A Four-Straight-Line Model for the Proteinase-Binding Characteristics of Human Blood Serum

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Kinetic evaluation of the capacity of human blood serum to form complexes with bovine trypsin generated partition profiles that may be approximated by a series of four intersecting straight lines. Such profiles are suggested to reflect the binding of trypsin to α_2 -macroglobulin in a kinetically preferred mode (α -sites), followed by a subsidiary mode (β -sites) and finally to α_1 -antitrypsin. The form of the profile, in addition to revealing a hitherto unreported proteinase-binding capability of α_2 -macroglobulin (β -sites), also indicates that saturation of α -sites corresponds to a molar binding ratio of α_2 -macroglobulin/trypsin of 1:2. Finally the profile provides, for certain pathological states, a clinically valuable characteristic.

Proteolytic enzymes play a crucial role in the regulation of a wide variety of biochemical processes essential to health. They are involved in the processes of coagulation, fibrinolysis, immunodefence via complement, kinin liberation, mobilization of tissue proteins, cleavage of food proteins, enhanced cell permeability, fertilization and defence against infection (leucocyte proteinases). In addition, unwanted proteolytic activity may develop as a result of natural leakage across membrane barriers or by spillage from damaged cells or from invasion by micro-organisms. An important part of the mechanism of control of this proteolytic activity is provided by a group of proteins, the proteinase inhibitors, which constitutes approx. 10% by weight of human serum protein and which is capable of inhibiting proteinases whether derived from animal, plant or bacterial sources. The disturbance of an acceptable balance between the activity of the proteinases and control exerted by the protein proteinase inhibitors has obvious pathological significance (Reich *et al.*, 1975; Fritz *et al.*, 1974). Our particular concern is to examine, by using kinetic methods, the fate of a series of proteolytic enzymes (initially those most likely to modify the alveolar interstitium) when they individually encounter human serum obtained from both normals and from patients suffering from pulmonary disability. This paper describes our model for the interaction based on the binding of bovine trypsin to human serum protein.

Human serum is known to contain at least six proteinase inhibitors (Heimburger *et al.*, 1971) of which two, α_1 A [180–280mg/100ml, approx. $36\mu\text{M}$ (mol.wt.=52000; Cohen & Fallat, 1974)] and α_2 M

[150–420mg/100ml, approx. $2.7\mu\text{M}$ (mol.wt.=725000; Jones *et al.*, 1972)], predominate both in mass and in inhibitory range. The kinetic partition of the serine proteinase, bovine trypsin, between the available competing inhibitory sites on these two inhibitors in fresh human serum (non-pooled) will be shown to yield new information on the nature of the enzyme-inhibitor complexes formed.

α_1 A is known to bind to trypsin to form a complex in which the active-site serine of the proteinase has been suggested (Bode *et al.*, 1976) [by analogy with functionally similar inhibitors (Bode *et al.*, 1976; Sweet *et al.*, 1974)] to provide the fourth element in the formation of a tetrahedral adduct about a susceptible peptide carbonyl group of the inhibitor. As a result both the proteolytic and esterolytic activity of the complexed enzyme is decreased to zero. If as a result of a homozygous (phenotype ZZ) deficiency of α_1 A in which both the quality (α_1 A manifests complex patterns of electrophoretic heterogeneity characteristic of the genotype and is the only inhibitor of which genetic variants are known) and quantity (decreased to approx. 20mg/100ml) of α_1 A are affected, then such complexes are formed with lower efficiency. A growing body of evidence supports a suggested relationship between the genetic disorder and severe pulmonary emphysema and hepatic diseases (Laurell & Eriksson, 1963), and, more recently, with other diseases (Kueppers & Black, 1974). Thus, for example, the pathogenesis of emphysema may involve uninhibited proteolytic digestion of lung by lysosomal proteinases from inflammatory cells such as leucocytes and lung macrophages.

