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POLYMERIC COLLAGEN FIBRILS

AN EXAMPLE OF SUBSTRATE-MEDIATED STERIC OBSTRUCTION OF ENZYMIC DIGESTION

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Summary

Polymeric collagen fibrils have been reacted with fluorescein and rhodamine isothiocyanates to produce fluorescent dye-labelled fibrils, containing seven dye substituents per molecule of tropocollagen within the polymeric collagen fibrils. Two dye-labelled peptides per molecule of tropocollagen were solubilised by trypsin (EC 3.4.21.4) from the telopeptide regions and four dye-labelled peptides were located in the helical regions solubilised by bacterial collagenase (EC 3.4.24.3). The solubilisation of dye-labelled peptides from these insoluble substrates were employed to measure the kinetics of trypsin and collagenase digestion of the telopeptide and helical regions, respectively, of the insoluble polymeric collagen fibrils. These studies demonstrated an apparent excess of enzyme for the readily available substrate under conditions when it was known that a vast excess of substrate existed in the reaction mixture calculated in terms of a molecular ratio. A point of equivalence was established for both trypsin and bacterial collagenase, approximately one enzyme molecule per 870 substrate molecules. On either side of this point the quantity of products formed was controlled by either the enzyme concentration or the substrate concentration. The results can be explained in terms of the inaccessibility of tropocollagen molecules within the molecular architecture of the polymeric collagen fibrils. The external layer of tropocollagen molecules obstruct collagenolytic enzymes penetrating to, and forming enzyme-substrate complexes with, the bulk of the substrate within the interior of the fibrils.

Introduction

Collagen fibres present in mature connective tissue consist of bundles of polymeric collagen fibrils [1,2]. Techniques have been developed for the prepa-

ration of dispersions of polymeric collagen fibrils [1,3] from which the insoluble polymeric collagen fibrils may be obtained in a pure form.

Kinetic studies on the bacterial collagenase (EC 3.4.24.3) digestion of polymeric collagen fibrils pre-labelled with fluorescein or rhodamine, clearly demonstrated marked differences in the rates of digestion and the substrate behavior of these polymers when compared with the digestion of tropocollagen monomers in solution or to the digestion of insoluble fibrils freshly reconstituted from tropocollagen monomers [4]. It was observed that the enzyme appeared to be in excess of the substrate when it was known that a vast excess of substrate was actually present in the reaction mixture [4]. This result could be explained in terms of the inaccessibility of tropocollagen molecules within the molecular architecture of the polymeric collagen fibrils.

Studies with trypsin (EC 3.4.21.4) and a trypsin-like neutral protease [5] also indicated a similar pattern of digestion kinetics to those observed with bacterial collagenase for polymeric collagen fibrils. Further studies have now established that a similar ratio exists between the number of enzyme molecules and the number of readily available molecules of substrate in the polymeric collagen fibrils, whether trypsin or collagenase is employed as the enzyme. The kinetics of enzyme digestion are controlled by this ratio; the numerical determination of this ratio can readily be achieved by experimentation. This is of considerable interest, since bacterial collagenase cleaves the helical regions of tropocollagen at very many sites to produce tripeptides, whilst trypsin cleaves the telopeptide regions of tropocollagen within the polymer without solubilising the fibrils to shortened tropocollagen molecules, i.e. trypsin does not depolymerise polymeric collagen fibrils and has a very limited attack on native tropocollagen molecules. Thus, the ratio of enzyme to substrate molecules which controls the kinetics of enzymic digestion of polymeric collagen fibrils must be related to the organisation of tropocollagen molecules within the polymer rather than being related directly to the number and location of sites for enzymic cleavage within the individual tropocollagen molecules.

The present study is confined to illustrating this unusual behavior and presenting an example of substrate mediated obstruction of enzymic digestion, exhibited by polymeric collagen fibrils which may be explained in terms of the molecular architecture of these fibrils.

Materials

Cystalline trypsin (EC 3.4.21.4) and L-(tosylamido-2-phenyl)ethyl chloromethylketone-treated trypsin free of chymotryptic activity were supplied by Worthington. Bacterial collagenase (EC 3.4.24.3) was also supplied by Worthington. A preparation of protease-free bacterial collagenase prepared by column chromatography (Lee-Own and Anderson, ref. 6) was kindly provided by these authors. A similar preparation of bacterial collagenase was prepared by affinity chromatography, first on an elastin column [7] followed by a column of polymeric collagen [4], to remove contaminating neutral proteases.

