Volume 268, number 1, 129–132 FEBS 08684 July 1990

The epidermolytic toxins are serine proteases

Stephanie J. Dancer[1], Richard Garratt[2], Jose Saldanha[3], Harren Jhoti[4] and Robert Evans[1]

[1]*Department of Biochemistry, UMDS, Guy's Campus, St. Thomas's Street, London SE1 9RT, UK,* [2]*Institute de Fisica e Quimica de Sao Carlos, Departamento de Fisica en Ciencias dos Materiais, Universidade de Sao Paulo, Caixa Postal 969, 13560 Sao Carlos SP, Brazil,* [3]*Biomedical Computing Unit, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK* and [4]*Laboratory of Molecular Biophysics, The Rex Richards Building, South Parks Road, Oxford OX1 3QU, UK*

Received 21 March 1990; revised version received 31 May 1990

Certain strains of *Staphylococcus aureus* usually belonging to phage group II produce epidermolytic toxins (ETA and ETB) which cause intraepidermal splitting in mice, neonates and occasionally adults. Amino acid sequences of ETA and ETB have been reported but the mechanism of epidermolysis remains unknown. A search of the NBRF-PIR computer database showed the toxins to have significant sequence similarity with staphylococcal V8 protease and that the catalytic triad of V8 protease is present in ETA and ETB. Comparison of ETA, ETB and V8 protease with other members of the trypsin-like serine protease family revealed little homology save for the immediate vicinity of the residues constituting the catalytic triad. The toxins, therefore, exhibit a distant relationship to mammalian serine proteases. A potential Ca^{2+}-binding loop was identified in ETA (but not ETB) on the basis of sequence similarity with the second calcium-binding loop of rat intestinal calcium-binding protein. Epidermolysis produced by ETA in the mouse bioassay was shown to be inhibited by the presence of EDTA consistent with a Ca^{2+}-dependent mechanism.

Epidermolytic toxin; *Staphylococcus aureus*; Serine protease

1. INTRODUCTION

The clinical term for the condition induced by epidermolytic toxins (ET) is the Staphylococcal Scalded Skin Syndrome (SSSS) whereby fluid-filled blisters form, coalesce, and rapidly rupture leaving confluent patches of reddened skin resembling a first degree burn [1,2]. Staphylococci, colonising the nose or umbilical stump, produce ET which are absorbed and circulate systemically [3]. Clinical isolates may be screened for the production of toxin by an in vivo mouse bioassay [4].

Outbreaks occur in maternity units as newborn babies are particularly susceptible to the toxins [5]. The toxins reach the epidermis by diffusing from dermal capillaries and separation occurs in the stratum granulosum [6]. Histological appearance and site of action distinguish SSSS from other causes of blistering [7] but the mechanism of epidermolysis is unknown although it is assumed that intercellular cohesive forces, mostly generated by desmosomes, are disrupted. Beneath and attached to the desmosome lies a condensation of filaments which help maintain the cytoskeleton by forming a framework within the cell. The toxins bind to the filaggrin group of proteins [8] which support these filaments [9], and are therefore indirectly associated with the desmosome. In cantharide acantholysis (whereby intraepidermal blisters are produced), dissolution of the desmosomal plaque occurs, which can be inhibited by neutral serine protease inhibitors [10].

Original theories on the action of ET included possibilities that disappearance of intercellular vesicles heralded the release of a proteolytic enzyme which caused desmosomal disruption [6], or that the toxins themselves acted as trypsin-like enzymes [11]. However, addition of protease inhibitors to both in vitro and in vivo models of epidermolysis failed to inhibit epidermal splitting [12–15], and until now it was generally agreed that the toxins have no proteolytic activity [15,16]. This paper reports the finding of sequence similarity between the epidermolytic toxins and staphylococcal V8 protease and the subsequent attempt to link observed structural characteristics with biological function.

2. MATERIALS AND METHODS

2.1. *Databank search*

As the primary structures of the two toxins [17–19] share 40% sequence identity, it was decided to look for homology with proteins of known function. This was done by rapid scanning of the National Biomedical Research Foundation – Protein Identification Resource (NBRF-PIR) databank (version 20) using the program FASTP [20] with a *k*-tuple parameter of 1. Fig. 1 shows the final sequence alignment achieved with the program MULTALIGN [21] and small manual adjustments.

