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Purification and characterisation of a plasminogen-binding protein from *Haemophilus influenzae*. Sequence determination reveals identity with aspartase

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Abstract

Plasminogen binding proteins have been described both for Gram positive and Gram negative bacteria. In the present work we describe the purification and characterization of a plasminogen binding protein from *Haemophilus influenzae* (strain HI-23459). Bacteria were sonicated in order to solubilize plasminogen-binding proteins. The supernatant was subjected to affinity chromatography on plasminogen kringle-4 fragment bound to Sepharose 4B and subsequently processed by ion-exchange chromatography on DEAE-Sepharose CL-6B. Characterization of the protein by SDS-PAGE displayed a single band with a molecular mass of about 55 000, both prior to and after reduction. The purified protein stimulates tPA (tissue plasminogen activator) catalysed plasminogen activation by a factor of approximately 300, mainly due to a decrease in K_m. Antibodies were raised in rabbits and used in quantitative and qualitative analysis. However, using a FITC-conjugate we failed to demonstrate the presence of the purified protein on the surface of intact bacteria. The corresponding gene was isolated from a λ EMBL3 phage library prepared from chromosomal DNA from the same *H. influenzae* strain, using an oligonucleotide probe based on the NH_2-terminal amino acid sequence. An open reading frame corresponding to 472 amino acid was found. The amino acid sequence of the translated gene demonstrates 97% identity with the recently published sequence from aspartate ammonia lyase (aspartase) from *H. influenzae*. Enzymatic analysis of the purified protein revealed a high aspartase activity.

Keywords: Plasminogen; Plasminogen activation; Aspartase; (*H. influenzae*)

1. Introduction

During the last decade it has been found that many somatic cells of different origin have the property to specifically bind plasminogen [1]. This binding is believed to be mediated through specific structures, which have been called receptors, although their physiological function is far from understood. A few years ago, our group was able to identify specific binding of plasminogen to many different types of bacteria, including both Gram-positive and Gram-negative species [2–6], following the initial observation by Lottenberg et al. of plasmin-binding to a strain of *Streptococcus pyogenes* [7,8]. Plasminogen binds to several bacterial species with high affinity and the dissociation constants, K_d, have been esti-

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mated as about 20–200 nM. The interactions are in most cases abolished by 6-aminohexanoic acid (6-AHA), suggesting that the lysine-binding sites in the plasminogen molecule are involved in the binding.

All strains of *H. influenzae* which have been investigated so far, have displayed this kind of property. In the present work we describe the purification and functional characterization of a plasminogen-binding protein from *H. influenzae* strain HI-23459. The corresponding gene was cloned and sequenced in both strands. The effect of the purified protein on the activation of plasminogen by urokinase or tPA was also studied.

2. Materials and methods

2.1. Reagents

CNBr-activated Sepharose 4B and DEAE-Sepharose CL-6B were purchased from Pharmacia AB (Uppsala, Sweden). Acrylamide, sodium dodecylsulfate (SDS), 4-chloro-1-naphthol and nitrocellulose sheets were from BioRad (Richmond, CA, USA). Aprotinin (Trasylol®) was from Bayer AG Leverkusen (Germany). Haemin and BHI broth was from Difco Laboratories (Detroit, MI, USA). Isovitalex® was from Becton Dickinson Microbiology System (Cockeysville, MD, USA). Flavigen-pli and tPA (lyophilised) were from Biopool (Umeå, Sweden). Urokinase (Ukidan®) was from Laboratories Sereno (Aubonne, Switzerland). The chromogenic substrate D-Val-Leu-Lys-pNA (S-2251) was from Chromogenix (Mölndal, Sweden). All other chemicals were of analytical grade and most of them were from Merck (Darmstadt, Germany).

2.2. Plasminogen and α_2-antiplasmin

Plasminogen was purified from human plasma [9], and plasminogen fragments was obtained from plasminogen as described elsewhere [10]. Kringle-4 from plasminogen was insolubilized on CNBr-activated Sepharose 4B as recommended by the manufacturer. About 5 mg of kringle-4 was bound per ml of settled gel. Plasmin was obtained from purified plasminogen and α_2-antiplasmin was purified from human plasma as described elsewhere [11].

