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Monoclonal-antibody studies of creatine kinase

The proteinase K-cleavage site

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Proteinase K cleaves a small peptide from native muscle-specific creatine kinase. We present evidence, from the binding of two monoclonal antibodies to formic acid-cleavage fragments and proteinase K-digest fragments of chick muscle creatine kinase, that the proteinase K-cleavage site is in the *C*-terminal region of the molecule. This specificity of proteinase K, which is not normally a highly specific enzyme, and the continued association of the two peptide fragments after cleavage suggest an unusual conformational feature in the cleavage-site region. By applying predictive methods for hydrophobicity and secondary structure to an amino acid sequence in this region, we suggest possible structural features at the cleavage site that are evidently conserved across avian and mammalian species. The most likely site is next to, or within, a β-turn on the surface of the molecule.

Native muscle-specific creatine kinase (40kDa) is generally resistant to attack by proteolytic enzymes, but it is attacked in a highly specific manner by fungal proteinase K (Williamson *et al.*, 1977). This enzyme removes a 4–5kDa fragment from one end of rabbit MM-CK (Williamson *et al.*, 1977; Price & Stevens, 1982), but it is not known which end.

In the present paper we show that the proteinase K-cleavage site is conserved across avian and mammalian species, insofar as chick and pigeon MM-CK enzymes are cleaved in the same way as the rabbit enzyme. The availability of monoclonal antibodies against specific regions of the MM-CK molecule (Morris & Head, 1982; G. E. Morris & L. C. Frost, unpublished work) has enabled us to show that cleavage occurs near the *C*-terminal end. By using a published sequence of the *C*-terminal region of rat MM-CK (Benfield *et al.*, 1984), we attempt to predict some of the structural characteristics of MM-CK enzymes in the cleavage-site region and suggest that cleavage occurs at aliphatic-side-chain amino acids within, or adjacent to, β-turn tetrapeptides separating two strongly hydrophobic sequences. All the available data are consistent with a large loop on the surface of the molecule, accessible to proteinase K but not to other proteinases.

Abbreviations used: MM-CK, muscle-specific creatine kinase (native, dimeric form); M-CK, subunit of the MM-CK dimer; SDS, sodium dodecyl sulphate.

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Materials and methods

Materials

Peroxidase-conjugated rabbit anti-(mouse Ig) antibody was obtained from DAKOpatts (Mercia–Brocades, West Byfleet, Surrey, U.K.). Creatine kinases from chicken and pigeon breast muscle were purified by the method of Eppenberger *et al.* (1967), and those from ox heart and rabbit muscle were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Proteinase K from *Tritirachium album* was obtained from Sigma Chemical Co. or Boehringer, Lewes, Sussex, U.K., and AnalaR formic acid (98–100%) was from BDH Chemicals, Poole, Dorset, U.K. Blue-dye-stained protein M_r markers were obtained from BRL Ltd., Cambridge, U.K. Diaminobenzidine (Sigma Chemical Co.) was handled as a possible carcinogen. The preparation and properties of some of the monoclonal antibodies against creatine kinases were as described by Morris & Head (1982, 1983); further monoclonal antibodies were prepared by G. E. Morris & L. C. Frost (unpublished work).

Creatine kinase digestions

Treatment of native MM-CK with proteinase K was carried out at 30°C by the method of Williamson *et al.* (1977), with CK at 20mg/ml and proteinase K at 4μg/ml. The reaction was terminated by incubation with 0.1mM-phenylmethanesul-

phonyl fluoride, and samples were boiled for 2min with 1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (w/v) sucrose before electrophoresis.

Formic acid digestion was adapted from the method of Sonderegger *et al.* (1982). MM-CK at 6mg/ml in 75% (v/v) formic acid was incubated at 37°C for 22h and then diluted and freeze-dried. Further cycles of redissolving in water and freeze-drying were performed to remove traces of acid. The final freeze-dried material was boiled for 2min in the sample buffer (Laemmli, 1970) containing 1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (w/v) sucrose.

