Immunological detection of degradation intermediates of skeletal-muscle glycogen phosphorylase *in vitro* and *in vivo*

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Over 95 % of the pyridoxal phosphate (PLP) in skeletal muscle is bound to one protein, glycogen phosphorylase. This, and the fact that phosphorylase constitutes approx. 5 % of the soluble protein in skeletal muscle, introduce the possibility that PLP might be used as a specific label to identify degradation intermediates of the enzyme. In this investigation, we have developed immunological methods, using a monoclonal antibody to PLP and polyclonal antibodies to phosphorylase, to detect degradation intermediates *in vitro* and *in vivo*. We have identified a family of degradation intermediates of glycogen phosphorylase in the high-speed-supernatant fraction of mouse skeletal muscle. These peptides react with both types of antibodies and are in the size and concentration range expected for degradation intermediates in a model in which the committed step is followed by rapid clearance of the products. Changes in amounts of degradation intermediates are examined in physiological or pathological conditions in which the rate of degradation of phosphorylase is altered.

INTRODUCTION

Intracellular protein degradation converts a native protein into its composite amino acids, and it is implicit that this process must require the sequential action of proteinases, initially generating large fragments but eventually yielding free amino acids. It follows that during the degradative processes intermediates must be formed by partial proteolysis (Beynon *et al.*, 1985*b*). In general, such intermediates have not been observed, implying that the first, committed, step in intracellular protein catabolism is considerably slower than subsequent steps (Beynon *et al.*, 1985*a*). The late steps in degradation represent the clearance of functionally inactivated material and, as such, would be expected to be rapid. However, many studies *in vitro* have demonstrated that proteolytic attack on native proteins can proceed via discrete transient intermediates that contain relatively resistant domains (Price & Johnson, 1989). Such investigations establish a precedent for low concentrations of discrete degradation intermediates *in vivo*.

Knowledge of the degradation intermediates could provide important information on the route(s) of catabolism of an intracellular protein, identifying proteolytically vulnerable areas of the molecule. Further, the intracellular localization of these intermediates would be valuable in identifying the subcellular sites of early stages of degradation. Identification of degradation intermediates *in vivo* is difficult. First, many of the peptides that are produced are devoid of properties that allow their identification. Secondly, it is to be expected that these intermediates exist at low levels in the cell, and therefore sensitive detection methods are needed. Western blots, with antibodies of the required affinity and selectivity, may provide that sensitivity.

There have been few studies that have attempted to identify degradation intermediates produced *in vivo*. The degradation of biotin-containing enzymes can be monitored with radiolabelled cofactor, and in cultured cell systems it has proved possible to demonstrate a family of biotinylated peptides, putatively derived from these enzymes (Chandler & Ballard, 1988). However, the four biotin-enzymes are present in cells at similar levels, and it was not possible to assign the intermediates to one particular

enzyme. Polyclonal antibodies have been used to identify degradation fragments of liver aldolase (Reznick *et al.*, 1985) and phosphofructokinase (Toda & Ohashi, 1986). The aldolase study may have been susceptible to degradation artefacts such as we have described (Cookson & Beynon, 1987), whereas the phosphofructokinase study used purified enzyme, radiolabelled *in vitro*, to demonstrate the lack of artefactual degradation products.

Glycogen phosphorylase (EC 2.4.1.1; phosphorylase) has a number of properties that facilitate the analysis of its degradation. First, it is present at high levels in skeletal muscle, constituting as much as 5 % of the soluble protein. Second, the enzyme is well understood in terms of structure (Fletcher & Madsen, 1980; Dombradi, 1980; Sansom *et al.*, 1985). The three-dimensional structures of the rabbit phosphorylases *a* and *b* are known to less than 0.3 nm resolution (Sprang & Fletcher, 1979; Sansom *et al.*, 1985). Finally, over 95 % of the pyridoxal phosphate (PLP) in skeletal muscle is associated with this enzyme. The cofactor can therefore be a specific probe for this enzyme, and we have used radiolabelled PLP to monitor the rate of degradation of the enzyme (Butler *et al.*, 1985; Cookson & Beynon, 1989). The PLP is attached to a lysine residue (Lys-680 of a total of 841 amino acids in the rabbit muscle enzyme) approx. 80 % along the primary sequence, and is buried deep within the phosphorylase structure. Removal of PLP from the holoenzyme is difficult (Shaltiel *et al.*, 1967), and thus PLP may remain attached to degradation intermediates. We have used a monoclonal antibody to PLP (Viceps-Madore *et al.*, 1983), in combination with polyclonal antibodies to muscle phosphorylase to search for degradation intermediates of this enzyme.