In contrast with α_1 A, the binding of α_2 M to enzymes such as trypsin appears not to involve covalent forces. Instead, the ability of α_2 M to inhibit the

Abbreviations used: α_1 A, α_1 -antitrypsin; α_2 M, α_2 -macroglobulin; Bz-Arg-OEt, *N*- α -benzoyl-L-arginine ethyl ester.

proteolytic activity of the whole class of endopeptidases is believed to derive from steric shielding of the active site of the enzyme. The operation of the inhibitor has been likened to that of a trap which may be sprung by initial scission of a peptide link in the inhibitor resulting in the enzyme being swallowed whole and intact (Barrett & Starkey, 1973). Whatever the subtlety of the shielding, however, the inherent activity of the complexed proteinase is not lost provided that the substrate is small enough to negotiate the steric restriction (Haverback *et al.*, 1962). Reports (Gentou *et al.*, 1968; Twumasi *et al.*, 1977) suggest that the activity of the bound enzyme

may, under some circumstances, even be enhanced. Furthermore, the activity of the α_2 M-bound trypsin towards small substrates is preserved in the presence of large inhibitors (such as α_1 A) while being destroyed by small inhibitors (such as benzamidine) that can negotiate access to the active site of the enzyme. No established relationship between inadequate α_2 M and consequent disease, such as exists for α_1 A, has yet been described.

In the present work, the properties of the enzyme-inhibitor complexes, outlined above, have been used in the construction of over 100 human partition profiles as exemplified in Fig. 1. In such a profile, each

Fig. 1. Selection of typical experimentally observed partition profiles that indicate the capacity of individual samples of human serum to form complexes with bovine trypsin

A given profile comprises 21 measurements of the activity of a sample of a solution of trypsin (0.10 ml, 1.25 nmol) in Tris buffer (0.086 M-Tris/0.02 M- Ca^{2+} , pH 8.14) after addition to a mixture of the buffer solution and human serum to a total volume of 0.5 ml at 30°C. After a period of equilibration (not less than 10 min) the activity of the mixture was determined by addition of a sample (0.1 ml) to a solution in Tris buffer of *N*- α -benzoyl-L-arginine ethyl ester (3.0 ml, 1.49 μ mol, 30°C) and the measurement of rate of change in A_{253} over a period of not less than 1 min. Profiles (a)–(f) correspond to serum samples 53, 127, 48, 54, 24 and 64 respectively (Topping & Craven, 1979) and comprise four normals (a, d, e and f) and patients with sarcoid fibrosis (b) and fibrosing alveolitis with rheumatoid arthritis (c) (B. G. Rigden & C. W. G. Turton, personal communication).

point is a separate rate measurement and represents the effectiveness of a mixture of trypsin and various amounts of human serum under controlled conditions of pH, temperature and ionic strength to hydrolyse the substrate, *N*- α -benzoyl-L-arginine ethyl ester. Such points are thus a measure of the total capability of the enzyme in its various forms, free and bound, to hydrolyse the substrate.

Experimental

Materials

N- α -Benzoyl-L-arginine ethyl ester hydrochloride, Tris and $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ were the purest grades available from BDH Chemicals, Poole, Dorset, U.K. Trypsin (bovine pancreatic) was obtained from Boehringer Corp., Lewes, Sussex, U.K., and bovine albumin (30%) from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Serum samples, prepared by centrifugation of whole human blood that had been allowed to clot, were obtained from the Royal Sussex County Hospital, Brighton, Sussex, U.K., the Victoria Hospital, Lewes, Sussex, U.K., and the Cardiothoracic Institute, Brompton Hospital, London, U.K. Solutions were prepared in deionized water that had been distilled under N_2 in all-glass apparatus. Buffer solutions were prepared by appropriate addition of HCl to aqueous mixtures of Tris and CaCl_2 .

