Plasma Arginine Esterase in Cystic Fibrosis: Kinetics of Activation, Identification as Plasma Kallikrein, Reaction with α_2 -Macroglobulin and Comparison with Levels in Normal Plasma

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Summary

Treatment of normal plasma with chloroform and ellagic acid yielded esterase activity against tosylarginine methyl ester, which reached a maximum within 2 h. After 2 h most or all of the activity was resistant to inhibition by soybean trypsin inhibitor (STI), but was still sensitive to the low molecular weight inhibitors di-isopropyl fluorophosphate, aprotinin and prolyl-phenylalanyl-arginyl chloromethane. The activity ran in gel chromatography with α_2 macroglobulin (α_2 M), as if it were due to an α_2 M proteinase complex.

The generation of the arginine esterase activity by chloroform and ellagic acid was apparently dependent on the activation of factor XII, being blocked by Polybrene. In plasma pretreated with methylamine-HCl (an inactivator of α_2 M), the arginine esterase was 95% sensitive to inhibition by STI. With regard to substrate specificity, inhibition characteristics, and gel chromatographic behaviour, it was indistinguishable from plasma kallikrein (EC 3.4.21.34, formerly 3.4.21.8). The chloroform and ellagic acid treatment of plasma resulted in a disappearance of prokallikrein simultaneous with the appearance of the arginine esterase. By these criteria, the arginine esterase activity was attributable entirely to plasma kallikrein either in its free form (methylamine-treated plasma) or bound to α_2 M (buffer-treated plasma).

Comparisons of STI-sensitive and STI-resistant arginine esterase activities of plasma samples from cystic fibrosis patients, obligate heterozygotes or other groups showed no significant differences in levels of activity, kinetics of activation or gel chromatographic behaviour.

We conclude that cystic fibrosis is unrelated to any abnormality in plasma arginine esterase activity, contrary to some previous reports.

It has been reported that plasma of cystic fibrosis patients and heterozygous carriers of the disease is partially deficient in the arginine esterase activity that appears following "activation" of the plasma with chloroform and ellagic acid (34). Such a deficiency might be helpful in diagnosis and genetic counselling for cystic fibrosis if it were consistently and unequivocally detectable. Some workers have gone so far as to suggest that the abnormality in plasma arginine esterase may be the basic defect in cystic fibrosis (12, 33). The reported deficiency has already been the subject of several independent studies (Table 1) but the results have been contradictory.

The treatment of plasma with ellagic acid and chloroform is a procedure that was used by Colman *et al.* (11) to activate plasma prokallikrein. Nevertheless, Rao and Nadler (33) suggested that the activity they measured in this way may not have been kallikrein. Two types of arginine esterase activity were distinguished by Rao *et al.* (34), one susceptible to and one resistant to inhibition by soybean trypsin inhibitor (STI); it was the STI-inhibitable

fraction that was reported to be significantly decreased. It has been suggested that the activity is due to a serine proteinase, on the basis of evidence for a deficiency in cystic fibrosis plasma of activity hydrolysing dinitrophenyl-labelled-protamine sulphate (33, 37), and of the reaction of the plasma with an active-site titrant of certain proteases, methylumbelliferyl guanidinobenzoate (42).

In a preliminary communication (5), we reported that the enzyme hydrolysing Tos-Arg-OMe in activated normal and cystic fibrosis plasmas can be identified as plasma kallikrein (free or complexed with α_2 macroglobulin). We now describe that work in full, and extend it to show that the activity is within the normal range in the majority of samples from individuals homozygous or heterozygous for cystic fibrosis.

MATERIALS

Blood. Fresh normal blood was obtained from a local blood transfusion service as previously described (4), or from male and female control donors (*i.e.*, non-cystic fibrotics with no family history of the disease), or people heterozygous or homozygous for cystic fibrosis. Controls were in two groups: pediatric controls who were sick children comprising two epileptics, one kidney disease, one liver disease, one bronchiectasis and one asthma, and adult controls who were normal healthy individuals. Cystic fibrosis patients varied widely in fitness with varying degrees of pulmonary or bowel involvement. One patient had severe hepatic complications.

Other materials. Pure human plasma kallikrein was kindly provided by Dr. H. Nagase of our Department after preparation as recently described (29).

Tos-Arg-OMe was from Sigma (London) Chemical Co., Poole, Dorset BH17 17NH, U.K., D-Pro-Phe-Arg-NPhNO₂ (S-2302), D-Val-Leu-Arg-NPhNO₂ (S-2266) and D-Val-Leu-Lys-NPhNO₂ (S-2251) were obtained from Kabi Diagnostica, S-11287, Stockholm, Sweden; Pro-Phe-Arg-NMec, Boc-Val-Pro-Arg-NMec and Boc-Ile-Glu-Gly-Arg-NMec were obtained from the Protein Research Foundation, Minoh-Shi, Osaka, Japan.

