The esterolytic activity of epidermolytic toxins

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The two epidermolytic toxins were shown to have intrinsic *N*-t-butyloxycarbonyl-L-glutamic acid α -phenyl esterase activity. The activity was dependent on free toxin pK_a values of 6.6 and 6.8 for ETA and ETB respectively. ETB incorporated 0.97 mol of radiolabelled di-isopropyl phosphorofluoridate/mol of protein with loss of esterolytic and epidermolytic activities. The correspondence of epidermolytic and esterolytic activities in ETA and ETB during thermal inactivation and reaction with di-isopropyl phosphorofluoridate, together with the inactivity of the mutant protein ETA S195G, demonstrates that the two activities are dependent on a single active serine residue in each protein.

INTRODUCTION

The epidermolytic toxins are two proteins from *Staphylococcus aureus*, ETA (M_r 26951) and ETB (M_r 27318), that cause skin-splitting in man and other animals [1]. A clinical manifestation observed in neonatal animals is the 'staphylococcal scalded skin syndrome', a collective term that describes a range of blistering conditions, characterized by painful erythema, widespread loss of epidermis and denudation of the body [2].

Modern studies of the toxins followed rapidly on the development of a mouse assay [3], which requires observation of the 'Nikolsky sign' (an irreversible skin-splitting on stroking the epidermis). Such an end-point assay is very sensitive, certainly to less than 0.5 μ g [1,2], but by its nature is slow and imprecise. For example, it has been shown that ETA and ETB have the same specific epidermolytic activity [4], but a confidence interval for the measurement cannot be readily given. The need for large numbers of experimental animals has deterred experimenters from making the animal assay exact. The epidermolytic effect can also be observed on neonatal mouse skin *in vitro* [5]. Such an assay may be more precise, and it has now been shown that a 2-fold difference in epidermolytic activity in the range corresponding to 0.5–10 μ g of toxin is detectable with greater than 99 % confidence [6]. Since ETA and ETB are indistinguishable in the assay performed *in vitro*, the result implies that their specific activities differ by less than 2-fold. Such an assay is only semi-quantitative. The assay *in vitro* is more suitable for studying the activity requirements of the toxins. It has been used to demonstrate that toxicogenesis is remarkably indifferent to inhibitors of energy metabolism, central metabolic pathways, receptor binding and proteolysis [6].

Following isolation, the nucleotide sequences of the toxins were established by three groups of workers [7–9]. The sequence similarity between ET and staphylococcal serine proteinase [10,11] became apparent when the sequences of all the proteins were incorporated into the standard data bases. ETA and ETB share about 40 % identity in amino acid sequence; between each of the toxins and staphylococcal proteinase there is about 25 % identity. Evidence for a homologous relationship is that the catalytic triad His-51, Asp-93 and Ser-169 of staphylococcal proteinase [10,11] align respectively with His-72, Asp-120 and Ser-195 of ETA and His-65, Asp-114 and Ser-186 of ETB [12]. The existence of a relationship was confirmed in one case [12] by demonstrating that the ETA putative active-site serine-195 did

react specifically, although incompletely, with radiolabelled di-isopropyl phosphorofluoridate, and in another [13] by observing an inhibitory effect with that active-site reagent. The importance of serine-195 in epidermolysis has now been demonstrated unequivocally by constructing the inactive mutant form ETA S195G [14].

In spite of the strong circumstantial evidence associating ET with proteolysis, the direct evidence, on the basis of biochemical assays with casein substrate [12,15] or sensitive plate tests [16,17] is that the toxins have no proteolytic, or other enzymic, activity. In these circumstances it occurred to us that it might be easier to establish an esterolytic rather than a proteolytic activity, since proteinases are always esterases and serine proteinases usually have a higher catalytic efficiency towards esters [18]. We now demonstrate that ETA and ETB will hydrolyse Boc-GluOPh and that the esterase and epidermolytic activities are dependent on a single serine residue in each toxin.

EXPERIMENTAL

Materials

ETA was purified from extracts of *S. aureus* 8325-4 transformed with the shuttle plasmid pETA5 [19]; ETB was purified from the organism after transformation with the chimeric plasmid pSetb [20]. Small amounts of ETA and ETB from strains TC7 and TC115 [17] and from strains TA and DI [4] respectively were also used. ETA S195G was purified from extracts of *S. aureus* 8325-4 carrying plasmid pETA11 [14]. All the preparations have been shown to be homogeneous after PAGE and Coomassie Blue staining. The concentrations of toxins were measured by using $A_{280}^{1\%}$ values of 8.2 and 8.0 for ETA and ETB respectively [21].