Western blotting

After slab-gel electrophoresis in the presence of SDS with 15% polyacrylamide in the system of Laemmli (1970), gels were soaked for 30–60min in 4M-urea before being blotted between a sandwich of nitrocellulose papers (Schleicher und Schüll) for 18h as described by Bowen *et al.* (1980), with the gel and nitrocellulose held vertically. The method produces two identical, though mirror-image, blots. In some cases, the sample was loaded as a continuous band, rather than in slots, and the final blot was cut into strips, which could be re-aligned exactly after development with different antibodies.

Gels were stained with Coomassie Blue, and nitrocellulose blots with Amido Black (Bowen *et al.*, 1980).

Nitrocellulose blots, or strips, were blocked with 3% (w/v) bovine serum albumin in phosphate-buffered saline (0.14M-NaCl/0.02M-sodium phosphate buffer, pH7.2), and then incubated successively with monoclonal antibody, peroxidase-labelled second antibody and diaminobenzidine substrate as described by Morris & Head (1982). To decrease background staining further, 0.05% Triton X-100 was included in both antibody steps and 1% (v/v) each of horse and foetal-calf serum were included in the second antibody step.

Results and discussion

Proteinase K removes a C-terminal fragment from native MM-CK

Formic acid hydrolysis cuts proteins at Asp–Pro bonds (Sonderegger *et al.*, 1982). Fig. 1(*a*) shows that chick, pigeon, rabbit and ox MM-CK enzymes, subjected to this treatment, all give two main fragments of similar sizes, indicating that the positions of two Asp–Pro bonds are highly conserved in birds and mammals. Comparison with the M_r markers α-chymotrypsinogen (26kDa) and intact M-CK (40kDa) suggests that the two fragments are between 27 and 33kDa (Fig. 1*b*).

In the published sequence of the *C*-terminal 80% of rat M-CK (Benfield *et al.*, 1984), the only two Asp–Pro bonds are located near the *N*-terminus, and the two *C*-terminal fragments produced by cutting these two bonds would have M_r values of 32666 and 29076, consistent with the observed size of formic acid-cleavage fragments in Fig. 1. On

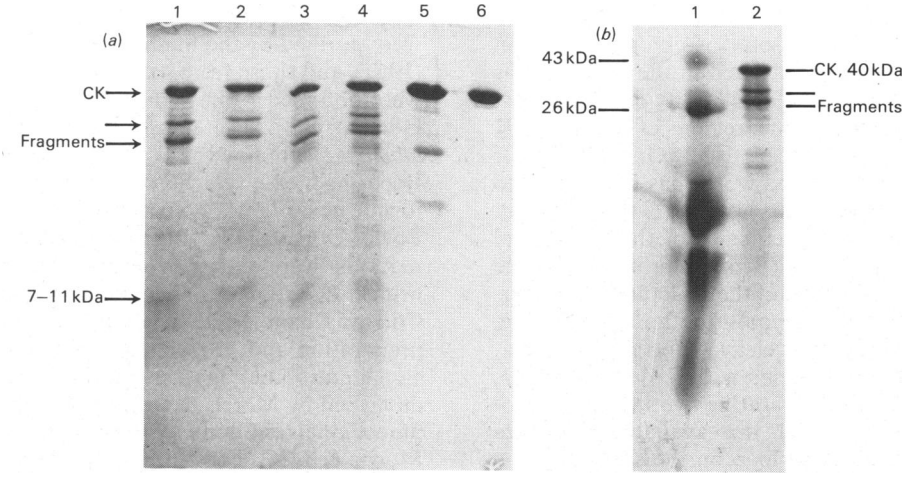

Fig. 1. *Formic acid digestion of MM-CK enzymes and electrophoretic analysis on SDS/15%-polyacrylamide gels*
(*a*) Lanes 1–4 show formic acid-treated M-CK subunits from chick, pigeon, rabbit and ox respectively. Lanes 5 and 6 are the untreated M-CK preparations from ox and chick. A faint band between the two main *C*-terminal digestion products is more prominent in the ox (lane 4), but this is the least pure of the four enzymes. Material in the 7–11kDa region, corresponding to the *N*-terminal peptides, is also indicated by an arrow. (*b*) Lane 1 shows M_r markers at 43kDa (ovalbumin) and 26kDa (chymotrypsinogen). The three main bands in lane 2 are chick M-CK (40kDa) and its two formic acid-cleavage fragments, all of which lie between the two markers.

