Monoclonal antibody studies of creatine kinase

The ART epitope: evidence for an intermediate in protein folding

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Chemical cleavage at cysteine residues with nitrothiocyanobenzoic acid shows that the last 98 amino acids of the 380-amino-acid sequence of chick muscle creatine kinase are sufficient for binding of the monoclonal antibody CK-ART. Removal of the last 30 amino acids by cleavage at methionine residues with CNBr results in loss of CK-ART binding. CK-ART binding is also lost when these C-terminal methionine residues are oxidized to sulfoxide, but binding is regained on reduction. Proteinase K 'nicks' native CK at a single site near the C-terminus and two fragments of 327 amino acids and 53 amino acids can be separated by subsequent SDS or urea treatment. CK-ART still binds normally to 'nicked' CK, which is enzymically inactive. After treatment with either urea (in a competition enzyme-linked immunosorbent assay) or SDS (on Western blots), however, CK-ART binds to neither of the two fragments, although these treatments do not affect binding to intact CK. This suggests that parts of both CK fragments contribute to the CK-ART epitope. CK-ART is both species- and isoenzyme-specific, binding only to chick M-CK. The only C-terminal regions containing chick-specific sequences are residues 300–312 and residues 368–371, the latter group being close to the essential methionine residues. We suggest that one, or possibly both, of these regions is involved in forming the conformational epitope on the surface of the CK molecule which CK-ART recognizes. Native CK is resistant to trypsin digestion. The C-terminal half of urea-treated and partly-refolded CK is also resistant to trypsin digestion, whereas the N-terminal half is readily digested. The results suggest a C-terminal region which can refold more rapidly than the rest of the CK molecule and provide evidence for an intermediate in CK refolding.

INTRODUCTION

Amino acid sequences for creatine kinase (CK) are available now for a number of isoenzymes and species, but no three-dimensional structures are known. Can monoclonal antibody studies provide information on CK structure or structure–function relationships? Can monoclonal antibodies be used to study the protein folding processes through which the three-dimensional structure is acquired?

Current ideas about protein folding mechanisms have been reviewed by Kim & Baldwin (1982), Goldberg (1985) and Pain (1987). The first stage is thought to be the rapid formation of short stretches of secondary structure, accompanied by the 'collapse' of the polypeptide chain into a 'globule' with a hydrophobic core. The process is slowed down by the occurrence of proline isomerization in some of the unfolded polypeptide chains and the necessity for recovery of the native proline configuration before normal folding can occur. A multi-domain protein would presumably collapse into a string of globules. Standard physical measurements (c.d., n.m.r., etc.) indicate that some, but not all, aspects of the native conformation exist at this stage. This inactive 'globule' is then thought to undergo relatively slow structural rearrangements to acquire the native conformation and enzymic activity.

In an earlier study (Morris *et al.*, 1987), chemical and proteolytic cleavage methods were used to locate the binding sites on CK for two monoclonal antibodies

which appear to recognize local sequences in the denatured or unfolded enzyme. These antibodies do not bind to native CK, so they can be used to detect and assay incompletely-folded CK. Since surface epitopes on native globular proteins usually involve widely-separated amino acids brought together by folding of the polypeptide chain (Barlow *et al.*, 1986), the majority of such epitopes would be disassembled by chemical cleavage mapping.

The monoclonal antibody CK-ART, however, recognizes an unusual surface epitope which appears to be unaffected by denaturation, since it will bind both to native CK and to urea-treated, enzymically inactive CK (Morris & Head, 1983). I now show that the epitope is nevertheless 'assembled' or 'conformational' but that it refolds much more readily than the whole protein. The results are consistent with a rapidly-folding region of CK which is present as an intermediate in the normal refolding process. It has been possible to map the epitope to the C-terminal region of CK and to identify three amino acids, one or more of which are involved in forming the epitope.

MATERIALS AND METHODS

Materials

Peroxidase-labelled rabbit anti-(mouse Ig) antibody was obtained from DAKO Ltd., High Wycombe, Bucks., U.K. Ultra-pure urea (Schwartz–Mann, Rahway, NJ, U.S.A.) was used for enzyme refolding experiments.

Abbreviations used: CK, creatine kinase; e.l.i.s.a., enzyme-linked immunosorbent assay; PMSF, phenylmethanesulphonyl fluoride; NTCB, nitrothiocyanobenzoic acid; IBA, *o*-iodosobenzoic acid.

Cleavage reagents, proteinase K from *Tritirachium album*, trypsin (DPCC-treated) and chymotrypsin (3 × crystallized) were obtained from Sigma and prestained protein markers from Gibco/BRL, Paisley, U.K. The peroxidase substrates *o*-phenylenediamine and diaminobenzidine (Sigma) were handled as possible carcinogens, while CNBr was handled in a fume hood and destroyed with bleach. Microtitre plates for e.l.i.s.a. (M25) were obtained from Dynatech, Billingshurst, Sussex, U.K.

