/[\mathrm{Plg}]_i' = c/c'$, and the following is defined:

$$\beta = \frac{H_i}{H'_i} = \frac{k_{cat,uPA}}{K_{M,uPA} + [Plg]_i} \frac{K'_{M,uPA} + [Plg]'_i}{k'_{cat,uPA}} \frac{[Plg]_i}{[Plg]'_i}$$

$$\beta = \frac{H_i}{H'_i} = \frac{k_{cat,uPA}/K_{M,uPA}}{k'_{cat,uPA}/K'_{M,uPA}} \frac{1 + [Plg]'_i/K'_{M,uPA}}{1 + [Plg]_i/K_{M,uPA}} \frac{[Plg]_i}{[Plg]'_i} \quad (8)$$

In this study, every reaction was compared with that of u-PA without any ligands, so the H_i always was presented by H_0. The ratio of catalytic efficiencies of the two reactions (F) was expressed as follows:

$$F = \frac{k_{cat,uPA}/K_{M,uPA}}{k'_{cat,uPA}/K'_{M,uPA}} \quad (9)$$

So, according to eq 8

$$F = \beta \frac{1 + [Plg]_i/K_{M,uPA}}{1 + [Plg]'_i/K'_{M,uPA}} \frac{[Plg]'_i}{[Plg]_i} \quad (10)$$

$$F = \beta/p\left[1 + \frac{c}{[(1-\alpha)/(\alpha - n^{-1})]c}\right] \Big/ \left[1 + \frac{c'}{[(1 - \alpha')/(\alpha' - n^{-1})]c'}\right] =$$

$$\beta/p\left[\frac{1 - \alpha + \alpha - n^{-1}}{1 - \alpha}\right] \Big/ \left[\frac{1 - \alpha' + \alpha' - n^{-1}}{1 - \alpha'}\right] = \beta/p\frac{1 - \alpha'}{1 - \alpha} \quad (11)$$

The difference in Gibbs free energy between the two reactions was derived from eq 12 in which R is the gas constant and T is the absolute temperature.

$$\Delta\Delta G_T^{\ddagger} = -RT \ln \frac{k_{cat,uPA}/K_{M,uPA}}{k'_{cat,uPA}/K'_{M,uPA}} = -RT \ln F \quad (12)$$

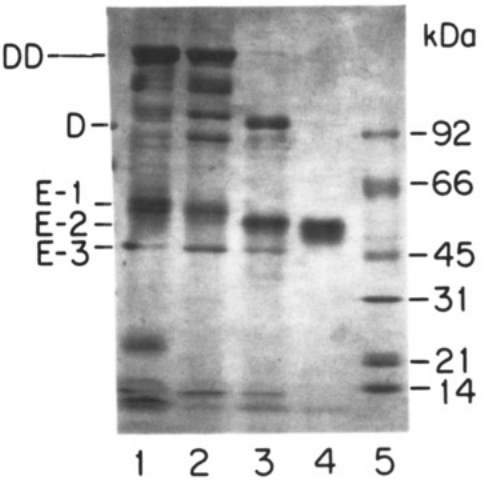

FIGURE 1: Gradient (5–15%) SDS–PAGE of the fragment E-2 preparation. Lane 1: fibrin digest containing the DD–E complex which partially dissociated in SDS buffer and fragment D and E. Lane 2: fractions from column 1 pool A containing the DD–E complex. Lane 3: fractions from column 1 pool B containing fragments D, E-2, and E-3. Lane 4: fractions from column 2 pool B containing purified fragment E-2.

RESULTS

Characterization of the Fragment E-2 Preparation by SDS–PAGE. The fibrin digestion mixture containing the DD–E complex and fragments D and E (lane 1) and selected fraction pools from column 1 containing the DD–E complex which partially dissociated in the SDS buffer (lane 2) and fragments D, E-2, and E-3 (lane 3) and from column 2 containing the purified fragment E-2 were visualized by gradient (5–15%) SDS–PAGE (Figure 1).

