Biochimica et Biophysica Acta, 659 (1981) 38—47
Elsevier/North-Holland Biomedical Press

BBA 69254

IMPROVED PURIFICATION AND FLUORESCENCE CHANGES UPON ACTIVATION OF HUMAN SEMINAL PLASMA ACIDIC PROTEASE PROENZYME

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(Received October 14th, 1980)

Key words Protease activation, Fluorescence change, (Human seminal plasma)

Summary

Modifications have been made to the previous purification procedure so that electrophoretically homogeneous acidic protease (EC 3.4.23.—) proenzyme of specific activity 800 units/mg may be isolated from human seminal plasma with a yield of over 50%. The intrinsic fluorescence of the proenzyme shows maximum excitation and emission wavelengths at 280 and 340 nm, respectively, typical of proteins containing tryptophan. Complete activation causes a 30—35% decrease in intrinsic fluorescence, accompanied by a shift in λ_{max} to the blue of 4—6 nm. Time course studies indicate that acidification of proenzyme to pH 3.1 leads to a sudden large decrease in fluorescence that precedes both the appearance of active enzyme band on sodium dodecyl sulphate (SDS)-polyacrylamide gels and the generation of enzyme activity as detected by the turbidimetric milk clotting assay. These results suggest that acidification causes a rapid conformational change which promotes the release of the activation peptide from the proenzyme to yield the active enzyme.

Introduction

Previous reports from this laboratory [1—4] have described the purification and properties of an acidic protease (EC 3.4.23.—) from human seminal plasma. This enzyme resembles gastric pepsin in many respects, for example, in amino acid composition, low pH optimum and inhibition by active-site-directed diazo and epoxy compounds. Furthermore, the acidic protease is present in seminal plasma as the inactive proenzyme form, which may be converted to the active form by exposure to acid. Physiologically this might occur when semen is

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deposited into the slightly acidic (pH 3.5—4.5) environment of the vagina [5].

From the study of the activation kinetics, a two-step activation mechanism has been proposed involving a pH-dependent change in the conformation of the native proenzyme into a form capable of self-activation, followed by the release of an activation peptide from the latter to form the active enzyme [3]. In this paper, we have used intrinsic fluorescence to demonstrate the occurrence of an acid-induced conformational change and have studied the time sequence of such a change compared to the generation of enzyme activity and to structural changes detected by SDS-polyacrylamide gel electrophoresis. To overcome problems in obtaining enough proenzyme for such studies, we have also modified the published purification procedure [2,6] so that a yield close to 50% may be obtained rather than the 10% previously described.

Methods

Materials

Pooled semen was collected from healthy volunteers and was centrifuged at $1000 \times g$ for 30 min at 4°C to remove spermatozoa The cell-free seminal plasma was stored at —20°C until use, whereupon it was thawed and clarified by centrifugation at $20\,000 \times g$ for 20 min at 4°C.

Improved purification procedure for acidic protease proenzyme

The following steps were performed as rapidly as possible at 0—4°C.

Step 1 Dialysis Pooled human seminal plasma was dialyzed against 0.02 M sodium phosphate buffer, pH 6.5 and clarified by centrifugation at $30\,000 \times g$ for 30 min.

Step 2 DEAE-cellulose chromatography. The dialysed, clarified seminal plasma was applied to a DEAE-cellulose column (4.2 × 45 cm), equilibrated with 0.1 M NaCl/0.02 M sodium phosphate buffer, pH 6.5. The column was washed at a flow rate of 50 ml/h firstly with the same buffer until the absorbance at 280 nm was less than 0.05 units, and then with a linear gradient (600 ml + 600 ml) of 0.1—0.7 M NaCl in the same phosphate buffer. Fractions of 8.5 ml were taken and analyzed by measuring absorbance at 280 nm and milk clotting activity [7] (after activation). Fractions containing protease activity were pooled, dialyzed against distilled water and lyophilized.

