Serine protease inhibitors (serpins) form enzymatically inactive, 1:1 complexes (denoted $E^*I^*$) with their target proteinases that release free enzyme and cleaved inhibitor only very slowly. The mechanism of $E^*I^*$ formation is incompletely understood and continues to be a source of controversy. Kinetic evidence exists that formation of $E^*I^*$ proceeds via a Michaelis complex ($E$-$I$) and so involves at least two steps. In this paper, we determine the rate of $E^*I^*$ formation from $\alpha$-antichymotrypsin and $\alpha$-antitrypsin using two approaches: first, by stopped-flow spectrofluorometric monitoring of the fluorescent change resulting from reaction of $\alpha$-antichymotrypsin with a fluorescent derivative of $\alpha$-antichymotrypsin (derivatized at position P7 of the reactive center loop); and second, by a rapid mixing/quench approach and SDS-polyacrylamide gel electrophoresis analysis. In some cases, serpins are both substrates and inhibitors of the same enzyme. Our results indicate the presence of an intermediate between $E1$ and $E^*I^*$ and suggest that the partitioning step between inhibitor and substrate pathways precedes P1–P1$^*$ cleavage.

$\alpha_1$-Antichymotrypsin (ACT)$^3$ is a human serine protease inhibitor (serpin), a superfamily of proteins believed to have evolved from a common ancestral gene over ~500 million years (1–3). The involvement of ACT in Alzheimer’s disease (4, 5) and in the regulation of the inflammatory response (6) as well as of prostate-specific antigen activity (8) makes it a particularly interesting protein for study. As is typical of serpins, ACT (I) forms an enzymatically inactive, 1:1 complex (denoted $E^*I^*$) with its target proteinases (for example, chymotrypsin) that releases free enzyme and cleaved ACT ($I^*$) only very slowly (9, 10). The complex is designated $E^*I^*$ to indicate that conformational change has taken place in both the enzyme (10, 11) and inhibitor (12, 13) moieties. For some serpin-protease interactions, full inhibition of proteinase requires >1 eq of serpin. The ratio of moles of inhibitor required per mole of proteinase for 100% inhibition is defined as the stoichiometry of inhibition (SI). Values of SI > 1 reflect a partitioning of serpin between inhibitor and substrate pathways, giving rise to “suicide inhibi-

or”-type kinetics (9, 14–16). A characteristic feature of the serpin-proteinase complex is that it is stable to both heat and SDS treatment, implying covalent bond formation between enzyme and serpin. The non-lability of $E^*I^*$ may be due either to distortion of the enzyme active site within the complex (10, 11) or to inaccessibility of the covalent $E$–I linkage toward attacking nucleophilic water, or both. The position of cleavage in released $I^*$ occurs within a so-called “reactive center loop,” which in intact I extends out from the rest of the molecule, contains a segment of modified $\alpha$-helix (17–21), and is the primary interaction site between the inhibitor and the target proteinase. Following standard nomenclature (22), the position of cleavage takes place between the P1 and P1’ sites of the inhibitor, which in ACT corresponds to positions 358 and 359. Residues proceeding toward the N and C termini from P1 and P1’, respectively, are labeled with higher P and P’ numbers. The reactive center loop extends from approximately P17 to P9’. Cleavage of intact I to form $I^*$ is accompanied by a large decrease in free energy and a substantial gain in stability toward denaturation by either heating or denaturing agents (23, 24). In $I^*$, residues P1–P14 are inserted into $\beta$-sheet A, the dominant structural element in ACT, as strand 4A. $\beta$-Sheet C is also reinforced, with the result that the P1 and P1’ residues are separated by 70 Å (25).