Characterization of A-pro-UK—A Comparative Study with pro-UK. The A-pro-UK had activities against synthetic substrate or against plasminogen which were ≈0.1% and ≈ 0.5% that of UK, respectively, consistent with other Lys-158

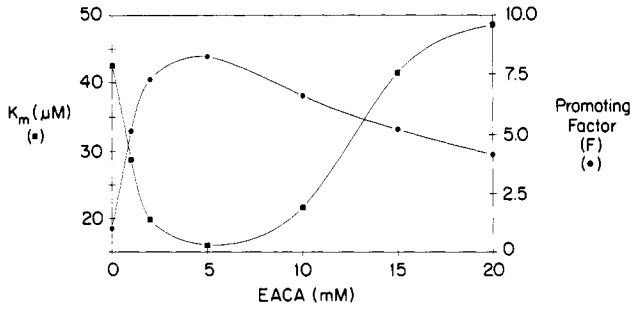

FIGURE 7: Glu-plasminogen activation by UK. The effect of EACA (0–20 mM) on K_M (■) and on the promotion factor (F) (●) (see legend to Figure 3).

plasminogen concentration, the slope remained linear up to the maximum ratio tested (Figure 6).

When fragment E-2 was replaced by EACA (0–20 mM), the highest F value obtained was 18, which was at 2.0 mM EACA (Table 2 in the supplementary material). At higher concentrations of EACA or EACA/plasminogen ratios, lower F values were found (Figure 4).

Lys-plasminogen: Against Lys-plasminogen (1.2 and 2.4 μM), A-pro-UK had a 19.7-fold higher catalytic efficiency than it had against Glu-plasminogen, calculated from eqs 8–11.

When fragment E-2 (6 μM) was added, however, inhibition rather than promotion was observed with an F value of 0.713 (±0.014) (Table 3 in the supplementary material). Inhibition of plasminogen activation was also seen with EACA where the F was ≈0.70 and plateaued at an EACA concentration of >1.0 mM (Figure 5).

Plasminogen Activation by UK with and without the Ligands. Glu-plasminogen: Urokinase has a 195.2-fold (±14.1) higher catalytic efficiency against Glu-plasminogen than did A-pro-UK (therefore the F for UK vs A-pro-UK was 195.2). The addition of fragment E-2 (20 μM) induced a reduction in K_M from 42.5 (±0.21) to 8.90 (±0.46) μM. This was associated with a slight increase in catalytic efficiency (F = 396.3 for UK + fragment E-2 vs A-pro-UK). Therefore, there was a 2.03-fold increase in catalytic efficiency induced by the ligand (396.3/195.2) (Table 1 in the supplementary material).

The addition of EACA (0–20 mM) also induced some reduction in K_M, but only at concentrations <15 mM. The peak effect (K_M 16 ± 1.20 μM) was seen at 5 mM EACA. This was associated with an 8.25-fold increase in catalytic efficiency. No promotion was seen at ≥15 mM EACA (Figure 7) and Table 4 in the supplementary material).

Lys-plasminogen: Against Lys-plasminogen, the catalytic efficiency of UK was 13.2-fold higher than against Glu-plasminogen, not dissimilar to the 19.7-fold for A-pro-UK against Lys- vs Glu-plasminogen. When EACA was added, only a slight promotion (1.15-fold) of the activation of Lys-plasminogen was seen associated with a slight reduction in K_M (1.33 ± 0.15 to 0.94 ± 0.06 μM) (Table 3 in the supplementary material).

Summary of the Promotion (F) by the Ligands on the Catalysis of Plasminogen Activation. The predominant promotion found was that of fragment E-2 on Glu-plasminogen activation by A-pro-UK, which was promoted at least 450-fold, since promotion had not yet saturated at the highest ratio of fragment E-2 to Glu-plasminogen used. By contrast, there was only an 18-fold promotion of this same reaction by EACA at the optimal concentration. Little promotion by the ligands was seen of UK-induced activation of Glu- or Lys-plasminogen. Lys-plasminogen activation by A-pro-UK was inhibited by the ligands (Table I).