Trypsin inhibitors (soybean type I-S, STI, or lima bean, type II-L), 4-chloromercuribenzoate, EDTA, 6-aminohexanoic acid, diisopropyl fluorophosphate were from Sigma, Polybrene (1,5-dimethyl-1,5-diaza-undecamethylene polymethobromide) was from Aldrich Chemical Co., Gillingham, Dorset SP8 4JL, U.K.; aprotinin (bovine pancreatic Kunitz trypsin inhibitor) as Trasylol was from Bayer AG, Wuppertal, Elberfeld, West Germany; Tos-Lys-CH₂Cl and 7-amino-4-methylcoumarin were from Bachem Feinchemikalien AG, CH-4416, Bubendorf, Switzerland; Factor-XII_a inhibitor (from popcorn) was a gift of Dr. Y. Hojima of the National Heart, Lung and Blood Institute, Bethesda, MD; Pro-Phe-Arg-CH₂Cl was the gift of Dr. C. Kettner, Brookhaven National Laboratory, New York, U.S.A.

Streptokinase (Kabikinase) was from Kabi Diagnostica. Dextran sulphate was from Pharmacia, and ellagic acid, bovine serum albumin, egg albumin, and cytochrome *c* from Sigma. Ultrogel AcA-34 was from LKB Instruments Ltd. Sheep immunoglobulin G and human α_2 M were prepared in the laboratory by published methods.

METHODS

MEASUREMENT OF ACTIVATABLE ARGININE ESTERASE IN PLASMA

Preparation of plasma. Each 2.5 ml sample of venous blood drawn into a polypropylene syringe was transferred to a polystyrene citrated pediatric tube (Catalog No. 173, type 2.5 ml, trisodium citrate-prothombin, Sterilin Products, Sterilin Ltd., Teddington, Middlesex, U.K.) and mixed with anticoagulant. The tube was centrifuged ($2500 \times g$ for 15 min at 5°C). The plasma was drawn off with a 1-ml plastic pipette tip and transferred to another plastic tube for assay or storage (at 4°C overnight or -20°C for longer periods). All subsequent handling of the plasma was also carried out with plastic pipette tips and tubes.

Treatment with methylamine-HCl or buffer. Portions (0.5 ml) of plasma were transferred to polypropylene tubes (10 ml) containing 50 μl of 1 M-sodium phosphate buffer, pH 7.6, in 1.5 M-NaCl (which maintained the buffer and NaCl concentration approximately the same as in the subsequent activation and assay) with or without 2 M methylamine-HCl. The capped tubes were incubated at 25°C for 1 h.

Chloroform treatment. At the end of the incubation with or without methylamine, an equal volume of chloroform (at -20°C) was added to each tube which was then capped and mixed by 20 cycles of inversion and return during 30–60 sec. Five min after addition of chloroform, the tube was placed in a centrifuge at 5°C and spun at $2500 \times g$ for 15 min. The bulk of the supernatant was then transferred to a clean tube.

Treatment with ellagic acid. Ellagic acid suspension (3 mg/100 ml of "assay buffer" which was 0.1 M sodium phosphate buffer, pH 7.6, containing 0.15 M NaCl) was prepared by heating at 70 – 80°C with stirring for 30 min and cooled before use.

Exactly 30 min after addition of the chloroform, 0.4 ml of chloroform-treated plasma was mixed with an equal volume of the ellagic acid suspension and incubated at 25°C . After appropriate times, samples of the plasma were removed for determination of activity against Tos-Arg-OMe. Assays were normally made after 1 h (methylamine-treated) or 2 h (buffer-treated samples).

Assay of arginine esterase activity. The hydrolysis of Tos-Arg-OMe (in the presence or absence of STI) was followed by determination of the liberated methanol. Reaction mixtures contained 50–200 μl of activated plasma, 0.6–0.75 ml (to make 0.8 ml total) of assay buffer, 0.1 ml of Tos-Arg-OMe (150 mM stock in assay buffer), and 0.1 ml of either STI (2 mg/ml stock in assay buffer) or assay buffer only. The tubes were incubated at 37°C for 2 h (or 15 min for purified kallikrein) before the reactions were stopped with 1 ml of 10% (w/v) trichloroacetic acid. The tubes were then centrifuged for 20 min at $2500 \times g$ at 5°C .

The concentration of methanol in the supernatant was determined by the method of Siegelman (39).

EFFECTS OF INHIBITORS ON ARGININE ESTERASE ACTIVITY

Plasma (up to 3 ml) was activated as usual and after 1 h (methylamine-treated plasma) or 2 h (buffer-treated plasma), samples (50 μl or 100 μl , respectively) were preincubated for 10 minutes at 37°C in the presence of the potential inhibitor in assay buffer (final volume 0.9 ml). Subsequently, the activity against Tos-Arg-OMe was determined as described above, without STI. Stock Dip-F (200mM) was dissolved in isopropanol and any inhibition was determined relative to another control containing the same amount of isopropanol (0.5% final concentration). Units of activity for the controls ranged between 0.5–1.9 $\mu\text{mole/h}$ for plasma added or 11.4 $\mu\text{mole/h}$ for purified kallikrein (0.1 ml, 8 μg).