Apparatus

Kinetic measurements were made in a Unicam SP.8000 double-beam spectrophotometer fitted with a thermostatically controlled eight-cell block or a Cary 14 spectrophotometer with a two-cell block. Constant temperature was maintained ($30 \pm 0.1^\circ\text{C}$) by using Grant circulating-water baths. Stoppered silica cells were used for all measurements. Measurements of pH were made with a Pye Dynacap pH-meter with a Pye-Ingold combined glass-calomel electrode maintained at $30 \pm 0.1^\circ\text{C}$.

Methods

Construction of a partition profile. A partition profile comprises 21 rate measurements of the effectiveness of trypsin to hydrolyse the substrate Bz-Arg-OEt, in the presence of various amounts of an individual serum. Each measurement was made as follows.

(a) A portion of a solution of trypsin (0.10ml, 1.25nmol) in Tris buffer (0.086M-Tris/0.02M- Ca^{2+} , pH8.14) was added with rapid mixing to a mixture of the buffer solution and human serum to a total volume of 0.5ml. All components were thermostatically maintained at 30°C before being mixed and allowed to equilibrate at 30°C for a period of 10–50min after mixing.

(b) After equilibration, the activity of the mixture was determined by addition of a portion (0.10ml) to a solution in the Tris buffer of Bz-Arg-OEt (3.0ml, 1.49 μmol , 30°C) and the measurement of rate of change in A_{253} (1cm cells) over a period of not less than 1 min.

The measurement of rate was facilitated by the use of the substrate Bz-Arg-OEt under the above conditions, which resulted in zero-order kinetics with respect to substrate.

Effect of added bovine serum albumin on the characteristics of a partition profile. Experiments in which bovine serum albumin was added to the buffer/serum mixture to maintain a 'constant protein' background before addition of trypsin, followed by measurement of tryptic activity as described above, resulted in no significant change in the form of the profile.

Effect of using partially deactivated trypsin on the characteristics of a partition profile. A solution of trypsin in buffer (30mg/100ml, in 0.086M-Tris/0.02M- Ca^{2+} , pH8.14) was immersed in a water bath at 30°C for 48h, after which the activity of the trypsin as measured against Bz-Arg-OEt had fallen to approximately half of its original value. The form of the partition profile that resulted from the use of 50%-deactivated trypsin was similar to the profile generated by a simple 50% decrease in trypsin concentration.

Results and Discussion

The capacity of an individual sample of human serum to form complexes with bovine trypsin may be described in terms of a partition profile (Fig. 1), which is a measure of the total hydrolytic effectiveness of trypsin, both free and bound to serum protein, versus the substrate Bz-Arg-OEt at 21 trypsin/serum ratios. Early in the generation of such profiles from both normal donors and from hospitalized patients it became apparent that the experimental points could be closely described by a series of four intersecting straight lines, AB, BC, CD and DE (Fig. 2). Attempts to generate a better (or comparable) description of the experimental points based on a smooth curve utilizing polynomial, logarithmic or exponential functions were unsuccessful (A. H. Craven, personal communication). The line AB, produced at low serum/trypsin ratios, is a common clinical laboratory measurement taken to represent the trypsin-inhibitory capacity of the serum. Less effective inhibition at higher serum/trypsin ratios (B to E) is readily interpreted in terms of the preferential capture of trypsin within a complex that retained residual hydrolytic activity (Ganrot, 1967a; Mehl *et al.*, 1964). The pattern of the change in activity (lines BC, CD, DE), which together with the rest of the profile appears to be reproducible with good

Fig. 2. Schematic description of a partition profile based on the four-straight-line model

A schematic profile derived from a consideration of eqns. (4)–(6) (see the text) after decoupling by making no allowance for effective competition between available serum inhibitory sites (i.e. $k_{+1} \gg k_{+2} \gg k_{+3}$). Extrapolation of lines ED and DC to the ordinate give respective values for the hydrolytic activity of trypsin (substrate Bz-Arg-OEt) when bound in the α -mode (r_a) and the β -mode (r_b) to α_2 M, relative to free trypsin. Line CB can be extrapolated through the origin when it is assumed (a) that the original serum sample contains no agent capable of manifesting comparable hydrolytic activity and (b) that trypsin bound to α_1 A is completely inhibited. Predictable deviations from the four-straight-line model result when, for a given sample of serum, either of these assumptions or the decoupling procedure becomes untenable.

precision for any given sample of human serum, is best interpreted in terms of hitherto unsuspected binding capabilities of α_2 M.