Rhodamine and fluorescein-labelled polymeric collagen fibrils were prepared from three year old bovine tendon polymeric collagen as described [4,5]. Each tropocollagen molecule within the polymer contained seven fluorescent substit-

uents attached to lysine or histidine residues. Four of these fluorescent substituents were located in the helical regions and three within the telopeptide regions of the tropocollagen molecules within the polymeric collagen fibrils [5].

Methods

All the methods used in this study have been described in detail elsewhere [4,5]; it is necessary, however, to give a brief description of the assay procedure here to facilitate the understanding of the present paper. The kinetics of enzymic digestion of polymeric collagen fibrils can be conveniently followed by measuring the quantity of fluorescent-labelled peptides solubilised from the insoluble substrate suspended in 5.0 ml 0.05 M Tris buffer containing 0.005 M $CaCl_2$ pH 7.5 at 37°C in a shaking water bath. Samples may be removed by means of a microsyringe at suitable time intervals; each sample is suitably diluted (e.g. 50-fold) with distilled water prior to fluorimetric analysis. Alternatively, the soluble labelled peptides may be estimated by direct spectroscopic measurement. In these experiments either a fixed quantity of substrate (7.0 mg of labelled polymeric collagen fibrils) was incubated with a variable quantity of enzyme or the situation was reversed by employing a fixed quantity of enzyme and variable quantity of labelled substrate. In one experiment (Fig. 4) with trypsin, the reaction temperature was maintained at 30°C rather than 37°C in order to avoid any possibility of thermal denaturation resulting in the exposure of helical regions which might then be digested by trypsin.

Results and Discussion

The effect of increasing the concentration of bacterial collagenase in the presence of a fixed quantity of substrate (7.0 mg rhodamine and fluorescein-labelled polymeric collagen fibrils) is shown in Fig. 1. Fig. 2 presents the results obtained for the initial period of digestion of fluorescein-labelled polymeric collagen fibrils, the collagenase being inhibited by the additions of ethylenediamine tetraacetate to give a final concentration of 20 mM, five minutes after mixing the enzyme with the substrate. Similar results to those in Fig. 2 were obtained by measuring the solubilisation of hydroxyproline-containing peptides and the appearance of aminogroups by the fluoram assay system [4] in the soluble fraction.

The evidence presented in Figs. 1 and 2 indicates that with 7.0 mg of substrate in the reaction mixture, the solubilisation of labelled peptides is linearly related to the amount of enzyme added to the system until 6—7 μg of enzyme have been added. At this point (P on Figs. 1 and 2), referred to as the point of equivalence [4], the addition of further quantities of enzyme have little effect on the quantity of products solubilised from the insoluble substrate. It was observed that when tropocollagen molecules in solution or freshly reconstituted collagen fibrils were used as substrate, no such point of equivalence was demonstrated [4].

It has been stated that enzyme concentrations above the point of equivalence produce little more soluble products. It was therefore of interest to ob-

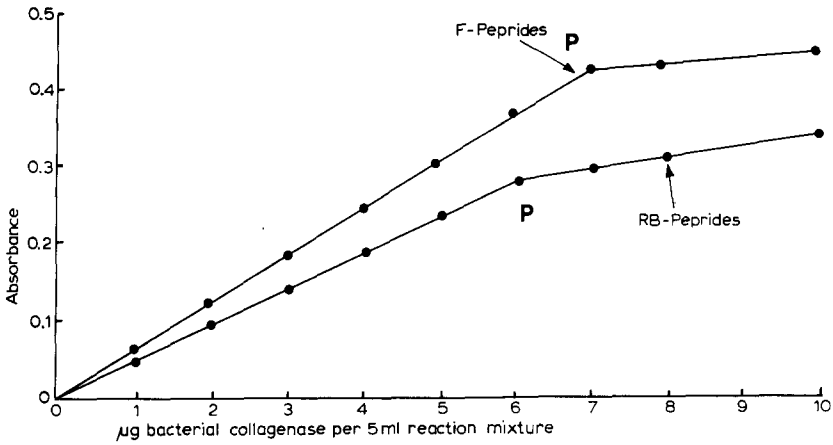

Fig. 1. Solubilisation of rhodamine- and fluorescein-labelled peptides from 7.0 mg rhodamine- and fluorescein-labelled polymeric collagen fibrils, respectively. Absorbances of rhodamine-labelled peptides measured at 365 nm and fluorescein-labelled peptides at 495 nm, after 1-h incubation at 37° C in 5 ml Tris/$CaCl_2$ buffer pH 7.5 with increasing quantities of bacterial collagenase. P denotes the point of equivalence.

serve that under these circumstances (e.g. 50 µg collagenase and 0–20 mg substrate), the quantity of substrate controlled the quantity of soluble products formed with a fixed amount of enzyme in the reaction mixture [4].

