Requirement of Zymogen Modification for Activation of Porcine Plasminogen†

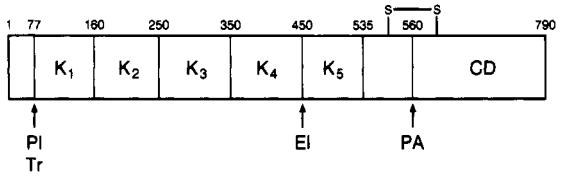
Raymund Machovich,‡ Robert D. Litwiller,§ and Whyte G. Owen*,§

Section of Hematology Research and Department of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, Minnesota 55905, and the Department of Biochemistry II, Semmelweiss University of Medicine, Budapest, Hungary

Received May 14, 1992; Revised Manuscript Received September 14, 1992

ABSTRACT: In physiological salt solutions, porcine plasminogen is refractory to activation by urokinase or trypsin and to proteolysis at Lys_{77} by plasmin or trypsin. Plasminogen becomes a substrate for urokinase (at Arg_{560}), plasmin (at Lys_{77}), and trypsin (at both bonds) if chloride ion is removed or if 6-aminohexanoate (2.5 mmol/L) is added. Irrespective of salts, activation of des(1–77)plasminogen is as efficient as activation of des(kringle$_{1-4}$)plasminogen and is inhibited 50% by 2.5 mmol/L 6-aminohexanoate. In solutions lacking chloride or containing 6-aminohexanoate, plasminogen, des(1–77)plasminogen, and des(kringle$_{1-4}$)plasminogen show no tendency to saturate urokinase in physiologically relevant concentrations (10 μmol/L). The findings are interpreted as indicating that plasminogen requires modification, either by proteolysis or by ligands, for activation.

Control of plasminogen activation is associated with macromolecular associations and proteolysis of plasminogen, with secretion, localization, activation, and inhibition of activators and with inhibition of plasmin.[1] Substrate-level control may be visualized in the context of the linear structure of plasminogen:



where K = kringle microdomain, CD = catalytic domain, PA = plasminogen activators, Pl = plasmin, Tr = trypsin, El = elastase, and the numbers identify residue positions.

Activators hydrolyze plasminogen at Arg_{560} to yield the two-chain protease (Robbins et al., 1967), while Lys_{77}-Lys_{78} is hydrolyzed autocatalytically by plasmin (Wiman & Wallen, 1973; Suenson & Thorsen, 1988). During human plasminogen activation, marked hysteresis occurs because the des(1–77)-zymogen is the more sensitive substrate than the native zymogen for activators. The rate of proteolysis at Lys_{77} (by plasmin) relative to that at Arg_{560} (by activators) could be a basis for regulation. However, limited proteolysis of human plasminogen by plasmin and the net conversion of plasminogen to plasmin have kinetics that are insufficiently disparate to enable clear resolution of the two processes in activation assays. Owing at least in part to contamination with des(1–77)-plasminogen, kinetic constants for plasminogen activation measured among laboratories have differed by more than an order of magnitude (Petersen et al., 1985), and the relative susceptibilities of the virgin and modified zymogens to activators remain unclear. The rate of plasminogen activation can also be enhanced by degradation of plasminogen with elastase to yield des(kringle$_{1-4}$)plasminogen (Sugiyama et al., 1987; Takada et al., 1988; Machovich & Owen, 1989) and by effectors such as amino acids, fibrin, fibrin(ogen) degradation products, denaturated proteins, and endothelium (Markus et al., 1978; Radcliffe & Heinze, 1981; Hoylaerts et al., 1982; Machovich & Owen, 1988; Hajjar et al., 1986).

Interpretation of kinetic data, especially with the addition of effectors, is obfuscated further by the finding that plasminogen activation is inhibited strongly by chloride ions (Violand et al., 1978; Machovich & Owen, 1990a), but in an effector-dependent manner. Some of the difficulties inherent in the system would be minimized if the feedback action of plasmin were avoided. Native porcine plasminogen bears an arginyl residue at position 78, which is lysyl in the human zymogen (Schaller et al., 1987). The correlation of this difference with a substantially lower sensitivity of porcine plasminogen to urokinase (Machovich & Owen, 1990a) prompts the hypothesis that the porcine system is relatively (to human) insensitive to feedback hydrolysis by plasmin. In the present work we find that, in physiological salt solutions, porcine plasminogen is refractory to hydrolysis by plasmin and further is refractory to activation by urokinase.