SUBSTRATE SPECIFICITY

Tripeptide nitroanilide hydrolysis. Hydrolysis of S-2302, S-2266 (relatively specific for plasma and glandular kallikrein, respectively, ref. 7), or S-2251 (plasmin, ref. 15), all at 0.25 mM, was measured in assay buffer (total volume 0.2 ml). Diluted activated plasma (10 μl of 10-fold diluted-activated plasma pretreated with methylamine-HCl) or purified kallikrein (16 ng in 10 μl) was assayed for 15 minutes at 37°C . The reaction was stopped by addition of an equal volume of 10% acetic acid. A_{405} was read after centrifugation. Control incubations were included in which enzyme was added after the acetic acid. The molar extinction coefficient (ϵ_{405}) assumed was 11,000 (average from Kabi Diagnostica practical sheets for S-2302, S-2266, S-2251).

Tripeptide-4-methyl-7-coumarylamide hydrolysis. Hydrolysis of Pro-Phe-Arg-NMec, Boc-Val-Pro-Arg-NMec and Boc-Ile-Glu-Gly-Arg-NMec (0.25 mM) substrates selective for kallikreins, thrombin and factor Xa, respectively (27) was measured with activated plasma (10 μl of 10-fold diluted) or pure kallikrein (16 ng in 10 μl) was measured in assay buffer (final volume 20 μl) for 15 min at 37°C . Reactions were stopped by addition of 1.5 ml of 5% acetic acid. The relative fluorescence intensity was measured in a Locarte MK4 single sided fluorimeter using a filter (LF/2 340–380 nm) for excitation and measuring the emission at 460 nm. The activity of plasma or pure plasma kallikrein was calculated with reference to standard solutions (up to 2 μM) of 4-methyl-7-aminocoumarin in 5% acetic acid.

DETERMINATION OF PROKALLIKREIN

The method was based on that of Kluft (24). Plasma samples (100 μl) were treated at 0°C with an equal volume of dextran sulfate solution (2.5 mg/100 ml of 50 mM Tris-HCl containing 12 mM NaCl) for 7 min. Samples (50 μl) were assayed for hydrolysis of D-Pro-Phe-Arg-NPhNO₂ (0.6 mM, final volume 0.5 ml) for 1 min at 37°C . The reaction was stopped by addition of 0.1 volume of 50% acetic acid and any precipitate was centrifuged ($2500 \times g$, 15 min). A_{405} measurements were taken for 4-nitroaniline release. Controls were included in which enzyme was added after the acetic acid. Substrate hydrolysis by plasma not treated with dextran sulfate was also measured and the difference between this and dextran sulfate-generated activity was taken to represent prokallikrein.

DETERMINATION OF PLASMINOGEN

The method was based on that recommended by Kabi Diagnostica for the use of D-Val-Leu-Lys-NPhNO₂. Buffer (75 μl of 50 mM Tris-HCl, pH 7.4, with 12 mM NaCl) and streptokinase (50 μl , 10,000 U/ml in water) was added to the sample (25 μl) and this was incubated for 10 min at 37°C . Substrate (350 μl of D-Val-Leu-Lys-NPhNO₂ in buffer) was added to give a final concentration of 0.6 mM substrate in the 0.5 ml assay volume. After 2 min incubation (37°C), 0.1 volume of 50% acetic acid was added, and A_{405} of the supernatant after centrifugation was measured. Control incubations were included in which enzyme was added after the acetic acid. Substrate hydrolysis by samples not treated with streptokinase (but preincubated at 37°C for 10 min with 50 μl of water) was also measured in a similar way. The difference between this and the streptokinase-generated activity was taken to represent plasminogen.

GEL CHROMATOGRAPHY

A column (type K15/90; Pharmacia (GB) Ltd.) was packed with Ultrogel AcA-34 (LKB Instruments Ltd.) in buffer (0.1 M sodium phosphate, pH 7.6, containing 0.15 M NaCl) containing EDTA (1 mM), 6-aminohexanoic acid (10 mM) to inhibit activation of zymogens, and toluene (0.03%) as preservative. The flow rate was 8–10 ml/h, and 2.0–2.6 ml fractions were collected.

The column was calibrated with samples (4 ml) containing Blue dextran (void volume, V_0), α_2 M (M_r 725,000), sheep immunoglobulin G (M_r 160,000), bovine serum albumin (M_r 68,000), egg

albumin (M_r 45,000) and cytochrome *c* (M_r 12,400). Pure human kallikrein (3.2 mg in 4 ml) was also run on the column, and 0.1 ml samples of the fractions assayed (30 min, 37°C) for hydrolysis of Tos-Arg-OMe. Elution volumes (V_e) of all peaks were expressed relative to the void volume, i.e., as V_e/V_o .