MATERIALS AND METHODS

Materials

Pure rabbit skeletal-muscle glycogen phosphorylase was provided by Professor P. Cohen, University of Dundee. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins were from Dako Ltd., High Wycombe, Bucks., U.K. Nitrocellulose (pore size 0.2 µm) was from Schleicher and Schuell,

Abbreviations used: PBS, phosphate-buffered saline (10 mM-sodium phosphate/0.15 M-NaCl, pH 7.4); PBS-T, PBS containing 0.2 % (v/v) Tween 20; PLP, pyridoxal phosphate.

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D-3354 Dassel, West Germany. All other chemicals were of analytical grade.

Preparation of tissue samples

Adult C57BL/6J and dystrophic C57BL/6J^{dy/dy} mice of either sex were bred in the Departmental facility. C57BL/10J and dystrophic C57BL/10J^{mdx/mdx} mice were a gift from Dr. P. McLennan, Department of Medicine, University of Liverpool. Mice were maintained at 22–25 °C on a 12 h-light/12 h-dark cycle and were allowed free access to food and water. In some animals, sciactomy was performed on one hind limb as described previously (Leyland *et al.*, 1990); the contralateral limb acted as control. Animals were killed by cervical dislocation, and liver or skeletal muscle from hind limbs and back was rapidly dissected and placed on ice. Day-old male broiler chicks (White Plymouth Rock) were obtained from Vale Royal Hatcheries, Frodsham, Cheshire, U.K., and 1-day-old male layer chicks (Hi-Sex Brown) were obtained from Farm Fresh Hatcheries, Tarleton, Lancashire, U.K. Chickens were housed in a heated brooder in a room with continuous lighting and were fed *ad libitum* on a commercial broiler starter diet containing 19% protein and 10.2 MJ of metabolizable energy/kg. Chickens (8 weeks old) were killed by cervical dislocation, and the pectoralis muscle was rapidly dissected and placed on ice. All tissues were homogenized in 4 vol. of 5 mM-Hepes/1 mM-EDTA/14 mM-mercaptoethanol, pH 7.4 (mouse), or 25 mM-sodium phosphate buffer, pH 7.4 (chicken), and the homogenate was centrifuged at 50000 g-h. The supernatant fraction was carefully decanted and was filtered to remove any fat particles.

Antibodies

The monoclonal antibody E6(4)1, highly specific for PLP, was prepared as described previously (Viceps-Madore *et al.*, 1983). Ascites fluid was used as a source of IgG without further purification. Affinity-purified rabbit antibodies to mouse phosphorylase were prepared as described previously (Cookson & Beynon, 1987). A polyclonal antibody to pure chicken phosphorylase was raised in rabbits by an initial subcutaneous injection of 100 µg of chicken phosphorylase, purified to homogeneity by AMP–Sephacryl affinity chromatography, followed by MonoQ ion-exchange chromatography. The antigen was injected in Freund's complete adjuvant; subsequently the animal was boosted by subcutaneous injection at two 6-weekly intervals, each with 100 µg of protein in Freund's incomplete adjuvant.

PAGE

SDS/PAGE was performed by the method of Studier (1973). The 1.5 mm-thick gels consisted of a 12.5% running gel and a 5% stacking gel. Electrophoresis was for 3–4 h at 40 mA at 10 °C. After electrophoresis, parts of the gel were stained for protein or electroblotted on to nitrocellulose. In some instances, samples for electrophoresis were treated with 160 mM-NaCNBH₃ for 10 min at 4 °C in the presence of 0.25 M-imidazole/citrate buffer, pH 6.0 (Shaltiel *et al.*, 1967), to reduce and thus to stabilize the aldimine linkage between cofactor and protein. Samples were mixed with an equal volume of sample buffer [0.08 M-Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 0.1 M-dithiothreitol, 10% (v/v) glycerol and 0.001% (w/v) Bromophenol Blue]. Unreduced samples were treated identically other than for the omission of NaCNBH₃. Samples were kept on ice, or heated to 60 °C or 100 °C according to the experiment.