2.2. *Preparation of toxin*

Toxin was prepared from an epidemic strain of *Staphylococcus aureus* isolated during a hospital outbreak of SSSS [5]. The strain

Correspondence address: S.J. Dancer, Department of Biochemistry, UMDS, Guy's Campus, St Thomas's Street, London SE1 9RT, UK

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

typed Group II (3A, 3C) and was partially purified from a single colony inoculum by ammonium sulphate fractionation and ion-exchange chromatography [22]. 2 mg partially purified toxin product tested positive within 60 min in the in vivo mouse bioassay.

2.3. *Bioassay*

Bioassay was carried out by subcutaneous injection into the epidermis of 18-day-old hairless mice [4,23]. A positive Nikolsky sign (wrinkling or peeling of the skin) appearing 10–120 min later signified the presence of ET. A negative control was provided by *S. aureus* strain NCTC 8325 (10^9 cfu in 0.2 ml nutrient broth). Inhibitors di-isopropylfluorophosphate (DFP) (10 μl of 50 mM DFP in isopropanol), phenylmethanesulphonylfluoride (PMSF) (30 μl of 2 mg/ml PMSF in isopropanol) and chelating agent ethylenediaminetetraacetic acid (EDTA) (0.2 ml of 50 mM EDTA, pH 7.5) were added to solutions of toxin (2 mg partially purified toxin in 0.2 ml Tris 50 mM, pH 7.5) in an attempt to inhibit epidermolysis. Staphylococcal V8 protease (250 IU in 0.2 ml of Tris 50 mM, pH 7.5) was also inoculated into mice in a similar attempt to inhibit its effect on skin by adding DFP (10 μl of 50 mM DFP in isopropanol).

Inhibitors were added to toxin and V8 protease solutions 12 h before inoculation and allowed to stand at room temperature until injected. The mice originated from one litter, and the experiment repeated with different litters over a period of 3 months. Failure to observe epidermolysis within 240 min was regarded as a negative result, and the experiment terminated.

3. RESULTS AND DISCUSSION

The computer search revealed 25% sequence identity of both toxins to the sequence of V8 serine protease also from *Staphylococcus aureus*. Significant scores [21] for the alignment (after removal of the C-terminal tail of V8 protease, residues 217–268) were 11.4 and 10.6 standard deviations from the mean for ETA and ETB, respectively, after 100 sequence randomisations. V8 protease is a member of the trypsin-like serine protease family unusual in its specificity for cleavage on the C-terminal side of acidic amino acid residues and in possessing no disulphide bridges [24].

The residues of the catalytic triad, His-51, Asp-93 and Ser-169 (V8 protease numbering) are present in both toxins and are located in three of the regions showing the highest sequence conservation. The region of greatest sequence identity surrounds the catalytic serine and includes Gly-167 and Gly-170, which occupy regions of (ϕ, ω) space disallowed to residues possessing side chains in the 3-dimensional structures of known