2.3. Determination of protein concentration

The protein concentration was determined according to the method of Lowry [12] using bovine serum albumin (BSA) as a standard.

2.4. Purification of plasminogen-binding proteins

The *H. influenzae* strain HI-23459 [3] was cultivated over night in BHI broth, supplemented with 20 mg/l Haemin and 1% Isovitalex®. Bacteria (about 4 l of culture) was centrifuged, washed twice and suspended to a bacterial concentration of $2 \cdot 10^{11}$ cells/ml (typically about 100 ml) [13]. Subsequently, the bacterial suspension was sonicated for 3×4 min at 0°C, using a Sonifer cell disruptor B-30 (Branson, Switzerland) and thereafter centrifuged for 40 min at 4°C and $33\,000 \times g$. After addition of aprotinin (final concentration 500 KIU/ml) the supernatant was stored frozen at −70°C until processed further.

Prior to the affinity chromatography on plasminogen kringle-4-Sepharose, NaCl was added to the supernatant (final concentration 0.5 M). The column (5 $cm^2 \times 5$ cm) was equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl, 0.1 g/l Tween-80 and 5 units aprotinin/ml. The supernatant was applied to the column and the washing was continued with the equilibration buffer until $A_{280} < 0.1$. In order to remove NaCl the column was further washed with 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1 g/l Tween-80 and 5 units aprotinin/ml. Elution was carried out by 0.01 M 6-AHA in the phosphate buffer.

The eluted material from the affinity column was dialysed against 0.12 M sodium phosphate buffer, pH 7.3, containing 0.1 g/l Tween-80. The material was subsequently subjected to ion exchange chromatography on a DEAE-Sepharose CL-6B column (2.0 $cm^2 \times 5$ cm). Elution was performed with a linear gradient 0–0.3 M NaCl in 0.12 M phosphate buffer. The chromatograms were monitored by the absorbance at 280 nm. Screening for plasminogen-binding protein was also performed, using the slot-blot method as described. After getting a specific antiserum towards the 55 000 M_r plasminogen-binding protein, the chromatograms were also routinely screened by electroimmunoassay.

2.5. Dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by an established procedure [14]. The gels were stained with Commassie Brilliant Blue.

2.6. Immunochemical Analyses

Rabbit antiserum was produced by immunization of rabbits with the purified plasminogen-binding protein (about 200 μg in Freunds complete adjuvant). Booster doses with the same amount in Freunds incomplete adjuvant were given every third month. Double diffusion was performed in order to demonstrate that antisera were specific. Electroimmunoassay was performed according to Laurell [15].

2.7. Semiquantitative method for estimation of plasminogen-binding capacity

Each sample (200 μl) was applied to a nitrocellulose membrane, using a Bio-Dot SF apparatus for Protein Slot Blotting (Bio Rad). Subsequently it was treated with 100 mg/l dried milk dissolved in 0.01 M Tris-HCl, pH 7.3, containing 0.15 M NaCl. The membrane was incubated with a plasminogen solution (50 mg/l in 0.1 M sodium phosphate buffer, pH 7.3) for 1 h. Thereafter positive spots were detected with the aid of HRP-conjugated [16] goat anti-plasminogen IgG, properly diluted in the Tris-HCl buffer as above, but also containing 1 g/l BSA. After washing the membrane with the Tris-HCl buffer, peroxidase activity was visualized [17].

2.8. NH_2-terminal amino acid sequence analysis

NH_2-terminal amino acid sequence analysis [18] was performed in a pulsed liquid phase sequencer (model 477A, Applied Biosystems, Foster City, CA, USA) equipped with an on-line PTH 120 analyser.