The kinetics of trypsin solubilisation of fluorescent peptides from the telopeptide regions of polymeric collagen fibrils are presented in Figs. 3–5. Increasing quantities of trypsin digesting 7.0 mg of fluorescein-labeiled (Fig. 3) polymeric collagen fibrils show a point of equivalence when 1.6 µg of trypsin is

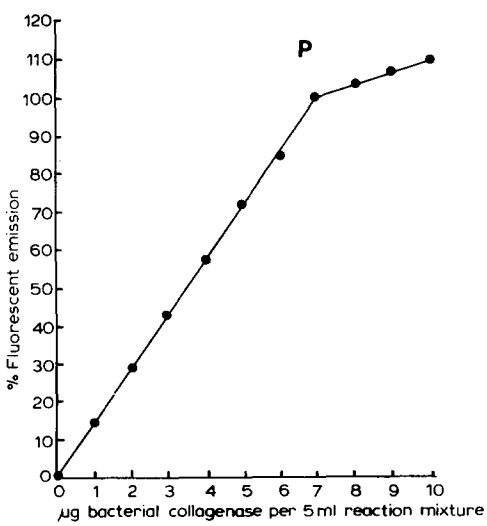

Fig. 2. Solubilisation of fluorescein-labelled peptides from 7.0 mg fluorescein-labelled polymeric collagen fibrils by bacterial collagenase after 5 min at 37°C; the reaction was stopped by the addition of EDTA. The fluorescent emission was measured on the undiluted supernatant fractions employing the 0.1 emission scale. P denotes the point of equivalence.

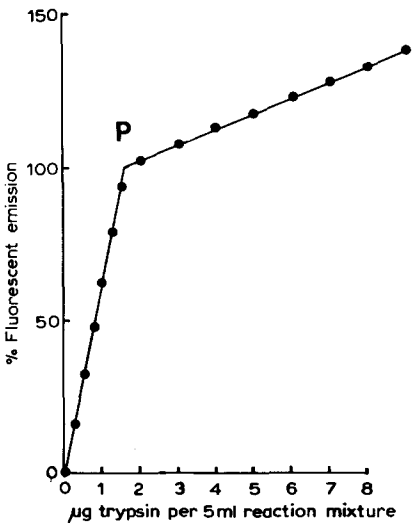

Fig. 3. Solubilisation of fluorescein-labelled peptides from 7.0 mg fluorescein-labelled polymeric collagen fibrils by a range of 0—8 μg trypsin acting for 1 h at 37°C in 5 ml buffer. The fluorescent emission was measured on the supernatant fractions, diluted fiftyfold, employing scale 0.03 and an excitation wavelenth of 495 nm with emission at 520 nm. P denotes the point of equivalence. Exactly similar results were obtained with rhodamine-labelled polymeric collagen fibrils as substrate.

reached; this system corresponds to the collagenase digest of Fig. 1. Exactly similar results were obtained with trypsin digestion of rhodamine-labelled polymeric collagen fibrils, again the point of equivalence was reached with 1.6 μg trypsin reacting with 7.0 mg polymeric collagen fibrils.

When the trypsin concentration was maintained at a constant value in excess of the point of equivalence, and the substrate concentration was increased, a linear relationship was obtained between the quantity of products and the substrate concentration similar to that reported when collagenase was employed [4].

If the point of equivalence really reflects the ratio of enzyme and available substrate molecules which can form an enzyme substrate complex, then this equilibrium should also be demonstrable if conditions are chosen sufficiently carefully. For a fixed quantity of enzyme and a variable quantity of substrate the point of equivalence should be reached when the substrate is in excess of the added enzyme. At this point the quantity of products should remain constant when the substrate concentration is increased. This situation is illustrated in Fig. 4, using trypsin as the enzyme.