Western blotting

Proteins separated by SDS/PAGE were electroblotted on to nitrocellulose by a modification of the method of Towbin *et al.* (1979). The transfer was performed at 10 °C for 20 h, with a

current of 70 mA. The nitrocellulose membrane was immunostained with anti-PLP monoclonal antibody or polyclonal antibodies to mouse or chicken phosphorylase.

All incubations were at room temperature with continuous shaking. The nitrocellulose was first incubated for 1.5 h in phosphate-buffered saline (PBS; 10 mM-sodium phosphate/0.15 M-NaCl, pH 7.4) containing 0.2% (v/v) Tween 20 (PBS-T) and a suitable dilution of antibody preparation. The monoclonal antibody was used at a dilution of ascites fluid of 1:250000, the rabbit anti-mouse phosphorylase serum at a dilution of 1:500 and the rabbit anti-chicken phosphorylase serum at a dilution of 1:1000. The nitrocellulose was then washed three times in PBS-T (5 min/wash). Second antibody, i.e. horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin or goat anti-rabbit immunoglobulin, diluted 1:1000 in PBS-T, was incubated with the nitrocellulose for 1.5 h, followed by a further three washes in PBS-T (5 min/wash). Second antibody was detected by incubating the nitrocellulose for 10 min in PBS-T, containing 0.02% (w/v) diaminobenzidine and 0.03% (v/v) H₂O₂. Faint degradation products are barely visible when the blot is viewed in reflected light, and have been emphasized by photography in transmitted light and by deliberate over-exposure. This treatment also enhances bands corresponding to non-specific reactions, which are readily identified by comparison with unreduced samples.

Limited-proteolysis studies

Pure preparations of rabbit glycogen phosphorylase were digested with trypsin. At appropriate stages in the digestion the reaction was terminated by inhibition of the proteinase with soybean trypsin inhibitor, immobilized on CNBr-activated Sepharose 6B. The course of the proteolytic reaction was monitored by measurement of the activity of glycogen phosphorylase (Carney *et al.*, 1978b) and by densitometric measurement of the phosphorylase band after SDS/PAGE.

RESULTS AND DISCUSSION

Detection of degradation intermediates

The premises on which our study is based are (a) that limited proteolysis of phosphorylase generates discrete degradation intermediates, and (b) that PLP will remain attached to those intermediates that include the PLP-binding residue. Proteolysis of native phosphorylase *in vitro* generates discrete proteolytic fragments, irrespective of the nature of the proteinase (Raibaud & Goldberg, 1973; Carney *et al.*, 1978a; Dombradi *et al.*, 1983). Thus the native protein consists of regions that are vulnerable to proteolysis, but the products of attack at these sites possess or acquire a greater resistance to proteolysis, such that intermediates accumulate. Fig. 1 describes the effect of trypsin on phosphorylase. Tryptic attack is much more restricted than would be expected from the primary specificity of this proteinase. Several degradation products of 50–90 kDa are generated, but none of them retain glycogenolytic activity, as the pseudo-first-order rate constant for inactivation of phosphorylase was 0.023 min⁻¹, which compares with a value of 0.024 min⁻¹ for the loss of 97.4 kDa phosphorylase band on SDS/PAGE. There are no intermediates between 40 kDa and low-molecular-mass peptidic material (< 5 kDa) at the bottom of the gel, which implies that material smaller than 40 kDa has lost sufficient higher-order structure to become vulnerable to rapid and extensive proteolysis. The family of degradation intermediates generated by trypsin has been used as a model system for this study.