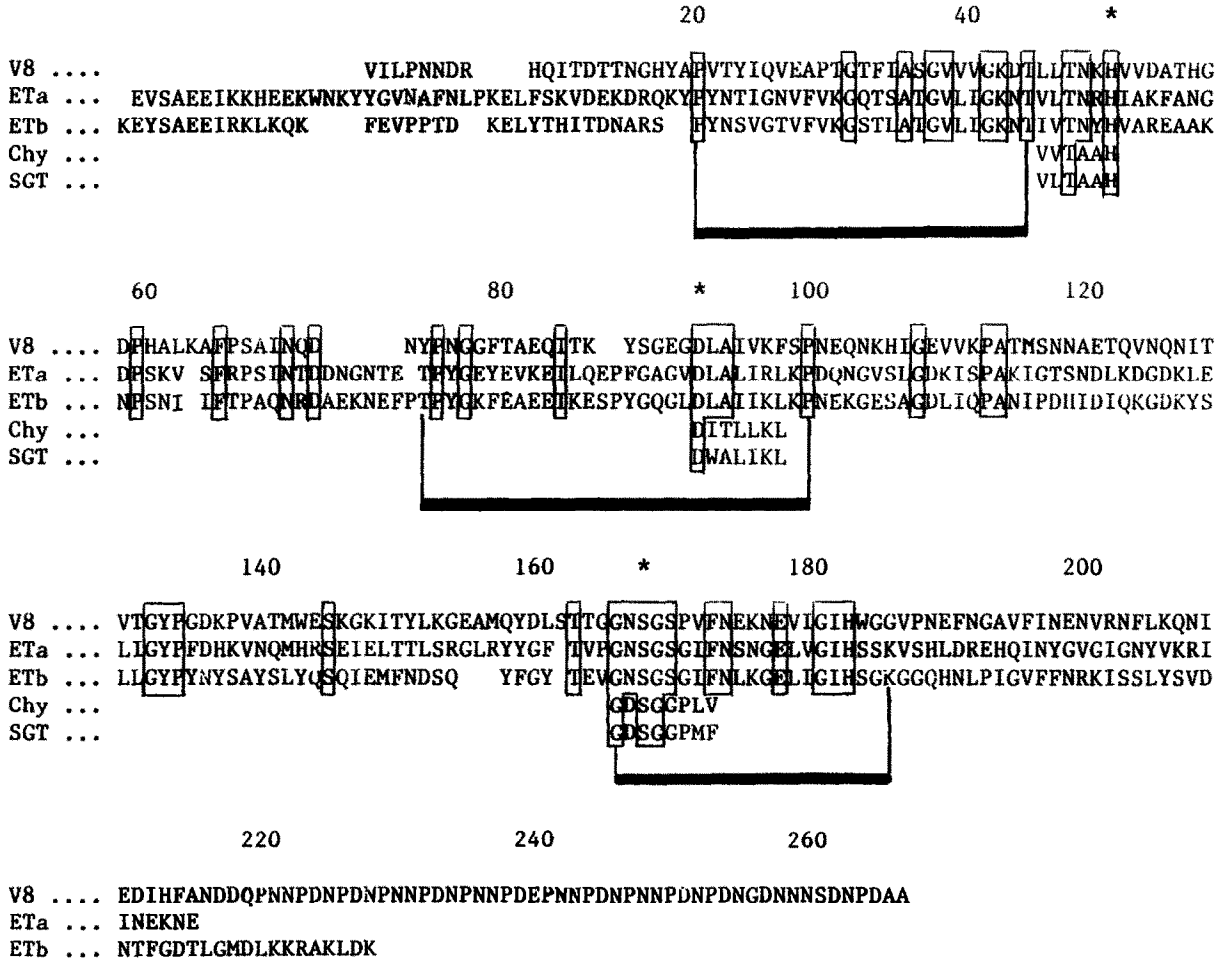

Fig. 1. Sequence alignment of ETA and ETB with the staphylococcal V8 serine protease. Many conservative substitutions are observed throughout the length of the molecules. Identical residues in all three sequences are boxed. Three highly conserved regions in the two toxin sequences are indicated by horizontal bars. Residues comprising the catalytic triad are indicated by an asterisk and the alignment with bovine chymotrypsin (Chy) and *Streptomyces griseus* trypsin (SGT) shown in the vicinity of these residues. Sequence similarity was identified using the NBRF-PIR protein sequence data bank using the program FASTP.

proteases. The catalytic triad of residues lies within or close to three regions originally identified as showing strong conservation between the two toxins (solid bars in Fig. 1) [17,18]. It is now possible to rationalise the existence of these conserved regions in terms of preservation of the protease active site. Little sequence similarity is observed to the sequences of mammalian serine proteases such as chymotrypsin or to other bacterial proteases such as *Streptomyces griseus* trypsin, save for the immediate vicinities of the catalytic residues (Fig. 1). However, this is sufficient to suggest a common evolutionary origin and it was concluded that ETA and ETB could be serine proteases.

Previous work has confirmed that the biological action of ETA, but not ETB, is dependent upon the presence of Ca^{2+} ions [25,26]. We therefore studied the structures of ETA and ETB in order to identify a possible calcium binding site present in ETA but not ETB. Scanning the sequence database revealed in the top 50 hits that residues 93–107 in ETA (residues 73–87 in Fig. 1) showed similarity with a region of rat intestinal/placental Ca^{2+}-binding protein (ICB) which contains its second EF-hand Ca^{2+}-binding loop [27,28] (see Fig. 2). All of the calcium ligating positions which are normally coordinated through side chain oxygens in EF-hand motifs [29] are similarly occupied by oxygen-bearing side chains of appropriate length in ETA, the only exception being position 9 which is frequently replaced by a water molecule [28]. Threonine at position 6 in ETA replaces glycine observed in the majority of such motifs [28,30], but variation can occur at this position, e.g. the first EF-hand loop of ICB (glycine replaced by proline and asparagine). In such a case, glycine at position 4, also seen in ETA, is considered obligatory [28] in order for the loop to bind calcium. The ETB sequence fulfills less criteria for an EF-hand motif and is not likely to bind Ca^{2+}.