As it is known that the point of equivalence is reached when approximately 1.6 μg trypsin are incubated with 7.0 mg fluorescein-labelled polymeric collagen fibrils an experiment can be designed in which 2 μg of trypsin is incubated with increasing quantities of substrate. The resultant graph of products formed plotted against substrate concentration (Fig. 4) consists of two distinct portions. The portion (A), in which region the enzyme concentration is in excess of the readily available sites on the substrate; in this region the quantity of products is controlled by the quantity of substrate used and where the

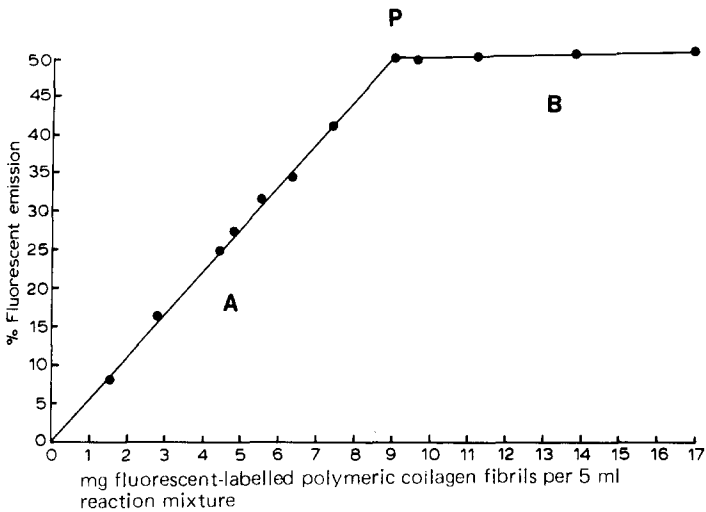

Fig. 4. Solubilisation of fluorescein-labelled peptides from fluorescein-labelled polymeric collagen fibrils (0—17 mg) by 2 μg trypsin acting for 1 h at 30°C in 5 ml of buffer. Fluorescent emission of the supernatant fractions, diluted fiftyfold, on scale 0.03 was measured under the standard conditions of Fig. 3. P denotes the point of equivalence. Regions A and B are referred to in the text.

enzyme concentration exceeds the value (P) found at the point of equivalence. The portion (B) of Fig. 4 indicates saturation of the enzyme with readily available molecules of substrate and exhibits no increase in product formation with increase in substrate concentration. In Fig. 4, at the point of equivalence, 2 μg of trypsin is equivalent to approximately 9.0 mg of substrate, or 1.6 μg trypsin: 7.0 mg substrate as found in Fig. 3 when the point of equivalence was approached from the side of excess substrate and increasing concentrations of enzyme. Since the results were obtained for Fig. 4 at 30°C, the argument that trypsin is attacking denatured helical regions can be refuted and it can be stated that the point of equivalence exhibited by polymeric collagen is a feature of the native collagen fibrils and fibres composed of these fibrils in vivo.

Similar results to those presented in Fig. 4 were obtained with bacterial collagenase, confirming the fact that the point of equivalence could be demonstrated by employing a fixed quantity of enzyme and variable quantities of substrate.

The results discussed above should make it clear that the point of equivalence is a real equilibrium position, experimentally approached from both directions and that it is a feature of the native polymeric collagen fibrils but not of tropocollagen molecules in solution or of freshly reconstituted collagen fibrils [4].

It is possible to calculate the approximate molar ratio of the enzyme to substrate at the point of equivalence, provided that the molecular weights of the enzymes and substrate molecules are known and the quantity of enzyme actually associated with the insoluble substrate is known. It was demonstrated [4] that 46% of the collagenase added to the reaction mixture was actually associated with the solid substrate at the point of equivalence when 7.0 mg of substrate was used. On the basis of approximate molecular weights of 270 000

for tropocollagen [8] if the point of equivalence was reached at 6 μg collagenase (approximate molecular weight 100 000 [9]) the molecular ratio of enzyme to substrate would be approximately 1 : 939; with 7 μg collagenase equivalent to 7 mg substrate, this ratio would be approximately 1 : 805. This means that as long as the molecular ratio is less than one enzyme to 800 or 900 tropocollagen molecules within the insoluble polymeric collagen fibrils, the degree of digestion of the fibrils measured by the solubilisation of labelled peptides is linearly related to the quantity of added enzyme. However, above this ratio, the enzyme appears to be in excess of the readily available substrate and further increases in enzyme concentration have little effect on the quantity of product formed.

Under the experimental conditions it was found that 39% of the added trypsin was bound to the substrate at the point of equivalence and 61% remained in the supernatant fraction. If the molecular weight of trypsin is 23 317 (calculated by Dr. G. Tomalin of the Biological Chemistry Department on the data presented in ref. 10), and if 1.6 μg of trypsin was equivalent to 7.0 mg of substrate at P in Fig. 3 then the enzyme to substrate ratio at P was approximately 1 : 872. This value is in good agreement with ratio of 1 : 805 and 1 : 935 obtained for 7 and 6 μg bacterial collagenase respectively, combined with 7.0 mg of substrate at the point of equivalence (Figs. 1, 2). The average of these two ratios obtained for collagenase is 1 : 870, compared to 1 : 872 for trypsin.