EXPERIMENTAL PROCEDURES

Porcine pancreatic elastase, lysine–agarose, and iPr_2PF were obtained from Sigma Chemical Company, St. Louis, MO. Human leukocyte elastase, with a specific activity of 18–20 units/mg, was a gift from Dr. J. Travis (Baugh & Travis, 1976). Spectrozyme PL (H-D-norleucyl-hexahydro-tyrosyl-lysine-*p*-nitroanilide) and Spectrozyme UK [Cbo-L-(*r*)Glu-(α-*t*-BuO)-Gly-*p*-nitroanilide] were the products of American Diagnostica, Hartford, CT. Urokinase was obtained from Abbott Laboratories.

Porcine plasminogen was prepared by lysine–agarose affinity chromatography (Summaria et al., 1976). Des(kringle$_{1-4}$)-

† This work was supported in part by Grants PO-1-HL-17430 and RO-1-HL47469 from the National Heart, Lung, and Blood Institute, by a grant from Marion Laboratories (Division of Merrill-Dow), by Hungarian Grant OTKA-1066, ETT 1-2/1991, and by the US–Hungarian Joint Fund Grant 081/91.

* Author to whom correspondence should be addressed.

‡ Semmelweis University of Medicine.

§ Mayo Clinic and Foundation.

[1] Nomenclature for zymogens of plasmin has traditionally been keyed to the amino terminal residues of human plasminogen (Glu) and its major product of degradation with plasmin (Lys). As illustrated in the present article, other species inevitably will yield other termini and, should the convention be followed, awkward nomenclature. For this paper, we define zymogens as follows: plasminogen denotes undegraded (plasma) plasminogen; des(1–77)plasminogen lacks the 77 amino terminal residues consequent to hydrolysis by plasmin at Lys_{77}; des(kringle$_{1-4}$)plasminogen has been termed "miniplasminogen" and lacks residues 1–447 owing to hydrolysis by elastase at Ala_{447}-Ile_{448}.

0006-2960/92/0431-11558$03.00/0

plasminogen was obtained from limit elastase digests: plasminogen (500 μg/mL) was incubated with elastase (20 μg/mL) at 37 °C for 2 h. Reactions were terminated by addition of iPr_2PF and dialyzed, and kringle domains 1–4 were removed by chromatography on lysine–agarose. Des(1–77)plasminogen was prepared from limit plasmin digests: plasminogen (1 mg/mL) was incubated with plasmin (18 μg/mL) in the presence of 6-aminohexanoate (2.5 mmol/L) at room temperature for 3 h. Reactions were terminated with iPr_2PF and dialyzed against 100 mmol/L HEPES (pH 7.4).

We confirmed that plasminogen activation in the presence of 6-aminohexanoate yielded two amino terminal sequences of RIYLSE (cleavage at Lys_{77}) and VVGG-V (cleavage at Arg_{560}) in addition to the zymogen N-terminal sequence of DSLDDY. Complete activation yielded one active site equivalent as titrated with *p*-nitrophenyl guanidinobenzoate. Treatment of plasminogen with elastase and urokinase yielded the expected amino terminal sequences of IAQVPS and VVGG-V.

Polyacrylamide gel electrophoresis in $NaDodSO_4$ was carried out with a Pharmacia Phast system. Amino acid sequence analysis was performed with an Applied Biosystems gas-phase sequencer.

Plasminogen activation reactions with urokinase were carried out at 22 °C in 100 mmol/L Na–HEPES (pH 7.4). For assay of plasmin activity, samples (2.5–5 μL) were diluted into 300 μL of 0.2 mmol/L peptide plasmin substrate (Spectrozyme PL) in 0.1 mol/L Tris-HCl–0.15 mol/L NaCl (pH 7.4) at 30 °C; the change in A_{405} was measured with an Abbott ABA-100 bichromatic analyzer. Active site titrations were carried out as described (Chase & Shaw, 1969).