The three-dimensional structure of trypsin-like serine proteases is based on two domains each containing a β-barrel usually of six strands and little α-helical content. The ETA Ca^{2+}-binding loop is probably not flanked by α-helices as in the classical EF-hand, but by β-strands or β-turns as recently observed in galactose-binding protein [30].

Incubation of toxin with serine protease inhibitors DFP and PMSF prior to subcutaneous inoculation in mice failed to inhibit epidermal splitting, but the time taken for epidermolysis to occur was prolonged when an inhibitor was present (Table I). Larger doses of DFP/PMSF, which may have produced total inhibition, caused toxic effects in the mice and death occurred before a positive/negative result could be obtained. Complete inhibition was obtained using ethylenediaminetetracetic acid (EDTA) and this effect was reversed with the addition of calcium chloride. The epidemic strain of *S. aureus* from a hospital outbreak thus produces ETA. Inoculation of V8 protease into mouse skin caused a non-specific breakdown of the epidermis which was completely inhibited by DFP.

In conclusion, we suggest that the epidermolytic toxins are members of the trypsin-like serine protease

```
                1  2  3  4  5  6  7     8  9  10  11  12
                *     *     *     +        #         |
ICB 57          D  K  D  G  D  G  E     V  S  Y   E   E   F  E  V   71
ETA 93          D  D  N  G  N  T  E     T  P  Y   G   E   Y  E  V   107
ETB 86          D  A  E  K  N  E  F  P  T  P  Y   G   K   F  E  A   101
```

Fig. 2. Sequence similarity of residues 57–71 of the rat intestinal/placental Ca^{2+}-binding protein (ICB) to a region of ETA as identified by scanning the NBRF-PIR databank using the program FASTP. Positions 1–12 constitute the Ca^{2+}-binding loop and the symbols indicate Ca^{2+}-binding residues. *, indicates a monodentate side chain ligand. +, indicates coordination through a main chain carbonyl. #, indicates coordination through a side chain oxygen or frequently a water molecule. —, indicates generally bidentate coordination through a glutamate side chain. A shorter or uncharged side chain at this position is unable to coordinate Ca^{2+} through two oxygen molecules. The sequence for ETB is given for comparison. It is unlikely to bind calcium due to a longer side chain at position 3 and the absence of glutamic acid at position 12. The loop in ETB also includes an insertion after position 7.

Table I

The table shows the effect of protease inhibitors DFP, PMSF and chelating agent EDTA on the time taken in minutes for epidermolysis to occur using the in vivo mouse bioassay following subcutaneous inoculation with partially purified toxin [4]. Also included is the time taken for skin reaction to occur following inoculation with V8 protease and the effect produced by adding DFP

	Epidermolysis		Non-specific skin reaction
	Control NCTC 8325[a]	Toxin[b] (2 mg)	V8 protease[c] (250 IU)
Test	>240	60	100
DFP			
10 μl of 50 mM DFP in isopropanol	not tested	120	>240
PMSF			
30 μl of 2 mg·ml^{-1} PMSF in isopropanol	not tested	150 (poor positive)	not tested
EDTA			
0.2 ml of 50 mM EDTA pH 7.5	not tested	>240	not tested
EDTA/Ca^{2+}			
0.2 ml of 50 mM EDTA + 50 mM $CaCl_2$, pH 7.5	not tested	65	not tested

[a] Non-toxin producing *Staphylococcus aureus* (10^9 cfu in 0.2 ml nutrient broth)

[b] Partially purified toxin (2 mg in 0.2 ml Tris buffer (50 mM) pH 7.5) from epidemic strain isolated during hospital outbreak [5]

[c] 250 IU V8 protease in 0.2 ml of Tris buffer (50 mM) pH 7.5

No skin reaction occurred following subcutaneous inoculation with the following: 0.2 ml of EDTA 50 mM/$CaCl_2$ 50 mM, pH 7.5; 0.2 ml of Tris 50 mM, pH 7.5 + each of 40 μl isopropanol/5 μl of 50 mM DFP in isopropanol/5 μl of 1 mg·ml^{-1} PMSF in isopropanol

family, and together with V8 protease represent some of the most distinct relations to mammalian enzymes so far identified. They possess all the catalytic apparatus necessary for protease activity. We therefore propose that the toxins exert a proteolytic effect which is ultimately responsible for the disruption of the cohesive elements necessary for cell adhesion, although as yet, knowledge of the natural substrate is unavailable. These results also have implications for the physiological mechanisms involved in cellular adhesion and are therefore relevant to dermatological studies on other blistering diseases.