Table I: Summary of the Maximum Promotion Factors Induced by Fragment E-2 and by EACA on Plasminogen Activation by A-pro-UK or UK Expressed as a Fraction of the Buffer Control Value

	Glu-plasminogen			Lys-plasminogen		
	E-2	EACA	buffer	E-2	EACA	buffer
A-pro-UK	>450	18	1	<1	<1	1
UK	2.0	8.25	1	ND	1.15	1

The relative Gibbs free energy for the reactions was calculated from eqs 11 and 12. The biggest differential in free energy was between A-pro-UK against Glu-plasminogen in buffer which was essentially zero and this reaction in the presence of fragment E-2 which was 150. The free energy of the latter reaction was greater than that of UK against Glu-plasminogen, which was about 130. The reaction of A-pro-UK in the presence of fragment E-2 was close to the maximum of the relative free energy in u-PA-induced plasminogen activation which was ≈180 and which was represented by the reaction of UK against Lys-plasminogen.

Amidolytic Activities of pro-UK, UK, and A-pro-UK in the Presence and Absence of Fragment E-2. No stimulation was observed in the amidolytic activities of pro-UK, UK, or A-pro-UK by fragment E-2 (20 μM), suggesting that fragment E-2 did not directly interact with these activators.

Discussion

The mechanism responsible for the fibrin selectivity of pro-UK-induced fibrinolysis remains unexplained. Previous studies have shown that fibrin-dependent plasminogen activation by pro-UK in plasma was invariably preceded by a lag phase which was significantly attenuated by plasmin pretreatment of the fibrin (Pannell et al., 1988). Plasmin preferentially cleaves fibrin at the carboxy side of lysyl residues (Weinstein & Doolittle, 1972) thereby exposing carboxy-terminal lysines (Violand et al., 1975), which have been shown to be important secondary plasminogen binding sites (Bok & Mangel, 1984; Fleury & Angles-Cano, 1991). Recently, it was shown that the three carboxy-terminal lysines in fragment E, a fibrin degradation product, selectively promoted plasminogen activation by pro-UK, whereas fragment D, a constituent of intact fibrin, promoted plasminogen activation by tissue plasminogen activator (t-PA) (Liu & Gurewich, 1991).

A Lys-158 plasmin-resistant rpro-UK mutant was used in the present study to avoid interference by UK generation during the reactions. The mutant was validated as a surrogate for pro-UK by studies with pro-UK itself in which measurements were limited to time points before significant (<2%) UK generation by plasmin occurred (Figure 2). A method of analysis was used on the basis of an equation of Chibber et al. (1985) to describe plasmin generation in the presence of the synthetic substrate S2251 in a continuous or accumulative system. By this method, the Michaelis constant (K_M) and catalytic efficiency can be calculated with only two concentrations of substrate, assuming Michaelis–Menten kinetics. This assumption is valid since the activations of Glu-plasminogen either by pro-UK in the presence of CNBr-2 fragments (Kirchheimer et al., 1987) or by two other plasmin-resistant mutants of pro-UK (Nelles et al., 1987) or by t-PA in the presence of various fibrin(ogen) fragments (Hoylaerts et al., 1982) all follow Michaelis–Menten kinetics.

The analytic method was tested by using it to measure the K_M of UK and of A-pro-UK against Glu- or Lys-plasminogen. The values obtained (42.5 μM and 8.50 μM, respectively) were representative of several of those in the literature for UK and for other Lys-158 pro-UK mutants (Table 5 in the supple-

mentary material). In addition, the relative catalytic efficiency of A-pro-UK obtained by the method was about 0.5% that of UK, which is comparable to the 0.4% figure reported by two groups for the intrinsic of pro-UK (Pannell & Gurewich, 1987; Petersen et al., 1988).