Cystic fibrosis or normal adult plasma (2.5–3 ml), either pretreated or not with 0.1 volume of methylamine-HCl for 1 h at 25°C, were subjected to activation with an equal volume of ellagic acid suspension (for 15 minutes to 2 h at 25°C) and 4 ml was applied to the column (1.5 × 87 cm). Samples (0.25 ml) from the fractions were assayed (2–4 h at 37°C) for Tos-Arg-OMe esterase activity.

RESULTS

EXPERIMENTS WITH NORMAL PLASMA: DEVELOPMENT OF THE STANDARD PROCEDURE FOR MEASUREMENT OF ARGININE ESTERASE ACTIVITY OF ACTIVATED PLASMA

Reproducibility. We examined the enzyme activation procedure exactly as it was used in previous studies of cystic fibrosis (34). The procedure was as in the "Methods" section except that there was no pretreatment with methylamine or buffer. Plasma (2.5–5 ml) was treated with an equal volume of cold chloroform, then 1–2.5 ml of the chloroform-treated plasma was mixed with an equal volume of ellagic acid in water (G.J.S. Rao, personal communication) or assay buffer (11) and then was incubated for 15 min at 25°C before 0.3 ml was taken for assay with Tos-Arg-OMe (15 min, 37°C). In seven experiments (involving plasma from six different individuals), we carried out three or four acti-

vations of a single plasma sample as above, followed by duplicate assays of each activated sample for Tos-Arg-OMe hydrolysis. The results of six of the seven experiments showed poor reproducibility (coefficients of variation > 15%). Duplicate determinations of esterase activity for a single activated plasma sample always agreed well, but there were large variations between replicate activation mixtures. It did not seem to matter whether the ellagic acid were dissolved in water or buffer (but buffer was adopted for our standard procedure, see "Methods"). The average ΔA_{580} was 0.075 (range 0.005–0.109), which was low compared to the average blank of 0.246. This value corresponded to an activity of 22 $\mu\text{mole/h/ml}$ of plasma which was lower than previously reported (Table 1).

Kinetics of activation of arginine esterase. The assay for Tos-Arg-OMe hydrolysis in unactivated plasma showed little activity: in two adult controls, 1.3, 2.3 $\mu\text{mole/h/ml}$; in three pediatric controls, 4.2, 2.2, 2.0 $\mu\text{mole/h/ml}$.

Activation of normal adult plasma with ellagic acid after treatment with chloroform (see the "Methods" section), was followed by assays over a period of 3 h. Representative results are shown in Fig. 1A. Similar results were obtained in individual experiments with at least six different plasma samples. Activity always reached a maximum within 2 h of activation and sometimes as early as 15 min. Addition of STI to the assay showed that most or all of the activity was resistant to this inhibitor at 2 h. At earlier times, the proportion of STI-sensitive activity was greater.

As is explained below, the partial STI-resistance of the activity was suggestive of the formation of an $\alpha_2\text{M}$ -enzyme complex. To test this possibility, plasma was pretreated with methylamine-HCl for 1 h before activation, because methylamine inactivates $\alpha_2\text{M}$ in plasma (4). Activation of arginine esterase reached a maximum by 1 h followed by a decline in activity (Fig. 1A). The amount of STI-sensitivity conferred on activated arginine esterase increased with time of preincubation with methylamine-HCl (results not shown), and after 1 h of pretreatment (followed by 1 h of activation), 95% of the activity remained sensitive to STI (average for 12 control plasmas). There was little effect of prolonging the preincubation period beyond 1 h. At this time much of the $\alpha_2\text{M}$ ran as the inactive 'fast' form in 5% polyacrylamide electrophoresis gels (c.f. ref. 4).

Treatment of plasma with chloroform did not inactivate the inhibitors of the esterase, since the activity in methylamine-HCl treated plasma still declined after 1 h (Fig. 1A).

As a routine we increased the time of activation to 1 h (for STI-sensitive activity of methylamine-treated plasma) or 2 h (for STI-resistant activity of buffer-treated plasma). We also increased the time of assay with Tos-Arg-OMe to 2 h, and decreased the amount

Table 1. Previous comparisons between arginine esterase levels in cystic fibrosis and normal plasma

Authors	Deficiency reported?
Tos-Arg-OMe as substrate	
Rao, Posner and Nadler (34)	Yes
Talamo, Colman and Milunsky (40)	No
Coburn, Coburn and Solomon (8)	Yes (in serum)
Wilson and Fudenberg (43)	Yes
Chan, Applegarth and Davidson (6)	Yes
Goldsmith, Stern, Saito and Ratnoff (17)	No
Dann and Blau (12)	Yes
Bz-Arg-OEt as substrate	
Lieberman (26)	No
Shapira, Rao, Wessel and Nadler (38)	Yes

