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Biochimica et Biophysica Acta 1207 (1994) 80–87

BBA Biochimica et Biophysica Acta

Heparin-induced structural and functional alterations of bovine trypsin

Paola Finotti *, Sabrina Manente

Department of Pharmacology, University of Padova, Largo E. Meneghetti 2, 35131 Padova, Italy

Received 20 January 1994

bstract

To investigate the mechanism whereby heparin can modulate the activity of serine proteinases, bovine trypsin was chosen as reference d treated with heparin at 10, 100 and 200 μg/ml, in buffer solvents, with and without incubation at 37°C. Heparin caused rapid, ffer- and pH-dependent decrease in trypsin solubility due to the generation of insoluble fragments from proteinase. Desalting treatments riously restored solubility by removing insoluble material. UV absorption and fluorescence emission spectra revealed significant parin-induced conformational alterations in the trypsin molecule, the maximal effect being apparent at a proteinase-to-heparin molar tio ranging from 1.6 to 1.0. The involvement of the catalytic sites of trypsin by heparin was further confirmed by the significant duction in the difference absorption spectra of proflavine. Both proteolytic and esterolytic activities of trypsin were shown to be arkedly decreased by heparin, especially after 5 h incubation at 37°C. However, when the proteolytic and esterolytic activities of trypsin ere measured on fresh solutions not submitted to desalting treatments, variable activation instead of inhibition of both activities was bserved in the presence of heparin, this effect waning spontaneously in time or after desalting treatment. The paradoxical increase in nctional activities was not inhibited by soybean trypsin inhibitor and was accompanied by denaturation and fragmentation of the oteinase as demonstrated by spectroscopic analyses and SDS-PAGE of fresh solutions. The results obtained indicate that heparin causes rapid, time- and temperature-dependent conformational alteration of trypsin with irreversible denaturation and degradation of the oteinase. The underlying mechanism appears to be heparin-catalyzed oxidative degradation of trypsin due to liberation of oxygen dicals which are also responsible for the temporary increase in catalytic functions.

ey words: Heparin; Trypsin; Inactivation; (Bovine)

. Introduction

The well-known antithrombotic effects of heparin are ostly linked to its ability to accelerate the reaction of erine proteinase inhibitors on circulating coagulation fac-ors [1–3]. The postulated mechanism involves heparin inding to both proteinase and inhibitor to form a stable ernary complex [2]. Differences in rate enhancement of roteinase inhibition are found related to either the struc-ural specificity of the inhibitor [4] or to heparin concentra-ions [4,5]. However, it has become apparent that heparin's effects on coagulation are more complex than expected on he basis of modulatory activity on serpins. Thus, it has been observed that heparin can also accelerate the inactiva-tion of antithrombin III by neutrophil elastase [6] and form a tight complex with it followed by either reduction in the elastolytic activity or impairment of the capacity of α1-proteinase inhibitor to inhibit this proteinase [7].

It has been proposed that a high-affinity binding site(s) is involved in the heparin-proteinase complex, similar to the heparin-binding site(s) on serpins [8,9]. Although with differences in affinity, binding to heparin has been demon-strated for thrombin [3,10], pancreatic elastase, cathepsin G and trypsin [7]. The activities of these proteinases have variously been found to be affected by heparin, and both activation and inhibition have been described in 'in vitro' experiments, depending on either heparin and substrate concentrations or experimental conditions [11,12]. Thus, the modulatory effects of heparin have been observed in kinetic studies on the amidolytic activity of thrombin, trypsin and plasmin [11]. Leucocyte elastase is either inhib-ited [7,8,12,13] or enhanced by heparin 'in vitro' [14]. The trypsin-like activity of the multicatalytic proteinase turns out to be greatly stimulated by heparin in a specific

Abbreviations: Serpins, serine proteinase inhibitors; Tos-Arg-OMe, tosyl-sulfonyl-L-arginine methyl ester; DNPP, dinitrophenylprotamine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide.

* Corresponding author. Fax: +39 49 831878.

SSDI 0167-4838(94)00049-M

manner [15], whereas inhibition of leucocyte-derived cathepsin G has been described over a wide range of heparin concentrations [9]. Additional 'in vivo' findings support the capacity of heparin significantly to lower circulating elastase activity in animals [12] and thrombin-induced fibrinopeptide A production in man [16].

Recently, the purification of a plasma-derived trypsin-like substance through heparin-affinity chromatography has raised the question of whether heparin changes the functional properties of the substance during the purification procedure [17]. To shed light on this intriguing problem an investigation was undertaken which started with 'in vitro' analysis of possible heparin-induced structural and functional modification of proteinase. Trypsin was therefore chosen as reference proteinase and treated with heparin in different experimental conditions. The results show that heparin rapidly induces a time- and temperature-dependent conformational alteration of trypsin, associated with its denaturation and fragmentation.