RESULTS

The kinetics of porcine plasminogen activation, and particularly the marked inhibitory effect of Cl^- (Machovich & Owen, 1990a), suggested that activation of unmodified porcine plasminogen might be remarkably inefficient in physiologic salt solutions. Accordingly, activation was carried out in Hanks' balanced salt solution supplemented with physiological albumin concentration to simulate the environment of plasminogen activation in vivo. In this system, native porcine plasminogen was essentially refractory to activation with urokinase (Figure 1A). Proteolytic modification of plasminogen with plasmin or elastase prior to activation each effected relatively efficient activation. Addition of 6-aminohexanoate likewise effected activation at a measurable rate. Relative to the human system (Wiman & Wallen, 1973; Suenson & Thorsen, 1988), porcine plasminogen activation exhibits little hysteresis. Resistance to activation in physiological salts persisted at urokinase:plasminogen (mol/mol) ratios approaching 1:5 (Figure 1B).

In Hanks' solution porcine plasminogen is likewise insensitive to plasmin (Figure 2). Addition of 15 mol % plasmin to plasminogen in physiological concentrations (2 μmol/L) yielded no des(1–77)plasminogen after 5 h. Hydrolysis at Lys_{77}-Arg_{78} was apparent when 6-aminohexanoate was added, but the reaction appeared less than half-complete (0.4 by densitometry) after 300 s; the observed second-order rate constant ($\ln 2/t_{1/2}/[\text{plasmin}]$, pseudo-first-order conditions) must lie below 200 M^{-1} s^{-1}.

Refractoriness of plasminogen in Hanks' solution to proteolysis is largely the consequence of Cl^- (Violand et al., 1978). In 100 mmol/L of Na-HEPES (Figure 3, lane 1), plasminogen hydrolysis was apparent in 3 h (compare Figure 2); without Cl^-, proteolysis was enhanced further by 6-aminohexanoate

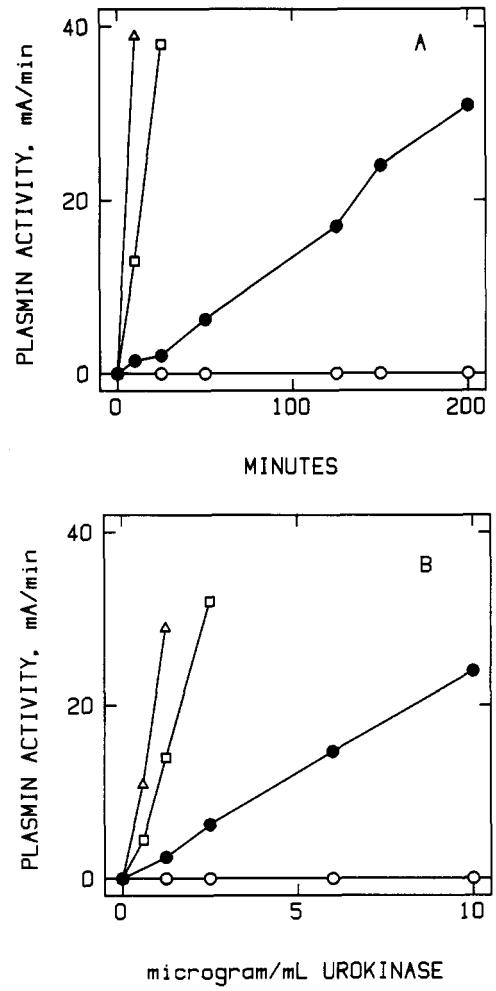


FIGURE 1: Activation of porcine plasminogens in physiological salt solution. Plasminogen (100 μg/mL) and des(1–77)plasminogen (90 μg/mL) were incubated for the indicated times with 2.5 μg/mL urokinase (A) or for 60 min with the indicated concentrations of urokinase (B) in Hanks' balanced solution (138 mmol/L NaCl, 5.3 mmol/L KCl, 1.3 mmol/L $CaCl_2$, 0.8 mmol/L $MgSO_4$, 0.34 mmol/L KH_2PO_4, and 0.3 mmol/L Na_2HPO_4) plus 5.5 mmol/L D-glucose, 0.5 mmol/L bovine albumin, 20 mmol/L HEPES, pH 7.4. Activation mixtures contained plasminogen (○), plasminogen plus 2.5 mmol/L 6-aminohexanoate (●), plasminogen plus 2.5 μg/mL leukocyte elastase (□), and des(1–77)plasminogen (△). Data are representative of two plasminogen preparations.