The calculations presented here are approximations based on approximations for molecular weights, coefficients for the binding of enzyme and substrate and a limited range of enzyme concentration at which the point of equivalence is reached, i.e. 6—7 μg for bacterial collagenase and 1.6 μg for trypsin acting on 7.0 mg of substrate. For the purposes of the present discussion it is not essential to know the exact molar ratios at P, provided that the approximate ratio is very large and in favour of the substrate.

Clearly, these two enzymes which attack either the helical regions or the telopeptide regions of tropocollagen molecules within polymeric collagen fibrils have a critical range of enzyme : substrate ratios, above which the enzymes lack readily available sites on the polymeric collagen fibrils to form productive enzyme-substrate complexes. At P there is a vast total excess of tropocollagen molecules within the insoluble polymeric collagen fibrils which remain inaccessible to the enzyme until such a time as the external layers of tropocollagen molecules have been attached. Thus the polymeric collagen fibrils exhibit a form of substrate mediated steric obstruction of collagenolytic enzymes which may be present in excess of the number of readily available sites of binding to tropocollagen molecules on the surface of the fibrils (see Fig. 5). In order to illustrate this substrate mediated steric inhibition of collagenolytic enzymes, experiments were set up in which a constant amount of collagenase or trypsin was allowed to react with a range of weights of substrate. These experiments showed a linear relationship between the quantity of substrate used and the quantity of products formed when the enzyme concentration was in excess of the molar ratio at P. Under these circumstances the enzyme appeared to be in excess of the number of readily available sites for complex formation with tropocollagen molecules in the substrate. Since the polymeric collagen fibrils are in the form of long cylinders (Fig. 6) with roughly uniform diameter it is

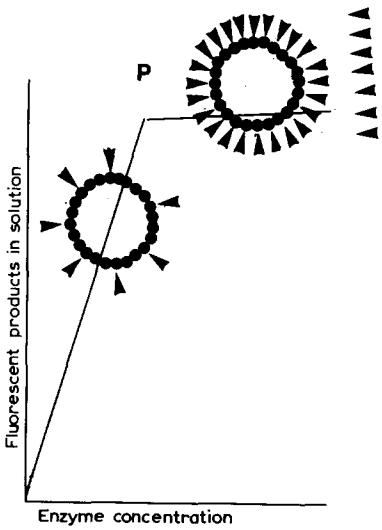

Fig. 5. This diagram represents a schematic view of the cross section of a polymeric collagen fibril. The tropocollagen molecules within the interior of the polymeric collagen fibril are not shown, only tropocollagen molecules situated at the circumference of the fibril are shown as black dots. Molecules of trypsin are indicated by arrowheads. P denotes the point of equivalence on the graph. At very low concentrations of enzyme, below P, a linear relationship exists between the products formed and the quantity enzyme in the reaction mixture, due to the excess of available substrate molecules on the surface of the polymeric fibril. With enzyme concentrations above (P), little more products are formed by increasing the enzyme concentration since all the readily available sites for an enzyme substrate complex on the surface of the fibril have been filled. Thus, the enzyme appears to be in excess of the substrate, although a vast excess of the substrate is present within the interior of the fibril. The circumferential tropocollagen molecules exhibit steric hindrance to the penetration of the enzyme and cause obstruction to collagenolytic enzymes.

understandable that the number of readily available substrate molecules within the fibrils is directly related to the surface area of the fibrils and as a consequence is directly proportional to the weight of substrate used in the enzymic digestion mixture. This relationship provides the explanation for the apparent anomalous situation observed when the quantity of substrate employed controlled the rate of product formation in the presence of a fixed amount of collagenase or trypsin.

It must be understood that at pH 3.5 individual polymeric collagen fibrils can be distinguished when these have been prepared as a dispersion in acetic acid solutions (Fig. 6) but that at pH 7.5 in Tris buffer the polymeric collagen fibrils are in the form of insoluble aggregates (or bundles found in vivo) thus the surface area readily available to collagenolytic enzymes at pH 7.5 will be greatly reduced compared to that of the dispersion shown in Fig. 6.