Kinetic evidence has existed for some time that formation of the nonlabile serpin-proteinase complex involves at least two steps (26–28): the second-order association of $E$ and $I$ in an “encounter” or Michaelis complex ($E$-$I$), followed by its conversion, in a first-order process, to $E^*I^*$ (Scheme 1).

\[
E + I \rightleftharpoons E \cdot I \rightleftharpoons E^*I^*
\]

**Scheme 1**

For at least some serpin-proteinase pairs, there is evidence that complex formation is reversible (29–31), although the equilibrium constant strongly favors $E^*I^*$. Recent results that $E^*I^*$ formation coincides with liberation of a new N terminus at P1’ (32, 33) demonstrate that within $E^*I^*$, I has been cleaved at the P1–P1’ bond. These results, coupled with the observation that $E^*I^*$ dissociates on treatment with hydroxylamine (34), provide strong evidence that the covalent bond within $E^*I^*$ is between the proteinase active site serine hydroxyl (Ser-195 in $\alpha$-chymotrypsin) and the liberated P1 carboxyl of the serpin and corresponds either to acyl-enzyme or to the tetrahedral intermediate (35) resulting from water attack on the acyl-enzyme. Indeed, these species may be in mobile equilibrium with each other.

Formation of the $E^*I^*$ complex requires completion of a minimum of three processes: 1) establishment of P1 interaction with the SI-binding site of the enzyme and of perhaps subsite interactions as well, necessitating at least partial unwinding of the helical portion of the reactive center loop; 2) partial or full insertion of strand 4A into $\beta$-sheet A (18, 36); and 3) P1–P1’
cleavage and formation of the covalent acyl-enzyme (or tetrahedral intermediate). However, the relative timing of these processes is unknown.

In this paper, we determine the rate of \( E^*1 \) formation from \( E1 \) using two approaches. In the first, we derivatize the A352C-rACT variant (at position P7) with the fluorescent reagent 4-bromomethyl-7-methoxycoumarin (BMMC), demonstrate that the resulting (7-methoxycoumaryl-4)-methyl derivative of A352C-rACT (MCM-A352C-rACT) inhibits \( \alpha \)-chymotrypsin in the normal manner, and measure its rate of complex formation with \( \alpha \)-chymotrypsin by stopped-flow spectrophotofluorometry. In the second, we apply a rapid mixing/quench approach and SDS-PAGE analysis to determine the rate of \( E^*1 \) formation on reaction of either MCM-A352C-rACT or rACT with \( \alpha \)-chymotrypsin. Our results indicate the presence of an intermediate between \( E \)-I and \( E^*1 \) and suggest that the partitioning step between inhibitor and substrate pathways precedes P1–P1' cleavage.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine N\(_2\)-p-tosyl-L-leucine chloromethyl ketone-treated chymotrypsin and human neutrophil elastase were obtained from Calbiochem or Sigma. The concentrations of these enzymes and of rACT were determined as described earlier (16). All chromophoric proteinase substrates, dithiothreitol, and phenylmethylsulfonyl fluoride were obtained from Sigma. BMMC was acquired from Molecular Probes, Inc. (Eugene, OR). SDS-PAGE analysis was performed according to Laemmli (37). Standard proteins were from Bio-Rad.

**Construction, Expression, and Purification of rACTs**—A352C-rACT was constructed using sequence overlap expression polymerase chain reaction (38, 39) and the ACT expression vector described previously (9, 16), gel-purified, and inserted in the XI, gel-purified, and inserted in the expression vector described previously (9, 16). 400 nm. First-order rate constants were obtained by fitting a single exponential to unsmoothed traces. 2

**Mechanism of Serpin-Proteinase Complex Formation**

**RESULTS**

**MCM-A352C-rACT**— Virtually complete derivatization of A352C-rACT was achieved under the conditions used for preparing MCM-A352C-rACT. By contrast, reaction of rACT with BMMC under exactly the same conditions gave only minor derivatization, reflecting the low reactivity of the single Cys residue (Cys-237) within wild-type ACT, which is buried within \( \beta \)-sheet B of rACT (20), as well as slow nonspecific reaction with carboxylate residues in the protein (43).

MCM-A352C-rACT inhibits \( \alpha \)-chymotrypsin with a stoichiometry of inhibition of \(-I\) (data not shown). The MCM-A352C-rACT-\( \alpha \)-chymotrypsin complex is stable toward SDS denaturation, as shown by SDS-PAGE analysis (see below), in the usual manner of serpin-proteinase complexes and is cleaved by catalytic amounts of human neutrophil elastase, similar to what is found for wild-type rACT.

