The Susceptibility of Muscle Phosphorylases *a* and *b* to Digestion by a Neutral Proteinase from Rat Intestinal Muscle

COMPARISON WITH THE EFFECTS PRODUCED BY PANCREATIC TRYPSIN AND CHYMOTRYPSIN

By IAN T. CARNEY,* ROBERT J. BEYNON,*† JOHN KAY* and NIGEL BIRKET†

*Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, and

†Miles Research Laboratories, Western Avenue, Bridgend Industrial Estate, Bridgend, Wales, U.K.

(Received 10 January 1978)

1. Phosphorylase *b* was inactivated three times more rapidly than phosphorylase *a* by a neutral, trypsin-like proteinase from rat intestinal muscle. Digestion of phosphorylase *a* produced a modified form which was deactivated by AMP. Removal of the pyridoxal phosphate cofactor increased the rate of inactivation of the *b* form by about 3-fold but the susceptibility of apophosphorylase *a* was no different from the holo form. 2. The extent of proteolysis of both holoenzyme forms, as gauged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, was limited and similar digestion patterns were obtained in both cases. 3. With ^{32}P -labelled phosphorylase *a* as substrate, the initial event in the inactivation was the release of a trichloroacetic acid-soluble peptide from the *N*-terminus of the enzyme, leaving the original 100000 subunit form essentially unchanged. Subsequent proteolysis was restricted, producing derivatives of mol.wt. 85000, 70000 and 65000, none of which contained any radioactive label. 4. By treatment of inactivated phosphorylase *b* with carboxypeptidase B, it was shown that the intestinal muscle proteinase had cleaved approximately 3 -Lys-X and 3 -Arg-X bonds in the polypeptide. 5. The protective effects of various allosteric modulators of phosphorylase on the inactivation of the *a* and *b* forms were generally in agreement with the known roles of the modifiers. Glucose increased the susceptibility of phosphorylase *a*. 6. Inactivation of phosphorylase *b* by trypsin and chymotrypsin also resulted in limited proteolysis but, in both cases, the digestion patterns obtained on sodium dodecyl sulphate/polyacrylamide gels were different from each other and from the pattern obtained with the intestinal muscle proteinase. 7. Inactivation of phosphorylase *b* by the muscle proteinase is about 100 times more rapid than the effects produced by trypsin or chymotrypsin when the activities are compared on an equimolar basis. 8. Consideration is given to regulation of the rate of enzyme degradation intracellularly by modulation of the conformation and susceptibility of the enzyme via factors such as covalent modification, allosteric ligands and state of aggregation.

The enzyme glycogen phosphorylase initiates glycogen breakdown and can comprise up to about 3% of the protein present in skeletal muscle (Fischer & Krebs, 1958). It is subject to precise regulation both by allosteric modifiers and by a phosphorylation-dephosphorylation cycle (for a review see Graves & Wang, 1972). The phosphorylation of the enzyme is mediated by a specific kinase and results in the incorporation of a single phosphate residue per subunit into a serine residue at position 14 from the *N*-terminus (Titani *et al.*, 1975). This converts inactive phosphorylase *b* into active phosphorylase *a* and changes the response of the enzyme to various allosteric modifiers, e.g. phosphorylase *b* is inactive

in the absence of AMP whereas the *a* form is active without this ligand; phosphorylase *b* is inhibited by glucose 6-phosphate while phosphorylase *a* is not. Both forms are pyridoxal phosphate-dependent enzymes, although the role of this cofactor has not been established with certainty. However, removal of the cofactor causes a number of physical changes in the enzyme and results in the loss of activity.

In a previous report (Beynon & Kay, 1978) we described the partial purification from intestinal muscle of a trypsin-like proteinase with a marked ability for inactivating native enzymes *in vitro* at neutral pH. It was postulated that such a proteinase might have a role in initiating the degradation of enzymes *in vivo*. Thus it was of interest to examine the effect of the intestinal muscle proteinase on a major muscle enzyme such as phosphorylase and to see

† Present address: Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

whether the extent and rate of proteolysis might be affected by cofactor removal, ligand binding or the degree of covalent modification (phosphorylation). Such a model system might provide information on how the susceptibility of a native enzyme to neutral proteolysis *in vivo* could be regulated.