2. Materials and methods

2.1. Materials

Trypsin (TPCK treated, from bovine pancreas), heparin (from porcine intestinal mucosa, average molecular weight 14 000), 2,4-dinitrofluorobenzene, fluorescamine, protamine sulfate, Tos-Arg-OMe and proflavine were from Sigma (St. Louis, MO, USA). All other chemicals were reagent grade from Sigma.

2.2. Solution preparation and analysis

Trypsin (250 μg/ml 10.4 μM) was dissolved in both 20 mM Tris-HCl and 0.1 M phosphate buffer (pH 6.0 and 7.4), without (control) or with the addition of 10, 100 and 200 μg/ml (final concentrations) of heparin. Identical aliquots were either processed immediately (fresh solutions) or incubated for 5 h at 37°C in a stirring bath before analysis.

In separate experiments, aliquots of both fresh and incubated solutions were either filtered on Millipore Millex-FG filters (0.2 μm) or ultrafiltered (Amicon Centricon membrane mol. wt. cut-off 3000) before being analysed. Proteins were measured by the spectrophotometric method [18] and by Micro BCA protein assay (Pierce) [19]. Ultraviolet absorption and fluorescence spectra were taken on a Shimadzu UV-160 visible recording spectrophotometer and on Perkin-Elmer LS-3 spectrofluorimeter, respectively. Corrections for Raman bands and the buffer background signal were made by subtracting buffer spectra. No heparin fluorescence was noted in the measurement conditions.

The visible difference absorption spectra of proflavine plus trypsin, with or without heparin, were recorded on a Shimadzu UV-160 spectrophotometer at wavelengths of 500 to 350 nm. Experiments were performed in which variations of the fluorescence emission spectra of trypsin were recorded continuously at 25°C after adding increasing concentrations of heparin (titration curves). The initial volume was 2.5 ml and the heparin concentration ranged from 0.30 to 600 μg/ml.

2.3. Proteolytic activity

The proteolytic activity of trypsin was tested by the sensitive fluorescent assay for trypsin-like proteinases [20], as described previously [17]. The incubation medium comprised 50 μl of protamine sulfate whose amino terminal group was blocked by dinitrofluorobenzene (DNPP 10 mg/ml in phosphate buffer 0.1 M (pH 7.5)), 1 mM HCl, 4 and 8 μl of trypsin solution (10 μg/ml) to a final volume of 0.1 ml. After incubation at 37°C for 20 min, a 30 μl aliquot of each sample was added to 1.8 ml phosphate buffer (0.1 M (pH 7.0)). The addition of fluorescamine (0.6 ml of a 0.1 mg/ml acetone solution) developed fluorescence which was measured on a Perkin-Elmer LS-3 spectrofluorimeter at 390 nm excitation and 470 nm emission, after subtracting the fluorescence of incubated reagents alone. Control experiments were also performed with trypsin and heparin, added either separately or together to the fluorescamine solution without incubation on the DNPP substrate, in order to exclude the spontaneous liberation of amino groups. Incubation of heparin alone on DNPP was used as control to exclude the interfering fluorescence of heparin. Experiments were run in duplicate and proteolytic activity expressed as relative fluorescence (arbitrary units)/ng protein. The rate of hydrolysis was linear up to 20 min of incubation and for the range of protein concentrations used.

In separate experiments, soybean trypsin inhibitor was added to the incubation medium before the addition of trypsin and trypsin plus heparin and allowed to react throughout 20 min incubation at 37°C. Controls with the inhibitor alone were made to rule out interference by DNPP substrate or fluorescamine.

2.4. Esterolytic activity

Esterolytic activity was determined by the method of Siegelman et al. [21] modified as specified previously [22]. The reaction mixture (in duplicate) contained in a final volume of 1 ml was: Tos-Arg-OMe substrate (38–45 μmol in 0.1 M sodium phosphate buffer (pH 7.6) with NaCl 0.15 M), sodium phosphate buffer, 0.5 and 1.0 μg of trypsin. After 30 min incubation at 37°C in a stirring bath, the reaction was terminated by the addition of 0.5 ml of 15% (w/v) trichloroacetic acid. The formaldehyde formed by the oxidation of methanol released by the hydrolysis of Tos-Arg-OMe was measured colorimetrically at 580 nm vs. a blank with trichloroacetic acid and chromotropic acid

working reagent. Standard curves were made with 0.1, 0.2, 0.5 and 1.0 μmol of absolute methanol. Activity was expressed as μmol MeOH released/mg protein per min. The reaction was linear up to 30 min of incubation.

2.5. Electrophoresis

Electrophoresis was performed according to the method of Laemmli [23] on homogeneous (20%) and gradient SDS gel acrylamide stained with Coomassie brilliant blue. Sample proteins per lane were 25 μg and marker proteins were ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and lactalbumin (14.2 kDa).

2.6. Assay for superoxide production

Superoxide generation by trypsin (250 μg/ml) with 100 μg/ml heparin was measured as the reduction of acetylated cytochrome *c* (0.2 mg/ml) in 20 mM Tris-HCl (pH 7.4) followed at 550 nm using an extinction coefficient of 19.6 $mM^{-1} cm^{-1}$ for the reduced cytochrome [24]. The reaction was followed for 3 min on a Shimadzu UV-160 spectrophotometer.