2.9. Effect of the purified plasminogen binding protein on the activation of plasminogen

Activation of plasminogen in the presence or absence of plasminogen-binding protein was performed utilizing a coupled enzymatic system. Plasminogen (24 nM–13.2 μM), plasmin substrate (flavigen-pli, 0.4 mM), plasminogen activators (tPA or urokinase) and plasminogen-binding protein in various concentrations (0–0.22 mg/ml) were mixed in the wells of a quartz microtiter plate (HELLMA, Baden, Germany). The buffer used for dilutions was in all instances 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1 g/l Tween-80. The final concentration of urokinase was 2 IU/ml, whereas in the experiments with tPA the final concentration was 20 IU/ml. The absorbance at 405 nm was measured (Anthos labtec reader 2001) every 10 min for about 100 min. When the absorbance at 405 nm was plotted against time squared almost straight lines were obtained after a short lag phase (typically less than 10 min). The slopes of the curves were taken as a measure of the plasminogen activation rate.

2.10. Determination of aspartase activity

Aspartase activity was measured spectrophotometrically at 240 nm by determination of fumarate formation [19]. The assay system contained a mixture of $MgCl_2$ (3 mM) and sodium L-aspartate (0.1 M) in a Tris-HCl (pH 9) buffer and the reaction was initiated by the addition of the enzyme. The increase in absorbance at 240 nm was determined [20]. Aspartase activity was expressed in units, one unit being the amount converting 1.0 μmol of L-aspartase to fumarate per min at 30°C. The molar extinction coefficient of fumarate at 240 nm is 2530 $M^{-1} \times cm^{-1}$ [21].

2.11. Effect of the purified plasminogen binding protein on the plasmin / α_2-antiplasmin reaction

The influence of the purified plasminogen-binding protein on the rate of the reaction between plasmin and α_2-antiplasmin was studied by mixing plasmin (final concentration 4.1 nM) and α_2-antiplasmin (final concentration 5.9 nM) in the presence of a plasmin substrate (0.3 mM D-Val-Leu-Lys-pNA in 0.1 M sodium phosphate buffer, pH 7.0). The absorbance at 410 nm was continuously recorded (Uvikon 810 Kontron). The apparent rate constants were calculated from the classical formula for second-order reactions using the slopes of the curves at different time intervals [22].

2.12. Cloning and DNA sequencing

DNA was isolated by the method of Marmur [23] from *H. influenzae* cells which were cultivated and harvested as described earlier [3,13]. A genomic DNA library was constructed using the bacteriophage λ EMBL 3 (Stratagene, La Jolla, CA, USA). A mixture of 256 oligonucleotides, 17 bases long with the sequence 5′-G-C-C/T-T-C-T/C/A/G-G-C-T/C/A/G-G-G-T/C/A/G-A-C-G/A-T-C-3′ derived from the NH_2-terminal protein sequence, residues 14–19, was used as a hybridization probe both in Southern blot [24] and plaque hybridization techniques [25]. The hybridization and washing conditions was essentially as described by Sambrook et al. [26]. A 3.4 kb *Bam*HI/*Sal*I restriction enzyme fragment hybridizing to the oligonucleotide probe was isolated from one of the positive λ recombinants and subcloned into the plasmid vector pUC 18. DNA sequencing of the fragment was performed using the Taq DyeDeoxy™ terminator cycle sequencing kit for the 373 DNA sequencing system from Applied Biosystems, USA. The sequence was determined in both strands using the gene walking strategy.

3. Results

3.1. Purification of plasminogen-binding proteins from H. influenzae strain 23459

Purification of plasminogen-binding proteins from *H. influenzae* strain 23459 was performed as outlined in Section 2. A typical chromatogram of the supernatant after sonication of bacteria on Sepharose-bound plasminogen kringle-4 is demonstrated in Fig. 1. Regularly less than 5% of the total protein by weight was adsorbed and could be eluted with 0.01 M 6-AHA. The material eluted with 6-AHA was then subjected to ion exchange chromatography on DEAE-Sepharose CL-6B. Elution was performed by a linear gradient 0–0.3 M NaCl. The elution profile is demonstrated in Fig. 2. Approximately 30% of the protein passed the column unadsorbed, while one main peak was obtained (at a NaCl concentration of about 0.1 M) on the gradient elution.