In the presence of fragment E-2, a linear promotion (F) of Glu-plasminogen activation by A-pro-UK was obtained which was 245.5-fold at the highest concentration of fragment E-2 (20 μM) (Figure 3). Promotion was a function of the ratio of fragment E-2 to plasminogen, probably reflecting that it was predominantly the fragment E-2-bound plasminogen, rather than free plasminogen, which was being activated. At the highest E-2/plasminogen ratio used (41.6/1), which was obtained by reducing the plasminogen concentration, promotion was 450-fold without saturating (Figure 6). Since there was only about a 10-fold reduction in the K_M of A-pro-UK against Glu-plasminogen, promotion was largely a function of the k_{cat} (Figure 3). A comparable promotion was also seen with pro-UK over early time points, before significant UK generation occurred (Figure 2). By contrast, fragment E-2 induced little promotion (2-fold) of UK-induced plasminogen activation. This may be in part related to UK being already close to the maximum for this reaction, as reflected by its relative Gibbs free energy against Glu-plasminogen ($\approx$130), compared with that against Lys-plasminogen ($\approx$180) which probably represents the upper limit.

By contrast, EACA, a ligand which binds to both the strong and weaker lysine binding sites on plasminogen (Markus et al., 1978a), induced a biphasic promotion of Glu-plasminogen activation by A-pro-UK which was 18-fold at the optimal EACA concentration (2 mM) (Figure 4). A similar promotion of other activators has been reported at the EACA concentration range used (Claeys & Vermylen, 1974; Markus et al., 1978b). At higher concentrations, EACA is an inhibitor of this reaction (Lorand & Condit, 1965). The contrasting effects of these ligands on pro-UK suggest that fragment E-2 induces a different modification in Glu-plasminogen than the open Lys-form which is induced by EACA (Alkjaersig, 1964; Castellino et al., 1973; Violand et al., 1975; Ramakrishnan et al., 1991). The special fragment E-2-induced conformation of Glu-plasminogen may be mediated by cooperativity of its three carboxy-terminal lysines which are responsible for the high affinity of this fragment for plasminogen (Varadi & Patthy, 1984). The alternative explanation that fragment E-2 increases the amidolytic activity of A-pro-UK or pro-UK was excluded.

An interaction between the N-peptide of Glu-plasminogen and pro-UK could also be involved since Lys-plasminogen activation was slightly inhibited rather than promoted by fragment E-2 (Figure 5, and Table 3 in the supplementary material). Lys-plasminogen is generated by removal of an N-peptide by plasmic cleavage of the Lys-76–Lys-77 bond, which opens the molecule, since this peptide maintains the closed conformation of Glu-plasminogen through intramolecular interaction with kringles (Cummings & Castellino, 1985; Urano et al., 1991) in the presence of Cl^- (Urano et al., 1987a,b). Similarly, when the lysine binding sites, principally kringle 4 (Sehl & Castellino, 1990) of Glu-plasminogen are occupied by certain ω-amino acids like EACA (Alkjaersig, 1964; Castellino et al., 1973; Violand et al., 1975; Ramakrishnan et al., 1991), its maximum dimension is increased by almost 2-fold (Mangel et al., 1990), and the plasminogen becomes more susceptible to activation (Claeys & Vermylen, 1974; Wiman & Wallen, 1975; Wallen, 1977; Markus et al., 1978b). In the present study, A-pro-UK had a 20-fold greater activity against Lys- than against Glu-plasminogen, an order of magnitude comparable to that reported for UK (Lucas et al., 1983b).

The finding that only native plasminogen activation was promoted by fragment E-2 explains a previous observation that the fibrin specificity of pro-UK-induced clot lysis was lost when Glu-plasminogen in the plasma was replaced by Lys-plasminogen (Pannell & Gurewich, 1986).