PLP does not remain attached to proteins under the denaturing conditions of sample preparation for SDS/PAGE, and it is necessary to reduce the Schiff base between the aldehyde group

Fig. 1. Generation of degradation intermediates by proteolysis of phosphorylase *in vitro*

Rabbit muscle glycogen phosphorylase (1.2 mg/ml) was digested at 30 °C with trypsin (5 µg/ml) in 0.02 M-Hepes buffer, pH 7.4, containing 0.1 M- Na_2SO_4 . At suitable time intervals, samples were removed for assay of phosphorylase activity (□). At the same time a further sample (60 µl) was added to 40 mg of soybean trypsin inhibitor-Sepharose 6B (equivalent to 100 µg of inhibitor). The mixture was centrifuged, and the supernatant (10 µg of protein) was subjected to SDS/PAGE. The gel was stained for protein (lower panel) and the phosphorylase band (■, 97 kDa) in each lane was quantified by densitometry.

on the cofactor and the ϵ -amino group on the protein. Extended treatment of phosphorylase with reducing agents such as NaBH_4 elicits low-level fragmentation of the protein. Preliminary experiments established that treatment with 160 mM- NaCNBH_3 for 10 min gave substantial fixation of cofactor without artefactual fragmentation. In this respect NaCNBH_3 is superior to the more reactive NaBH_4 . When phosphorylase is treated with either NaBH_4 or NaCNBH_3 , the apparent molecular mass on SDS/PAGE increases (Fig. 2). We have no explanation for this phenomenon, although retention of cofactor on the protein may interfere with SDS binding. The shift in mobility seems to be specific to phosphorylase (and has been observed with rabbit, mouse and chicken enzymes), and is a useful diagnostic tool for the identification of the protein and PLP-containing fragments.

Retention of PLP by degradation fragments

Detection of phosphorylase-derived peptides by the anti-PLP monoclonal antibody is only feasible if the cofactor remains attached to the products of proteolysis. A tryptic digest of phosphorylase (< 10% residual activity) was monitored by Fast Green staining of protein bands in the polyacrylamide gel and by

Fig. 2. Immunological detection of degradation intermediates of phosphorylase, generated *in vitro*

Rabbit muscle glycogen phosphorylase (2.0 mg/ml) was digested for 140 min at 30 °C with trypsin (30 µg/ml) in 0.02 M-Hepes buffer, pH 7.4, containing 0.1 M- Na_2SO_4 . The digest was split into two, and one half was treated with NaCNBH_3 (+[H]) as described in the Materials and methods section before SDS/PAGE. Identical pairs of lanes (one reduced sample, +[H]; one unreduced sample, -[H]) were either stained for protein in the gel or electroblotted on to nitrocellulose. Of the latter, one pair was probed with a polyclonal antibody to phosphorylase, and the other with a monoclonal antibody to PLP.

Western blots (Fig. 2), either with an affinity-purified polyclonal antibody to mouse phosphorylase ('anti-phosphorylase'), or with a monoclonal antibody to PLP ('anti-PLP'). All tryptic peptides can be detected by the polyclonal antibody to the protein. After NaCNBH_3 treatment most of the bands increase in mobility. Moreover, anti-PLP Western blotting demonstrates that many of these degradation intermediates contain PLP. The specificity of the monoclonal antibody is confirmed by the lane in which a sample was electrophoresed without reduction with NaCNBH_3 ; no bands are visible. Some bands react with anti-phosphorylase, but do not react with anti-PLP; these are peptides that have lost cofactor or which do not include the PLP-binding lysine. All peptides of molecular mass greater than about 78 kDa must contain the PLP-binding residue, and, as expected, peptides unreactive with the anti-PLP monoclonal antibody are all smaller than this. Fig. 2 also demonstrates the greater sensitivity of the anti-PLP antibody relative to the polyclonal antibody to the protein.

PLP remains attached to phosphorylase degradation products during proteolysis *in vitro*, and can be detected with the sensitivity and selectivity that might be applicable to studies of the degradation of the enzyme *in vivo*. However, the time scale of the proteolysis experiments *in vitro* is dramatically shorter (first-order rate constant for inactivation = 1.38 h^{-1}) than the time frame for degradation of the enzyme *in vivo*. The first-order rate constant for phosphorylase degradation in mouse skeletal muscle is approx. 0.1 day^{-1} (Cookson & Beynon, 1989; Leyland *et al.*, 1990; Leyland & Beynon, 1991). It was therefore necessary to examine the stability of the PLP-peptide linkage over a longer period. A tryptic digest of phosphorylase was treated with an excess of immobilized soybean trypsin inhibitor to remove the proteinase, and the peptide mixture was dialysed for 72 h against PBS containing a strong PLP-scavenging reagent (10 mM-hydroxylamine). After dialysis, the digestion mixture was re-