**Rate of MCM-A352C-rACT-\( \alpha \)-Chymotrypsin Complex Formation by Stopped-flow Spectrophotofluorometry**—Excitation of a solution of MCM-A352C-rACT at 330 nm gives the emission spectrum shown in Fig. 2, with \( \lambda_{max} = 399 \) nm. Human neutrophil elastase-induced cleavage leads to an increase in emission intensity and a slight blue shift (\( \lambda_{max} = 397 \) nm), consistent with the expected shift to a more hydrophobic environment as the chromophore moves from a solvent-exposed position to one that is at least partly buried within \( \beta \)-sheet A. The fluorescence spectrum of the MCM-A352C-rACT-\( \alpha \)-chymotrypsin complex is similar to that of cleaved MCM-A352C-rACT.

Mixing of \( \alpha \)-chymotrypsin and MCM-A352C-rACT led to an increase in fluorescence at 400 nm, which was used to measure the rate constant for complex formation in a stopped-flow spectrophotofluorometer. All stopped-flow reactions were carried out under pseudo first-order conditions, with \( \alpha \)-chymotrypsin (denoted \( E \)) present in large molar excess over MCM-A352C-rACT (denoted I). Results obtained at pH 7.0 and 40 °C are shown in Fig. 3. Rate constants for complex formation as a function of [\( E \)], temperature, pH, and ionic strength are presented in Table I. As predicted by Scheme 1, at high enough [\( E \)], the measured first-order rate constant becomes independent of [\( E \)] and reaches a saturated value. Under our conditions, [\( E \)] \( \geq 65 \) \( \mu \)M satisfies this condition.

Our results are similar to those of Shore et al. (13), who carried out an analogous study measuring the rate of complex formation between a fluorescent derivative of plasminogen activator inhibitor-1 (derivatized at P9) and plasminogen activator. These workers reported a value for \( k_{cat} \) (the rate constant determined by the change in fluorescence) of 4 s\(^{-1}\) (at pH 7.4 and 25 °C) and found that the large increase in fluorescence...
intensity and the 13-nm blue shift accompanying complex formation were virtually identical to the changes observed on cleavage of derivatized plasminogen activator inhibitor-1.

An Arrhenius plot of $k_f$ at pH 7 (15–40°C) gives a good straight line and an apparent activation energy of 19 kcal/mol, indicating a substantial energy barrier for the process leading to fluorescence change. The rate constant increases 2-fold in the pH range 6–8, but the addition of 1 M NaCl has little effect.

**Rate of MCM-A352C-rACT-$\alpha$-Chymotrypsin and rACT-$\alpha$-Chymotrypsin Complex Formation by Quenched Stopped Flow**—Monitoring the buildup of the SDS-stable complex $E^\ast I^\ast$ provides another way of measuring complex formation. $E^\ast I^\ast$ is stable toward 0.1 N HCl (data not shown). As a consequence, rates of $E^\ast I^\ast$ formation could be measured by 0.1 N HCl quenching at various times following rapid mixing of $E$ and $I$ and quantitative scanning of stained SDS-PAGE analyses of the quenched reaction mixtures (Fig. 1).

The results obtained are summarized in Table I. In terms of Scheme 1, our expectation was that $E-I$ would not survive the quench and would be measured as free $E$ and intact $I$. This expectation was confirmed by the result that the observed first-order rate constant for $E^\ast I^\ast$ formation ($k_{\text{on}}$) saturates as a function of $E$ concentration (Experiments 20–22). The slight downturn in rate constant at the highest enzyme concentration used (0.2 mM, Experiment 23) might be due to a nonspecific protein effect. All rate constants were determined at $[E]_o$ in excess over $[I]_o$ and could be measured by either $E^\ast I^\ast$ buildup or $I$ disappearance with similar results. $E^\ast I^\ast$ buildup was chosen for calculation of rate constants because it could be measured with greater precision.

Although the saturated rates of complex formation for MCM-A352C-rACT reaction with $\alpha$-chymotrypsin are not significantly different when measured by stopped-flow spectrofluorometry or by quenched stopped flow at 25°C (Table I, compare Experiments 1 and 2 with Experiment 3), at 40°C, the stopped-flow rate is clearly faster (~2.5-fold) (Fig. 3 and Table I, Experiments 4 and 6). This difference demonstrates that Scheme 1 is inadequate to explain the kinetics of $E^\ast I^\ast$ formation, as discussed below. We also note that the rate constants for $E^\ast I^\ast$ formation from both rACT and MCM-A352C-rACT are similar in magnitude and that the rate constant for rACT reaction increases 3–6-fold over the pH range 6.0–9.0 (Table I, Experiments 21, 27, and 28).