2.7. Reduction of Nitroblue tetrazolium (NBT)

The reduction of NBT was determined by the modified fructosamine assay [25]. After dialysis samples of untreated (control) and heparin-treated trypsin were lyophilized and rehydrated with 0.3 ml NaCl 0.9%, incubated at 37°C for 10 min with 0.2 M sodium carbonate (pH 10.3), 480 μM NBT and uricase (> 2.5 kU/l). Measurements were made against a fructosamine calibrator (Roche, Italy) treated with the same reagents as above. Absorbance was red at 550 nm on the Hitachi 747 automated analyzer (Boehringer-Mannheim, Germany) against reagent blanks. The standard curve was linear up to 1000 μmol/l.

3. Results

3.1. Heparin-induced alterations of UV and fluorescence spectra of trypsin

The addition of heparin, even at 10 μg/ml, instantaneously conferred turbidity on trypsin buffered solutions at acidic pH, despite the fact that both trypsin and heparin were soluble in either buffer at concentrations 50-times higher than those used. Because of light scattering, solutions were either ultrafiltered (membrane mol. wt. cut-off 3000) or filtered (Millex-FG filters 0.4 nm) for clarification. The former method was used to recover material if trypsin underwent degradation. After filtration, the UV spectra of acidic trypsin solutions showed a concentration-dependent reduction by heparin of UV peak absorbance, whose pick was also shifted from 278 nm to 272 and 275 nm in the presence of 100 and 200 μg/ml heparin, respectively (data not shown). Similar results were obtained with Tris-HCl or phosphate buffer.

After ultrafiltration, the UV spectra of ultrafiltrates of heparin-treated solutions showed the presence of material. After incubation, the turbidity of trypsin solutions with heparin at acidic pH decreased significantly; desalting on incubated solutions caused even more marked reduction in UV spectra compared to those seen in the absence of incubation (data not shown).

At pH 7.4, a buffer-dependent effect on solubility of trypsin in the presence of heparin was apparent, partly conditioned by heparin concentration. Thus, whereas in phosphate buffer solubility was preserved at each heparin concentration, in Tris-HCl turbidity still persisted at 10 μg/ml, decreased at 100 μg/ml, and disappeared at 200 μg/ml heparin. Desalting treatment on pH 7.4-buffered trypsin solutions always gave a UV-spectra absorbance decrease inversely related to heparin concentration (data not shown).

To investigate in detail alterations following the trypsin-heparin interaction, the fluorescence emission spectra of fresh and incubated buffered trypsin solutions were recorded after excitation at 295 nm, specifically to detect the emission of tryptophan residues (peak at 330 nm). Given the sensitivity of fluorescence measurement which needs very low protein concentrations, the analysis was performed on undesalted solutions. As shown in Fig. 1 (panel A), a significant increase in the peak emission spectrum of tryptophan was apparent in trypsin solutions at pH 6.0 in the presence of heparin (Fig. 1, curves b,c,d) regardless of its concentration or buffer. At pH 7.4 (Fig. 1, panel B), only 10 μg/ml heparin caused an increase in peak emission at 330 nm, whereas 100 and 200 μg/ml heparin caused a reduction in the intensity of peak emission (Fig. 1, curves b,c,d).

Further support for the direct effect of heparin on trypsin came from the fluorescence emission spectra of trypsin continuously recorded after the addition of increasing heparin concentrations. The maximal change in peak fluorescence emission was taken as the titration curve end-point. Fig. 2 shows the emission spectra of trypsin without (spectrum 1) and with the addition of heparin 0.31 μg/ml (spectrum 2), 0.4 μg/ml (spectrum 3) and 5.0 μg/ml (spectrum 4), corresponding to final trypsin to heparin concentration (w/w) ratios of 25, 19 and 1.5, respectively. The maximum of about 23% reduction in peak emission (spectrum 4) was not significantly modified by increasing heparin concentrations up to a final ratio of 1.0. When this was shifted in favour of heparin (trypsin to heparin ratio from 1:5 to 1:90), the reduction in fluorescence emission was accompanied by a progressive red shift (peak at 350 nm) (data not shown). Since the spectrum of trypsin alone did not change in time, nor did heparin have any detectable effects on the spectrum of the solvent alone, the reduction in peak emission is consistent