Materials and Methods

The intestinal muscle proteinase was purified to the ammonium sulphate fractionation stage and its concentration was determined by 'active site' titration with soya-bean trypsin inhibitor as described by Beynon & Kay (1978). The activity of this proteinase towards all substrates could be completely and instantaneously stopped at any stage by the addition of very small amounts of soya-bean or lima-bean trypsin inhibitors. Therefore, although the muscle proteinase preparation used was not homogeneous, it would appear unlikely that there was more than one type of proteolytic activity present.

Rabbit skeletal muscle phosphorylase *a* was obtained from Boehringer Corp. Ltd., London, U.K. Phosphorylase *b* was purified from rabbit skeletal muscle as described by Fischer & Krebs (1958) and crystallized three times in the presence of AMP and Mg^{2+} . The enzyme was freed of AMP by passage through charcoal and converted into phosphorylase *a* by the action of phosphorylase kinase and [γ - ^{32}P]-ATP as described by Antoniwi *et al.* (1977). The A_{260}/A_{280} ratio of this AMP-free material was 0.53. Phosphorylase *b* and ^{32}P -labelled phosphorylase *a* were the kind gifts of Dr. P. Cohen. Agarose-soya-bean trypsin inhibitor, chymotrypsin and trypsin were products of Miles Laboratories Ltd., Stoke Poges, Slough, U.K. α -Trypsin was prepared from the commercial product by chromatography as described by Schroeder & Shaw (1968). Bovine serum albumin, glycogen, glucose 1-phosphate, AMP, soya-bean trypsin inhibitor and carboxypeptidases A and B were obtained from Sigma (London) Ltd., Kingston-upon-Thames, Surrey, U.K. Imidazole and sodium glycerophosphate were obtained from BDH Ltd., Poole, Dorset, U.K., and were recrystallized from ethyl acetate and ethanol/water (1:1, v/v) respectively before use. All other chemicals were obtained from Sigma (London) Ltd. PCS scintillant was a product of Amersham-Searle Ltd., U.K.

Samples for determination of radioactivity were placed in 10 ml of PCS scintillant and measured in a Beckman LS100 liquid scintillation counter.

Phosphorylase activity was measured in an assay which enables the initial rate of phosphate release from glucose 1-phosphate to be followed (Carney *et al.*, 1978). Phosphorylase *b* was assayed in the presence of 1 mM-AMP, whereas phosphorylase *a* was assayed without AMP.

Removal of pyridoxal phosphate from phosphorylases *a* and *b*

Phosphorylase *b* was freed of tightly bound AMP by treatment with an equal amount of charcoal (Fischer & Krebs, 1958). Apophosphorylase *b* was prepared from this as described by Shaltiel *et al.* (1966) and apophosphorylase *a* was obtained as described by Shaltiel *et al.* (1969). Reconstitution of the holoenzymes was accomplished by incubation at 30°C with 6.4×10^{-5} M-pyridoxal phosphate for 30 min (*b* form) or 5 min (*a* form).

Proteolytic inactivations

Phosphorylase (0.5 mg) was dissolved in 0.1 ml of 0.1 M-sodium maleate buffer, pH 6.8 (for *b* form), or 0.05 M-sodium glycerophosphate buffer, pH 6.8, containing 1 mM-EDTA (for *a* form), and added to 0.2 ml of 0.05 M-sodium pyrophosphate buffer, pH 8.5. The final pH was 8.4. Digestions at 30°C were started by addition of the intestinal muscle proteinase preparation in a small volume (1–5 μ l, containing about 20 ng of proteinase as determined by active-site titration). Samples were removed at various times for assay of the phosphorylase activity remaining. For sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) samples containing approximately 30 μ g of protein were removed and added to sufficient agarose-soya-bean trypsin inhibitor (suspended in 0.2 ml of water) to inhibit the proteinase completely. The insoluble proteinase-inhibitor complexes were removed by centrifugation and the supernatants were freeze-dried before redissolving for application to the gels. Molecular-weight markers used were phosphorylase, serum albumin, glutamate dehydrogenase, malate dehydrogenase, soya-bean trypsin inhibitor and cytochrome *c*. Inactivations by trypsin and chymotrypsin (5 μ g) were carried out in an identical manner.