In the presence of fragment E-2, A-pro-UK had a plasminogen-activating activity comparable to that of UK, so that there was near full activity without activation by proteolytic cleavage. This is not necessarily inconsistent with certain reports that pro-UK has no significant intrinsic activity (Kasai et al., 1985; Urano et al., 1988; Husain, 1991), since these kinetic studies did not include ligands. Rather, the present study shows that pro-UK has a substantial latent intrinsic activity against plasminogen which is triggered by a fibrin fragment. Therefore, the findings illustrate a mechanism for fibrin-dependent plasminogen activation independent of fibrin affinity, which contrasts with the well-established paradigm illustrated by t-PA in which fibrin specificity is mediated by fibrin binding (Hoylaerts et al., 1982). Since the fragment E region in fibrin is created by plasmin degradation and is not available in intact fibrin, the present findings are also consistent with both the characteristic lag phase of pro-UK-induced clot lysis (Pannell & Gurewich, 1986) and its attenuation by limited plasmin treatment of a clot (Pannell et al., 1988).

However, the comparability of the plasminogen-activating activities of pro-UK and UK in the presence of fragment E-2 appears to contradict previous findings that fibrinolysis is considerably amplified by the activation of pro-UK on the clot surface (Gurewich et al., 1988; Declerk et al., 1990). This paradox has been explained by the fact that UK is unrestricted whereas pro-UK is restricted to the activation of fragment-E-bound plasminogen on the clot (Pannell & Gurewich, 1992).

It has not been shown that the soluble system used in these investigations reproduces the conditions present on the fibrin surface, particularly with reference to the availability of C-terminal lysines in the fragment E region. However, certain ex vivo and in vivo observations suggest that the necessary conditions to express the intrinsic activity of pro-UK do exist on the fibrin surface. For example, a Lys-158 mutant pro-UK with little amidolytic or plasminogen-activating activity and which was essentially inactive against intact clots induced effective lysis when clots were plasmin pretreated or when a small dose of t-PA was added to plasma to initiate fibrinolysis (Gurewich et al., 1988). Similarly, Collen et al. (1989) showed that two Lys-158 pro-UK mutants (Ala-158 and Glu-158), which had little activity in vitro, had surprising thrombolytic activity in rabbits with jugular vein thrombosis. This discrepancy between the in vitro and in vivo activities may be explained by the presence of sufficient t-PA in vivo to initiate fibrin degradation so that the new plasminogen-binding sites required by the Lys-158 mutants would be made available. Finally, when fibrin clots were pretreated with plasmin or when a small amount of t-PA was added to the plasma in which they were suspended, pro-UK-induced clot lysis was greatly accelerated (Pannell et al., 1988).

In conclusion, plasminogen activation by A-pro-UK, a pro-UK surrogate, was shown to be promoted more than 450-fold by fibrin fragment E-2. This promotion gave it an activity comparable to that of UK and was largely related to a change in k_{cat}. Promotion was mediated by an interaction between Glu-plasminogen and fragment E-2, since the ligand had no affect on the amidolytic activity of A-pro-UK or

pro-UK. Therefore, it was postulated that fragment E-2 induces a novel conformational change in Glu-plasminogen that differs from that induced by EACA which caused relatively little promotion. The phenomenon by which a selective, fibrin-mediated conformational change in native plasminogen promotes its activation illustrates a mechanism for fibrin specificity which is unrelated to fibrin affinity of the activator. The mechanism helps to explain the fibrin specificity of pro-UK and contrasts it with that of t-PA.

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Supplementary Material Available

Four tables (1–4) giving the rates of Glu- and Lys-plasminogen activation by pro-UK and UK in the presence and absence of fragment E-2 and EACA and Table 5 summarizing the values in the literature for the K_M of UK, pro-UK, and Lys-158 pro-UK mutants against Glu- and Lys-plasminogen (5 pages). Ordering information is given on any current masthead page.