Step 3 Sephadex G-100 chromatography. The lyophilized DEAE-cellulose pool was dissolved in a minimal volume of water and applied to a Sephadex G-100 column (3.9 × 95 cm), equilibrated with 0.1 M NaCl/0.02 M sodium phosphate buffer, pH 6.5. The column was eluted with the same buffer at a flow rate of 32 ml/h. Fractions of 8 ml were collected and analyzed by measuring absorbance at 280 nm and milk clotting activity (after activation). Fractions containing protease activity were dialyzed against distilled water and lyophilized.

Aliquots from each purification step were kept at —20°C for further analysis of enzyme activity by the acid-denatured hemoglobin assay [8], protein content by the method of Lowry et al. [9], and purity by SDS-polyacrylamide gel electrophoresis [10].

Fluorescence spectroscopy

Fluorescence spectroscopy was carried out in an Aminco-Bowman spectrofluorimeter. Excitation spectra were scanned at an emission wavelength of 340 nm and emission spectra were scanned at an excitation wavelength of 280 nm. In some experiments fluorescence emission intensity at 340 nm was measured with an excitation wavelength of 280 nm. Fluorescence increased linearly with protein concentration up to a concentration of 0.2 mg/ml.

Excitation and emission spectra of acidic protease before and after activation

1 ml purified proenzyme (approx. 0 12 mg/ml) in sodium phosphate buffer, pH 6.5 was pipetted into two matched fluorescence cuvettes. To the experimental cuvette, 0.1 ml 0.2 M H_3PO_4 was added to give a final pH of 2.7, and to the control cuvette, 0.1 ml 0.2 M sodium phosphate buffer, pH 6.5 was added. After a 30 min incubation at room temperature, the fluorescence excitation and emission spectra of both samples were scanned. Four 0 1-ml aliquots were taken from each sample and stored in ice for measurement of milk clotting activity. Then the experimental sample was neutralized by pipetting 0.5 ml acidified proenzyme into 4.5 ml 0.2 M sodium phosphate buffer, pH 6.5, and the control sample was treated in the same manner. Fluorescence spectra were again scanned and samples were also used to assay for milk clotting activity.

Time course of activation of acidic protease at pH 3 1

A stock solution of proenzyme was prepared by dissolving approx. 2 mg purified proenzyme in 1 ml 0.02 M sodium phosphate buffer, pH 6.5. A 0.3 ml sample of the stock proenzyme solution was added to 4.2 ml 0.05 M sodium phosphate buffer, pH 3.0, mixed and incubated at room temperature (final pH was 3.1). A control was prepared by adding 0.1 ml stock proenzyme solution to 1.4 ml 0.05 M sodium phosphate buffer, pH 6.5. At various times of incubation, the fluorescence emission intensity at 340 nm of the acidified proenzyme solution and of the control were measured. In addition, at various times, 0.5 ml of the acidified proenzyme solution was pipetted into 0.6 ml 0.2 M sodium phosphate buffer, pH 6.8 to stop activation and stored in ice for measurement of milk clotting activity. The milk clotting activity of unactivated proenzyme (control) and fully active enzyme (incubated at pH 2.7 for 30 min at 37°C), at the same protein concentrations as the experimental samples, were also measured.

Structural changes accompanying activation of acidic protease

Activation of proenzyme was carried out by adding 0.165 ml 0.05 M H_3PO_4 to 1.25 ml proenzyme stock solution (approx. 1 mg/ml) in 0.01 M sodium phosphate, pH 6.5, so that the final pH was approx 3.1. Activation was allowed to proceed at room temperature. At various times, samples were removed in triplicate. One 0.05 ml sample was 'neutralized' by transfer into 0.075 ml 0.2 M sodium phosphate buffer, pH 6.5, stored in ice and used for estimation of milk clotting activity. Another 0.05 ml sample was transferred into 0.95 ml 0.2 M sodium phosphate buffer, pH 6.5, stored in ice and used for measurement of fluorescence emission at 340 nm. A third 0.05 ml sample was added to 0.035 ml 2% SDS/4 M β-mercaptoethanol/20% glycerol/0.002%

bromophenol blue in 0.125 M Tris-HCl buffer, pH 6.8, heated at 100°C for 2 min and used for polyacrylamide gel electrophoresis. Controls, at the same protein concentration as that of the experimental, were also performed, consisting of unactivated proenzyme (obtained by incubation of 0.015 sodium phosphate, pH 6.5) and of fully active enzyme (obtained by incubation with 0.25 N HCl for 30 min)