Western blots, the monoclonal antibody 2A7 reacts with both M-CK and B-CK of most non-rodent species tested (G. E. Morris & L. C. Frost, unpublished work). On Western blots of formic acid-treated chick MM-CK, it fails to bind to the two large fragments, but does bind to material in the 7–11 kDa region, corresponding to the *N*-terminal peptides (Fig. 2, lane 6). The antibody CK-ART, in contrast, binds to the large fragments (Fig. 2, lane 1) rather than to this broad band of *N*-terminal material, which is also visible on the stained gel (Fig. 1). [Three smaller fragments, including a narrow band at 10 kDa, are detected by CK-ART only and are believed to be traces of *C*-terminal peptides, since bonds other than Asp–Pro show some sensitivity to formic acid (Sonderegger *et al.*, 1982). They are barely detectable by staining for protein (Fig. 1), and none of the other antibodies bind to them.] This experiment establishes that the 2A7 binding site lies within about 8 kDa of the CK *N*-terminus, whereas the CK-ART site lies within 29 kDa of the *C*-terminus. Of the four other monoclonal antibodies shown in Fig. 2, lanes 2–5, one behaves like 2A7 and the other three are similar to CK-ART.

Proteinase K treatment of native CK produces two fragments, of about 4 kDa and 36 kDa (Fig. 3*a*). Since CK-ART binds to 29 kDa and 32.5 kDa *C*-terminal formic acid-cleavage fragments, it would also bind to the even larger 36 kDa proteinase K-digest fragment if this were *C*-terminal. The fact that it does not (Fig. 3*b*) shows that the large fragment is an *N*-terminal peptide and that the proteinase K-cleavage site is near the *C*-terminus. This conclusion is supported by the fact that 2A7 does bind to the 36 kDa proteinase K-digest fragment (Fig. 3*c*). Incidentally, this experiment also suggests that a sequence very close to the *C*-terminus is required for CK-ART binding.

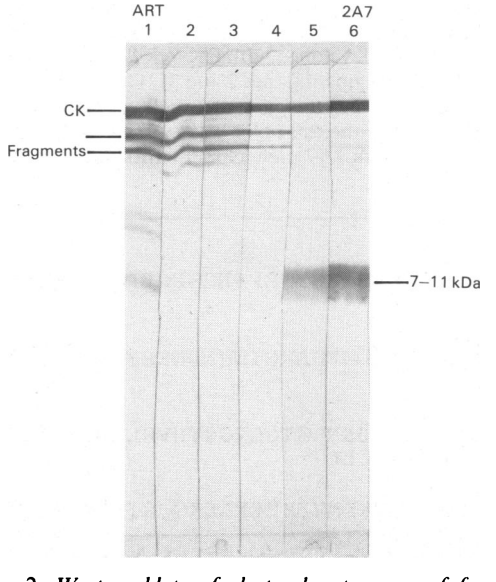

Fig. 2. *Western blots of electrophoretograms of formic acid-digested chick MM-CK developed with six different monoclonal antibodies*

Lane 1, CK-ART; lane 2, G11; lane 3, F11; lane 4, 2B1; lane 5, 5H5; lane 6, 2A7. The other antibodies (G. E. Morris & L. C. Frost, unpublished work) are included to emphasize the contrast between those binding on the *N*-terminal side of the Asp–Pro bonds and those binding on the *C*-terminal side.