The effects of ligands on the proteolysis reactions were examined by adding the ligand at an appropriate concentration to the pyrophosphate buffer.

The proteolytic reactions were first-order throughout their course and rate constants for the inactivations were derived from the slopes of semilogarithmic plots of percentage activity remaining against time. Experiments were carried out at five concentrations of proteinase and the first-order constants obtained were directly proportional to the amount of proteinase over the range used (8–40 ng). Since the concentrations of proteinase used were known, values are expressed in terms of second-order rate constants.

Treatment with carboxypeptidases

A sample of phosphorylase *b* (5.5 mg) was exposed to the intestinal muscle proteinase in 1.1 ml of 0.1 M-

sodium maleate plus 2.2 ml of 0.05 M-sodium pyrophosphate buffer, pH 8.4 (final volume 3.3 ml), until all of its activity had just been removed. Agarose-soya-bean inhibitor was then added and the insoluble inhibitor-proteinase complex was removed by centrifugation.

Samples of this inactivated phosphorylase (2.5 mg), as well as of the native enzyme, were digested with carboxypeptidases A or B at 37°C as described by Ambler (1967) at substrate/carboxypeptidase ratios of 40:1. Samples (0.3 ml containing 0.5 mg of protein) were removed at various times from the digestion mixtures, added to 0.1 ml of 1 M-HCl and, after centrifugation to remove the insoluble material, the supernatants were freeze-dried. They were then redissolved in 0.1 M-sodium citrate buffer, pH 2.2, and loaded into a Locarte amino acid analyser.

In digestions involving ^{32}P -labelled phosphorylase *a*, samples (20 μl) for radioactivity counting were removed into 0.2 ml of 20% trichloroacetic acid and soya-bean trypsin inhibitor (0.5 mg) was added as a carrier to ensure complete precipitation of the protein. The samples were centrifuged and the super-

natants were counted in 10 ml of PCS scintillant. The pellets were extensively washed with 4% trichloroacetic acid before being dissolved in 0.2 ml of 0.1 M-sodium hydroxide. Samples of the dissolved pellets (0.05 ml) were placed in 10 ml of PCS scintillant and stored overnight at room temperature in the dark before radioactivity counting.

The sodium dodecyl sulphate/polyacrylamide gels obtained after staining with Coomassie Blue were sliced into the various protein bands and these were swollen in 0.5 ml of NCS tissue solubilizer (Amersham-Searle) for 6 h at 50°C. PCS scintillant (10 ml) was added to each extract and the samples were again kept overnight in the dark before radioactivity counting.

Results

Inactivation of phosphorylases a and b by the intestinal muscle proteinase

Representative inactivations of holophosphorylases *a* and *b* (Fig. 1) show that the *b* form was

Fig. 1. Susceptibilities of apo- and holo-phosphorylases *a* and *b* to the intestinal muscle proteinase

Holophosphorylases *b* (■) and *a* (□) were incubated (0.5 mg of each) at 30°C in 0.3 ml of maleate/pyrophosphate or glycerophosphate/pyrophosphate buffers, pH 8.4, respectively with 2 μl of the intestinal muscle proteinase preparation (containing 20 ng = 0.6 pmol of proteinase). Samples (20 μl) were removed at various time intervals and the phosphorylase activity remaining was assayed in the presence of 1 mM-AMP (for *b*) but without ligands for phosphorylase *a*. Samples for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were removed from the digests of holophosphorylase *b* ($b_0 - b_5$) and *a* ($a_0 - a_3$) at the indicated times. Apophosphorylases *b* (▲) and *a* (△) were incubated (0.5 mg of each) at 25°C in 0.3 ml of 0.05 M-sodium pyrophosphate buffer, pH 8.4, containing 15 mM-mercaptoethanol with 2 μl (20 ng) of proteinase. Samples (20 μl) were removed and added to 0.18 ml of assay buffer [0.1 M-sodium maleate, pH 6.8, plus 40 mM-mercaptoethanol and serum albumin (1 mg/ml)] containing soya-bean trypsin inhibitor (40 μg) and 0.06 mM-pyridoxal phosphate. After preincubation for 30 min (for phosphorylase *b*) or 5 min (for *a*) to reconstitute the holoenzymes, the activity remaining was assayed in the presence or absence of 1 mM-AMP for the *b* and *a* forms respectively. Apophosphorylases *b* (●) and *a* (○) were stable without added proteinase.