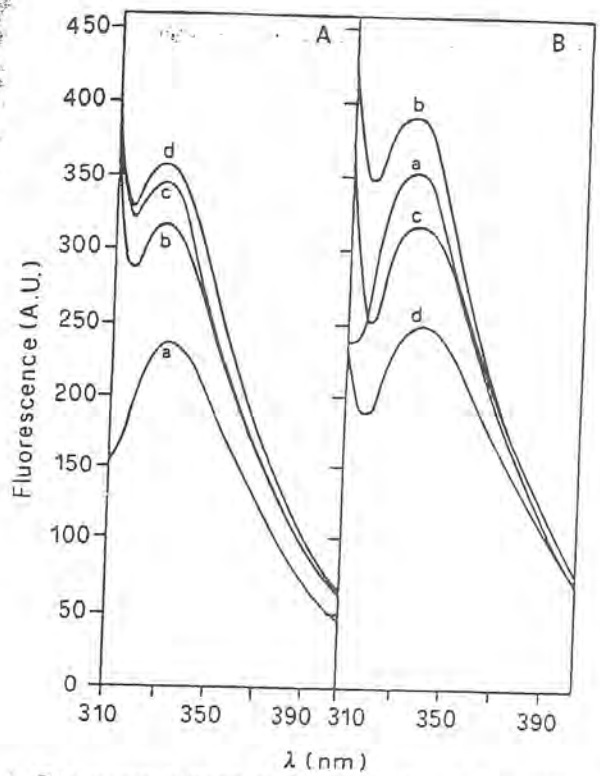

Fig. 1. Fluorescence emission spectra of trypsin solutions with and without addition of heparin. Fluorescence emission spectra (wavelenghts ranging from 310 to 400 nm) of fresh, undesalted solutions of trypsin (250 μg/ml) without (a, control) and with heparin at final concentrations of 10 μg/ml (b), 100 μg/ml (c) and 200 μg/ml (d) were recorded after excitation at 295 nm. Before analysis solutions were diluted to a final protein concentration of 15 μg/ml. (A) trypsin buffered solutions at pH 6.0. (B) trypsin buffered solutions at pH 7.4.

with heparin directly affecting the structural conformation of trypsin.

3.2. Effects of heparin on catalytic sites of trypsin

Since spectroscopic analyses showed marked alterations of trypsin after the addition of heparin, it was rational to suppose that the catalytic sites of trypsin were also involved in the interaction of heparin with trypsin. For this reason, both proteolytic and — the more specific — esterolytic activities of trypsin were measured both before and after solutions were submitted to desalting, in order to evaluate the influence of the latter variable on functional activity. Percentage variations of the proteolytic and esterolytic activities of trypsin in the presence of heparin at acidic pH are reported in Tables 1 and 2. The proteolytic activity of fresh trypsin solutions not submitted to desalting was found to be significantly increased after the addition of heparin, especially at 10 μg/ml (Table 1). Either desalting or incubation reversed the heparin-induced increase in activity into inhibition: this was about 23% and 53% after desalting and incubation, respectively, regardless of heparin concentrations (Table 1). A further dose-dependent inhibitory effect by heparin was observed when the solutions were submitted to both desalting and incubation (Table 1, inhibition 62–82%).

A similar trend of heparin-catalysed increase of trypsin activity on fresh undesalted solutions, converted into inhibition when the solutions were desalted or incubated, was observed on esterolytic activity (Table 2). However, unlike what was observed on proteolytic activity, incubation and

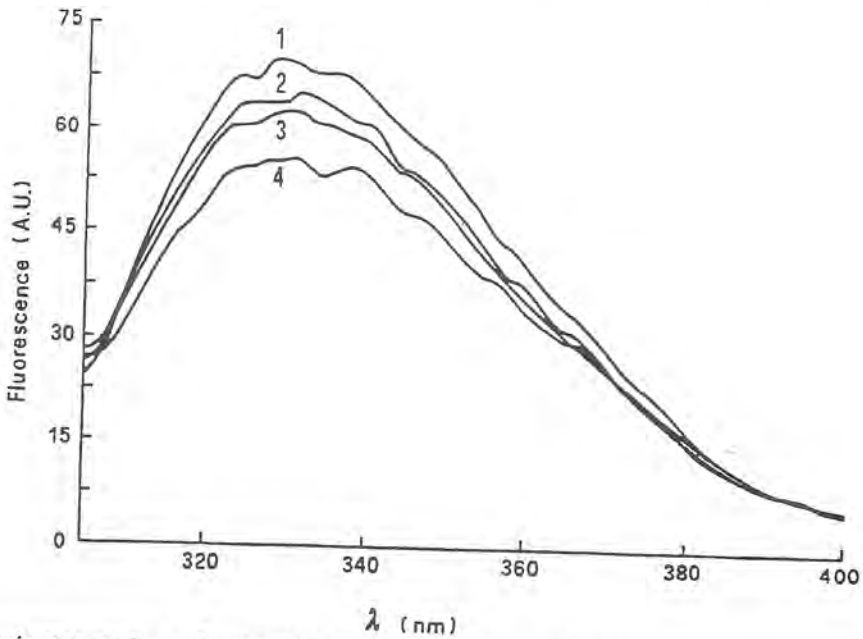

Fig. 2. Effect of varying heparin concentration on fluorescence emission spectra of trypsin. Fluorescence emission spectra were recorded continuously at wavelengths of 305 to 400 nm for excitation at 295 nm on trypsin (7.72 μg/ml) without (1, control) and after addition of increasing concentrations of heparin. Spectra 2, 3 and 4: trypsin with heparin 0.3 μg/ml, 0.4 μg/ml and 5.0 μg/ml, respectively.