The purification procedure was followed by the slot-blot method for semiquantitative estimation of

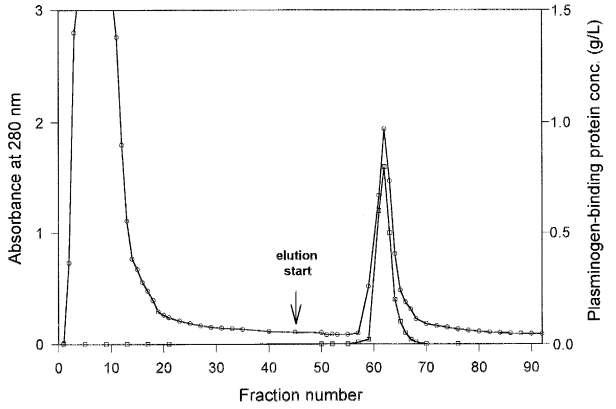

Fig. 1. Affinity chromatography on plasminogen kringle-4-Sepharose. The flow rate was 25 ml/h and 2.5 ml fractions were collected. Elution start with 6-AHA is marked by (↓). The absorbance at 280 nm was recorded (○) and the concentration of the 55 000 M_r plasminogen-binding protein was determined by electroimmunoassay (□).

plasminogen-binding proteins (see Section 2). Almost all plasminogen-binding capacity was adsorbed to the plasminogen kringle-4-Sepharose column (Fig. 3). Furthermore, more than 80% of the plasminogen-binding capacity found in the 6-AHA-eluate was adsorbed on the DEAE-Sepharose column and eluted by the gradient with NaCl. For this reason we have concentrated on investigating the properties of the purified major plasminogen-binding protein found in this eluate.

SDS-PAGE of the different fractions obtained during the purification procedure is demonstrated in Fig. 4. The protein peak eluting at about 0.1 M NaCl in the DEAE-Sepharose chromatography constitutes a single band with an apparent molecular mass of about 55 000 both prior to and after reduction. The main compound in the DEAE-Sepharose break-through fraction is a protein with a molecular mass of about 25 000. It is contaminated, however, with several low M_r compounds.

The purification procedure was monitored quantitatively using a specific rabbit antiserum. During the affinity chromatographic step on plasminogen kringle-4-Sepharose a 30-fold purification is obtained with a yield of about 50%. After this step the 55 000 M_r plasminogen-binding protein is more than 50% pure. After the final chromatography on DEAE-Sepharose the protein is completely purified with a total yield of about 20%. The results obtained during the

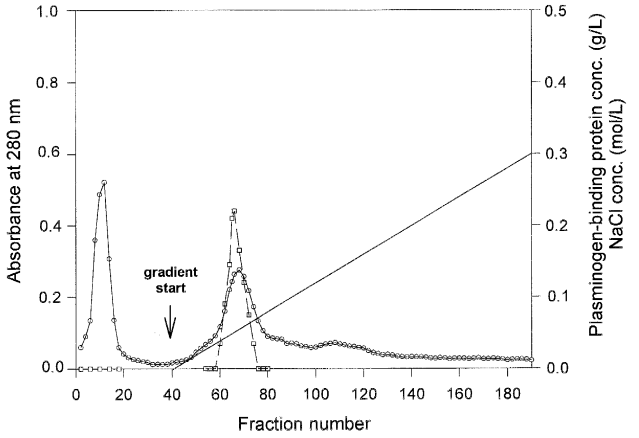

Fig. 2. Ion exchange chromatography on DEAE-Sepharose CL 6B. Elution was performed with a linear gradient 0–0.3 M NaCl in the equilibration buffer. The flow rate was 10 ml/h and 1.8 ml fractions were collected. The absorbance at 280 nm was recorded (○) and the concentration of the 55 000 M_r plasminogen-binding protein was determined by electroimmunoassay (□).

purification procedure are summarized in Table 1. Using a FITC-conjugate of the same antiserum we however failed to demonstrate the purified protein on the surface of intact bacteria.

NH_2-terminal sequencing by automatic Edman degradation for 35 steps displayed the following sequence: Thr-Gln-Phe-Arg-Lys-Glu-Val-Asp-Leu-Leu-Gly-Glu-Arg-Asp-Val-Pro-Ala-Glu-Ala-Tyr-Trp-Gly-Ile-His-Thr-Leu-Arg-Ala-Val-Glu-Asn-Phe-Asn-Ile-Ser-.