inactivated approximately three times more rapidly than the *a* form at 30°C. Since the concentration of proteinase used (20 ng/0.3 ml of digest) was known and it had been found that the observed first-order rate constants ($k_{\text{obs.}}$) were proportional to proteinase concentration (see the Materials and Methods section), comparisons of the rates of inactivation of the various forms of phosphorylase will be made in terms of second-order constants ($k_{\text{obs.}}/[E]$). The values obtained for inactivation of the *b* and *a* forms are 0.37×10^6 and $0.13 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively. It was not possible to carry out digestions of the

apoenzyme forms at 30°C owing to their instability. However, by incubating apophosphorylases *b* and *a* with the proteinase at 25°C, samples could be removed at various times for reconstitution of the holoenzyme form with pyridoxal phosphate before assaying the activity remaining (Fig. 1). Removal of the pyridoxal cofactor from phosphorylase *a* had little effect on the rate of inactivation ($K_2 = 0.12 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 25°C), whereas apophosphorylase *b* at 25°C was about three times more susceptible to inactivation ($K_2 = 0.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) than the holo-form was at 30°C. All four forms of phosphorylase were stable at their respective temperatures without added proteinase and mercaptoethanol (15 mM) had no effect on the rates of inactivation of holophosphorylases *a* and *b*.

Samples were removed at various times during the inactivations of holophosphorylases *b* and *a* (b_0 – b_5 ; a_0 – a_3 respectively; Fig. 1) for analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after inhibition and removal of the proteinase by the addition of agarose–soya-bean trypsin inhibitor. The patterns obtained (Fig. 2) were similar for both holophosphorylases and the extent of digestion was limited, generating in both cases products of approximate mol. wts. 85 000, 70 000 and 65 000 (as determined by comparison with marker proteins). Even when virtually all of the enzymic activity had been removed (b_5 ; a_3), a large amount of protein persisted in the position of the original 100 000 subunit.

Fig. 2. Extent of digestion of phosphorylases *a* and *b* by the intestinal muscle proteinase

Incubations of holophosphorylases *a* and *b* with the intestinal muscle proteinase were carried out as described in the legend to Fig. 1, and samples (20 μ l) were removed at the indicated times and added to 0.2 ml of insolubilized soya-bean trypsin inhibitor suspended in water. After centrifugation the supernatants were freeze-dried before analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Inactivation of ^{32}P -labelled phosphorylase *a* by the intestinal muscle proteinase

Fig. 3 shows the digestion of ^{32}P -labelled holophosphorylase *a* by the proteinase. It can be seen that the release of ^{32}P -containing material into a trichloroacetic acid-soluble form occurred at a parallel rate to the loss in enzymic activity. Samples were removed during the inactivation for analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and a pattern identical with that shown for phosphorylase *a* in Fig. 2 was obtained. The radioactivity in all of the protein bands obtained at the various times was determined and it was found that the label was rapidly lost from the material in the 100 000-mol.wt. subunit position (Fig. 3) at a similar rate to the loss in enzymic activity. No radioactive label could be detected in any of the protein bands resulting from digestion.

Digestion with carboxypeptidases A and B

Carboxypeptidases A and B were unable to release any free amino acids from native phosphorylase *b*. A sample of phosphorylase *b* was treated with the muscle proteinase until all of its activity had just been removed and then agarose–soya-bean trypsin inhibitor was added to inactivate the proteinase. Carboxy-

Fig. 3. Inactivation of ^{32}P -labelled phosphorylase *a* by the intestinal muscle proteinase