Table 1
Percentage variations of proteolytic activity of acidic trypsin solutions in presence of heparin before and after incubation. Effects of desalting treatment.

Incubation/ desalting	Percentage activity variation + heparin (μg/ml)		
	10	100	200
No/No	+127	+81	+81
No/Yes	−23	−22	−24
Yes/No	−57	−50	−53
Yes/Yes	−62	−74	−82

Desalting comprised ultrafiltration performed as specified in Section 2 either before (fresh solutions) or after trypsin solutions (250 μg/ml) at pH 6.0 had been incubated for 5 h at 37°C in a stirring bath. Protein concentrations in incubation medium were 5.0 and 10 ng/ml. Percentages are calculated over controls of trypsin alone. Activities (A.U. fluorescence/ng prot) of controls ($\mu \pm$S.D. of three separate experiments in duplicate with either protein concentration) were: 208 ($\pm$3.3) and 150 ($\pm$1.5) for non-incubated solutions, without and with desalting respectively; 157 ($\pm$1.8) and 137 ($\pm$4.7) for incubated solutions, without and with desalting. Other experimental details in Section 2.

desalting did not worsen the heparin-induced inhibition of esterolytic activity observed after desalting without incubation (Table 2).

To shed light on the unexpected result of a marked heparin-dependent increase in trypsin activity when it was measured on fresh, undesalted solutions, experiments were set up in which the proteolytic activity of trypsin without (control) and with 100 μg/ml heparin was measured in the presence of three different concentrations of soybean trypsin inhibitor (Table 3). The inhibitor yielded similar dose-dependent enzyme activity inhibition in the absence of heparin, in both incubated and non-incubated trypsin solutions (Table 3), thus demonstrating that incubation did not substantially affect the availability of catalytic sites to react with the inhibitor. Instead, the proteolytic activity of

Table 2
Percentage variations of esterolytic activity of acidic trypsin solutions in presence of heparin before and after incubation. Effects of desalting treatment

Incubation/ desalting	Percentage activity variation + heparin (μg/ml)		
	10	100	200
No/No	+5	+36	+50
No/Yes	−42	−49	−56
Yes/No	−16	−33	−41
Yes/Yes	−27	−49	−50

Desalting was performed by ultrafiltration as specified in Section 2 either before (fresh solutions) or after trypsin solutions (250 μg/ml) at pH 6.0 had been incubated for 5h at 37°C in a stirring bath. Protein concentrations in incubation assay were 0.5 and 1.0 μg/ml. Percentages are calculated over controls of trypsin alone. Activities (μmol/mg prot. per min) of controls ($\mu \pm$S.D. of two separate experiments performed in duplicate with either protein concentration) were: 230 ($\pm$14) and 274 ($\pm$14) for non-incubated solutions, without and with desalting respectively; 171 ($\pm$13) and 189 ($\pm$15) for incubated solutions without and with desalting. Other experimental details in Section 2.

Table 3
Percentage inhibition by soybean trypsin inhibitor of proteolytic activity of acidic trypsin solutions with and without heparin

Trypsin (250 μg/ml)		Activity inhibition (%)		
Addition	Incubation	2:1	4:1	8:1
None (control)	No	21	61	80
	Yes	22	63	81
Heparin (100 μg/ml)	No	23	37	67
	Yes	21	51	70

Proteolytic activity was measured as specified in Section 2 on either fresh or incubated trypsin solutions at pH 6.0 without any desalting treatment. Soybean trypsin inhibitor (in water) was added to trypsin in incubation medium at final concentrations of 10, 20 and 40 ng/ml to obtain inhibitor to trypsin concentration ratios of 2:1, 4:1 and 8:1, respectively. Percentages are calculated over controls of trypsin without inhibitor. Activities of controls ($\mu \pm$S.D. of three separate experiments in duplicate with 5 ng/ml trypsin) were (A.U.fluorescence/ng prot.): 287 ($\pm$4.5) and 216 ($\pm$3.9) for trypsin without heparin in absence and presence of incubation, respectively; 433 ($\pm$5.2) and 179 ($\pm$4.4) for trypsin with heparin in absence and presence of incubation.

heparin-treated trypsin solutions, even in the presence of the higher concentrations of the inhibitor, was never found to be inhibited to the same extent as that observed in the absence of heparin, especially in non-incubated solutions (Table 3: inhibitions of 37% and 67% vs. 61% and 80%, respectively). Thus, the increase in catalytic activity observed in fresh, heparin-treated trypsin solutions is not related to the increased functioning of catalytic sites which, instead, appear to be reduced by heparin addition.