Acknowledgements: S.J. Dancer is supported by a grant from the Special Trustees of Guy's Hospital. The authors would like to acknowledge the help given by Professor W.C. Noble with the mouse bioassay.

REFERENCES

[1] Melish, M.E. and Glasgow, L.A. (1971) J. Paediatr. 78, 958–967.
[2] Elias, P.M. (1974) Arch. Dermatol. 110, 295–296.
[3] Melish, M.E., Chen, F.S., Sprouse, S., Stuckey, M. and Murata, M.E. (1981) in: Staphylococci and Staphylococcal Infections (Jeljasziewicz, J. ed.) pp. 287–298 (Zbl. Bakt. Suppl. 10), Gustav Fischer-Verlag, Stuttgart, New York.
[4] Melish, M.E. and Glasgow, L.A. (1970) N. Engl. J. Med. 282, 1114–1119.
[5] Dancer, S.J., Simmons, N.A., Poston, S.M. and Noble, W.C. (1988) J. Infect. 16, 87–103.
[6] Lillibridge, C.B., Melish, M.E. and Glasgow, L.A. (1972) Paediatrics 50, 728–738.
[7] Lyell, A. (1979) Br. J. Dermatol. 100, 69–86.
[8] Smith, T.P. and Bailey, C.J. (1986) FEBS Lett. 194, 309–312.
[9] Dale, B.A., Holbrook, K.A. and Steinert, P.M. (1978) Nature 276, 729–731.
[10] Bertaux, B., Prost, C., Heslan, M. and Dubertret, L. (1988) Br. J. Dermatol. 118, 157–165.
[11] Wuepper, K.D., Dimond, R.L. and Knutson, D.D. (1975) J. Invest. Dermatol. 65, 191–200.
[12] McLay, A.L.C., Arbuthnott, J.P. and Lyell, A. (1975) J. Invest. Dermatol. 65, 423–428.
[13] Elias, P.M., Fritsch, P. and Epstein, E.H. (1977) Arch. Dermatol. 113, 207–219.
[14] Nishioka, K., Katayama, I. and Sano, S. (1981) J. Dermatol. 8, 7–12.
[15] Takiuchi, I., Kawamura, M., Teramoto, T. and Higuchi, D. (1987) J. Infect. Dis. 156, 508–509.
[16] Kondo, I. (1976) in: Staphylococcal Exfoliatin A and B (Kondo, I. ed.) pp. 82–95 (Saikin dokuso Kenkyu) 1st edn, Kyouritsu Press, Tokyo.
[17] O'Toole, P.W. and Foster, T.J. (1987) J. Bacteriol. 169, 3910–3915.
[18] Lee, C.Y., Schmidt, J.J., Johnson-Winegar, A.D., Spero, L. and Iandolo, J.J. (1987) J. Bacteriol. 169, 3904–3909.
[19] Sakurai, S., Suzuki, H. and Kondo, I. (1988) J. Gen. Microbiol. 134, 711–717.
[20] Lipman, D.J. and Pearson, W.R. (1985) Science 227, 1435–1441.
[21] Barton, G.J. and Sternberg, M.J.E. (1987) J. Mol. Biol. 198, 327–337.
[22] Dancer, S.J., Ogwang, W. and Evans, R.W. (1990) Purification of Epidermolytic Toxin using Fast Protein Liquid Chromatography (in preparation).
[23] Arbuthnott, J.P., Kent, J. and Noble, W.C. (1973) Br. J. Dermatol. 88, 481–485.
[24] Drapeau, G.R. (1978) Can. J. Biochem. 56, 534–544.
[25] Sakurai, S. and Kondo, I. (1978) Jpn. J. Med. Sci. Biol. 31, 208–211.
[26] Kondo, I., Sakurai, S. and Sarai, Y. (1976) in: Staphylococci and Staphylococcal Diseases (Jeljaszewicz, J. ed.) pp. 489–498, Gustav Fischer-Verlag, Stuttgart.
[27] MacManus, J.P., Watson, D.C. and Yaguchi, M. (1986) Biochem. J. 235, 585–595.
[28] Szebenyi, D.M.E. and Moffat, K. (1986) J. Biol. Chem. 261, 8761–8777.
[29] Kretsinger, R.H. and Nockolds, C.E. (1973) J. Biol. Chem. 248, 3313–3326.
[30] Vyas, N.K., Vyas, M.N. and Quiocho, F.A. (1987) Nature 327, 635–638.