^{32}P -labelled phosphorylase *a* (0.33 mg, 1.54×10^4 d.p.m.) was incubated at 30°C in 0.15 ml of 0.05 M-glycerophosphate / 0.05 M-pyrophosphate buffer, pH 8.4, with 5 μl of intestinal muscle proteinase (50 ng). Samples were removed: (1) for assay of phosphorylase *a* activity remaining ($\circ$) and (2) added to 0.2 ml of 20% trichloroacetic acid containing 0.5 mg of carrier soya-bean trypsin inhibitor. After centrifugation, the supernatants ($\bullet$) were counted for radioactivity and, after washing with 4% trichloroacetic acid, the insoluble material ($\blacktriangle$) was taken up in 0.2 ml of 0.1 M-NaOH for scintillation counting; (3) samples for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were treated as described in the legend to Fig. 2. After staining with Coomassie Blue and destaining electrophoretically each protein band was sliced from the gel, swollen and counted for radioactivity. The radioactivity in the position of the 100000-mol.wt. subunit is shown ($\blacksquare$). No radioactivity could be detected in any of the other bands.

Fig. 4. Digestion of inactivated phosphorylase *b* by carboxypeptidase *B*

Phosphorylase *b* previously inactivated by the muscle proteinase was incubated at 37°C with carboxypeptidase *B* (2.5 mg of phosphorylase/1.5 ml of 0.1 M-sodium maleate/0.05 M-sodium pyrophosphate buffer, pH 8.4; substrate/carboxypeptidase ratio of 40:1). Samples (0.3 ml) were removed at various time intervals and added to 0.1 ml of 1 M-HCl containing norleucine (20 nmol) as an internal standard. After centrifugation, the supernatants were freeze-dried before amino acid analysis.

peptidase A did not remove any amino acids from this digested phosphorylase. However, treatment with carboxypeptidase B (Fig. 4) released approx. 3 lysine and 3 arginine residues/molecule of phosphorylase.

Effects of ligands on the rate of inactivation of holophosphorylases *a* and *b*

Since both forms of phosphorylase are susceptible to allosteric regulation, the effects of various ligands on the rates of inactivation were examined (Table 1). For phosphorylase *b*, the ligands can be grouped into three classes: those with no effect ($0.47 \times 10^6 > K_2 > 0.37 \times 10^6$), those which reduced the rate by about one half ($0.25 \times 10^6 > K_2 > 0.18 \times 10^6$) and those which virtually stabilized the enzyme against inactivation ($K_2 \approx 0.04 \times 10^6$).

Table 1. Effect of various ligands on the rate of inactivation of phosphorylases *a* and *b* by rat intestinal muscle proteinase

Values for the second-order rate constants are given. n.d., Not determined.

Ligand	$10^{-6} K_2 \text{ (M}^{-1}\cdot\text{s}^{-1}\text{)}$	
	Phosphorylase <i>b</i>	Phosphorylase <i>a</i>
None	0.37	0.13
UDP-glucose (10 mM)	0.38	0.04
NADH (1 mM)	0.47	n.d.
Glucose 6-phosphate (3 mM)	0.18	0.12
IMP (3 mM)	0.22	0.03
Adenine (3 mM)	0.22	n.d.
Phosphate (10 mM)	0.25	0.03
AMP (2 mM)	0.045	0.03
CMP (2 mM)	0.04	0.06
ATP (5 mM)	n.d.	0.04
Maltotriose (0.2 M)	n.d.	0.115
Glucose (50 mM)	n.d.	0.30

The effect of each individual ligand on the inactivation of phosphorylase *a* was not necessarily the same as had been found with phosphorylase *b*. However, in general, it was noticeable that in addition to ligands that protected, partially protected or had no effect, glucose actually increased the susceptibility of the *a* form to a rate approaching that obtained with phosphorylase *b*.

Digestion of phosphorylase b with trypsin and chymotrypsin

Holophosphorylase *b* was rapidly inactivated by trypsin (Fig. 5), but, since much more of this proteinase was added ($5\mu\text{g}/0.3\text{ml}$) in comparison with the intestinal muscle proteinase, the calculated second-order rate constant is $0.0033 \times 10^6 \text{M}^{-1} \cdot \text{s}^{-1}$. Chymotrypsin ($5\mu\text{g}/0.3\text{ml}$) was slightly less effective ($K_2 = 0.023 \times 10^6 \text{M}^{-1} \cdot \text{s}^{-1}$).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of samples removed from the digests (Fig. 6) showed that the patterns of degradation obtained with trypsin and chymotrypsin were different. Both of these patterns were different, in turn, from that obtained with the intestinal muscle proteinase. Digestion with trypsin or chymotrypsin led to the rapid disappearance of the 100000-mol.wt. subunit but the overall extents of degradation were still relatively limited. Only small amounts of low-molecular-weight material appeared on the gels.