3.3. Measurement of difference absorption spectrum of proflavine

It is known that the acrydinic dye proflavine binds to catalytic sites of trypsin [26] and chymotrypsin [27], the enzyme-dye complex yielding an absorption spectrum with λ_{max} at a longer wavelength compared with the free dye, so that the maximum difference in optical density between free and complexed dye occurs at about 470 nm. Substances which bind to the same catalytic sites on proteinases displace the dye, causing an increase in free proflavine and reduction of the difference spectrum [26,27]. If heparin binds to trypsin, the loss of the trypsin–dye difference spectrum would be expected to occur in the presence of heparin. To perform this measurement, the lowest heparin concentration (10 μg/ml) was employed in order to avoid the known interference of heparin with proflavine [28]. As shown in Fig. 3, the characteristic difference absorption spectrum of proflavine-trypsin (a) was almost completely lost in the presence of heparin (b), this result confirming the profound alteration of the catalytic sites on trypsin.

3.4. Electrophoretic analysis

When samples of trypsin with and without heparin were processed by SDS-PAGE, it was apparent that, regardless

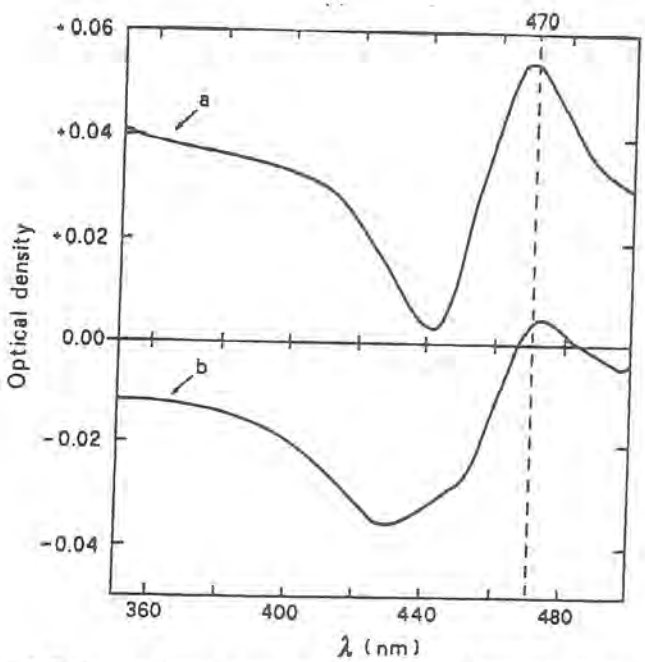

Fig. 3. Difference absorption spectra of proflavine with trypsin in absence and presence of heparin. Reference cuvette contained 10 μM proflavine in 0.1 M phosphate buffer (pH 8.0), 25°C. Samples contained in the same buffer solvent: proflavine plus either 10.4 μM trypsin (a) or trypsin (as in a) plus 10 μg/ml heparin (b). Analysis performed on fresh undesalted solutions.

of the buffer and pH of solutions, heparin caused concentration-dependent alterations in the number and mobility of trypsin bands (Fig. 4). Thus, whereas at low heparin concentrations high molecular weight bands of trypsin were observed together with disappearance of the main 24 kDa band (Fig. 4, lanes 2 and 5), the highest heparin

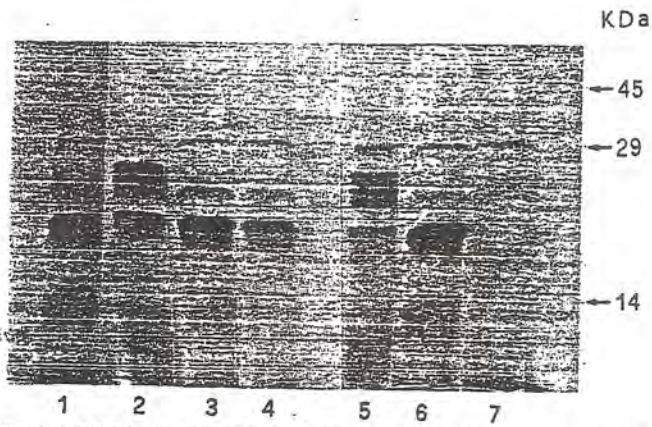

Fig. 4. SDS-PAGE of trypsin-buffered solutions in absence and presence of heparin. 25 μg proteins of buffered trypsin solutions at both pH 6.0 (lanes 2–4) and 7.4 (lanes 5–7) without (control, lane 1) and with 10 μg/ml (lanes 2 and 5), 100 μg/ml (lane 3 and 6) and 200 μg/ml heparin (lanes 4 and 7) were subjected to homogeneous 20% SDS-PAGE in nonreducing conditions and stained with Coomassie brilliant blue. Molecular mass markers (kilodaltons) on right: ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and lactalbumin (14 kDa).

concentration always yielded significant reductions in band number (Fig. 4, lanes 4 and 7): at pH 7.4 an almost empty lane was apparent (lane 7). The SDS-PAGE of incubated solutions showed even more significant heparin-related reductions of trypsin bands, suggestive of trypsin degradation into low molecular weight fragments which may elute from the bottom of the gel (data not shown).