References

Alkjaersig, N. (1964) *Biochem. J. 93*, 171–182.
Bachmann, F., & Tran-Thang, C. (1990) *Fibrinolysis 4* (Suppl. 3:75), Abstract no. 192.
Bok, R. A., & Mangel, W. F (1985) *Biochemistry 24*, 3279–3286.
Castellino, F. J., & Powell, J. R. (1981) *Methods Enzymol. 80*, 365–378.
Castellino, F. J., Brockway, W. J., Thomas, J. K., Laio, H., & Rawitch, A. B. (1973) *Biochemistry 12*, 2787–2791.
Chibber, B. A. K., Morris, J. P., & Castellino, F. J. (1985) *Biochemistry 24*, 3429–3434.
Christensen, U. (1978) *Biochim. Biophys. Acta. 526*, 194–201.
Christensen, U. (1985) *FEBS Lett. 182*, 43–46.
Claeys, H., & Vermylen, J. (1974) *Biochim. Biophys. Acta 342*, 351–359.
Collen, D., Mao, J., Stassen, J. M., Broeze, R., Lijnen, H. R., Abercrombie, D., Puma, P., Almeda, S., & Vovis, G. (1989) *J. Vasc. Med. Biol. 1*, 46–49.
Cummings, H. S., & Castellino, F. J. (1985) *Arch. Biochem. Biophys. 236*, 612–618.
Declerck, P. J., Lijnen, H. R., Verstraete, M., Morean, H., & Collen, D. (1990) *Blood 75*, 1794–1800.
Fleury, V., & Angles-Cano, E. (1991) *Biochemistry 30*, 7630–7638.
Gurewich, V., Pannell, R., Louie, S., Kelley, P., Suddith, S. L., & Greenlee, R. (1984) *J. Clin. Invest. 73*, 1731–1739.
Gurewich, V., Pannell, R., Broeze, R. J., & Mao, J. I. (1988) *J. Clin. Invest. 82*, 1956–1962.
Hoylaerts, M., Rijken, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem. 256*, 2912–2919.
Husain, S. S. (1991) *Biochemistry 30*, 5787–5805.
Ichinose, A., Fujikawa, K., & Tadakazu, S. (1986) *J. Biol. Chem. 261*, 3486–3489.
Kasai, S., Arimua, H., Nishida, M., & Suyana, T. (1985) *J. Biol. Chem. 260*, 12377-12382.
Kirchheimer, J. C., Resch, I., Christ, G., Wojta, J., & Binder, B. R. (1987) *Eur. J. Biochem. 166*, 393–397.
Lijnen, H. R., Zamarron, C., Blaber, M., Winkler, M. E., & Collen, D. (1986) *J. Biol. Chem. 261*, 1253–1258.
Lijnen, H. R., Van Hoef, B., Nelles, L., & Collen, D. (1990) *J. Biol. Chem. 265*, 5232–5235.
Liu, J., & Gurewich, V. (1991) *J. Clin. Invest. 88*, 2012–2017.
Lorand, L., & Condit, E. V. (1965) *Biochemistry 4*, 265–270.
Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983a) *J. Biol. Chem. 258*, 4249–4256.