Fig. 3. PLP-binding proteins in soluble fractions of mouse skeletal muscle and liver

Skeletal muscle or liver was homogenized in 4 vol. of 20 mM-Hepes/0.14 M-NaCl, pH 7.4. The homogenate was centrifuged at 100 000 *g* h and a portion of each supernatant fraction was treated with NaCNBH₃ (+[H]). Reduced and unreduced (–[H]) samples (liver 60 µg of protein; muscle 45 µg of protein) were separated by SDS/PAGE, and the gel was stained for protein or electroblotted on to nitrocellulose and probed with monoclonal anti-PLP antibody.

solved on SDS/PAGE and stained for protein, or immunoblotted with the anti-PLP monoclonal antibody. After exhaustive dialysis against hydroxylamine there was no further proteolytic digestion of phosphorylase, and PLP was still attached to phosphorylase and its degradation products (results not shown). Thus, degradation products possess sufficient higher-order structure to retain PLP even in the presence of a reagent that promotes dissociation. The requirement for higher-order structure was confirmed by dialysis against the same buffer containing 0.1% (w/v) SDS; PLP was lost from all peptides (results not shown).

Specificity of labelling of phosphorylase *in vivo* by PLP

PLP-labelled degradation intermediates of phosphorylase would be observed against a background of bands attributed to other PLP-binding proteins present in the tissue preparations. Thus the size distribution of PLP-containing proteins *in vivo* was assessed in liver and skeletal muscle, the two largest pools of PLP in the body. Soluble proteins from both tissues were prepared, immediately treated with NaCNBH₃, separated by SDS/PAGE and blotted with the monoclonal antibody to PLP (Fig. 3). Some non-specific binding of the antibody is seen both in liver and muscle preparations, but this is identifiable by the duplication of the bands in lanes that have not been treated with NaCNBH₃. Comparison of NaCNBH₃-reduced samples with unreduced samples is a useful control that identifies non-specific bands on Western blots. Phosphorylase is not a major PLP-binding protein in liver, and the region of the blot in which degradation fragments would be expected is occupied by many other bands, attributable

to both specific and non-specific effects. This contrasts with skeletal muscle, in which the great majority of PLP is bound to glycogen phosphorylase. In muscle preparations, some PLP-proteins were identified at the lower molecular mass of 40–45 kDa; these are putatively aminotransferases.

PLP-labelled degradation intermediates of phosphorylase in skeletal muscle

In some blots, faint PLP-containing bands (for example, at approx. 70 kDa) could be seen (results not shown). These could be the products of intracellular degradation of phosphorylase. However, the heating of samples during preparation for SDS/PAGE can cause low-level fragmentation of proteins, the products of which might be misconstrued as products of proteolysis *in vivo* (Cookson & Beynon, 1987). Accordingly, a muscle supernatant was exposed to different temperatures, and for various times, during sample preparation for SDS/PAGE (Fig. 4a). Incubation at 100 °C caused detectable fragmentation of phosphorylase, resulting in the progressive appearance of bands on the Western blot using anti-PLP (extreme right-hand lane of Fig. 4a). At 60 °C these PLP-peptides are not generated, but several bands in the 40–90 kDa range decrease in intensity during incubation at 60 °C. When a muscle supernatant fraction was treated with sample buffer at 4 °C and loaded at room temperature, the same peptides were present at higher concentrations and are therefore heat-labile. Although the non-specific staining is worse in the absence of heat treatment, there was clear evidence of one strongly staining band of the same mobility as that seen at 60 °C (see lower arrow on Fig. 4a), and a number of other faint bands that we believe are present in skeletal muscle *in vivo*. These PLP-containing bands are also visible on blots probed with affinity-purified anti-phosphorylase IgG and elicit the same response on heating (Fig. 4b), which confirms that they are derived from phosphorylase *in vivo*. The anti-phosphorylase IgG also identifies bands on both NaCNBH₃-treated and untreated samples, and these show the shift in mobility on reduction that is consistent with their derivation from phosphorylase.