Inspection of Fig. 1 reveals a small buildup with time of cleaved $I$, migrating just ahead of intact $I$. Such a band was observed in reactions of both MCM-A352C-rACT and rACT
we (9) and others (14, 15, 47, 48) proposed the existence of an intermediate between E-I and E*1* from which partitioning could occur between the inhibitor pathway, leading to E*1* formation, and the substrate pathway, leading to enzyme and cleaved inhibitor (I*) release, to account for the finding that some serpin-proteinase pairs have SI values > 1 (Scheme 2). For reasons of parsimony, we equate the intermediate we demonstrate kinetically in this work with E*I* in Scheme 2.

As noted above, the time course for the increase in fluorescence (Fig. 3) could be well fit with a single exponential, implying that the emission spectrum of E*I* is similar to that of E*1*. Analysis of the time dependence of the fluorescence spectrum over the wavelength range 350–450 nm was consistent with this conclusion. Thus, in applying the simulation program HopKINSIM (49) to estimate microscopic rate constants in Scheme 2 from the data in Fig. 3, the rate of fluorescence change was equated with the rate of disappearance of E-I. As the rate of complex formation measured by quenched stopped flow reflects E*1* formation, the difference between the two curves in Fig. 3 measures the buildup and decay of E*I*. Satisfactory simulations were obtained with the following rate constant values: $k_2 = 30–45 \text{ s}^{-1}$; $k_{-2} < 10 \text{ s}^{-1}$; and $k_3 = 20–25 \text{ s}^{-1}$. Both $k_4$ and $k_{-3}$ were assumed to be negligible. It is clear that $k_3 \gg k_4$ for both rACT and MCM-A352C-rACT since, in both cases, SI is little different from 1. $k_{-3}$ may be ignored given that the overall equilibrium between E*1* and E-I very much favors E*1*.

These simulations predict a lag in the formation of E*1*, which we have been unable to demonstrate unequivocally, for two reasons. First, the predicted lag is most obvious over a time scale (0–10 ms) that brackets the dead time (~5 ms) of our instrument. Making the lag more obvious by lowering the temperature is not an option because the buildup of E-I depends on the higher temperature. Second, background in the gel lowers the precision with which low levels of protein can be quantified.

At 40°C, $k_3$ (the rate constant for acylation of α-chymotrypsin within the complex) is similar in magnitude to $k_{-2}$. By contrast, $k_3$ must be considerably larger than $k_2$ at 25°C, given the similarity of $k_2$ and $k_{-2}$ (~7 2 s⁻¹) at this temperature. We infer that $k_3$ has a small activation energy between 25 and 40°C. This is reminiscent of the temperature dependence of the rate constants for formation of acyl-α-chymotrypsin from the Michaelis complex of the enzyme with either p-nitrophenyl acetate or p-nitrophenyltrimethyl acetate (50). These rate constants display a dramatic drop in activation energy from 21 kcal/mol (measured from 6 to 20°C) to 1 kcal/mol (measured from 23 to 36°C), reflecting a thermally induced transition (at ~25°C) between two forms of enzyme with different catalytic activities.

Acyl-chymotrypsin formation on reaction with model amide substrates depends on His-57 being in the neutral form. The relevant $pK_a$ within the enzyme-substrate complex varies between 6 and 7, depending on the substrate (7). The pH dependences we observe for both $k_3$ and $k_{-3}$ are consistent with the need for a neutral His-57, although other ionizable group(s) within the E-I complex may also modulate these processes. The decrease in rates observed at lower pH suggests that clear demonstration of a lag in E*1* formation might be possible for rates measured at 40°C below pH 6.