The proteinase K-cleavage site is in a 'random-coil' region separating two hydrophobic sequences

Proteinase K cleaves preferentially at amino acids with hydrophobic side chains (Ebeling *et al.*, 1974). Since there are many of these in CK, the high specificity of the cleavage site in the native molecule must be due to a conformational feature. Such a feature might be a random-coil region on the surface of the native molecule, since the denatured and unfolded enzyme is attacked at many additional sites (Price & Stevens, 1982). This suggests that we should look for a hydrophilic 'random-coil' sequence, containing residues with hydrophobic side chains and within 37–46 amino acid residues from the *C*-terminus (to generate a small peptide of 4–5 kDa). Since this feature is conserved, at least between birds and mammals, we are justified in looking for it in the partial sequence of rat M-CK (Benfield *et al.*, 1984) in Fig. 4.

By using the criteria of Chou & Fasman (1978), the ten-residue sequence at 37–46 amino acid residues from the *C*-terminus cannot be β-sheet because of the abundance of glycine, serine, aspartic acid and glutamic acid residues, and the presence of glycine and asparagine and the lack of strong α-helix formers makes α-helix unlikely. The region also contains three proteinase K-sensitive sites at isoleucine, alanine and leucine (Fig. 4), but the remaining and adjacent residues make the region hydrophilic overall. All three residues are either near to or within tetrapeptides with considerable β-turn potential (Fig. 4), and β-turns tend to be exposed on the surface of proteins (Chou & Fasman, 1978). Cleavage of rat M-CK at these three potential sites would yield small peptides of appropriate M_r (4938, 4666 and 4282 respectively).

We also need to explain why the two peptides remain firmly associated after proteinase K treatment, requiring urea or SDS treatment to dissociate them (Price *et al.*, 1981). Since there are no disulphide bridges in CK (Watts, 1973), such a strong interaction is likely to be hydrophobic. The hydrophobicity profile of the partial rat M-CK sequence is shown in Fig. 5. This was obtained