Fig. 1. Progress of activation of arginine esterase activity after the addition of ellagic acid. Plasma (1–4 ml) from four individuals [two normals (A), two cystic fibrosis (B)] was treated as described in the "Methods" section, and at the time intervals shown samples (0.1–0.3 ml) were taken for assay (15 min–2 h incubation) against Tos-Arg-OMe. Treatments were as follows: □, buffer only, assayed without STI; ■, buffer only, assayed with STI (200 $\mu\text{g/ml}$); ○, methylamine assayed without STI, and ●, methylamine, assayed with STI.

of plasma to 50–200 μ l. This allowed us to measure separately the two types of activity and also increased the ΔA /blank ratio and allowed us to obtain activity values in our control plasmas similar to those obtained by other workers (34). The reproducibility of our activations and subsequent assays was thus improved to give coefficients of variation < 12.5% (on the basis of experiments with four different plasmas, each with triplicate activations).

IDENTIFICATION OF THE ARGININE ESTERASE OF ACTIVATED NORMAL PLASMA

Involvement of coagulation factor XII in the activation. We found that adding Polybrene (0.01 vol. of 2 mg/ml solution) to methylamine-HCl treated plasma (1–2 ml) before addition of chloroform and then a further 0.01 vol. of Polybrene before the ellagic acid (0.5 ml) resulted in a 77% decrease in activity against Tos-Arg-OMe detectable after 1 h of activation. Polybrene introduced after activation did not inhibit the arginine esterase. These results suggested that activated factor XII was involved in the activation of arginine esterase in a manner similar to that shown previously for activation of arginine esterase with chloroform and ellagic acid (11).

Substrate specificity. We assayed activated plasma (methylamine-HCl-treated) with tripeptide nitroanilide and methylcoumarylamide substrates selective for various trypsin-like enzymes that occur in plasma (as described in the "Methods" section). The results are shown in Table 2. The plasma showed greater activity against D-Pro-Phe-Arg-NPhNO₂, a highly specific substrate for plasma kallikrein (7) than against the other substrates. Accordingly, a similar specificity profile was determined for pure plasma kallikrein, the activities being normalized with reference to those with Tos-Arg-OMe. Similar results were obtained in a second experiment with a different plasma sample taken from the same individual but using different times of incubation and amounts of enzyme samples. The results with pure kallikrein proved to be very similar to those for the plasma enzyme. Moreover, the relative activities with the nitroanilides and methylcoumarylamides were similar to those previously reported for plasma kallikrein (14, 27).

Inhibition characteristics. The effects of potential inhibitors on the arginine esterase were assessed as described in the Methods section. The results are shown in Table 3. Again, a comparison was made with pure plasma kallikrein. In a control experiment with standard methanol solution, the chromotropic colour reaction was itself shown to be 14% inhibited by 4-chloromercuribenzoate, but not by STI, aprotinin, Pro-Phe-Arg-CH₂Cl or di-isopropyl-fluorophosphate (relative to the isopropanol solvent control). An appropriate correction was therefore made in the calculation of inhibition of the enzyme by 4-chloromercuribenzoate.

Table 2. Comparison of the substrate specificity of the arginine esterase in activated, methylamine-treated normal plasma with purified kallikrein

Substrate	Ratio of substrate hydrolysis compared to Tos-Arg-OMe	
	Crude plasma arginine esterase	Purified plasma kallikrein
Tos-Arg-OMe ¹	1	1
D-Pro-Phe-Arg-NPhNO ₂	0.910	0.912
D-Val-Leu-Arg-NPhNO ₂	0.224	0.222
Pro-Phe-Arg-NMec	0.084	0.125
Boc-Val-Pro-Arg-NMec	0.005	0.008
Boc-Ile-Glu-Gly-Arg-NMec	0.004	0.007
D-Val-Leu-Lys-NPhNO ₂	0	0

¹ The absolute activities of the enzymes with Tos-Arg-OMe were 48 μ mole/h/ml plasma and 24.6 μ mole/min/mg kallikrein. Assays, in duplicate, gave values which did not differ by more than 5% from the given means.

It can be seen that the inhibition spectrum of the arginine esterase in methylamine-HCl treated plasma was closely similar to that of plasma kallikrein.

Activation of plasma not treated with methylamine-HCl gave an arginine esterase which was resistant to STI but was sensitive to the same low molecular weight inhibitors as the STI-sensitive enzyme. We concluded that the results were consistent with this activity being due to an esterase- α_2 M complex in the untreated or buffer-treated plasma. Binding of proteinases by α_2 M is well known selectively to restrict their sensitivity to high molecular weight inhibitors (3).