(Figure 3, lane 2). Phosphate ion, often used as a buffer in this system, had no effect (lane 3), whereas Cl^- reproduced the behavior observed in Hanks' solution (lanes 4 and 5). Neither porcine des($kringle_{1-4}$)plasmin nor human plasmin was more effective than porcine plasmin at hydrolysis of the porcine plasminogen (not shown). Chloride ion (up to 230 mmol/L) inhibited neither plasmin nor urokinase activities on peptide nitroanilide substrates.

In solutions containing 2.5 mM 6-aminohexanoate but no Cl^-, plasminogen and des(1–77)plasminogen are activated with similar kinetics (Figure 4). This similarity arises because activation of des(1–77)plasminogen is inhibited slightly (35–60% in four measurements) by 2.5 mmol/L of 6-aminohexanoate. In contrast to the human system (Ellis et al., 1987; Urano et al., 1987), the rate of activation of porcine plasminogen (in the absence of Cl^-) showed no saturation with respect to substrate (not shown), whether 6-aminohexanoate was present or not, or to proteolytically modified plasminogens; the efficiency of activation of des(1–77)-plasminogen was nearly that of des($kringle_{1-4}$)plasminogen.

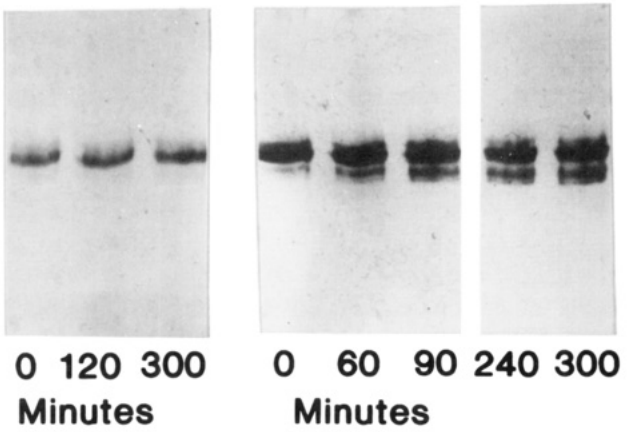


FIGURE 2: Proteolysis of plasminogen by plasmin. Plasminogen (200 μg/mL in Hanks' balanced salt solution) was incubated with plasmin (14 μg/mL) at 22 °C (left panel) or with 6-aminohexanoate (2.5 mmol/L) and plasmin (right panel). At the indicated intervals, samples were added to 1:10 volume of 10% $NaDodSO_4$ and analyzed by polyacrylamide gel (10–15%) electrophoresis.

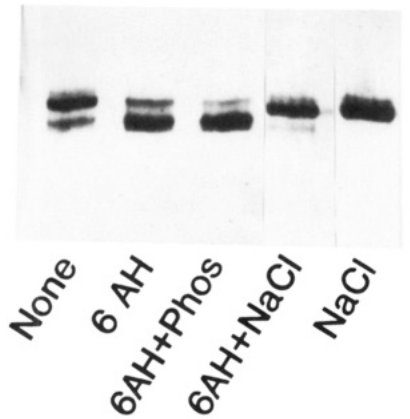


FIGURE 3: Effect of NaCl on proteolysis of plasminogen by plasmin. Plasminogen (90 μg/mL) in 100 mmol/L Na-HEPES (pH 7.4) was incubated at 22 °C with porcine plasmin (3 μg/mL) plus, as indicated, 2.5 mmol/L 6-aminohexanoate (6AH), 125 mmol/L NaCl, and 38 mmol/L sodium phosphate (Phos). At 180 min samples were analyzed by polyacrylamide gel (12.5%) electrophoresis in $NaDodSO_4$.