Di-[1,3- 3 H]isopropyl phosphorofluoridate was from NEN Research Products. Boc-GluOPh (lot no. 48F5818), α -chymotrypsin (type II), α -chymotrypsinogen (type II), staphylococcal V8 proteinase (type XVII-B) and di-isopropyl phosphorofluoridate were obtained from Sigma Chemical Co.

Esterase assays

Boc-GluOPh was made up in dry dioxane and assayed by spectrophotometry after complete hydrolysis, by NaOH or by esterase-dependent catalysis, by using a molar absorption coefficient of 1.5 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ at 270 nm [22].

Abbreviations used: ET, epidermolytic toxin; ETA and ETB, serotypes A and B respectively; Boc-GluOPh, *N*-t-butyloxycarbonyl-L-glutamic acid α -phenyl ester.

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Reaction was carried out at 28 °C in a cuvette containing 1 ml of 0.2 M-Tris/phosphate buffer, pH 7.80, at 21 °C, substrate in dioxane (4–20 μ l) and toxin (1–10 μ g). The pH-dependence of $k_{\text{cat.}}/K_m$ was measured in buffers prepared by the adjustment of 0.2 M- KH_2PO_4 with Tris. Data-fitting to the Michaelis–Menten equation, or to the relevant equation from pH-dependence:

$$k_{\text{cat.}}/K_m = \tilde{k}/(1 + [\text{H}^+]/K_a) \quad (1)$$

was carried out by non-linear-regression procedures.

Radiolabelling experiments

After incubation with radioactive di-isopropyl phosphorofluoridate, the labelled protein was isolated after precipitation in 10% (w/v) trichloroacetic acid at 2 °C. The protein pellets were washed twice with 5% trichloroacetic acid in water and finally with 5% trichloroacetic acid in 50% (v/v) ethanol. Co-precipitation with BSA (250 μ g; pretreated with di-isopropyl phosphorofluoridate) was used to achieve quantitative recovery of small amounts of protein (< 50 μ g). The incorporated radioactivity was measured by liquid-scintillation counting (efficiency about 20%, determined by a channels-ratio procedure) after dispersal of the protein precipitate in NCS tissue solubilizer and dissolution in toluene/Triton-X-100 scintillation cocktail. The specific radioactivity (d.p.m./nmol) of di- ^3H isopropyl phosphorofluoridate was determined by titrating the radioactivity incorporated into α -chymotrypsin as a function of enzyme inhibition [23]. Chymotryptic activity was measured spectrophotometrically with *N*-carboxypropionyl-L-phenylalanine *p*-nitroanilide [24]. Chymotrypsin concentrations were measured spectrophotometrically by using a value of 20.4 for $A_{280}^{1\%}$.

Neonatal mouse assay for epidermolytic activity

Sha-sha mice at 3 days of age were injected (50 μ l) subcutaneously with ETB control, treated ETB or buffer solution. The animals were examined for the Nikolsky sign at hourly intervals [25].

RESULTS

Esterase

ETA and ETB readily demonstrated activity towards Boc-GluOPh. The activity was strictly dependent on protein concentrations in the range 1–20 μ g for ETA and ETB, and the mutant

ETA S195G was completely inactive (Fig. 1). At a convenient ester concentration of 0.2 mM, the specific activities were 1.1 and 1.7 μ mol/min per mg for ETA and ETB respectively. The esterolytic activity was present in all other samples of epidermolytic toxin tested: the corresponding relative specific activities were 96% and 92% for ETA from strain TC7 and ETB from strain TC115 respectively, and 65% and 72% for ETA from strain TA and ETB from strain DI respectively. The activities were insensitive to the presence of 1 mM-EDTA.

The Michaelis–Menten parameters (Table 1) were obtained from measurements of initial rate at various substrate concentrations. Within the limited concentration range available spectrophotometrically, there were no deviations from the simple behaviour.

The pH-dependence of $k_{\text{cat.}}/K_m$ (Fig. 2) requires a single ionization of each free enzyme at $\text{p}K_a$ 6.6($\pm$ 0.06) and $\text{p}K_a$ 6.8($\pm$ 0.1) for ETA and ETB respectively. These pH values are typical of the histidine-associated $\text{p}K_a$ found with all serine proteinases.