These putative degradation intermediates are all in the range 60–70 kDa, although the NaCNBH₃-induced shift in mobility precludes accurate estimates of size. These must be resistant phosphorylase fragments that retain sufficient structure to bind cofactor, and to survive in muscle. Proteolysis of phosphorylase *in vivo* yields fragments of the same sizes as are seen *in vitro* with purified proteinases, and it is likely that there are regions of the phosphorylase molecule that are susceptible to proteolytic attack, irrespective of the nature of the proteinase. These fragments, in common with phosphorylase, were only present in the muscle high-speed supernatant fraction. No other PLP-labelled peptides were detectable in either homogenates or sedimented fractions (E. J. Cookson, unpublished work).

Degradation intermediates in different muscle systems

The levels and pattern of degradation intermediates were assessed under a number of different conditions in which we have shown that the rate of degradation of phosphorylase is altered. The steady-state levels of intermediates reflect the relative activities of the proteolytic processes that are responsible for both their generation and removal. Additionally, new degradation products might imply the activity of a previously inactive proteolytic system. We have previously measured the rate of degradation of phosphorylase in normal and dystrophic (C57BL/6J^{dy/dy}) mouse skeletal muscle. In normal muscle, the rate of degradation of the enzyme in C57BL/6J mouse muscle is 0.125 day^{–1}, but is lower (0.049 day^{–1}) in C57BL/6J^{dy/dy} mice (Leyland & Beynon, 1991). The rate of synthesis is even more

Fig. 4. Genuine and artefactual degradation intermediates of glycogen phosphorylase

Freshly prepared mouse muscle supernatant fraction (a portion of which was treated with NaCNBH₃; +[H]) was heated for the indicated time/temperature in SDS/PAGE sample buffer before SDS/PAGE. (a) The gel was electroblotted on to nitrocellulose and probed with monoclonal anti-PLP antibody. The sample labelled '25 °C' was left at room temperature for 20 min. Each lane contains 80 µg of protein. The top arrow identifies the strongly stained phosphorylase band (although, because of the NaCNBH₃ treatment, not at 97.4 kDa); the bottom arrow identifies the main degradation intermediate. (b) The gel was electroblotted on to nitrocellulose, and identical lanes were probed with a monoclonal antibody to PLP (Anti-PLP) and a polyclonal antibody to glycogen phosphorylase (Anti-ph.). The arrowhead (<) identifies the main degradation intermediate (that shifts on the gel after NaCNBH₃ treatment).

Fig. 5. Degradation intermediates of glycogen phosphorylase during denervation-induced muscle atrophy

Muscle samples from the gastrocnemius of control and 10-, 20- and 30-day left-sciatectomized mice were treated (with and without reduction by NaCNBH₃; +[H] and -[H] respectively) as described in the Materials and methods section and Western-blotted with a monoclonal antibody to PLP. Degradation intermediates are identified by arrowheads.

dramatically decreased, and thus the lower amount of phosphorylase in dystrophic muscle is attributable to generalized down-regulation of enzyme turnover. The pattern and level of degradation intermediates were the same in C57BL/6J and C57BL/6J^{dy/dy} mice. Further, there was no difference in pattern between the C57BL/10J and the dystrophin-deficient C57BL/10^{mdx/mdx} dystrophic mouse, in which the levels of phosphorylase are unaltered (A. V. Flannery, unpublished work). There is no evidence for alterations in the pattern of protein degradation in the two dystrophic phenotypes.

After section of the sciatic nerve in the mouse there is a marked wasting of the lower hind-limb muscles, and the specific activity of phosphorylase falls to approx. 30% of that of the contralateral control limb, which does not change. The decrease in phosphorylase content in the muscle is attributable in part to an accelerated degradation of the enzyme, from 0.12 to 0.20 day⁻¹