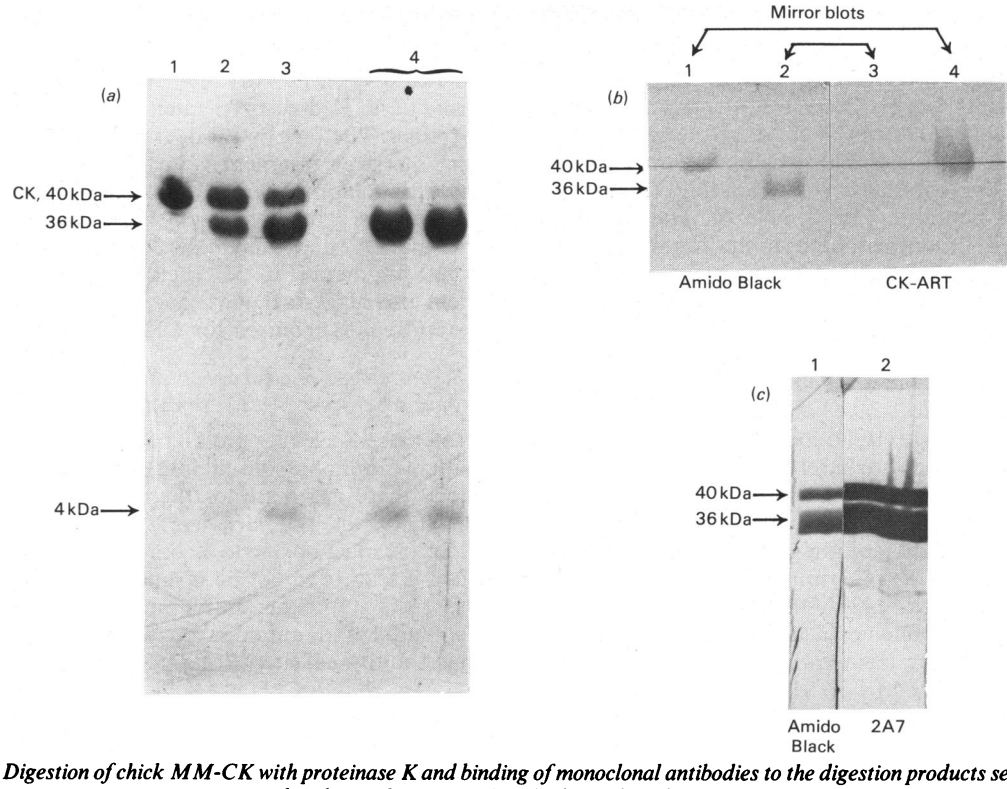

Fig. 3. *Digestion of chick MM-CK with proteinase K and binding of monoclonal antibodies to the digestion products separated by electrophoresis on SDS/polyacrylamide gels*

(*a*) Stained gel showing time course of digestion. Intact CK (40kDa) is gradually converted into two fragments of 36kDa and 4kDa. Lanes 1–4 are CK treated for 0, 2, 10 and 30min respectively. (*b*) Mirror-image replica blots from each side of the same gel, either stained with Amido Black (lanes 1 and 2) or developed with CK-ART (lanes 3 and 4). Thus CK-ART binds to intact CK (lanes 1 and 4), but not to the 36kDa fragment (lanes 2 and 3). The horizontal line marks the 40kDa position on both blots. The lower half of the gel is not shown, since the small peptide is not detectable on the blot. (*c*) CK was partially digested with proteinase K and the final blot was cut vertically into strips, which were either stained with Amido Black (lane 1) or developed with 2A7 monoclonal antibody (lane 2). Unlike CK-ART, 2A7 does bind to the 36kDa fragment.

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   ][33kDa                                  ][29kDa
NLFDPIIQDRHGGFKPTDKHKTDLNHENLKGGDDLDPNYVLSSRVRTGRSIKGYTLPPHCSRGERRAVEKLSVE

ALNSLTGEFKGKYYPLKSMTEQEQQQLIDDHFLFDKPVSPLLLASGMARDWPDARGIWHNDNKSFLVWVNEEDH

LRVISMEKGGNMKEVFRRFCVGLQKIEEIFKKAGHPFMWNEHLGYVLTCPSNLGTGLRGGVHVKLANLSKHPKF
                                                          tttt
                                    *[  *[  *[
EEILTRLRLQKRGTGGVDTAAVGAVFDISNADRLGSSEVEQVQLVVDGVKLMVEMEKKLEKGQSIDDMIPAQK
          --++++++++++----------++++++++++----------
     tttt      ββββββ    tttt tttt ββββββββhhhhhhhhhhhhhhhhhhhhhhhhhhh
                           tttt
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Fig. 4. *Identification of cleavage sites in the rat M-CK sequence*

About 20% is missing from this sequence at the *N*-terminus (Benfield *et al.*, 1984). The two Asp–Pro (DP) bonds near the *N*-terminus are indicated by][. Potential sites for proteinase K cleavage are indicated by *[. In the *C*-terminal region, hydrophobicity is indicated according to whether the hydropathic index in Fig. 5 is greater than (+) or less than (−) zero. Secondary-structure predictions indicate likely α-helical (h), β-sheet (β) and β-turn (tttt tetrapeptides) regions. The additional requirements for initiator and terminator amino acids in h and β regions (Chou & Fasman, 1978) have been applied. No assignment has been made where ambiguity exists in the prediction. The β-turn tetrapeptide containing the active cysteine residue is also shown.