Fig. 5. Inactivation of phosphorylase *b* by trypsin and chymotrypsin

Phosphorylase *b* (0.5 mg) was incubated at 30°C with $5\mu\text{g}$ of trypsin (●) or chymotrypsin (○) as described in the legend to Fig. 1. Samples for electrophoresis were removed from the tryptic ($T_0 - T_3$) and chymotryptic ($C_0 - C_3$) digests as indicated.

Production of an AMP-deactivated derivative of phosphorylase a

It has been shown by Nolan *et al.* (1964) that controlled digestion of phosphorylase *a* with trypsin releases a small peptide from the *N*-terminus of the

Fig. 6. Extent of digestion of phosphorylase *b* by trypsin and by chymotrypsin

Incubations and sample preparation for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were carried out as described in the legends to Figs. 5 and 2.

Fig. 7. Effect of AMP on the enzymic activity of the digestion products from phosphorylase *a* produced by the intestinal muscle proteinase

Holophosphorylase *a* (0.5 mg) was incubated at 30°C in 0.3 ml of 0.05 M-glycerophosphate/pyrophosphate buffer, pH 8.4, with the proteinase. Samples (20 µl) were removed for assay of the enzymic activity remaining with (●) and without (○) AMP (1 mM).

enzyme and produces a form (phosphorylase *b'*) that is inactive unless assayed in the presence of AMP.

Phosphorylase *a* was incubated with the intestinal muscle proteinase and duplicate samples were removed at various times and assayed for residual phosphorylase activity, one without AMP and the other with AMP (1 mM) added. Fig. 7 shows that the rate of inactivation of phosphorylase when the residual activity was assayed in the presence of AMP was apparently greater than the loss of phosphorylase *a* activity (i.e. when assays were performed in the absence of AMP). Thus, in contrast to the effect observed with trypsin, it would appear that the modified subunit produced by the muscle proteinase must be inhibited by AMP.

Discussion

Phosphorylase *b* was digested more readily than phosphorylase *a* by the intestinal muscle proteinase but the extent of digestion of these native substrates, as shown by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, was relatively limited. Only a few peptide bonds appeared to have been hydrolysed by the intestinal muscle proteinase since a virtually unchanged 100000 mol.wt. subunit persisted

throughout the course of the inactivations. This was confirmed by the studies with ^{32}P -labelled phosphorylase *a* in which the proteinase released the radioactive label into a trichloroacetic acid-soluble form at a parallel rate to the loss of enzymic activity. The loss of radioactivity from both the trichloroacetic acid-insoluble fraction and the original 100000 mol.wt. subunit (on gel electrophoresis) also paralleled the loss in enzymic activity. The phosphoserine residue is situated close to the *N*-terminus of the enzyme, in a basic region of the polypeptide chain which, from X-ray-crystallographic studies, is believed to be extended away from the main body of the subunit (Fletcher *et al.*, 1976). It might therefore be exposed, thus facilitating attack by a proteinase of trypsin-like specificity.

The data suggest that in phosphorylase *a*, and by implication phosphorylase *b*, the initial step in the proteolysis by the intestinal muscle proteinase is a cleavage close to the *N*-terminus of the phosphorylase, resulting in loss of activity and phosphopeptide, but leaving the molecular weight of the subunit essentially unchanged. An analogous situation has been described for the digestion of phosphorylase *a* with trypsin (Nolan *et al.*, 1964), except that the derivative obtained, phosphorylase *b'*, is activated by AMP. This contrasts with the deactivation observed with the product from the muscle proteinase treatment, suggesting that the sites of cleavage by trypsin and by the muscle proteinase are different.

This conclusion is further supported from the sodium dodecyl sulphate/polyacrylamide gels, which showed different patterns of digestion with all three of the proteinases tested.