Thermal stability

Samples of ETA, ETB and staphylococcal proteinase were held at 55 °C for the given time then assayed immediately at 28 °C (Fig. 3). No significant inactivation of ETA was detected over a period of 8 h; ETB lost activity after a few minutes, the loss of activity being associated with the precipitation of the protein; staphylococcal proteinase was inactivated rapidly ($t_{1/2} \sim 30$ s) and completely. In a further experiment, it was shown that treatment in a boiling-water bath for 2 min did not affect

Table 1. Michaelis–Menten parameters for ET esterases

The measurements were made in 0.2 M-Tris/phosphate buffer, pH 7.80, containing 2.0% (v/v) dioxane (reaction rates were very sensitive to dioxane concentrations; cf. [29]), at 28 °C with substrate concentrations in the range 0.175–1.4 mM.

Parameter	ETA	ETB
$k_{\text{cat.}}$ (s^{-1})	4.9 ± 0.4	5.3 ± 0.3
K_m (mM)	1.9 ± 0.3	1.1 ± 0.1
$k_{\text{cat.}}/K_m$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	2.5 ± 0.1	4.7 ± 0.3

Fig 1. Esterase activities of ETs

The concentration-dependence of ETA (□), ETB (○) and ETA S195G (◇) esterases was measured at 0.2 mM-Boc-GluOPh.

Fig 2. pH-dependence of $k_{\text{cat.}}/K_m$ for ET esterases

The data from ETA (□) and ETB (○) were obtained at 28 °C in 0.2 M-Tris/phosphate buffer. The fitted lines are the unweighted fits to eqn. (1).

Fig 3. Thermal stabilities of esterases

ETA (□), ETB (○) or staphylococcal proteinase (△), all at 0.2 mg/ml in 0.2 M-Tris/phosphate buffer, pH 7.8, were held in a water bath at 55 °C. Samples (50 µl of ETs, 4 µl of proteinase) were diluted into substrate solution at the given time and assayed immediately.

Fig 4. Reactivity of active-serine proteins towards di-isopropyl phosphorofluoridate

ETA (2 mg/ml) (□ and ■), ETB (2 mg/ml) (○ and ●), chymotrypsinogen (1.2 mg/ml) (▼) and chymotrypsin (1.2 mg/ml) (▲) were treated at room temperature with 0.07 mM di-³H]isopropyl phosphorofluoridate (9.5×10^5 d.p.m./nmol) in phosphate-buffered saline, pH 7.3. At the given time, samples (50 µl for radioactivity, 5 µl for esterase) were taken for assay. □ and ○, Enzymic activity; ■, ●, ▲ and ▼, radioactivity.

ETA ($102 \pm 2\%$ recovery of activity) but inactivated 94% of ETB activity.

Reaction with di-isopropyl phosphorofluoridate

The reactivities of ETA, ETB, α -chymotrypsin and chymotrypsinogen are compared in Fig. 4. ETA and chymotrypsinogen accumulated label slowly, but in a quasi-linear fashion over the period of the experiment, to levels of 0.016 and 0.015 mol of di-³H]isopropyl phosphorofluoridate/mol of protein respectively. Any associated change in esterase activity of ETA was too small to measure. Chymotrypsin reacted rapidly ($t_{1/2} < 30$ min) and stoichiometrically. ETB was intermediate in its response and lost about 50% of esterase activity coincidentally with incorporation of 0.45 mol of di-³H]isopropyl phosphorofluoridate/mol of protein.

Under slightly different reaction conditions, including an increased concentration of reagent (Fig. 5), a more complete reaction of ETB with di-isopropyl phosphorofluoridate took place. The radioincorporation pattern followed simple exponen-

Fig 5. Inactivation of ETs with di-isopropyl phosphorofluoridate

The proteins (0.2 mg/ml ETA, 0.4 mg/ml ETB) in 0.2 M-Tris/phosphate buffer, pH 7.80, were reacted at room temperature with 2 mM-di-isopropyl phosphorofluoridate (³H-radiolabelled to 1.9×10^4 d.p.m./nmol in the ETB mixture only). Symbols as in Fig. 4.

tial kinetics to an ultimate incorporation of $0.97 (\pm 0.02)$ mol of di-³H]isopropyl phosphorofluoridate/mol of protein. The inhibition of esterase activity, measured in the same reaction mixture, was superimposable on the radiolabelling data and confirms that inactivation is associated with a stoichiometry of 1:1 at all stages of reaction.