Proteolysis of plasminogen with trypsin was studied to address the minimum requirements for stimulation of activation by 6-aminohexanoate. As with urokinase, activation with trypsin is accelerated by 6-aminohexanoate or elastase (Figure 5); activation of plasminogen with trypsin is catalytically inefficient relative to that with urokinase (compare Figure 1). On the other hand, hydrolysis of the amino terminal peptide is more efficient with trypsin than with plasmin (Figure 6; compare 40-min sample with 300-min sample in Figure 2). With either enzyme, 6-aminohexanoate was required for significant hydrolysis. After activation with urokinase, sequence analysis yielded the expected sequences of RIYLS and VVGG-V while activation with trypsin yielded IYLSE and VVGG-V, to indicate that trypsin hydrolyzes Arg-Ile, one residue C-terminad to plasmin. Interestingly, no other degradation with trypsin was evident on the electropherograms (Figure 6) or N-terminal sequence analysis.

DISCUSSION

Although urokinase and tPA have been used effectively in thrombolytic therapy, their roles in physiological fibrinolysis have not been elucidated. The physiological significance of tPA has been inferred from its dependence on cofactors,

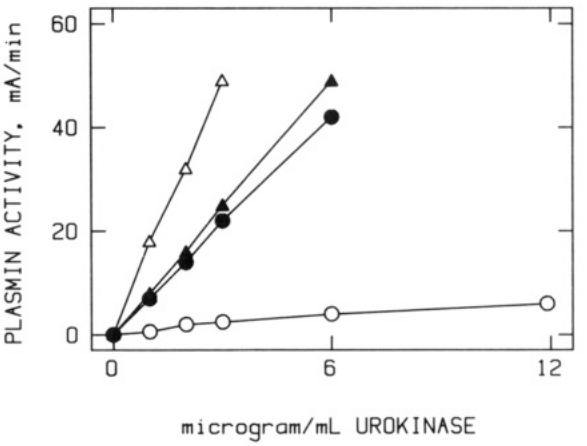


FIGURE 4: Effect of 6-aminohexanoate on activation of plasminogen and des(1–77)plasminogen. Plasminogen (200 μg/mL) and des-(1–77)plasminogen (180 μg/mL) in 100 mmol/L Na-HEPES (pH 7.4) were incubated for 25 min at 22 °C with urokinase at the indicated concentrations. Activation mixtures contained plasminogen (○), plasminogen with 2.5 mmol/L 6-aminohexanoate (●), des(1–77)-plasminogen (△), and des(1–77)plasminogen with 2.5 mmol/L 6-aminohexanoate (▲). Data are representative of duplicate independent measurements, each from two plasminogen preparations.

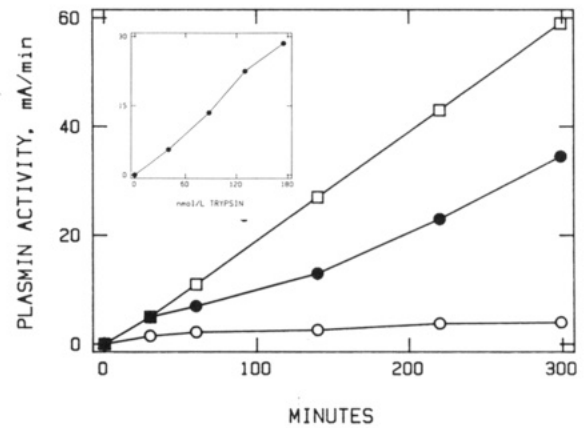


FIGURE 5: Activation of plasminogen by trypsin. Plasminogen (140 μg/mL) in Hanks' balanced salt solution buffered with 50 mmol/L HEPES (pH 7.4) was activated with trypsin (2 μg/mL) alone (○) or with addition of either 2.2 mmol/L 6-aminohexanoate (●) or 1 μg/mL elastase (□). Inset: Dependence on trypsin concentration of activation rate (with 2.2 mmol/L 6-aminohexanoate), measured at 140 min.