Any given point on a profile may be regarded as the overall result of the competition between an array of protein proteinase-inhibitor binding sites for a fixed amount of enzyme. The recorded resultant activity is then a measure of the sum of the products of the rates of interaction of the enzyme with a given inhibitor site and the residual activity of the enzyme when bound to that site, plus the activity of any free enzyme. The plateau region of the profile (DE) is thus

most simply regarded as the condition when all the enzyme is accommodated at inhibitor sites (α_2 M $_{\alpha(n)}$) that possess the largest specific reaction rate constants for attachment of enzyme to inhibitor and that result in bound enzyme with a residual activity corresponding to r_a (Fig. 2). For all the enzyme to be bound to ' α -sites' would require the corresponding rate constants to be infinitely larger than any associated with competing sites, but the linearity of the segments of the profile indicate that the competing inhibitor sites are in groups with very large separation of rate constants. Point D thus corresponds to virtually complete formation of a 1:n α_2 M-trypsin complex. The suggestion that has been made (Ganrot, 1967a), that trypsin partitions approximately equally between α_1 A and α_2 M in the presence of excess of both inhibitors, would lead to considerable curvature in the partition profiles. On the other hand our suggestion, that in the region DE most of the enzyme is bound to α -sites on α_2 M, requires an explanation for the observed variation in the height of the plateau not in terms of a corresponding variation in the partition of enzyme between α_2 M and other inhibitory sites but rather in a variation of the residual activity of the α_2 M-bound trypsin.

Many carefully performed studies described in the literature have attempted to establish the binding stoichiometry of α_2 M and a variety of proteinases. The problem is not trivial, and there are difficulties inherent in kinetic titration as there are in the far more numerous attempts based on preparative procedures used to isolate inhibitor. These difficulties have led to apparent conflict in establishing the molar combining ratios of α_2 M to proteolytic enzymes, and values of 1:1 (Ganrot, 1967b; Gentou *et al.*, 1968; Baumstark, 1970; Saunders *et al.*, 1971; Sugihara *et al.*, 1971; Barrett & Starkey, 1973; Hamberg *et al.*, 1973; Yamamoto, 1973), 1:2 (Mehl *et al.*, 1964; Ganrot, 1966a,b, 1967a,b; Lanchantin *et al.*, 1966; Berthillier *et al.*, 1968; Bieth *et al.*, 1968, 1970; Jacquot-Armand & Krebs, 1969; Dolovich & Wicher, 1971; Lebreton de Vonne *et al.*, 1971; Saunders *et al.*, 1971; Rinderknecht & Geokas, 1973; Schreiber *et al.*, 1973; Yamamoto, 1973; Fine *et al.*, 1975; Nakamura *et al.*, 1975; Ohlsson & Laurell, 1976; Twumasi *et al.*, 1977) and 1:3 (Bieth *et al.*, 1970; Nagasawa *et al.*, 1970) have been published. Even the 1:1 binding ratio of α_1 A to proteinase has been questioned (Johnson *et al.*, 1974). A variety of proteinases has been used in the binding studies and not all the α_2 M has been of human origin (although human α_2 M shows common antigenic determinants with many mammals; Steinbuch & Audran, 1974). Accepting this, the apparent ambiguity in binding ratios has been rationalized in a number of ways. The partial complexing of α_2 M by thrombin and/or plasmin during production of the serum (Szczeklik, 1971), calculations of binding ratios based on the