The treated protein had no epidermolytic activity at 10 µg dosage in the assay performed *in vivo* after 1 and 2 h. A control ETB sample (10 µg), untreated with di-isopropyl phosphorofluoridate, gave a positive reaction 45 min after injection.

ETA esterase was measurably inactivated under these conditions (Fig. 4), but reaction was incomplete even after 20 h.

DISCUSSION

The use of Boc-GluOPh was suggested because it is a suitable substrate for the related staphylococcal proteinase [22]. However, the specific esterase activities of the toxins are much lower than that of staphylococcal proteinase, and the possibility that the observed activities are the result of a contaminating activity has to be addressed. Staphylococcal proteinase (M_r 29023) was readily separable from ETs on SDS/PAGE in our experiments, and there is no question of gross contamination. The possibility of minor adventitious contamination by the proteinase, producing a significant fraction of the esterase activity found in the toxins, is ruled out by the following data. (a) All the toxin samples tested, prepared by a variety of procedures from different strains on different occasions, had the characteristic A or B specific activity. The slightly lower values from strains TA and DI may result from the considerably age of these samples. (b) The thermal stability of ETA esterase, particularly the result at 100 °C, rules out any possibility that measurable temperature-sensitive esterase activity is present. (c) The absence of esterolytic activity in ETA S195G, which is purified by a procedure identical with that for the wild-type ETs, demonstrates that no non-ET esterase remains after purification.

The low concentration of staphylococcal proteinase in preparations of ET may be understood in terms of the efficiency of strain selection and the differences in protein property. The

proteinase is a very acidic protein [26], with a $pI < 4$ predicted from the amino acid composition [11], whereas ETs have pI values close to 7 [17]. Since the purification procedures [4,17] rely on ion-exchange chromatography or isoelectric focusing, it is to be expected that a rigorous separation of ET and proteinase would be achieved.

The absolute values of k_{cat} and K_m indicate that the toxins are not very efficient catalysts. However, the activity is of the serine proteinase type from the pH-dependence of k_{cat}/K_m , which indicates pK_a values that should be associated with His-72 and His-65 of ETA and ETB respectively. A notable feature of the results (Fig. 2) is that the data reach a plateau. In most serine proteinases [18], including staphylococcal proteinase [27], such data show the histidine-associated pK_a plus the alkaline pK_a of a residue required to be in the acid form.

A wide variety of evidence links the esterase with epidermolytic activity and active serine residues. The correspondence of the thermal stabilities of esterase and epidermolytic activities [28], particularly the extraordinary stability of ETA to 100 °C, links the two activities by a classical procedure. The inactivity of ETA S195G confirms (Fig. 1) that esterolytic, as well as epidermolytic [14], activity is controlled by Ser-195. The reactivity of ETB with di-isopropyl phosphorofluoridate allows a similar demonstration (Fig. 5) that the esterase depends on a single serine residue, presumably Ser-186, but this has not yet been demonstrated. The inactivity of di-isopropyl phosphorofluoridate-treated ETB, at 10 µg in an assay that detects at least 1 µg [14], links the esterolytic and epidermolytic activities of ETB.

The studies presented here may explain some of the apparent contradictions as to whether di-isopropyl phosphorofluoridate inactivates epidermolytic toxin [12,13]. The data for ETA (Figs. 4 and 5) show that the reaction is incomplete, as previously claimed [12]. The maximum inhibition of 30% achieved at 20 h reaction of ETA with di-isopropyl phosphorofluoridate cannot be registered by the epidermolytic assay *in vitro* [6]. However, it seems plausible that such an inhibition was sufficient to perturb the assay system used by Dancer *et al.* [13], or that their toxin is ETB, which would explain the apparent contradiction.

The considerable difference in reactivities (Fig. 4) align ETA with the proenzyme chymotrypsinogen, and ETB towards the active chymotrypsin, although there is only a small difference in the respective esterolytic efficiencies (Table 1). The reactivity of 'active-serine' enzymes with di-isopropyl phosphorofluoridate is quite variable [29], and it may be that the reactivities of ETA and ETB are governed by otherwise-unimportant structural features. If we assume, as in other cases [18], that esterase activities will be greater than proteinase activities by catalytic efficiency factors of 10^4 -fold, then it is obvious that ETA and ETB will be very inefficient proteinases. It may be that there is some activation process, possibly associated with the very selective site of action of the toxins [1,2], which remains to be discovered. The data in this paper suggest that ETB, rather than the less reactive ETA,

may be a more suitable experimental subject in the search for a proteolytic effect on the epidermis.

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