Disappearance of prokallikrein from plasma during treatment with chloroform and ellagic acid. If the arginine esterase activity produced in plasma by the treatment with chloroform and ellagic acid were due to the factor-XIII-mediated conversion of prokallikrein to kallikrein, it should be possible to show the disappearance of prokallikrein by independent means. Kluft (24) has described a method for the determination of prokallikrein by rapid activation with dextran sulfate and specific assay of the resulting kallikrein with a tripeptide nitroanilide substrate (see the "Methods" section).

Plasma (5 ml) was treated with chloroform as usual. Samples (1 ml) were mixed with an equal volume of ellagic acid in water, or water alone, and incubated at 25°C. At intervals, 25 μ l samples were assayed with D-Pro-Phe-Arg-NPhNO₂ for 1 min. At the same time, 100 μ l samples were taken for dextran sulfate treatment and subsequent assay. The differences between the values with and without dextran sulfate represented the prokallikrein. The results are shown in Figure 2. It was found that prokallikrein disappeared with a half-time of 30–40 min after treatment with chloroform but no ellagic acid, whereas the half-time was of the order of 15 min with ellagic acid, too.

Since chloroform alone appeared to activate prokallikrein (Fig. 2), the time allowed between chloroform treatment and ellagic acid activation was carefully standardised. In a control experiment, plasma incubated alone at 25°C for 6 h showed little or no spontaneous activity (<5 μ mole/h/ml) and only a slight decrease (13%) in dextran sulfate activatable prokallikrein.

A similar study was made of the concentrations of plasminogen and plasmin in the plasma during the activation procedure. Little or no (<2 μ mole/h/ml) activity against D-Val-Leu-NPhNO₂ appeared in the activated plasma, and the activity against this substrate that appeared following treatment with streptokinase (representing plasminogen, approximately 133 μ mole/h/ml) remained constant even during a 26-h period of treatment of the chloroform-treated plasma with ellagic acid. These results made it seem unlikely that plasmin or trypsin (1) contributed any of the arginine esterase activity.

Molecular weight of arginine esterase. Samples of normal activated plasma were run on a column of Ultrogel Aca-34 as described in the Methods section. Representative results are shown in Figure 3 for normal plasma. For plasma not treated with methylamine-HCl, the STI-resistant enzyme (80–100% of the activity) always ran just behind the void volume ($V_e/V_o = 1.08$, average of four experiments). α_2 M was identified in the main peak fractions by SDS polyacrylamide gel electrophoresis (4), and a purified α_2 M sample eluted from the column with a similar relative elution volume (V_e/V_o , 1.07). These findings supported the idea of the association of the esterase with α_2 M. It was shown by Vogt (45) that treatment of plasma with acetone resulted in the activation of an arginine esterase which eluted with α_2 M from Sephadex G-200. Prior immunoadsorption of α_2 M from the plasma displaced the peak from the macroglobulin to the globulin region.

Further evidence for the involvement of α_2 M with the very high molecular weight arginine esterase was the finding that treatment of plasma with methylamine-HCl eliminated most or all (88–100%) of this very high molecular weight arginine esterase activity (Fig. 3A).

The proteinase-binding activity of α_2 M is known to be destroyed by methylamine (4). The STI-sensitive activity generated in plasma pretreated with methylamine-HCl eluted in a broad peak

Table 3. Test of potential inhibitors of the activated arginine esterase in normal plasma¹

Inhibitor (concentration)	Inhibition of Tos-Arg-OMe esterase activity (% relative to control)				Purified plasma kallikrein
	Methylamine-HCl-treated plasma		Buffer-treated plasma		
	A	B	B	C	
STI (200 µg/ml)	96	98	7	6	100
Lima-bean trypsin inhibitor (200µg/ml)	0	9	0	0	4
Aprotinin (200 µg/ml)	91	90	21	0	90
Factor XII _a inhibitor (from popcorn, 200 µg/ml)	1	4	10	N.D.	4
EDTA (1 mM)	N.D.	0	N.D.	0	0
4-Chloromercuribenzoate (1 mM)	42	53	N.D.	51	58
Di-isopropylfluorophosphate (1 mM)	87	100	80	83	100
Tos-Lys-CH ₂ Cl (1 mM)	0	0	0	0	5
Pro-Phe-Arg-CH ₂ Cl (1 µM)	92 (D)	100	100 ² 99 ^{2,3}	98 ² (D) 88 ^{2,3} (D)	100

¹ The results are from experiments with plasma samples from three individuals A, B, and C, with the exception of two results for Pro-Phe-Arg-CH₂Cl, which were obtained with plasma from a fourth individual, D.

² No preincubation at 25°C of plasma with buffer before activation. N.D., not determined.

³ Preincubation with inhibitor for 15 min at 25°C, and activation with ellagic acid for 15 min.