**DISCUSSION**

**Kinetics of E*1* Formation**—At 40°C, the saturated rate of fluorescence change on mixing of MCM-A352C-rACT with α-chymotrypsin exceeds the saturated rate of overall E*1* formation (Fig. 3). This result provides clear kinetic evidence for an intermediate between E-I and E*1*, which, unlike E*1*, does not survive the quench/SDS-PAGE analysis procedure. Earlier,
Mechanism of Serpin-Proteinase Complex Formation

TABLE I
First-order rate constants for complex formation

| Exp. | [I] | [E]/[I] | Temperature | pH | 1 mM NaCl | Method | k' |
|------|-----|---------|-------------|----|-----------|--------|----|
| 1    | MCM, a | 1.2 | 50 | 25.0 | 7 | – | F | 6.9 ± 0.1 (2) |
| 2    | MCM, 1.3 | 100 | 25.0 | 7 | – | F | 7.0 ± 0.1 (2) |
| 3    | MCM, 14 | 50 | 25.0 | 7 | – | Q | 9.8 ± 2.4 (2) |
| 4    | MCM, 1.3 | 50 | 40.0 | 7 | – | F | 33.3 ± 0.9 (3) |
| 5    | MCM, 1.3 | 100 | 40.0 | 7 | – | F | 33.5 ± 1.5 (3) |
| 6    | MCM, 13.5 | 50 | 40.0 | 7 | – | Q | 13.5 ± 3.0 (2) |
| 7    | MCM, 1.3 | 50 | 15.0 | 7 | – | F | 2.3 ± 0.1 (2) |
| 8    | MCM, 1.3 | 100 | 15.0 | 7 | – | F | 2.3 ± 0.1 (2) |
| 9    | MCM, 1.3 | 50 | 25.0 | 6 | – | F | 4.3 ± 0.1 (2) |
| 10   | MCM, 1.3 | 100 | 25.0 | 6 | – | F | 4.6 ± 0.1 (2) |
| 11   | MCM, 1.3 | 200 | 25.0 | 6 | – | F | 4.8 (1) |
| 12   | MCM, 1.3 | 400 | 25.0 | 6 | – | F | 4.8 (1) |
| 13   | MCM, 1.3 | 50 | 25.0 | 8 | – | F | 9.1 ± 0.1 (2) |
| 14   | MCM, 1.3 | 100 | 25.0 | 8 | – | F | 9.1 ± 0.1 (2) |
| 15   | MCM, 1.3 | 200 | 25.0 | 8 | – | F | 9.6 (1) |
| 16   | MCM, 1.3 | 400 | 25.0 | 8 | – | F | 9.6 (1) |
| 17   | MCM, 1.3 | 50 | 25.0 | 7 | + | F | 6.2 ± 0.1 (3) |
| 18   | MCM, 1.3 | 100 | 25.0 | 7 | + | F | 6.6 ± 0.2 (3) |
| 19   | MCM, 1.3 | 200 | 25.0 | 7 | + | F | 7.3 ± 0.1 (2) |
| 20   | WT, 15 | 1.6 | 25.0 | 7.5 | – | Q | 5.3 (1) |
| 21   | WT, 15 | 3.3 | 25.0 | 7.5 | – | Q | 6.8 ± 0.7 (3) |
| 22   | WT, 15 | 6.6 | 25.0 | 7.5 | – | Q | 6.9 ± 0.7 (3) |
| 23   | WT, 15 | 13.1 | 25.0 | 7.5 | – | Q | 4.9 ± 0.7 (3) |
| 24   | WT, 15 | 6.6 | 10.0 | 7.5 | – | Q | 2.1 ± 0.2 (2) |
| 25   | WT, 15 | 6.6 | 40.0 | 7.5 | – | Q | 17 ± 2 (3) |
| 26   | WT, 15 | 50 | 25.0 | 7.0 | – | Q | 8.0 (1) |
| 27   | WT, 15 | 6.6 | 15.0 | 7.0 | – | Q | 3.3 ± 0.4 (2) |
| 28   | WT, 23 | 6.6 | 25.0 | 9.0 | – | Q | 16 ± 6 (2) |

a Buffers used were as follows: pH 6 and 8, method F, 20 mM phosphate; pH 6.0, 7.5, and 9.0, method Q, 50 mM Tris and 50 mM KCl; and pH 7, methods F and Q, 20 mM phosphate.