Lucas, M. A., Straight, D. L., Fretto, L. J., & McKee, P. A. (1983b) *J. Biol. Chem. 258*, 12171–12177.
Mangel, W. F., Lin, B., & Ramakrishnan, V. (1990) *Science 248*, 68–73.
Markus, G., DePasquale, J. L., & Wissler, F. C (1978a) *J. Biol. Chem. 253*, 727–732.
Markus, G., Evers, J. L., & Hobika, G. H. (1978b) *J. Biol. Chem. 253*, 733–739.
Nelles, L., Lijnen, R., Collen, D., & Holmes, W. (1987) *J. Biol. Chem. 262*, 5682–5686.
Pannell, R., & Gurewich, V. (1986) *Blood 69*, 1215–1223.
Pannell, R., & Gurewich, V. (1987) *Blood 69*, 22–26.
Pannell, R., & Gurewich, V. (1992) *Fibrinolysis 6*, 1–5.
Pannell, R., Black, J., & Gurewich, V. (1988) *J. Clin. Invest. 81*, 853–859.
Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.
Petersen, L. D., Lund, L. R., Dano, K., Nielsen, L. S., & Skriver, L. (1988) *J. Biol. Chem. 263*, 11189–11195.
Ramakrishnan, V., Patthy, L., & Mangel, W. F. (1991) *Biochemistry 30*, 3963–3969.
Sehl, L. C., & Castellino, F. J. (1990) *J. Biol. Chem. 265*, 5482–5486.
Tran-Thang, C., Kruithof, E. K., Atkinson, J., & Bachmann, F. (1986) *Eur. J. Biochem. 160*, 599–604.
Urano, T., Chibber, B. A. K. & Castellino, F. J. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4031–4036.
Urano, T., de Serrano, V. S., Chibber, B. A. K., & Castellino, F. J. (1987b) *J. Biol. Chem. 262*, 15959–15964.
Urano, T., de Serrano, V. S., Gaffney, P. J., & Castellino, F. J. (1988) *Arch. Biochem. Biophys. 264*, 222–230.
Urano, T., Takada, Y., & Takada, A. (1991) *Thromb. Res. 61*, 349–359.
Varadi, A., & Patthy, L. (1983) *Biochemistry 22*, 2440–2446.
Varadi, A., & Patthy, L. (1984) *Biochemistry 23*, 2108–2112.
Violand, B. N., Sodetz, J. M., & Castellino, F. J. (1975) *Arch. Biochem. Biophys. 170*, 300–305.
Wallen, P. (1977) *Thrombosis and Urokinase*, pp 91–102, Academic Press, London.
Weinstein, M. J., & Doolittle, R. F. (1972) *Biochim. Biophys. Acta 258*, 577–590.
White, W. F., & Barlow, G. H. (1970) *Methods Enzymol. 19*, 665–672.
Wiman, B., & Wallen, P. (1975) *Eur. J. Biochem. 50*, 489–494.
Zamarron, C., Lijnen, H. R., van Hoef, B., & Collen, D. (1984) *Thromb. Haemostas. 52*, 19–23.