Turbidimetric milk clotting assay for acidic protease activity

Except where otherwise stated, acidic protease activities described in this paper were determined at room temperature by the turbidimetric milk clotting assay adapted from McPhie [7]. Stock milk solution was prepared by dissolving Carnation non-fat dry milk in 25 ml double-distilled water. 50 ml assay medium contained 1.0 ml stock milk solution, and 4.0 ml 0.1 M $CaCl_2$ in 0.2 M sodium acetate buffer, pH 5.3 (final concentration). The reaction was initiated by adding 0.05—0.2 ml enzyme solution to 3.0 ml assay medium in a Spectronic 20 tube, followed by thorough mixing. The time (T, s) required to reach an absorbance of 0.3 units at 510 nm was measured with a stopwatch. Activity was expressed as $1/T$ (s^{-1}). In some cases, proenzyme was activated prior to activity measurement. For routine column assays activation was achieved by preincubation in 0.1 M citric acid/sodium phosphate buffer, pH 2.5 for 20 min at room temperature.

Acid-denatured hemoglobin assay for acidic protease activity

The method of Kassell and Meitner [8] was used to determine acidic protease activity in the proenzyme pool obtained after each purification step (Table I), so that the results obtained here could be directly compared to previous results employing the same assay method [2,6]. The assay mixture (2.0 ml) contained 12.5 mg/ml acid-denatured hemoglobin in 0.1 M citric acid/sodium phosphate buffer, pH 2.5 plus an appropriate amount of enzyme. The reaction was carried out at 37°C for 30 min and was then stopped by addition of 2.0 ml 10% trichloracetic acid. After centrifugation at 30 000 × g for 20 min, the absorbance at 280 nm of the supernatant was measured in a Zeiss PMQ II spectrophotometer.

Protein determination

Protein concentration was measured by the method of Lowry et al. [9], using bovine serum albumin as a standard. For routine screening during column chromatography, the relative protein content was estimated by measuring the absorbance at 280 nm.

SDS-polyacrylamide gel electrophoresis

The purity of proenzyme was checked by electrophoresis in 0.1% SDS/7.5% polyacrylamide gel tubes (0.6 × 10 cm) according the procedure of Weber and Osborn [10]. Electrophoresis was performed at a constant current of 8 mA/gel for 6—7 h. Separation of proenzyme and active enzyme in time course studies was carried out by electrophoresis in discontinuous SDS-polyacrylamide gradient (7—15%) slab gels (13 × 15 × 0.1 cm) according to the procedure of Laemmli [11]. Electrophoresis was carried out at a constant voltage of 150 V

for 2—3 h. Both types of gel were stained with Coomassie brillant blue R-250. In some cases, gels were scanned in a Gilford model 2000 spectrophotometer at 550 nm.