including fibrin, denatured protein, and endothelium. Physiologically plausible factors regulating urokinase have not been established, but our findings imply that activation of native porcine plasminogen by urokinase either depends on an effector or does not occur physiologically. Expectation of an effector has a basis in the finding by Ellis et al. (1992) that efficiency of human plasminogen activation increases 6-fold upon binding of urokinase to a membrane factor. Furthermore, a fragment obtained from plasmin digests of fibrin has been found to enhance the activation of single-chain urokinase, likely the physiologically functional form of the activator (Liu & Gurevich, 1991). In undegraded porcine plasminogen, neither the Lys_{77} (plasmin-sensitive) nor Arg_{560} (activator-sensitive) scissile bond is susceptible to proteolysis in the presence of chloride ions. The effect of Cl^- appears more profound in the porcine system than in the human (Urano et al., 1987); however, the marked hysteresis observed in human plasminogen activation suggests that the differences may be quantitative and the consequence of an inherently higher sensitivity of human plasminogen to proteolysis by plasmin. A unified mechanism is obscured at present by marked inconsistencies

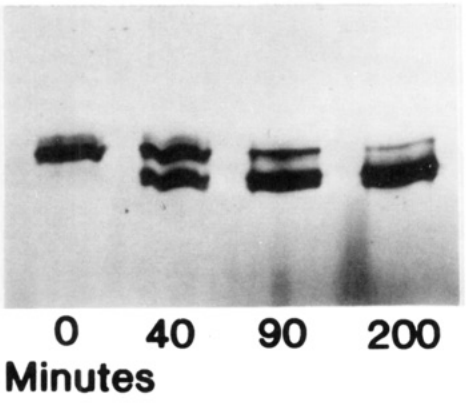


FIGURE 6: Proteolysis of plasminogen by trypsin. Plasminogen (140 μg/mL) in Hanks' balanced salt solution buffered with 50 mmol/L HEPES (pH 7.4) and containing 2 mmol/L 6-aminohexanoate was digested with trypsin (5 μg/mL) at 22 °C. At the indicated times, samples were added to 1:10 volume of 10% $NaDodSO_4$ and analyzed by polyacrylamide gel (10–15%) electrophoresis. A sample taken at 200 min was subjected to N-terminal sequence analysis.

in the literature on human plasminogen activation kinetics (Petersen, 1985) that may have arisen in part from chloride ion effects and in part from a propensity of human plasminogen to be contaminated with des(1–77)plasminogen.

At least in the porcine system, the kinetic consequence of proteolysis of amino terminal sequences by plasmin or elastase, or possibly other proteases, is of such a magnitude that a role for the process in regulation of fibrinolysis cannot be dismissed, even while mechanisms for effecting proteolysis cannot yet be identified. The reaction in principle could provide for exogenous (pharmacologic) as well as endogenous regulation by effectors. However, contradictory contentions that the "Lys–plasminogen intermediate does not occur" (Robbins, 1987) and "Lys–plasminogen formation is prerequisite for fibrinolysis" (Suenson & Thorsen, 1988) need resolution. Proteolysis of plasminogen by plasmin offers an inviting analogy to that of prothrombinase, where feedback proteolysis of prothrombin by thrombin has a major impact on activation kinetics and, in experimental settings, has obscured precursor–product relationships (Stenn & Blout, 1972; Esmon et al., 1974). The prothrombin pathway was effectively controlled experimentally by including thrombin inhibitors in activation reactions. With plasminogen, feedback proteolysis has been approached primarily by using saturating concentrations of plasmin substrates or inhibitors (Collen et al., 1986; Ellis et al., 1987; Gurewich, 1989). Together with studies of effectors, the findings indicate that hydrolysis by plasmin at Lys_{77} could be rate-controlling (Suenson & Thorsen, 1988). However, the problem is compounded not only by the effect of Cl^- but by the use of plasmin substrates as inhibitors (Gurewich, 1989). Because plasmin substrates bear lysyl residues, their use in high (inhibitory) concentrations may negate the assumed effect by superimposing enhancement of hydrolysis at Lys_{77} on plasmin inhibition.