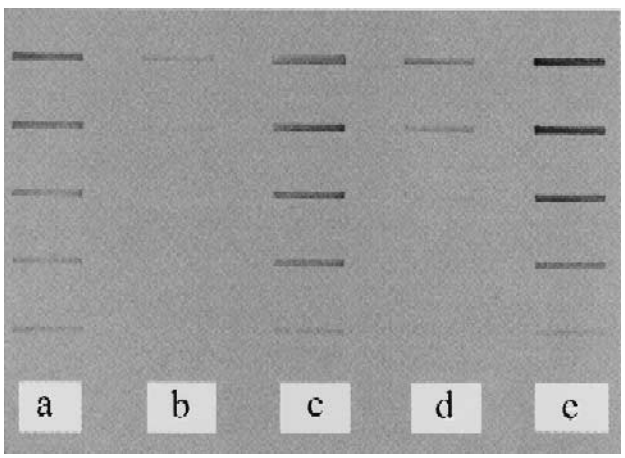

Fig. 3. Results from a typical slot-blot experiment. Samples in dilutions (1/10–1/1000) were applied to a nitrocellulose sheet. For experimental details, see Section 2. The following samples were applied: (a) starting material; (b) plasminogen kringle-4-Sepharose 'break through'; (c) 6-AHA eluate from this column; (d) DEAE-Sepharose chromatography 'break through'; (e) eluate from DEAE-Sepharose.

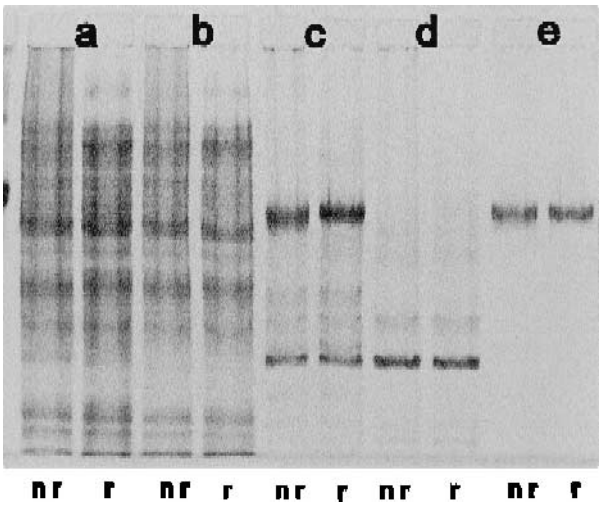

Fig. 4. SDS-PAGE of: (a) the starting material; (b) plasminogen kringle 4-Sepharose 'break through'; (c) 6-AHA eluate from this column; (d) DEAE-Sepharose 'break through'; (e) eluate from DEAE-Sepharose. The samples were run both non-reduced (nr) and reduced (r).

3.2. Effect of the purified plasminogen binding protein on the kinetics of plasminogen activation or on the reaction between plasmin and α_2-antiplasmin

The effect of purified plasminogen-binding protein on plasminogen activation by urokinase or tPA has been studied in coupled enzymatic systems. Addition of purified plasminogen-binding protein, in concentrations up to 0.22 mg/ml, resulted in a considerable, and concentration dependent, stimulatory effect when

Table 1
Protein distribution among the different fractions obtained during purification of the plasminogen-binding protein from *H. influenzae* strain HI-23459

Fraction	Total protein (mg)	PLG-binding protein (mg)	Aspartase activity (U)	Specific activity (U/mg [a])
Starting material	575	15.8	720 ± 36	1.2
K4-Sepharose	24.9	14.1	375 ± 43	15.1
DEAE-Sepharose	6.0	6.0	123 ± 7	20.5

[a] Specific activity (U/mg) is based on total protein.

tPA was used as activator (Fig. 5). In contrast, only a minor influence on urokinase mediated acivation was observed (data not shown).