Fig. 2. Prokallikrein determination in normal plasma during treatment with chloroform or chloroform and ellagic acid. Plasma (5 ml) was treated with chloroform as usual, then 1 ml samples were mixed with an equal volume of ellagic acid suspension or water, and incubated at 25°C. Samples taken periodically were assayed for D-Pro-Phe-Arg-NPhNO₂ hydrolysis directly or after dextran sulphate activation. Treatments were: □, ellagic acid, assayed after dextran sulphate; ■, ellagic acid, assayed directly; ●, water, assayed after dextran sulphate; and ●, water, assayed directly. From the differences, prokallikrein concentrations (in units of potential kallikrein activity) were calculated: △, chloroform/ellagic acid; ▲, chloroform/water.

($V_e/V_o = 1.40$, average of four experiments) indicative of an M_r slightly in excess of 160,000. Similar results were obtained for activation periods of 15 min, 1 h or 2 h. The ratio of hydrolysis of Pro-Phe-Arg-NMec/Tos-Arg-OMec for the eluted esterase was found to be similar to that for purified plasma kallikrein (Table 2), providing further evidence for the identification of the STI-sensitive arginine esterase as plasma kallikrein.

Fig. 3. Gel chromatography of activated tosyl arginine methyl esterase in normal and cystic fibrosis plasma. Normal adult (one different person each run, (A) and cystic fibrosis pooled plasma (2 different patients each run, (B) were either pretreated (○) or not (●) with methylamine-HCl before activation and application (4 ml) to an AcA-34 column ("Methods"). Fractions were assayed for Tos-Arg-OMe hydrolysis ("Methods"). V_e/V_o is elution volume relative to the void volume. Standard proteins were used to calibrate the column for molecular weight.

The apparent M_r of the esterase uncomplexed with α_2M was higher than that of free kallikrein, but data showing the association of plasma kallikrein with other proteins in plasma giving apparent molecular weights in excess of 180,000 have been reviewed by Wuepper (44), and Colman (9) suggested that a monomer-dimer relationship existed between two types of kallikrein recognisable in gel chromatography.

COMPARISONS BETWEEN NORMAL, CYSTIC FIBROSIS AND HETEROZYGOTE PLASMAS

STI-sensitive and -resistant arginine esterase activities in activated plasma. Our plasma samples from cystic fibrosis patients included

some that had been stored frozen, and others assayed without freezing. Preliminary experiments showed no effect of freezing on the results, so the data (Fig. 4, Table 4) were combined in comparisons with normal plasma.

There were no significant differences (even at the 5% level) in STI-sensitive activity (Fig. 4A, 1 h of activation, methylamine-HCl-treated plasma) between cystic fibrosis plasma and pediatric controls or heterozygote plasma and adult controls. Again, no significant difference was found in comparisons of the STI-resistant activity (Fig. 4B, 2 h of activation, buffer treated plasma), nor was there any significant difference between the two control groups (pediatric or adult) for either type of activity studied. The ranges of both types of activity were quite large. Within the group of cystic fibrosis samples, neither type of activity depended on the sex of the patient and there was no significant correlation with age for STI-sensitive ($r = 0.034$, $df = 26$) or STI-resistant ($r = 0.284$, $df = 24$) activities. The spontaneous total activity in untreated and unactivated plasma from three cystic fibrosis samples was 3.0, 3.0, and 1.3, which was not significantly different from the measurements on pediatric controls (see above).

Kinetics of activation. The shapes of the curves for the activation of STI-resistant and -sensitive enzymes (Fig. 1B) were similar to those of normal plasma. The apparent differences in total activity (1 h, methylamine-HCl treated, STI-sensitive, or 2 h, STI-resistant, untreated plasma,) were probably fortuitous in view of the wide ranges of activity seen in Figure 4.

Fig. 4. Arginine esterase (Tos-Arg-OMe) activities in cystic fibrotic, heterozygote and normal plasma. Arginine esterase was measured either as 'STI-sensitive' enzyme activity (A) in plasma pretreated with methylamine-HCl, then activated with chloroform and ellagic acid for 1 h ('free' enzyme), or as STI resistant activity (B, probably α_2 M-bound) in plasma similarly activated for 2 h with buffer pretreatment in place of methylamine-HCl.

Molecular weight of esterase. Fractionation of pooled cystic fibrosis plasma activated either with or without methylamine-HCl pretreatment (Fig. 3B) did not show any abnormalities in elution profile as compared to the normal adult plasma.

DISCUSSION

The identity of the arginine esterase. Plasma contains precursors of several trypsin-like enzymes liable to hydrolyse Tos-Arg-OMe or similar substrates, *i.e.*, thrombin, plasmin, coagulation factors IXa, Xa, XIa, XIIa, complement components C1s and D, and plasma kallikrein. Tissue kallikrein (EC 3.4.21.35) which has been postulated as the deficient enzyme in cystic fibrosis (12) also has a related specificity. The properties of all these enzymes have recently been summarised (2). On the basis of substrate specificity, inhibition characteristics, molecular weight and amount of enzyme present, we have attempted to identify the arginine esterase activated by chloroform and ellagic acid, and considered by some to be deficient in cystic fibrosis.