The finding that hydrolysis of the amino terminal peptide with trypsin, which occurs at a residue neighboring Lys_{77}, is enhanced by 6-aminohexanoate indicates that the effects of 6-aminohexanoate arise entirely through conformational modification of plasminogen. This conclusion is not surprising in light of the major changes in plasminogen hydrodynamics induced by the amino acid (Urano et al., 1987). Porcine plasminogen clearly requires modification, either proteolytic or conformational, to enable activation; although it has not yet been isolated, porcine urokinase merits investigation. While it is not established unequivocally that other species, human in particular, control the process in the same manner, the stringency of the porcine system as well as the conservation (in two species) of a plasmin cleavage site invite further analysis in other species, as well as a reexamination of human plasminogen.

ACKNOWLEDGMENT

Agnes Himer provided technical assistance, and Dr. Jack Henkin (Abbott Laboratories, Abbott Park, IL) supplied the urokinase preparation.

REFERENCES

Baugh, R. J., & Travis, J. (1976) *Biochemistry 15*, 836–843.
Chase, T., & Shaw, E. (1969) *Biochemistry 8*, 2212–2224.
Christensen, U. (1984) *Biochem. J. 223*, 413–421.
Collen, D. C., Zamarron, C., Lijnen, H. R., & Hoylaerts, M. (1986) *J. Biol. Chem. 261*, 1259–1266.
Ellis, V., Scully, M. F., & Kakkar, V. V. (1987) *J. Biol. Chem. 262*, 14998–15003.
Esmon, C. T., Owen, W. G., & Jackson, C. M. (1974) *J. Biol. Chem. 249*, 606–611.
Gurewich, V. (1989) *Fibrinolysis 3*, 59–66.
Hajjar, K. A., Harpel, P. C., Jaffe, E. A., & Nachman, R. L. (1986) *J. Biol. Chem. 261*, 11656–11662.
Machovich, R., & Owen, W. G. (1988) *Enzyme 40*, 109–112.
Machovich, R., & Owen, W. G. (1989) *Biochemistry 28*, 4517–4522.
Machovich, R., & Owen, W. G. (1990a) *Biochim. Biophys. Acta 1040*, 109–111.
Machovich, R., & Owen, W. G. (1990b) *Blood Coagulation Fibrinolysis 1*, 273–277.
Markus, G., DePasquale, J. L., & Wissler, F. C. (1978) *J. Biol. Chem. 253*, 727–732.
Petersen, L. C., Brender, J., & Suenson, E. (1985) *Biochem. J. 225*, 149–158.
Radcliffe, R., & Heinze, T. (1981) *Arch. Biochem. Biophys. 211*, 750–761.
Robbins, K. C. (1987) in *Hemostasis and Thrombosis* (Colman, R. W., Hirsh, J., Marder, V. J., & Salzman, E. W., Eds.) pp 340–357, Lippincott Company: Philadelphia.
Schaller, J., Marti, T., Rosselet, S. J., Kampfer, U., & Rickli, E. E. (1987) *Fibrinolysis 1*, 91–102.
Stenn, K. S., & Blout, E. R. (1972) *Biochemistry 11*, 4502–4515.
Suenson, E., & Thorsen, S. (1988) *Biochemistry 27*, 2435–2443.
Sugiyama, N., Iwamoto, M., & Abiko, Y. (1987) *Thromb. Res. 47*, 459–468.
Summaria, L., Spitz, F., Arzadon, L., Boreisha, I. G., & Robbins, K. C. (1976) *J. Biol. Chem. 251*, 3693–3699.
Takada, A., Takada, Y., & Sugawara, Y. (1988) *Thromb. Res. 49*, 253–263.
Urano, T., de Serrano, V. S., Chibber, B. A. K., & Castellino, F. J. (1987) *J. Biol. Chem. 262*, 15959–15964.
Violand, B. N., Byrne, R., & Castellino, F. J. (1978) *J. Biol. Chem. 253*, 5395–5401.
Wiman, B., & Wallen, P. (1973) *Eur. J. Biochem. 36*, 25–31.