3.5. Assay for superoxide production

The addition of heparin (100 μg/ml) to a Tris-buffered trypsin solution (pH 7.4) caused the reduction of acetylated cytochrome *c*, dA_{550}/dt being $3 \cdot 10^{-3}$ absorbance units per min. This value represents an increase of 0.5 nmol reduced cyt *c*/mg prot/min. The addition of superoxide dismutase (150 U/ml) to the mixture of trypsin, heparin and cyt *c* completely abolished the reduction of cyt *c* by heparin.

3.6. Reduction of NBT

Reduction of NBT is taken as indicating liberation of free oxygen radicals by reducing substances [29]. The addition of heparin enhanced NBT reduction, expressed as μmol fructosamine/μmol trypsin, which was 47%, 20% and 90% of control (untreated trypsin) with 10, 100 and 200 μg/ml heparin, respectively.

4. Discussion

The results of the present work show that heparin causes significant structural and functional alterations of trypsin, rapid in onset, the intensity of which is mostly dependent on buffer pH, time of incubation and, to a lesser extent, heparin concentration. Heparin causes an almost instantaneous reduction in trypsin solubility at pH 6.0 regardless of buffer, whereas solubility is restored either after solutions are submitted to desalting or at neutral pH dependent on buffer, and this fact indicates that insoluble, probably acidic, fragments of the proteinase rapidly form after the addition of heparin.

This possibility is also confirmed by separate observations (data not shown). First, after lyophilization, solutions of trypsin with heparin turned out to be insoluble even in the commonest solvents of trypsin such as formic and hydrochloric acid unless the solutions underwent prolonged dialysis. Comparison of the SDS-PAGE of these solutions before and after dialysis always showed a decrease in the number of trypsin bands in the latter. Second, after ultrafiltration, significant material was recovered in the filtrates of trypsin solutions with heparin. Third, the insoluble material separated by centrifugation from trypsin solutions with heparin turned out to be trypsin on amino-acid analysis and SDS-PAGE. Moreover, the solubility of this material was restored by the addition of 0.1 M phos-

phate buffer at pH 7.0. Thus, the absorbance decrease in UV spectra of trypsin after desalting and incubation is consistent with the loss of aromatic amino-acid residues due to the increased susceptibility to degradation conferred on trypsin by heparin. Likewise, the progressive reduction in the number and altered mobility of trypsin bands in SDS-PAGE in the presence of heparin (Fig. 4) is in keeping with the heparin-catalysed fragmentation of trypsin, with loss of lower molecular weight fragments from the bottom of the gel.

The apparent clarification of trypsin solutions with heparin in phosphate buffer at pH 7.4 cannot be explained in terms of a pH-dependent effect. Indeed, if an electrostatic interaction occurred between trypsin and heparin, then pH would be expected to restore solubility, independently of buffer. Instead, in Tris-HCl at neutral pH, precipitation still occurs, more significant at the lowest than at higher heparin concentrations. Moreover, both fluorescence emission spectra and SDS-PAGE heparin-induced alterations of trypsin either in Tris-HCl or phosphate at pH 7.4 did not differ significantly. This favours the possibility that, at neutral pH, buffer is mostly responsible for the observed differences. Thus, phosphate may simply mask or attenuate the heparin catalysed effects on trypsin by binding to sites to which heparin also binds, as demonstrated for antithrombin [30].

Separate results all indicate that heparin-trypsin interaction is rapid in onset and specific. First, the emission spectra of trypsin continuously recorded after the addition of increasing heparin concentrations (Fig. 2) show that at concentrations up to 100 μg/ml heparin directly modifies the structural conformation of trypsin, as evidenced by changes in tryptophan emission intensity. This experiment further confirms the results of fluorescence emission spectra made separately on different trypsin solutions (Fig. 1). Second, the difference absorption spectra of proflavine with trypsin measured after heparin was directly added to the proteinase (Section 2), prove that the catalytic sites of trypsin are no longer available for binding to proflavine in the presence of heparin (Fig. 3). The involvement of catalytic sites of trypsin in the conformational alteration induced by heparin was separately verified by the results of measurement of the functional activities of trypsin in the absence and presence of heparin. Both proteolytic and esterolytic activities were significantly reduced by heparin, this effect being crucially dependent on both incubation and desalting (Tables 1 and 2). The most striking result of these experiments was the paradoxical heparin-induced increase of functional activities of trypsin when measured on fresh, undesalted solutions (Tables 1 and 2). The possibility that this effect was due to increased functioning of catalytic sites on trypsin by heparin was contradicted not only by the spectroscopic and electrophoretic results (Figs. 1 and 4), but also by the finding that, at higher concentrations (20 and 40 ng/ml), the soybean trypsin inhibitor did not reduce proteolytic activity in the presence of heparin to the same degree as in the absence of heparin. This result, besides confirming the observation that availability of catalytic sites of trypsin is reduced, proves that this anomalous functional increase is unrelated to trypsin catalytic activity. Such a paradoxical effect may be explained by considering that heparin is able to catalyse trypsin degradation by a process similar to the oxidative degradation of proteins by reducing substances [31–33]. Results of experiments aimed at specifically investigating this topic demonstrate that solutions of trypsin with heparin reduce acetylated cytochrome and nitroblue tetrazolium, whereas inhibition is observed after the addition of superoxide dismutase. Thus, immediately after the heparin-trypsin interaction, reactive oxygen species may be liberated which start the oxidative degradation of trypsin; the same reactive species may also account for the apparent increase in the proteolytic activity of trypsin by enhancing the degradation rate of trypsin substrates. Desalting and incubation appear to enhance the rate of the denaturing process on the proteinase, also leading to more rapid decrease in free radical concentrations and to the removal of degradation products of trypsin.