b Method F, stopped-flow fluorescence; method Q, quenched stopped flow.

c For the rate constants, the error ranges shown are the larger of average deviation or average precision. Numbers of independent observations are in parentheses.

d MCM, MCM-A352C-rACT; WT, wild-type rACT.

kinetic intermediacy of E'I' between E-I and E'1', our results also permit the interesting inference that the P1–P1' linkage remains intact in E'I'. Only a minor amount of cleaved I is formed during the course of reaction of α-chymotrypsin and either rACT or MCM-A352C-rACT (Fig. 1). Furthermore, at 40 °C, the rate of cleaved I formation was clearly much slower than the rate of fluorescence change. These observations require that when E'I' dissociates during the quench/analysis procedure, it forms E and intact I, whereas cleaved I would have been expected if the P1–P1' linkage were cleaved in E'I'. Here it should be noted that E'I' rises to a level of ~35–40% of total I after 40 ms, so cleaved I arising from E'I' dissociation would have been easily detectable. Interpreted according to Scheme 2, maintenance of the P1–P1' linkage in E'I' requires that partitioning into substrate and inhibitor pathways occurs prior to acyl-envelope formation.

The small accumulation of cleaved I may reflect formation of cleaved I in solution (via reaction 4 in Scheme 2), due to partitioning of the E'I' complex in solution between inhibitor and substrate pathways, or may rather arise from partitioning of E'1' in solution into an SDS-stable complex (the major pathway) and an SDS-unstable complex as a consequence of the quench/analysis procedure. Given the evidence that E'1' may correspond to acyl-envelope or to the final tetrahedral intermediate (35) resulting from water attack on the acyl-envelope, an interesting possibility is that the observed cleaved I may arise from the breakdown of the final tetrahedral intermediate during quench/analysis.

A final speculation, based on the pH dependence of k', is that E'I' corresponds to a covalent adduct between E and I, as suggested by Olson et al. (48). More specifically, we propose the initial tetrahedral adduct formed by serine 195 attack on the P1 carbonyl. Such an adduct could reasonably be expected to break down to E and intact I on quench/analysis. Experiments underway to directly measure the rate of P1–P1' cleavage within the complex should permit direct testing of whether the P1–P1' linkage is intact within E'I'.

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REFERENCES
1. Huber, R. & Carrell, R. W. (1989) Biochemistry 28, 8951–8960
2. Gettina, P., Patou, P. A. & Schapira, M. (1993) Bioessays 15, 461–467
3. Schulze, A. J., Huber, R., Bode, W. & Engbl, R. A. (1994) FEBS Lett. 344, 117–124
4. Das, S. & Potter, H. (1995) Neurog. Aging 16, 747–753
5. Gauthier, T. W. & Loor, A. M. (1995) Shock 4, 251–256
6. Fersht, A. (1985) Enzyme Structure and Mechanism, 2nd Ed., pp. 168–169, W. H. Freeman & Co., New York
7. Plotnick, M. I., Mayne, L., Schechter, N. M. & Rubin, H. (1996) Biochemistry 35, 7586–7590
8. Mast, A. E., Engbl, J. D., Pizzo, S. V. & Salvesen, G. (1995) Biochemistry 30, 1723–1730
9. Shore, J. D., Do, D. E., Francis-Chmura, A. M., Verhamme, I., Kvassman, J., Lawrence, D. A. & Ginsburg, D. (1995) J. Biol. Chem. 270, 5285–5298
10. Bjork, I. & Fish, W. W. (1982) J. Biol. Chem. 257, 9487–9493
11. Bjork, I., Jackson, C. M., Jornvall, H., Lavine, K. K., Nordling, K. & Sansalv, W. J. (1992) J. Biol. Chem. 267, 2406–2411
12. Rubin, H., Wang, Z., Nickburg, E. B., McLean, S., Naidoo, N., Schoenberg, O. L., Johnson, J. L. & Cooperman, B. S. (1996) Biochemistry 35, 10608–10615
13. Plotnick, M. I., Mayne, L., Schechter, N. M. & Rubin, H. (1996) Biochemistry 35, 7586–7590
14. Stein, P. E., Fermi, G. & Wardell, M. R. (1994) Structure 2, 257–270
Mechanism of Serpin-Proteinase Complex Formation