Mechanism of Adenylate Kinase. Structural and Functional Roles of the Conserved Arginine-97 and Arginine-132†

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ABSTRACT: The structural and functional roles of two conserved active site residues, Arg-97 and Arg-132, in chicken muscle adenylate kinase (AK) were evaluated by site-directed mutagenesis in conjunction with one- and two-dimensional proton nuclear magnetic resonance (NMR), kinetics, and guanidine hydrochloride-induced denaturation. In addition, ^{31}P NMR analysis was used to evaluate the contribution of Arg-97 to the phosphorus stereospecificity of AK. The results and conclusions are summarized as follows: (i) Kinetic analysis of R97M reveals 6- and 28-fold increases in the dissociation constant K_i and Michaelis constant K of AMP, respectively, and a moderate 30-fold decrease in k_{cat}. The K_i and K values of MgATP are relatively unperturbed. The localized effect of AMP stabilization was independently confirmed by proton NMR titration, which showed a ca. 20-fold increase in the dissociation constant of AMP but not of MgATP. (ii) R132M affords a dramatic decrease in k_{cat} by a factor of 8.0×10^3, with unchanged dissociation and Michaelis constants for either substrate. The lack of perturbation in the affinities toward substrates was confirmed by proton NMR titration. (iii) Although small chemical shift changes were observed for the free mutants and their complexes with substrates, further analyses by nuclear Overhauser enhanced spectroscopy with the bisubstrate analogue inhibitor, P^1,P^5-bis(5′-adenosyl)pentaphosphate (AP_5A), indicated little perturbation in the global conformation. (iv) Contributions to conformational stability by Arg-97 and Arg-132 are negligible on the basis of the free energy of unfolding, $\Delta G_d^{H_2O}$. (v) R97M was predicted and demonstrated to exhibit enhanced stereospecificity at the AMP site by at least 10-fold relative to WT in the conversion of adenosine 5′-monothiophosphate to adenosine 5′-(1-thiodiphosphate). This result for R97M was predicted on the basis of the orientation of Arg-97 relative to Arg-44 and AMP in the active site as observed in available crystal structures and the stereospecificity results of R44M [Jiang, R.-T., Dahnke, T., & Tsai, M.-D. (1991) *J. Am. Chem. Soc. 113*, 5485-5486]. (vi) The above structural and functional analyses led us to conclude that Arg-97 interacts with the phosphoryl group of AMP, beginning at the binary complex (1-2 kcal/mol), continuing through the transition state (3.5 kcal/mol), and that Arg-132 stabilizes the transition state by greater than 5 kcal/mol. (vii) The functional importance of Arg-97 appears to be similar to that of Arg-44 [Yan, H., Dahnke, T., Zhou, B., Nakazawa, A., & Tsai, M.-D. (1990) *Biochemistry 29*, 10956-10964]. The results for R97M also clarify conflicting reports from analogous mutants in other types of AK and support conclusions based upon analysis of the mitochondrial matrix AK·AMP crystal structure [Diederichs, K., & Schulz, G. E. (1991) *J. Mol. Biol. 217*, 541-549].

While significant progresses have been made on the structure–function relationship of adenylate kinase (AK)[1,2] as reviewed recently by Tsai and Yan (1991), the functional roles of most active site residues remain to be established. The best characterized region of substrate sites is the phosphate binding region. Crystal structural analyses of the $MgAP_5A$ complexes of yeast AK (AKy) (Egner et al., 1987) and *Escherichia coli* AK (AKe) (Müller & Schulz, 1988) suggest that the phosphates are surrounded by several conserved arginine residues. The structure of the AK1·$MgAP_5A$ complex has not been reported; however, Egner et al. (1987) showed an overlay of the structure of free AK1 with that of AKy·$MgAP_5A$, as shown in Figure 1. The numbering of residues in Figure 1 is according to the "family numbering system" (Schulz et al., 1986); the arginine residues of AK1 addressed in this paper, 44, 97, 132, 138, and 149, correspond to residues 53, 106, 141, 178, and 189, respectively, in the family numbering system. While the structure of AKy·$MgAP_5A$ best represents the current knowledge on the substrate sites of AK, we consider it as a starting point to probe the *quantitative* structure–function relationship of AK.

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ADPαS, adenosine 5′-(1-thiodiphosphate); AK, adenylate kinase; AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-monothiophosphate; AP_5A, P^1,P^5-bis(5′-adenosyl)pentaphosphate; ATP, adenosine 5′-triphosphate; ATPαS, adenosine 5′-(1-thiotriphosphate); 1D, one-dimensional; 2D, two-dimensional; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; FID, free induction decay; Gdn-HCl, guanidine hydrochloride; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhanced spectroscopy; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; UV, ultraviolet; WT, wild type.

[2] The AK from different sources are abbreviated as follows: from muscle, AK1 (followed by the letters c, h, p, and r designating chicken, human, porcine, and rabbit, respectively); from *E. coli*, AKe; from yeast, AKy; from mammalian mitochondrial intermembrane space, AK2; from mammalian mitochondrial matrix, AK3. Unless otherwise specified, the numbering system used in this paper is the conventional system for AK1. Although cAK has one additional residue near the N-terminus (Kishi et al., 1986), the Met-1 residue is absent in the cAK expressed in *E. coli* (Tanizawa et al., 1987). This makes numbering of cAK consistent with other AK1.

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