Investigations are currently under way to reveal the fine mechanism underlying reactive oxygen species production after heparin addition to trypsin. Studies are also necessary to establish whether this heparin-catalysed mechanism of proteinase inactivation is specific for trypsin, or whether it may also account for the inactivation of other related serine proteinases by heparin.

References

[1] Heuch, C.C., Schiele, U., Horn, D., Fronda, D. and Ritz, E. (1985) J. Biol. Chem. 260, 4598–4603.
[2] Pletcher, C.H., Cunningham, M.T. and Nelsestuen, G.L. (1986) J. Biol. Chem. 261, 4143–4147.
[3] Olson, S.T. and Bjork, I. (1991) J. Biol. Chem. 266, 6353–6364.
[4] Pratt, C.W., Whinna, H.C. and Church, F.C. (1992) J. Biol. Chem. 267, 8795–8801.
[5] Pratt, C.W. and Church, F.C. (1992) J. Biol. Chem. 267, 8789–8794.
[6] Jordan, R.E., Nelson, R.M., Kilpatrick, J., Newgren, O., Esmon, P.C. and Fournel, M.A. (1989) J. Biol. Chem. 264, 10493–10500.
[7] Frommherz, K.J., Faller, B. and Bieth, J.C. (1991) J. Biol. Chem. 266, 15356–15362.
[8] Walsh, R.L., Dillon, T.J., Scicchitano, R. and McLennan, G. (1991) Clin. Sci. 81, 341–346.
[9] Evangelista, V., Piccardoni, P., Maugeri, N., De Gaetano, G. and Cerletti, C. (1992) Eur. J. Pharmacol. 216, 401–405.
[10] Olson, S.T., Halvorson, H.R. and Bjork, I. (1991) J. Biol. Chem. 266, 6342–6352.
[11] Petersen, L.C. (1983) Eur. J. Biochem. 137, 531–535.
[12] Redini, F., Lafuma, C., Hornebeck, W., Choay, J. and Robert, L. (1988) Biochem. Pharmacol. 37, 4257–4261.
[13] Redini, F., Tixier, J.-M., Petitou, M., Choay, J., Robert, L. and Hornebeck, W. (1988) Biochem. J. 252, 515–519.
[14] Lonky, S.A., Marsh, J. and Wohl, H. (1978) Biochem. Biophys. Res. Commun. 85, 1113–1118.
[15] Ohkubo, I., Gasa, S., Namikawa, C., Makita, A. and Sasaki, M. (1991) Biochem. Biophys. Res. Commun. 174, 1133–1140.

[16] Jones, R.L. (1985) Diabetes 34, 836–843.
[17] Finotti, P., Carraro, P. and Calderan, A. (1992) Biochim. Biophys. Acta 1139, 122–132.
[18] Stoscheck, C.M. (1990) Methods Enzymol. 182, 50–68.
[19] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[20] Brown, F., Freedman, M.L. and Troll, W. (1973) Biochem. Biophys. Res. Commun. 53, 75–81.
[21] Siegelman, A.M., Carlson, A.S. and Robertson, T. (1962) Arch. Biochem. Biophys. 97, 159–163.
[22] Finotti, P. and Verbaro, R. (1987) Clin. Chim. Acta 170, 121–134.
[23] Laemmli, U.K. (1970) Nature 227, 680–685.
[24] Azzi, A., Montecucco, C. and Richter, C. (1975) Biochem. Biophys. Res. Commun. 65, 597–603.
[25] Schleicher, E.D. and Vogt, B.W. (1990) Clin. Chem. 36, 136–139.
[26] Wong, R.F., Chang, T.-L. and Feinman, R. (1982) Biochemistry 21, 6–12.
[27] Bernhard, S.A., Lee, B.F., Tashjian, Z.H. (1966) J. Mol. Biol. 18, 405–420.
[28] Feinman, R.D. and Li, E.H.H. (1977) Fed. Proc. 36, 51–55.
[29] Mullarkey, C.J., Edelstein, D. and Brownlee, M. (1990) Biochem. Biophys. Res. Commun. 173, 932–939.
[30] Hall, P.K. and Roberts, R.C. (1989) Biochim. Biophys. Acta 993, 217–223.
[31] Stadtman, E.R. (1992) Science 257, 1220–1224.
[32] Hunt, J.V. and Wolff, S.P. (1990) FEBS Lett 269, 258–260.
[33] Wolff, S.P. and Dean, R.T. (1987) Biochem. J. 245, 243–250.