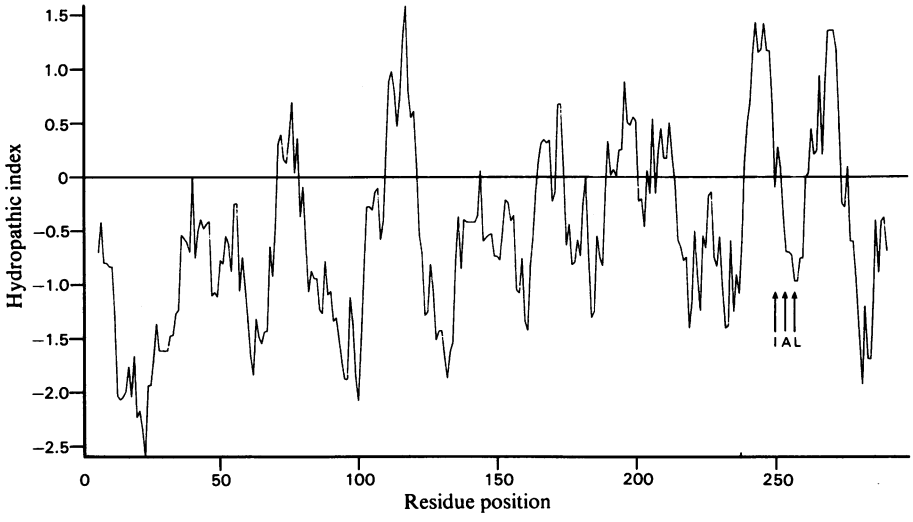

Fig. 5. *Hydrophobicity profile of the rat M-CK sequence*
The profile was generated by a computer program based on Kyte & Doolittle (1982) and incorporating Simpleplot on a Tektronix terminal. The three aliphatic side-chain amino acids (leucine, alanine and isoleucine) in the proposed proteinase K-cleavage region are indicated by arrows.

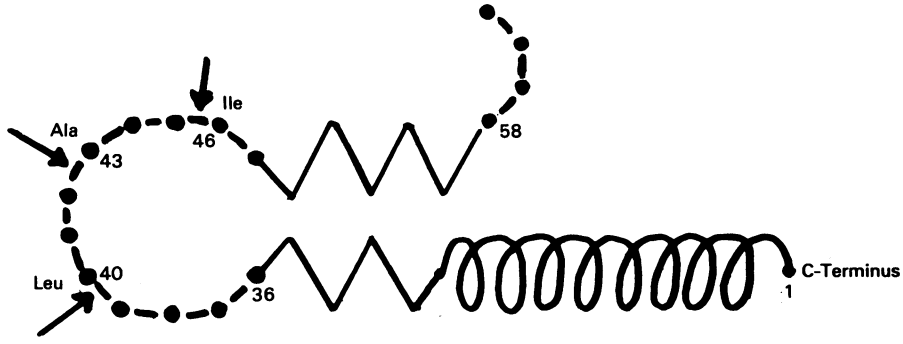

Fig. 6. *Hypothetical structure for the M-CK C-terminal 60 amino acid residues, based on Figs. 4 and 5*
Suggested proteinase K-cleavage sites are indicated by arrows. Residues are numbered from the *C*-terminus. Key: α-helix, ; β-sheet, ; coil or β-turn, .

from the rat M-CK sequence by computer-averaging over eleven residues (five on each side), with the use of the individual hydrophilicity values given by Kyte & Doolittle (1982). The principal features were the same when more or fewer adjacent residues were selected for averaging. Fig. 5 is translated into + or − values in Fig. 4, according to whether the hydropathic index (Kyte & Doolittle, 1982) is greater or less than zero, positive values being hydrophobic. The proposed cleavage site is flanked by two of the strongest hydrophobic regions in the whole sequence. Also shown in Fig. 4 are the results of a Chou & Fasman (1978) prediction of the secondary structure in this region. The hydrophobic regions immediately adjacent to the proposed cleavage region are predicted to be β-sheet structures, one of them having a possible β-turn tetrapeptide (Gly-Ser-Ser-Glu) immediately adjacent to it in the hydrophilic region. Most of the remaining *C*-terminal sequence is strongly predicted to be α-helix.

We therefore suggest the diagrammatic structure shown in Fig. 6 for the *C*-terminus of M-CK. The hypothesis is testable insofar as the essential features should be conserved in all MM-CK enzymes that show the same sensitivity to proteinase K. Furthermore, since chick BB-CK is not sensitive to proteinase K in this region (G. E. Morris, L. C. Frost & L. P. Head, unpublished work), it would not be expected to share the same characteristics of sequence and structure. We have avoided extending the prediction back to the

creatine-binding site, which has been known for some time to consist of a β-turn, forming a surface groove in the molecule (Watts, 1973). A pleated β-sheet with four β-turns is possible, but the prediction is more ambiguous closer to the active site, some sequences also having α-helix potential.