The activation of plasminogen by tPA in the absence or in the presence of the plasminogen-binding protein (0.22 mg/ml) was studied at different plasminogen concentrations (24 nM–13.2 μM). According to Lineweaver–Burk plot of the data the reactions obeyed Michaelis–Menten's kinetics. Thus, in the absence of plasminogen binding protein a K_m of about 40 μM and a V_{max} of 0.086 nM plasmin/min were found. In the presence of 0.22 mg/ml of the plasminogen binding protein a K_m of 0.25 μM and a V_{max} of 0.21 nM plasmin/min were found.

The influence of purified plasminogen-binding protein on the reaction between plasmin and α_2-antiplasmin is demonstrated in Table 2. A concentration dependent effect was observed resulting in a 3.2-fold decrease in rate constant ($19 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ vs $6.0 \cdot 10^6$ $M^{-1} \cdot s^{-1}$) at the highest concentration of the purified plasminogen-binding protein studied (0.15 mg/ml).

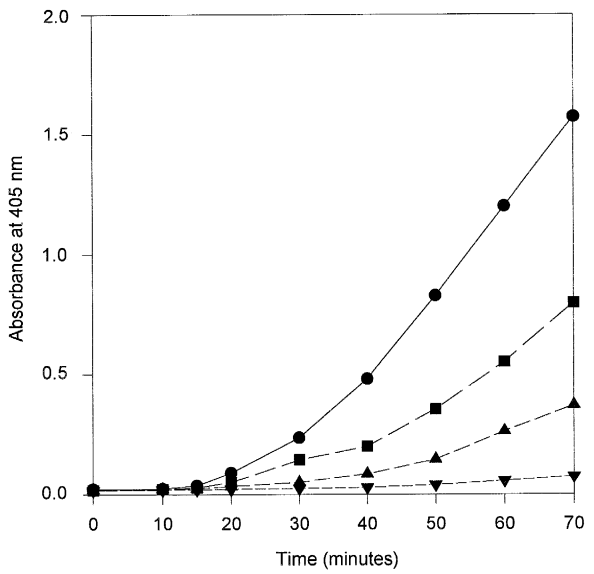

Fig. 5. Effect with the purified plasminogen binding protein on the activation of plasminogen by tPA. The experiments were performed using different concentrations of the plasminogen-binding protein: (▼) 0 mg/ml (▲) 0.02 mg/ml (■) 0.08 mg/ml (●) 0.22 mg/ml. The plasminogen concentration was 660 nM in all experiments.

Table 2
The influence of the reaction rate between plasmin and α_2-antiplasmin in presence of different concentrations of the purified plasminogen-binding protein

Plasminogen-binding protein (mg/ml)	Reaction rate $(M^{-1}\ s^{-1}) \cdot 10^6$ ($n = 4$)
0	19 ± 2.5
0.038	10 ± 1.5
0.075	7.0 ± 1.0
0.150	6.0 ± 0.9

3.3. Identification of the gene, transcriptional and translational signals

The cloning and sequencing of a 3.4 kb DNA fragment was performed as outlined in Section 2. A search for coding regions in the sequence was made using an option in the Staden package for computerized sequence analysis. An open reading frame of 1416 nucleotides was identified (Fig. 6). The reading frame was preceded by a short sequence resembling the ribosome binding site sequence of *Escherichia coli* [27]. The 5′- flanking region upstream of the proposed ribosome binding site shows the presence of a possible promoter. The sequence TATAAT at nucleotide number 68 in Fig. 6 show homology to the −10 region of the consensus sequence of *E. coli* promoters [28]. However no similarity could be observed in the −35 region. Aligning the putative promoter sequence with promoter sequences from other cloned genes from *H. influenzae* including the adenylate cyclase [29] and outer membrane protein P 1 [30] genes also indicate that the −10 region is conserved whereas the −35 region is not.