Results and Discussion

Turbidimetric milk clotting assay

With the exception of the data shown in Table I, which were obtained using the acid-denatured hemoglobin assay, all other data described in this paper were obtained using the turbidimetric milk clotting assay for the measurement of acidic protease activity. Our comparisons of this assay with the acid-denatured hemoglobin assay [8] used in earlier studies [1—4] indicate that both assays show a similar linear range with respect to enzyme concentration, are of similar sensitivity and correlate well with each other. In addition, the turbidimetric milk clotting assay offers two advantages, useful for the present studies, firstly it is more rapidly performed than the hemoglobin assay which requires a centrifugation step after trichloracetic acid precipitation to remove undigested substrate. Secondly, in contrast to the low pH (pH 2.5) used for the hemoglobin assay, the pH for the milk clotting assay is relatively high (pH 5.3), so that very little activation of proenzyme occurs making it possible to preferentially assay activated enzyme. In practice, unactivated proenzyme shows about 5—10% of the activity of fully activated enzyme, presumably because some activation takes place during prolonged incubation at pH 5.3. Minor corrections for the activity of unactivated proenzyme have been included in the data shown in Fig. 3 and Table II, by defining the activity of unactivated proenzyme and fully activated enzyme as 0 and 100% of maximum activity, respectively.

Improved purification of acidic protease proenzyme

Because previous procedures for the isolation of human seminal plasma acidic protease and its proenzyme gave very low yields [2,5], our first concern

TABLE I

IMPROVED PURIFICATION OF PROENZYME FORM OF HUMAN SEMINAL PLASMA ACIDIC PROTEASE

Acidic protease activity was assayed with acid-denatured hemoglobin as substrate [8] 1 unit is defined as the amount of enzyme required to cause an increase of 1 absorbance unit at 280 nm in 30 min Protein was quantitated by the method of Lowry et al [9]

	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Percent yield
Seminal plasma *	10 044	8289	1 2	(1)	(100)
Supernatant after dialysis	9134	5657	1 6	1 3	91
DEAE-cellulose ** column	6837	34	201	168	68
Sephadex G-100 ** column	5597	7	800	667	56

* Volume of seminal plasma used was 180 ml

** Routine assays of column fractions were performed by the turbidimetric milk clotting assay [7] After pooling, enzyme-containing pools were reassayed by the hemoglobin method [8]

TABLE II

STRUCTURAL CHANGES ACCOMPANYING ACTIVATION OF ACIDIC PROTEASE PROENZYME

The proenzyme (0 8 mg/ml) was incubated in 0 015 M sodium phosphate buffer, pH 3 1 at room temperature At the times indicated, samples were taken in triplicate One sample was neutralized and used for estimation of milk clotting activity [7] Another sample, after adjustment to pH 6 5, was used for measurement of fluorescence emission at 340 nm in an Aminco-Bowman spectrofluorimeter The third sample was subjected to SDS-polyacrylamide gel electrophoresis [11] gels are shown in Fig 4 Percent maximum milk clotting activity is shown as percent of activity of fully active enzyme obtained by preincubation of the same concentration of proenzyme with 0 01 N HCl for 30 min Proenzyme fluorescence is shown as a percent of the fluorescence emission intensity of a control sample preincubated with 0 015 M sodium phosphate buffer, pH 6 5 n d , not determined

Time (min)	Percent maximum milk clotting activity	Percent of proenzyme fluorescence
0 5	3 5	98 1
2	9 5	94 4
5	13 5	85 1
15	30 5	68 2
30	69	67 3
40	86	n d

has been to modify the previous procedure to obtain improved yields. Thus, preliminary experiments were carried out to evaluate the efficiencies of the steps that previously gave poor yields. The results obtained using seminal plasma indicated that $(NH_4)_2SO_4$ fractionation did indeed give a poor recovery (38%), but the yield from DEAE-cellulose chromatography was satisfactory (77%). The poor yields of 37% on DEAE-cellulose chromatography described previously [2,6] could be due to loss of activity during concentration with aquacide or time delays during chromatography.

From these preliminary studies, we have modified the published purification procedures [2] by omitting the $(NH_4)_2SO_4$ precipitation and Sephadex G-200 steps, by using dialysis and lyophilization for concentration, and by using the more rapid milk clotting assay (after activation) to screen columns, so that columns may be processed without any unnecessary delays. The resulting procedure involves three steps dialysis and clarification, DEAE-cellulose chromatography and Sephadex G-100 chromatography. The acid protease proenzyme is homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 1) and does not contain active enzyme unless previously activated (Fig. 4). Occasionally minor contaminants are present, but may be removed by repeating the Sephadex G-100 step.