Although hydrophobic β-sheets on either side of the cleavage site might explain the continued association of the two fragments, it is more difficult to explain the complete loss of enzyme activity after proteinase K treatment. The active cysteine residue in the creatine-binding site (Watts, 1973) is 60 amino acid residues closer to the *N*-terminus than the cleavage site, and the active histidine, tyrosine and lysine residues (Watts, 1973) could be those nearest to this cysteine residue (Fig. 4). Conformational changes affecting the active site as a result of proteinase K cleavage (Price *et al.*, 1981) remain the most likely explanation, but such changes are not apparent to us from the sequence alone.

Conclusions

Two recent advances have made the present analysis possible: first, the availability of monoclonal antibodies (G. E. Morris & L. C. Frost, unpublished work) with binding sites on different sides of the two Asp–Pro bonds in M-CK, and secondly the availability of the rat M-CK partial sequence given by Benfield *et al.* (1984), which has enabled us to locate these highly conserved Asp–Pro bonds to the *N*-terminal region of the molecule. Consequently, we have been able to locate the proteinase K-cleavage site at the *C*-terminal end of M-CK.

The known approximate sizes of the small and large fragments produced by proteinase K digestion enables us to locate the cleavage site to within 10–15 amino acid residues, and expectations of an unusual conformation in this area can be confirmed by examination of the rat M-CK sequence, since the specificity of proteinase K is also conserved between avian and mammalian CK enzymes. Williamson *et al.* (1977) estimated the cleavage site to be 38 amino acid residues from the *C*-terminus of rabbit M-CK. Cleavage of the Leu–Gly bond in rat CK would leave a 39-residue fragment (Fig. 4), and this bond also has the most hydrophilic environment of the three suggested (third arrow in Fig. 5). Trypsin and other proteolytic enzymes do not attack this region of native CK (or any other region), presumably for steric reasons, since the denatured enzyme is rapidly degraded to small peptides (Williamson *et al.*, 1977; Price & Stevens, 1982).

A picture emerges of proteinase K-sensitive hydrophobic amino acid residues exposed on the surface of the molecule by their presence in, or next to, hydrophilic β-turns, a possible loop being formed by the interaction of the two strongly hydrophobic β-sheet regions on each side (Fig. 6). This hypothesis predicts that the presence of β-turns with adjacent hydrophobic residues in this region will be conserved in all MM-CK enzymes (but not BB-CK enzymes), though the precise sequence in this region need not be conserved.

Although there is extensive evidence for the usefulness of the predictive method for secondary structure (Chou & Fasman, 1978), the predictions are not infallible. It is important to note, therefore, that the identification of the cleavage site does not depend on the accuracy of these predictions. Thus interaction between the hydrophobic regions around the cleavage site and exposure of the hydrophilic cleavage region on the CK surface would be expected whatever the secondary structure.

After this paper was submitted, a complete 380-amino-acid-residue sequence for chicken M-CK appeared (Kwiatkowski *et al.*, 1984). All the essential features of the rat enzyme *C*-terminal sequence are retained in the chicken enzyme, although there are five differences in the 60 *C*-terminal residues and one of the two or three β-turns predicted in the 'loop' (Fig. 6) is lost. The positions of the two Asp–Pro bonds are identical. The leucine residue in the cleavage region can now be identified as Leu-341 in chick M-CK by conventional numbering from the *N*-terminus. The 60 *C*-terminal residues of chick M-CK (with rat M-CK differences below) are:

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TGGVDTAAVGAVFDISNADRLGFSEVEQVQMVVDGVKLMVEMEKKLEQNQPIDDMIPAQK
                      S     L              KG S
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