It is important to establish whether this substrate mediated obstruction of collagenolysis is limited to the initial period of enzymic action on the polymer or whether it is typical of the whole process of connective tissue turnover. It was possible to design a sequence of enzymic digestions of a series of twelve weighed samples of fluorescein-labelled polymeric collagen fibrils (weights varying from 2 to 14 mg) employing the methods described above for trypsin and collagenase. In this experiment, 20% of the substrate was solubilised by the end

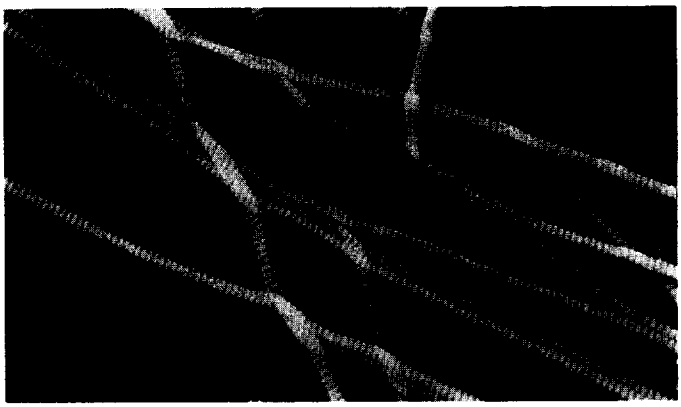

Fig. 6. Electron micrograph of polymeric collagen fibrils dispersed in 0.1 M acetic acid pH 3.5 and placed on an electron microscope grid, shadowed with gold/palladium, magnified by 14 040 and presented by courtesy of Dr. J.A. Chapman.

of the third enzymic digestion and one can be certain that the digestion kinetics at this stage reflected the extensive digestion of polymeric collagen fibrils. The twelve weighed samples of substrate were first digested with 2 μg trypsin for 3 h at 37°C, and the kinetic data showed the expected point of equivalence since the substrate was in excess of the enzyme (similar to Fig. 4).

The insoluble residues after trypsin digestion were washed four times with Tris/$CaCl_2$ buffer and then incubated at 37°C for 2 h with 7.0 μg collagenase. Again the expected point of equivalence for collagenase was obtained since the substrate was in excess of the enzyme at the higher range of substrate weights. At this stage, after a total of 5 h digestion with trypsin followed by collagenase, the insoluble residues were again extensively washed. Six samples of the trypsin-collagenase-treated fibrils were then digested with an excess of trypsin and six samples with an excess of collagenase. The trypsin digestion was carried out for 1.5 h with 10 μg of enzyme, i.e. in molar excess of the available substrate, and a linear relationship between the solubilised product and the initial weight of substrate was obtained as expected.

The remaining six residues of trypsin-collagenase-treated fibrils were then digested with a molar excess of bacterial collagenase (70 μg per tube) for 0.5 h. The kinetic data again demonstrated the linear relationship between the product formed and the weight of substrate used when the enzyme concentration (in molar terms) was in excess of the readily available substrate on the partially digested polymeric collagen fibrils. At the end of the third successive digestion 20% of the substrate had been solubilised by trypsin and collagenase.

The results described in the sequential enzymic digestion of fluorescein-labelled polymeric collagen fibrils exhibited (a) the expected points of equivalence for each enzyme used, (b) the expected behavior when each enzyme was present in molar excess of the point of equivalence, (c) independence of trypsin and collagenase digestion of the substrate at two geographically distinct sites, and (d) the kinetic patterns observed for the initial enzymic digestion of polymeric collagen fibrils was retained even after 20% of the substrate had been solubilised by the action of these enzymes. Clearly, the steric obstruction

towards enzymic digestion of polymeric collagen fibrils is a feature of the extensive digestion of the substrate rather than being confined to the initial period of digestion.

It may be concluded that the molecular architecture of tropocollagen molecules within the polymeric collagen fibrils provides a protective shield against attack by collagenolytic enzymes. The outer layers of tropocollagen molecules protect the bulk of the substrate within the individual polymeric collagen fibrils which compose the insoluble substrate at physiological pH. This accounts for the differences noted in the kinetics of collagenase digestion of tropocollagen and polymeric collagen fibrils [4] and also explains to some extent the very low turnover rate for mature collagen in vivo. Without such a substrate-mediated steric obstruction of collagenolytic enzymes it is difficult to conceive how mammalian connective tissue could withstand the attack of mammalian collagenases which are known to adhere to insoluble collagen fibres [11] in vivo.

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