19. Schreuder, H. A., de Boer, B., Dijkema, R., Mulders, J., Theunissen, H. J. M., Grootenhuis, P. D. J. & Hol, W. G. J. (1994) *Nat. Struct. Biol.* 1, 48–54
20. Wei, A., Rubin, H., Cooperman, B. S. & Christianson, D. W. (1994) *Nat. Struct. Biol.* 1, 250–257
21. Song, H., Lee, K., Kwon, K., Yu, M. & Suh, S. (1995) *FEBS Lett.* 377, 150–154
22. Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27, 157–162
23. Bruch, M., Weiss, V. & Engel, J. (1988) *J. Biol. Chem.* 263, 16626–16630
24. Carrell, R. W. & Owens, M. C. (1985) *Nature* 317, 730–732
25. Baumann, U., Huber, R., Bode, W., Grosse, D. & Laurell, C. B. (1991) *J. Mol. Biol.* 218, 595–600
26. Olson, S. T. & Shore, J. D. (1982) *J. Biol. Chem.* 257, 14891–14895
27. Stone, S. R., Nick, H., Hofsteenge, J. & Monard, D. F. (1987) *Arch. Biochem. Biophys.* 252, 237–244
28. Bruch, M. & Birth, J. G. (1989) *Biochem. J.* 259, 929–930
29. Shieh, B.-H., Potempa, J. & Travis, J. (1989) *J. Biol. Chem.* 264, 13420–13423
30. Longstaff, C. & Gaffney, P. J. (1991) *Biochemistry* 30, 979–986
31. Stone, S. R. & Hermans, J. M. (1995) *Biochemistry* 34, 5164–5172
32. Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I. M., Krassman, J.-O. & Shore, J. D. (1995) *J. Biol. Chem.* 270, 25309–25312
33. Wilczynska, M., Fa, M., Ohlsson, P.-I. & Ny, T. (1995) *J. Biol. Chem.* 270, 29652–29655
34. Owen, G. W. (1975) *Biochim. Biophys. Acta* 385, 380–387
35. Matheson, N. R., van Halbeek, H. & Travis, J. (1991) *J. Biol. Chem.* 266, 13489–13491
36. Hopkins, P. C. & Stone, S. R. (1995) *Biochemistry* 34, 15872–15879
37. Laemmli, U. K. (1970) *Nature* 227, 680–685
38. Hemsley, A., Arnheims, N., Toney, M. D., Cortonassi, G. & Galas, D. J. (1989) *Nucleic Acids Res.* 17, 6545–6550
39. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989) *Gene* (Amst.) 77, 51–59
40. Rubin, H., Plotnick, M., Wang, Z.-M., Liu, X., Zhong, Q., Schechter, N. M. & Cooperman, B. S. (1994) *Biochemistry* 33, 7627–7633
41. Kilpatrick, L., Johnson, J. L., Niekharg, E. B., Wang, Z., Clifford, T. F., Banach, M., Cooperman, B. S. & Rubin, H. (1991) *J. Immunol.* 146, 2388–2393
42. Krassman, J. O. & Shore, J. D. (1995) *Fibrinolysis* 9, 215–221
43. Farinotti, R., Siard, P., Bourson, J., Kirkiacharian, S., Valeur, B. & Mahuzier, G. (1983) *J. Chromatogr.* 269, 81–90
44. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
45. Johnson, K. A. (1986) *Methods Enzymol.* 134, 677–705
46. Johnson, K. A. (1992) *Enzymes* 20, 1–61
47. Patston, P. A., Gettins, P., Beechem, J. M. & Schapira, M. (1995) *Biochemistry* 34, 8876–8882
48. Olson, S. T., Rok, P. E., Krassman, J., Shore, J. D., Lawrence, D. A., Ginsburg, D. & Björk, I. (1995) *J. Biol. Chem.* 270, 30007–30017
49. Wachsstock, D. H. & Pollard, T. D. (1994) *Biophys. J.* 67, 1260–1273
50. Adams, P. A. & Swart, E. R. (1977) *Biochem. J.* 161, 83–92