For the purposes of comparison with earlier studies, samples of pools taken at each step were also analyzed for protein content by the method of

Fig 1 SDS-polyacrylamide gel electrophoresis of purified proenzyme (25 μg) Cylindrical 7 5% polyacrylamide gels were run according to the procedure of Weber and Osborn [10]

Lowry et al. [9] and for protease activity using the acid-denatured hemoglobin assay [8]. The results (Table I) indicate that by using the present modified procedure the specific activity of the final product (800 units/mg) is comparable to the 875 units/mg and 765 units/mg previously obtained for active enzyme [12] and proenzyme [6], respectively. In addition, a yield of up to 56% can be obtained (Table I), compared to yields of 9% and 10% previously described for active enzyme [2] and proenzyme [6]

Fluorescence spectra of acidic protease before and after activation

Amino acid analysis indicates that the proenzyme and active enzyme forms of human seminal plasma acidic protease contain 20 and 17 tyrosine residues/molecule, respectively, while the tryptophan content is still not known [3]. However, the fluorescence spectra of the proenzyme (Fig. 2) show maximum wavelengths for excitation and for emission at 280 and 340 nm, characteristic of proteins containing tryptophan [12]. When excited at 275, 290, 295 and 300 nm, the emission maximum remained at 340 nm. This suggests that emission is due to tryptophan alone, but it is likely that there is some energy transfer from tyrosine to tryptophan.

When the proenzyme is acidified to pH 2.7, there is a decrease in fluorescence intensity of approx. 31%, together with a decrease in the maximum wavelengths for excitation and emission of about 4—6 nm (Fig. 2). This fluorescence decrease upon acidification was accompanied by a 20.8-fold increase in milk clotting activity from 0.017 $s^{-1} \cdot ml^{-1}$ to 0.354 $s^{-1} \cdot ml^{-1}$. This fluorescence decrease observed upon activation is irreversible, since after neutralization to pH 6.5, the fluorescence of the activated enzyme was also approx. 30% less than that of control proenzyme incubated at pH 6.5 without previous exposure to acidic pH.

This irreversible quenching of intrinsic fluorescence suggests that the activa-

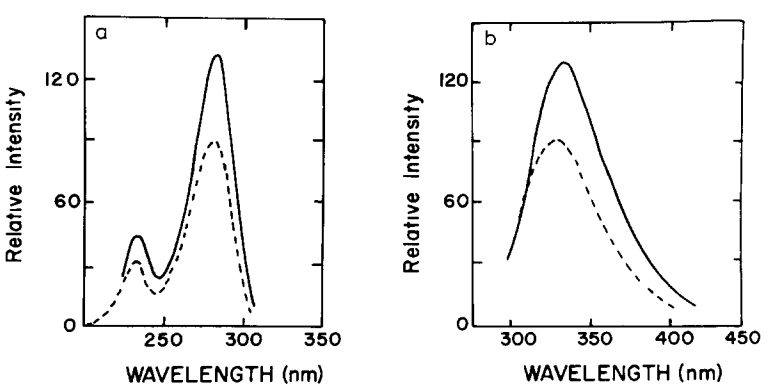

Fig 2 Fluorescence emission and excitation spectra of acidic protease proenzyme before and after activation Spectra were scanned in an Aminco-Bowman spectrofluorimeter an excitation wavelength of 280 nm was used for emission spectra and an emission wavelength of 340 nm was used for excitation spectra One sample of proenzyme was activated by incubation at pH 2 7 for 30 min, while another sample (at an identical protein concentration) was incubated at pH 6 5 a Excitation spectra ———, proenzyme after incubation at pH 6 5, - - - - - -, active enzyme formed after incubation of proenzyme at pH 2 7 b Emission spectra ———, proenzyme after incubation at pH 6 5, - - - - - -, active enzyme formed after incubation of proenzyme at pH 2 7