(Leyland *et al.*, 1990). Degradation intermediates were determined in muscle at 10, 20 and 30 days after denervation. At these times, the phosphorylase activity as a percentage of that of the contralateral limb was 49%, 30% and 15% respectively. There are no changes in phosphorylase expression in the contralateral limb over this period (Leyland *et al.*, 1990). At 10 days after denervation, the degradation intermediates were present in muscle at a higher concentration, but were of the same sizes as those in normal muscle (Fig. 5). This implies that the accelerated removal of phosphorylase from denervated muscle is due to an increase in the activity of the same degradative machinery that operates in normal muscle. Denervation-induced atrophy induces multiple proteolytic systems, and Furuno *et al.* (1990) have suggested that the degradation of cytosolic proteins in skeletal muscle is largely attributable to a non-lysosomal proteolytic system. This is consistent with our observation that there are no phosphorylase degradation intermediates in the particulate fraction of mouse skeletal muscle (E. J. Cookson, unpublished work). At 20 and 30 days after denervation, the levels of intermediates are much lower, consistent with the lower amount of phosphorylase in the muscle and a higher rate of degradation, which would combine to decrease the pool size of degradation fragments.

We have recently measured, by PLP-labelling, the rate of degradation of phosphorylase in the pectoralis muscle of broiler and layer chickens (Flannery *et al.*, 1992). This muscle grows extremely rapidly in broiler chickens, and over a 5-week period (from 3 to 8 weeks of age) the total pectoralis content of glycogen phosphorylase increases by 18-fold, from approx. 75 to 1400 mg. By contrast, in layer chickens, the same pool expands by 3-fold from approx. 100 to 270 mg over the same period. The major influence on accumulation of enzyme is the rate of synthesis, but the rate of degradation of the enzyme in 8-week-old animals in broilers is approximately twice that in layers. Accordingly, we examined the degradation intermediates in broiler and layer pectoralis muscle, using anti-PLP and a polyclonal antibody to chicken phosphorylase. Intermediates, similar to those seen in mouse muscle, were present in all samples (Fig. 6). The

Fig. 6. Degradation intermediates of glycogen phosphorylase in the pectoralis muscle of broiler and layer chickens

Pectoralis muscle from 8-week-old broiler and layer chickens was used to prepare a supernatant fraction as described in the Materials and methods section. A portion of this supernatant was treated with NaCNBH_3 ('+[H]') and kept at 25 °C before SDS/PAGE. The gel was electroblotted on to nitrocellulose, and identical lanes were probed with a monoclonal antibody to PLP (Anti-PLP) and a polyclonal antibody to chicken glycogen phosphorylase (Anti-phosphorylase). Degradation intermediates are identified by arrowheads.

confirmatory shift in mobility on NaCNBH_3 reduction, a feature of all animal phosphorylases that we have examined, is also evident for these samples when blotted with the anti-phosphorylase serum. The pattern of intermediates, which was essentially the same in broiler and layer muscle, implies that alterations in the rate of degradation of phosphorylase are elicited by changes in the activity of existing degradative processes, rather than by activation or suppression of complete systems. There were no obvious differences in the relative amounts of the intermediates in the muscle from the two strains.

The levels of these degradation products are very low. In isolated cells they can be increased by inclusion of proteinase inhibitors or lysosomotropic agents in media (Botbol & Scornick, 1983). Such manipulations are not possible in intact animals, and we estimate that *in vivo* the levels of phosphorylase-derived intermediates are substantially less than 1% of the amount of intact protein. This has implications for the degradation kinetics of the enzyme, and reinforces the concept of a 'committed step' that is followed by rapid clearance of the committed protein molecule. It has been suggested that the multi-catalytic proteinase, or proteasome, may act by co-operativity between individual catalytic subunits, and that this might confer a degree of 'processivity' on proteolysis which might lead to degradation that was essentially complete after the protein was committed to destruction (Dick *et al.*, 1991). At present, we have no knowledge of the nature of the committed step for phosphorylase, which might include limited proteolysis, ubiquitin-mediated conjugation/proteolysis, release from a glycogen particle or sequestration into a different subcellular compartment. However, irrespective of the nature of this step, it is apparent that the

products, including the 70 kDa peptide(s), are removed from the muscle cell at a rate constant that could be at least two orders of magnitude greater than the rate of degradation. Although this is expected on teleological grounds, the mechanism whereby a protein can be committed slowly to a very rapid process remains unclear, but is one of the keys to an understanding of protein degradation.

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