We can conclude that the substrate specificity of the STI-sensitive plasma enzyme is consistent with its being plasma kallikrein, and that thrombin, factor Xa and plasmin and trypsin made negligible contributions. Similar conclusions were reached about the identity of the arginine esterase elicited in plasma by kaolin (11). The inhibition by STI ruled out any significant contribution from tissue kallikreins (30), C1s (32), factor XIIa (18) or thrombin (25). The lack of significant inhibition by lima bean trypsin inhibitor ruled out any contribution by factor XIIa (18), plasmin (35) or trypsin (21). The inhibition by aprotinin ruled out any contribution by thrombin (22). The inhibition by 4-chloromercuribenzoate was probably due to an interaction away from the active site, since the enzyme was shown to be a serine proteinase (inhibited by di-isopropyl fluorophosphate). The selective inhibitor of plasma kallikrein, Pro-Phe-Arg-CH₂Cl (23), was a potent inhibitor of the enzyme in plasma, whether activated by our standard procedure, or by the procedure reported previously (33). The results of prokallikrein measurements during chloroform and ellagic acid treatment (Fig. 2) were consistent with the activation of arginine esterase being a conversion of prokallikrein, which is a factor XIIa-dependent (Polybrene-blocked) process. The molecular weight of the esterase was similar to that of plasma kallikrein, and greater than those of tissue kallikrein (30), factor D (28), factor IXa (31), factor Xa (13), thrombin (25) and trypsin (41).

The activation of plasma by chloroform and ellagic acid was necessary to reveal Tos-Arg-OMe esterase activity as the total spontaneous activity was low ($< 10\%$ of the potential activity). Once active this esterase (kallikrein) formed a complex with α_2 M as shown by its very high molecular weight and inhibition characteristics (*e.g.*, STI-resistance). The property of kallikrein of complexing with α_2 M as soon as it is generated from prokallikrein has been discussed (16, 29). Prior inactivation of α_2 M in plasma by treatment with methylamine-HCl prevented complex formation.

Factor XIa has been estimated to give only 4% of the kallikrein-like activity in plasma when activated, and furthermore, it does not react with α_2 M (19), so it was unlikely to account for any significant amount of the arginine esterase we measured.

Experiments with cystic fibrosis plasma. We have been unable to confirm the reported deficiency of arginine esterase activity even when measuring wholly STI-sensitive or -resistant enzymes. The ranges of activity found for normals, heterozygotes, and cystic fibrosis patients, and the fact that there were no significant differences between the groups and no significant correlation with age or sex (in the cystic fibrosis groups) made it seem unlikely that these measurements will be of clinical value. The results show that the arginine esterase is not directly related to the basic defect in cystic fibrosis.

Why then do our results differ from some previous reports? First, some apparent deficiencies may have been fortuitous. In our hands, the measurement of activity by the methods used previously showed poor reproducibility, and there is, in any case, a wide

Table 4. Arginine esterase activities in cystic fibrosis, heterozygote and normal plasma samples¹

	Group and description			
	(a) Cystic fibrosis	(b) Pediatric control	(c) Cystic fibrosis parent (heterozygote)	(d) Adult control
STI-sensitive arginine esterase				
Number analysed	29	6	12	6
Average age (years)	9.62	7.72	N.D.	N.D.
Age range	4–16.3	3.25–14.4	N.D.	N.D.
Esterase activity				
Mean	42.4	43.1	47.4	49.6
S.D.	16.5	9.9	21.2	28.7
S.E.	3.06	4.06	6.12	11.7
STI-resistant arginine esterase				
Number analysed	27	6	14	6
Average age (years)	9.29	7.72	N.D.	N.D.
Esterase activity				
Mean	18.0	23.7	19.6	27.1
S.D.	9.4	13.5	7.6	16.8
S.E.	1.82	5.51	2.02	6.86

¹ Activities were assayed as described in the text. The small differences between means for groups (a) vs (b), (c) vs (d) and (b) vs (d) were subjected to Student's *t* test: no difference was significant at the 5% level. The proportion of males was 2/6 for the pediatric controls, and 19/29 for the cystic fibrosis group. N.D., not determined.

scatter of normal values. The activation is mediated by factor XIIa, but ellagic acid is an inefficient activator of factor XII (as was reported by Kluft, 14) and at 15 min (the time used by other workers) maximum values were often not obtained. A second consideration is that some cystic fibrosis patients may have low (pro)kallikrein levels secondary to hepatic involvement. Indeed, out lowest activity was for the single patient who was hospitalized with liver disease. Prokallikrein is synthesized in the liver (10), and prokallikrein levels have been shown to be low in patients with hepatic cirrhosis (9, 36). Prokallikrein has been shown to be the factor missing in the genetically determined 'Fletcher' trait (20, 44). This condition has no clinical symptoms and the only abnormality is a prolonged *in vitro* clotting time. Deficiency of plasma kallikrein is thus quite unlike cystic fibrosis.

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