tion of the acidic protease proenzyme is accompanied by an irreversible change in the protein conformation, involving changes in the local environment of tryptophan residues in the molecule. A conformational change has also been shown to occur during the activation of pepsinogen [13—15]. Since structural data on acidic protease is still very limited, the precise nature of the conformational change is still a matter for speculation. However, it is interesting to note that the fluorescence quenching is associated with a blue shift, rather than a red shift as found in most proteins [6]. Two explanations for such a phenomenon come to mind. Firstly, there may be changes in the environment of more than one tryptophan residue, so that one or more may move to a more non-polar environment (giving a blue shift and fluorescence enhancement), while others move to a more polar environment (giving a red shift and fluorescence decrease). The latter tryptophan residues would contribute less in magnitude to the overall spectrum, so that the overall spectrum may shift to the blue and yet show decreased fluorescence. A second possibility is that certain tyrosine and tryptophan residues move further apart on activation, so that the efficiency of energy transfer is less, leading to decreased fluorescence. At the same time, because of the less efficient energy transfer, there may be some emission by tyrosine (which has a λ_{max} of 300—310 nm), so that the overall spectrum shifts to the blue. In this connection, it is interesting to note that amino acid analysis indicates that the acidic protease activation peptide contains three tyrosine residues [3].

Time course of activation of acidic protease at pH 3 1

The time course of the fluorescence and activity changes upon activation are shown in Fig. 3. The fluorescence intensity decreases very rapidly upon acidification during the first one or two minutes, and then decreases much more slowly over the next 30 min. In contrast, the amount of active enzyme formed increases quite steadily throughout the incubation period. Such a pattern suggests that the sudden fluorescence quenching reflects a conformational change that occurs before the acidic protease acquires activity.

Structural changes accompanying activation of acidic protease proenzyme

Another experiment was carried out to define more clearly the time interrelationships of various structural changes occurring during activation. Changes in tertiary structure were followed by measurement of intrinsic fluorescence, while the release of the activation peptide was followed by the appearance of the active enzyme band on gel electrophoresis. At the same time, the activation of enzyme, defined as percent of maximum milk clotting activity, was also followed. To ensure that the changes in intrinsic fluorescence observed are related to activation rather than due to general effect of acidity on protein conformation, solutions were readjusted to pH 6.5 before fluorescence measurement, so that only irreversible changes in fluorescence were detectable.

The results (Table II) again indicate a rapid decline in intrinsic fluorescence with time, so that within 5 min some 40—50% of the total fluorescence decrease has already occurred. During the same time period, less than 15% of the enzyme activity has been gained (Table II), while it was still not possible to detect the active enzyme band on gel electrophoresis (Fig. 4). However, by 15