Tryptic digestion has been used as a probe in previous studies which have attempted to determine the spatial separation of the binding sites for ligands in phosphorylases *b* (Kurganov *et al.*, 1972) and *a* (Varsanyi & Bot, 1973), by examining the effects of the ligands on the rates of inactivation. The aims of the present study were somewhat different but the protective effects of ligands on the muscle proteinase-catalysed inactivations of both forms of phosphorylase are in general agreement with the effects observed previously with trypsin. The rates of the muscle proteinase-catalysed inactivation of phosphorylases *a* and *b* in the presence of AMP were similar and these forms of the enzyme are supposedly very similar as judged by parameters such as specific activity, sedimentation constants and by electron spin resonance (Griffiths *et al.*, 1976). Glucose 6-phosphate did not affect the inactivation of phosphorylase *a* by the muscle proteinase but reduced the susceptibility of the *b* form by about one half. In contrast, glucose increased the rate of inactivation of phosphorylase *a* to a rate approaching that of the *b* form. It has been suggested that glucose promotes the dissociation of the tetrameric form of phosphorylase *a* *in vitro* into

dimers (Wang *et al.*, 1965). Varsanyi & Bot (1973) also found that glucose increased the trypsin-catalysed inactivation of phosphorylase *a*.

Thus whereas the ligand effects observed with the two proteinases are similar, it appears that the muscle proteinase is different in the proteolytic reactions that it produces with phosphorylases *a* and *b*. However, it has previously been shown (Beynon & Kay, 1978) that the intestinal muscle proteinase is sensitive to inhibitors of trypsin-like enzymes and the trypsin-like specificity of the proteinase is confirmed by the present study. From the data obtained by treatment of inactivated phosphorylase *b* with carboxypeptidase B it would appear that the intestinal muscle proteinase hydrolysed at least six bonds in the polypeptide chain, each one involving -Lys-X or -Arg-X. This is in reasonable agreement with the value that can be calculated for the minimum number of bonds that would have to have been split in order to account for the various products observed, i.e. phosphopeptide release leaving an essentially unchanged 100000 mol.wt. subunit along with the generation of degradation products of mol.wt. about 85000, 70000 and 65000.

Another factor to emerge from this comparison is the greatly enhanced efficiency of the muscle proteinase for inactivating phosphorylase. The preparation of muscle proteinase used was purified only to the ammonium sulphate fractionation stage, so that the possibility of this preparation containing more than one type of proteolytic activity must be considered. However, since the proteolysis of all enzyme, protein and peptide substrates (including phosphorylase) tested with this preparation could be completely stopped at any stage by the addition of small amounts of trypsin inhibitors (Beynon & Kay, 1978), if multiple proteinase activities were present in the intestinal preparation, they must have been very similar and therefore all the more interesting to study. Thus although direct comparison with pure trypsin must be viewed with some reservation, nevertheless, on an approximate equimolar basis, the intestinal muscle proteinase is about 100 times more efficient than trypsin for inactivating phosphorylase *b* ($K_2 = 0.37 \times 10^6$ and $0.0033 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively). This is in keeping with the earlier findings of Beynon & Kay (1978), who showed that trypsin and the intestinal muscle proteinase had similar activities towards denatured polypeptide substrates but the latter was 100–200 times more efficient in its capacity for inactivating native enzymes.

Katunuma *et al.* (1975) have described the purification from several rat tissues of 'group-specific' proteinases, specific for inactivating pyridoxal phosphate-dependent enzymes only. The proteolysis of apo- and holo-phosphorylases *a* and *b* (i.e. with and without the pyridoxal phosphate) by one of these, a chymotrypsin-like neutral proteinase purified from

rat skeletal muscle, has been described by Matsuda & Fischer (1975).

From their data, an approximate rate constant of $0.00005 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ can be calculated for the inactivation of phosphorylase *a*. Even allowing for differences in substrate concentration, temperature, pH etc., since the corresponding figure for the intestinal muscle proteinase is 0.13×10^6 , it would appear that the intestinal proteinase inactivates phosphorylase *a* about 2000 times more rapidly. Thus it would seem that the activity of the 'group-specific' proteinase towards one of its own 'specific' substrates is not particularly marked and is no more 'specific' than trypsin (K_2 approx. $0.002 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$).