assumption of 100% activity for commercial preparations of enzymes that may be as little as 50% active (Mehl *et al.*, 1964; Barrett & Starkey, 1973), the possibility of loose adsorption of excess enzyme on the surface of the α_2 M-enzyme complex (Baumstark, 1970), the modification of α_2 M by proteolytic enzymes in the process of complexing (Steinbuch *et al.*, 1968) or damage to the vulnerable α_2 M molecule by the use of particular methods or materials in the isolation procedures (Steinbuch & Audran, 1974); all are factors that could give rise to uncertain (often non-integral) binding ratios. A further complication may arise from the use of pooled sera, as we have found (Topping & Craven, 1979) the binding characteristics of α_2 M may, under certain circumstances, vary with the donor. With due regard to the above considerations and to the considerable inconsistency in reported normal values for α_2 M in human serum (Wilding *et al.*, 1967) and with the assumptions that (a) in the 'plateau' region DE α_2 M captures the major share of the enzyme and (b) only active forms of the enzyme bind effectively to the inhibitors, our calculations reveal a binding capacity of α_1 A that is approx. 6.5 times that of α_2 M at point D (i.e. only half the 'normal' molar ratio). This result correlates best with a binding ratio of α_1 A to bovine trypsin of 1:1 and a correspondence of point D (Fig. 2) to the formation of a 1:2 complex between α_2 M and trypsin. Although this suggests the presence of two α -sites on α_2 M, it does not exclude the possibility that progression along the plateau DE corresponds to the preferential formation of an α_2 M-enzyme complex in which only one of the α -sites is occupied. It follows that in practice a principal determinant in the formation of a 1:1 or a 1:2 complex could prove to be the rate of dispersion of the enzyme in the presence of a large excess of α_2 M.

Regions BC and CD clearly also relate to the formation of complexes with residual tryptic activity, but whether these complexes derive from subsidiary sites on α_2 M or from a different hitherto undetected inhibitor or whether the α -sites (α_2 M $_{\alpha(1,2)}$) are involved in complex-formation at different rates with various forms of the enzyme is not immediately apparent. The latter possibility, that the trypsin preparations used had suffered autolysis to some degree (Schroeder & Shaw, 1968; Hakim, 1972; Beardslee & Zahnley, 1973; Foucault *et al.*, 1974) and that the resulting modifications reacted with α_2 M both at different rates and to give complexes with different residual activities, seemed unlikely when it was found that treatment of trypsin under conditions calculated to promote autolysis (Beardslee & Zahnley, 1973) did not affect appreciably subsequent partition profiles.

The first possibility, that the B-to-E region corresponds to priority filling of α -sites on α_2 M by trypsin to give complexes with a resultant residual activity

r_α , followed subsequently by the binding of trypsin to a number of secondary sites on α_2 M (i.e. α_2 M $_{\beta 1}$, α_2 M $_{\beta 2}$ etc.) at rates that are between the rates of acquisition of trypsin by the α -sites and by α_1 A giving complexes in which trypsin attached to a β -site has a residual activity of r_β (Fig. 2), is compatible in all respects with the pattern of four intersecting straight lines that comprise a profile. The designation of distinct binding sites on α_2 M as α - and β -sites, however, is a notation of convenience only. There is no evidence for independent binding loci on the inhibitor, but only for a capability of α_2 M for binding proteinase in different modes that correspond to different degrees of inhibition. Why the kinetically apparent secondary binding of trypsin by α_2 M (β -mode) has not been apparent also by isolation of such complexes is not at present clear.

The basic model, outlined above, which describes the primary interactions between trypsin and human serum without diffusion limitations, may be represented by the competing processes (1)–(3):

where k_{+1} , k_{+2} and k_{+3} are the specific rate constants for the binding of trypsin (T) to vacant α - and β -sites of α_2 M [i.e. $\alpha_2\text{M}_{\alpha(1,2)}$ and $\alpha_2\text{M}_{\beta(1,2 \dots n)}$] and to α_1 A respectively. The quantities P_A and P_B are thus proportional to the concentrations of trypsin bound to α - and β -sites respectively of α_2 M and do not correspond to discrete products.