3.4. Protein structure and homology

The molecular mass of the predicted protein was calculated as 51.218. During the course of this work the entire sequence of the *H. influenzae* genome was published. The comparison of our sequence data with the published sequence of the *H. influenzae* aspartase gene revealed 95% identity, at the nucleotide level and 97% identity on the protein level. Pronounced identity on the protein level was also found with aspartases from other bacterial species including *E.*

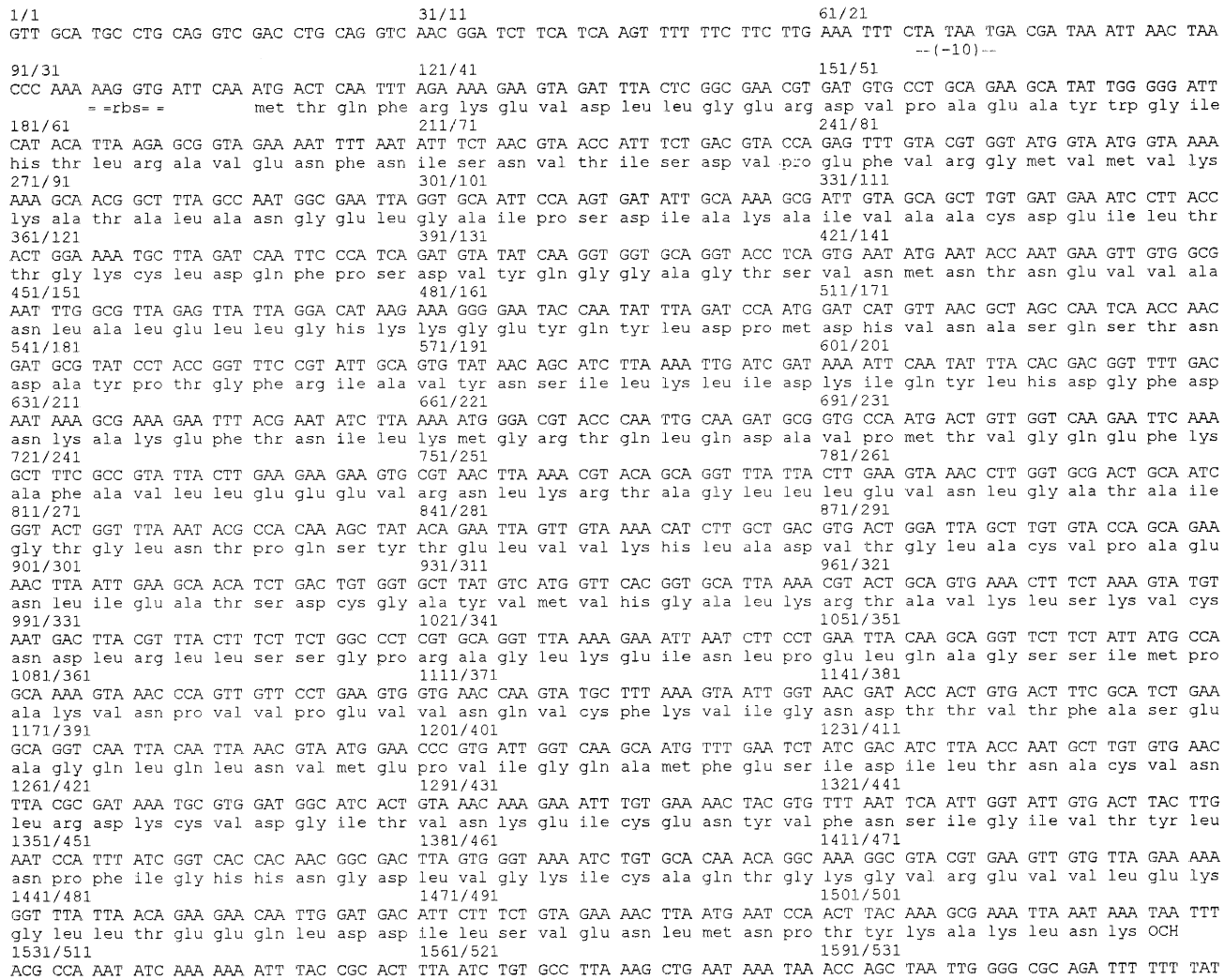

Fig. 6. Nucleotide sequence and the deduced amino acid sequence of the *phi* gene in *H. influenzae* strain HI-23459. Possible promoter region (---) and ribosome binding site (= = =) are shown.

coli (79%), *Serratia marcescens* (79%) and *Bacillus subtilis* (48%).