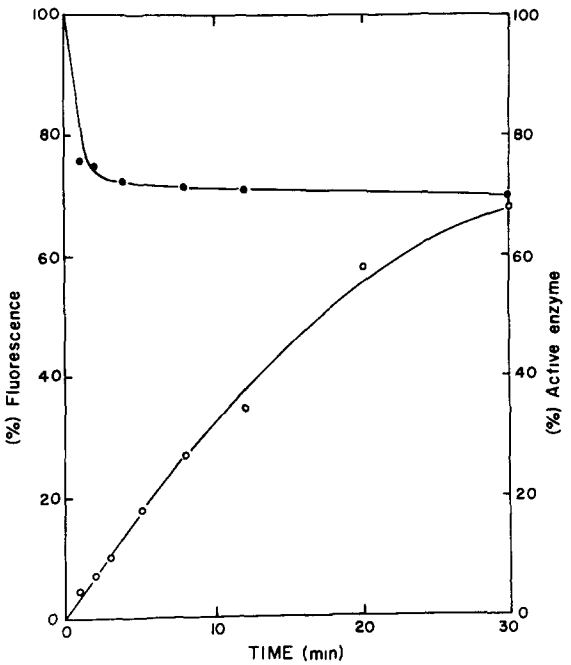

Fig 3 Time course of fluorescence and activity changes upon acidification of proenzyme Proenzyme solution was acidified to pH 3 1 At various times, fluorescence emission at 340 nm was measured at an excitation wavelength of 280 nm and samples were removed for assay of milk clotting activity [7] Fluorescence data are expressed as the fluorescence intensity at any given time as a percent of a control proenzyme solution incubated at pH 6 5. Percent maximum activity was calculated by assuming that proenzyme incubated at pH 6 5 had 0% of maximum activity and fully active enzyme obtained by incubation at pH 2 7 for 30 min and 100% of maximum activity ●———●, percent fluorescence relative to proenzyme, o———o, percent of maximum activity

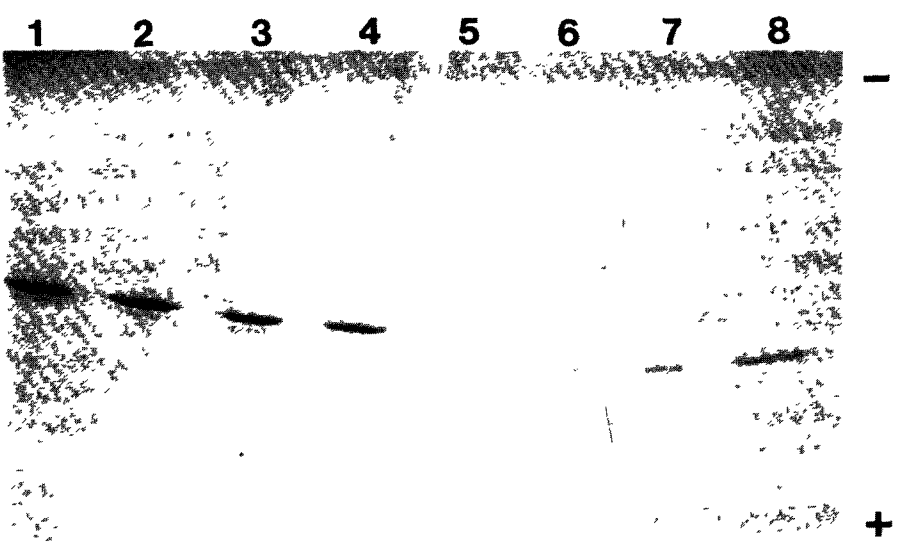

Fig 4 SDS-polyacrylamide gel electrophoresis of proenzyme after activation at pH 3 1 for various times Samples of proenzyme at various degrees of activation (from Table II) were run on a 7 5—15% polyacrylamide slab gel according to the procedure of Laemmli [11] 1 Unactivated proenzyme, 2 activated for 0 5 min, 3 activated for 2 min, 4 activated for 5 min, 5 activated for 15 min, 6 activated for 30 min, 7 activated for 40 min, 8 fully activated enzyme

min, when the fluorescence change is essentially complete and 30.5% of the maximum enzyme activity is obtained, some active enzyme is detectable by electrophoresis. Much of the gain in enzyme activity and much of the appearance of the active enzyme band on electrophoresis occurs between 15 and 30 min, a time period when there is very little fluorescence change.

The data reported here therefore indicate that acidification of human acidic protease proenzyme causes a change in protein conformation that precedes the release of the activation peptide. This conformational change presumably promotes peptide bond cleavage to yield the activation peptide and the active enzyme. During cleavage and release of the activation peptide, the conformation of the protein does not appear to change very much, since only very small changes in intrinsic fluorescence are observed. However, conformational changes that do not affect the environment of tyrosine or tryptophan residues cannot be ruled out.

Acknowledgements

This work was supported by a research grant from the Rockefeller Foundation. The assistance of Mrs. T. Vajrodaya in the preparation of this manuscript is also appreciated.

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