Inactivation of native enzymes by the intestinal muscle proteinase is very rapid but it has been suggested (Beynon & Kay, 1978) that the 'specificity' for attack by this (and other) proteinase(s) may be determined, primarily, by the vulnerability of the substrate enzyme to proteolysis rather than any inherent 'group specificity' of the proteinase. The present study shows that by changing the susceptibility of the substrate enzyme allosterically or through changes in primary, secondary, tertiary or quaternary structure, it is possible to modulate the rate of proteolysis. Beynon & Kay (1978) found a specific inhibitor of the intestinal muscle proteinase so that if the inactivating ability of this (and other) proteinase(s) is maintained *in vivo*, it would appear that adequate methods are available for controlling the expression of the activity. Thus although this study has been carried out with skeletal muscle phosphorylase rather than the smooth muscle enzyme, which seems to be a slightly different protein (Beuding *et al.*, 1964), it would seem to provide an excellent model from which to begin investigations into the intracellular location and role of the proteinase.

Supported by the M.R.C. (Grant no. G974/126/B). I. T. C. acknowledges the receipt of an SRC/CASE studentship in co-operation with Miles Laboratories; R. J. B. was in receipt of an S.R.C. studentship. We are very grateful to Dr. Philip Cohen, University of Dundee, for his interest in this work and for providing us with generous samples of radioactive and unlabelled phosphorylase. We thank Dr. R. A. John of this Department for his help in preparing this manuscript.

References

- Ambler, R. P. (1967) *Methods Enzymol.* **11**, 155–166
- Antoniw, J. F., Nimmo, H. G., Yeaman, S. J. & Cohen, P. (1977) *Biochem. J.* **162**, 423–433
- Beuding, E., Kent, N. & Fisher, J. (1964) *J. Biol. Chem.* **239**, 2099–2101
- Beynon, R. J. & Kay, J. (1978) *Biochem. J.* **173**, 291–298
- Carney, I. T., Beynon, R. J., Kay, J. & Birket, N. (1978) *Anal. Biochem.* **85**, 321–325

- Fischer, E. H. & Krebs, E. G. (1958) *J. Biol. Chem.* **231**, 65-71
- Fletterick, R. J., Sygusch, J., Semple, H. & Madsen, N. B. (1976) *J. Biol. Chem.* **251**, 6142-6146
- Graves, D. J. & Wang, J. H. (1972) *Enzymes 3rd Ed.* 435-482
- Griffiths, J. R., Dwek, R. A. & Radda, G. K. (1976) *Eur. J. Biochem.* **61**, 237-242
- Katunuma, N., Kominami, E., Kobayashi, K., Banno, Y., Suzuki, K., Chichibu, K., Hamaguchi, Y. & Katsunuma, T. (1975) *Eur. J. Biochem.* **52**, 37-50
- Kurganov, B. I., Lisovskaya, N. P. & Livanova, N. B. (1972) *Biokhimiya* **37**, 289-298
- Matsuda, Y. & Fischer, E. H. (1975) in *Intracellular Protein Turnover* (Schimke, R. T. & Katunuma, N., eds.), pp. 213-222, Academic Press, New York
- Nolan, C., Novoa, W. B., Krebs, E. G. & Fischer, E. H. (1964) *Biochemistry* **3**, 542-551
- Schroeder, D. D. & Shaw, E. (1968) *J. Biol. Chem.* **243**, 2943-2949
- Shaltiel, S., Hedrick, J. L. & Fischer, E. H. (1966) *Biochemistry* **5**, 2108-2116
- Shaltiel, S., Hedrick, J. L., Pocker, A. & Fischer, E. H. (1969) *Biochemistry* **8**, 5189-5196
- Titani, K., Cohen, P., Walsh, K. A. & Neurath, H. (1975) *FEBS Lett.* **55**, 120-123
- Varsanyi, M. & Bot, G. (1973) *Acta Biochem. Biophys. Acad. Sci. Hung.* **8**, 23-31
- Wang, J. H., Shonka, M. L. & Graves, D. J. (1965) *Biochem. Biophys. Res. Commun.* **18**, 131-135
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412