3.5. Aspartase activity

Due to the identification of the translated gene as aspartase, the aspartase activity was measured in the different fractions from the purification procedure. The results obtained are summarized in Table 1. Our results demonstrate that the plasminogen binding protein indeed is an aspartase.

4. Discussion

Many cells of different origins with invasive properties seem to trigger the fibrinolytic enzyme system using several different mechanisms. Mammalian cells such as macrophages or many tumour cells produce plasminogen activators (tPA or uPA) in high concentrations [31]. Certain bacteria such as β-haemolytic *Streptococci*, *Staphylococcus aureus* or *Yersinia pestis* produce very potent and unique plasminogen activators of their own [32,33]. For β-haemolytic *Streptococci* and *Yersinia pestis*, production of plas-

minogen activators seems to be related to virulence and the invasive behaviour of the bacteria [34,35].

Another mechanism seems to involve lysine-binding site mediated plasminogen-binding to the cell surface [36]. Regarding mammalian cells it has been found that carboxyterminal lysine residues in the receptor proteins play important roles in the interactions. The binding is typically quite weak with K_d values above 1 μM. Nevertheless, plasminogen bound to the surface of such cells seems to be somewhat more readily activated than free plasminogen. Also, the formed plasmin, which is still bound to the cell surface, is to some extent protected against inactivation by α_2-antiplasmin [36].

Recently we have demonstrated specific plasminogen-binding to a large number of bacterial species, both Gram-positive and Gram-negative [2–6]. In most cases plasminogen-binding is abolished in the presence of the lysine analogue 6-AHA, indicating that also here interactions via the lysine-binding sites in the plasminogen 'kringles' are involved [3,37]. The affinity between plasminogen and the presumed bacterial 'receptor proteins' seems to be much higher than for the corresponding proteins on mammalian cells, with dissociation constants, around 20–200 nM [2–6].

In the present work we have purified a plasminogen-binding protein from a strain of *H. influenzae* and cloned the corresponding gene. The purification was monitored by the use of specific rabbit antibodies enabling us to follow the purification procedure. The concentration of the purified protein in the starting material was almost 5% of the total protein content in the supernatant obtained after sonication of bacteria suggesting an important function. Sequence analysis identified the translated gene as aspartase which could also be verified by the demonstration of aspartase activity.

The identification of a plasminogen binding protein of *H. influenzae* as an enzyme is in agreement with the results with other plasminogen binding structures. Both plasmin binding of group A *streptococci* [8] and plasminogen binding of human cells [36] are mediated by membrane oriented metabolic enzymes. In all three cases the receptor molecule contains a C-terminal lysine residue that seems to be important for its plasminogen/plasmin binding function.

The purified 55 000 M_r plasminogen-binding protein is a potent stimulator of the tPA- but not of the urokinase-catalysed plasminogen activation, mainly through an about 200-fold decrease in K_m. This tPA-mediated plasminogen activation has also been found in experiments with whole bacteria [38]. Thus, the stimulation of plasminogen activation is much higher than what is achieved when plasminogen binds to mammalian cells. In fact, the data are quite similar to results obtained with the tPA mediated plasminogen activation using fibrin as a stimulator [39,40]. The data obtained suggest that plasminogen bound to plasminogen-binding protein through a lysine-binding site mediated interaction is more easily activated by tPA. The plasmin formed in this way, with some of its lysine-binding sites occupied, is in addition somewhat protected against the action of α_2-antiplasmin. This mechanism would provide the bacteria with a proteolytic tool, which might be of importance in bacterial invasion.

Using our specific antibodies we failed to detect the protein on the bacterial surface. This might imply that the protein is not exposed on the bacterial surface. Another possible explanation could be that our antibodies mainly recognise unexposed epitopes and that only a small region of the protein is exposed externally. The possible secretion of this protein has not been ruled out. However, the high cellular concentration of the protein will result in release of large amounts during bacterial lysis. Considering the impact of the protein in augmentation of fibrinolytic activity it could also this way play a role in bacterial virulence.

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