The rates of binding of trypsin to the various inhibitor sites are as shown in eqns. (4)–(6):

$$\frac{d\text{P}_\text{A}}{dt} = k_{+1}[\alpha_2\text{M}_{\alpha(1,2)}][\text{T}] \quad (4)$$

$$\frac{d\text{P}_\text{B}}{dt} = k_{+2}[\alpha_2\text{M}_{\beta(1,2 \dots n)}][\text{T}] \quad (5)$$

$$\frac{d\text{P}_\text{C}}{dt} = k_{+3}[\alpha_1\text{A}][\text{T}] \quad (6)$$

The considerable degree of linearity of each of the four segments of a large majority of the partition profiles suggests that an approximate solution to the set of non-linear differential eqns. (4)–(6) may be generated simply by decoupling the set by using the assumption that the relative specific reaction constants for binding trypsin to the several groups of available sites are infinitely separated.

From this simplifying assumption, the basic interpretation of a partition profile as shown in Fig. 1 is as follows.

Segment A-B

The maximum tryptic activity at point A (absence of inhibitors) is progressively decreased with increasing serum by successive saturation of $\alpha_2M_{\alpha(1,2)}$, $\alpha_2M_{\beta(1,2 \dots n)}$ and finally α_1A (Fig. 2). At point B no free trypsin remains and initial trypsin (T_0) is related to concentrations of inhibitor sites according to eqn. (7):

$$T_0 = \alpha_2M_{\alpha 1} + \alpha_2M_{\alpha 2} + \alpha_2M_{\beta 1} + \alpha_2M_{\beta 2} \dots + \alpha_2M_{\beta n} + \alpha_1A \quad (7)$$

Segment B-C

This region represents a progressive saturation of all available sites on α_2M at the expense of α_1A . The slope of the progression is proportional to the sum of the residual activities of trypsin bound to all available α_2M binding sites and the line extrapolates through the origin. At point C no α_1A -trypsin complexes are formed.

Segment C-D

Priority binding of trypsin to $\alpha_2M_{\alpha 1}$ and $\alpha_2M_{\alpha 2}$ at the expense of $\alpha_2M_{\beta(1,2 \dots n)}$ results in line CD, which has a gradient proportional to the difference between the residual activities of trypsins bound to an α - and a β -site. The line DC extrapolates to intersect the ordinate at a point r_β , which is a measure of the residual activity of trypsin bound to a β -site. At point D all α_2M_α sites are occupied and all other inhibitor sites are free.

Segment D-E

Plateau DE has zero slope (provided that the serum contains no agent capable of attacking the substrate) and corresponds to all trypsin accommodated at α_2M_α sites. At point D both α -sites on α_2M are bound to trypsin. Whether, at higher serum concentrations, this state is maintained or whether occupation of only a single α -site is preferred may well be determined by collision statistics. Even if there is no rate discrimination between the α -sites, irreversibility of complex-formation should assure the ultimate formation of a 1:1 complex. The line ED extrapolates to intersect the ordinate at a point r_α (Fig. 2), which indicates the residual activity of trypsin bound to an α -site.

The above interpretation of a partition profile provides a useful basis for describing the kinetic distribution of trypsin between inhibitory sites in human serum. A complete description, however, must allow for less than infinite separation of rate constants. The following paper (Topping & Craven, 1979) describes how this has been achieved to provide a close simulation of any given partition profile, thus confirming the basic tenability of eqns. (1)-(7).

Finally, early indications (R. M. Topping & B. G. Rigden, unpublished work) of the existence of a potentially valuable correlation between the shape of the human partition profile and the clinical condition of the donor are now being confirmed with particular regard to diseases of the chest. Whether the proteinase-binding characteristics of α_2M , which principally determine the shape of the profile, predispose to the clinical condition or whether they change in response to the condition or to any other stimulus are questions that remain to be finally answered.

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