MM-CK from chick breast muscle and BB-CK from chick heart were purified to homogeneity by the methods of Eppenberger *et al.* (1967). Human MM-CK was a gift from Dr. E. J. Milner-White (Department of Biochemistry, University of Glasgow, U.K.). Rat CKs were purified by Dr. G. Pay (CIBA/Geigy, Horsham, Sussex, U.K.) and an electric organ membrane preparation from *Torpedo marmorata* containing an MM form of CK was a gift from Dr. J. Cavanagh and Professor E. Barnard (MRC Centre, Cambridge, U.K.). Rabbit CKs were obtained from Sigma.

Urea treatment

CK was treated with 8.1 M-urea by adding 9 vol. of 9 M-urea, incubating at 37 °C for 10 min, and then adding excess (10–50 vol.) ice-cold e.l.i.s.a. incubation buffer [0.9% NaCl/25 mM-sodium phosphate (pH 7.2)/0.05% Triton X-100] containing bovine serum albumin (0.5 mg/ml); 80–90% of both enzymic and native immunological (radioimmunoassay) activity is lost after this treatment (Galatowicz & Morris, 1981). Renaturation of enzyme activity is prevented by keeping the sample at 0 °C (Morris *et al.*, 1987).

Competition e.l.i.s.a.

Minor modifications were made to the method described by Morris & Head (1983).

Microtitre plates were coated with 0.1 ml per well of a 5 µg/ml solution of protein in phosphate-buffered saline (0.9% NaCl/25 mM-sodium phosphate, pH 7.2) for 1 h at room temperature. In experiments involving urea treatment of competitor CK, the 1 h preincubation with CK-ART antibody was carried out at 0 °C on a separate plate in an attempt to minimize refolding during this period. Enzyme assays (not shown) have confirmed that there is no recovery of enzyme activity in the microtitre wells during the assay period.

Digestions

For cleavage at methionine residues, CK (3 mg) was treated with a 30-fold molar excess (over methionine) of CNBr (3.5 mg) in a final volume of 1 ml of 70% formic acid for 24 h at room temperature (Croft, 1980). Excess reagents, acids and salts were removed on a Sephadex G-15 column (12 cm × 1 cm) equilibrated with 8 M-urea/125 mM-Tris/HCl (pH 6.8)/0.1 mM-PMSF. The unretarded fraction, containing all fragments greater than 1–2 kDa, was used directly for electrophoresis after addition of 1% SDS, 5% 2-mercaptoethanol and 10% sucrose and boiling for 2 min. The urea ensures that all peptides remain in solution.

For cleavage at tryptophan residues, CK was treated with an equal weight of IBA for 24 h at room temperature in a final solution of 4-M-guanidinium chloride in 80% acetic acid, with 2% *p*-cresol to minimize cleavage at tyrosine (Mahoney & Hermodson, 1979; Fontana *et al.*,

1983). Preparation for electrophoresis on Sephadex G-15 was as for CNBr. In order to convert methionine sulfoxide produced by this treatment back to the reduced form, samples were incubated at 37 °C for 24–72 h with 700 mM-dithioerythritol (Houghton & Li, 1983) in 8 M-urea-containing buffer after the Sephadex G-15 step. This reduction can also be done in the presence of 1% SDS.

For cleavage at cysteine residues, creatine kinase (0.5 mg) in 0.5 ml of 5 M-guanidinium chloride/0.2 M-Tris/acetate, pH 8.0, was reduced with 1 mM-dithioerythritol for 30 min at room temperature. NTCB (30 µl of a 25 mg/ml solution) was added (Stark, 1977) and the mixture incubated at 37 °C for 15 min. The mixture was then loaded immediately onto a Sephadex G-15 column equilibrated at 4 °C with 8 M-urea/0.2 M-Tris/acetate (pH 9.0)/1 mM-PMSF to remove excess reagent and to raise the pH. Cleavage was completed by incubation at 37 °C for 16 h. The pH was adjusted to 6.8 with HCl and SDS (1%), 2-mercaptoethanol (5%) and sucrose (10%) were added before boiling for 2 min for electrophoresis.

Digestion of CK (20 mg/ml) with proteinase K (4 µg/ml) for 30 min at 30 °C was carried out by the method of Williamson *et al.* (1977) and the digest was used directly for electrophoresis after addition of SDS (1%), 2-mercaptoethanol (5%) and sucrose (10%) and boiling.

Western blotting

After slab-gel electrophoresis in the presence of SDS with 15% acrylamide in the system of Laemmli (1970), gels were blotted for 18 h onto two nitrocellulose sheets by diffusion to give two identical mirror-image blots as previously described (Morris & Head, 1985). Prestained marker proteins were also transferred to enable precise identification of antibody-binding bands. Markers are ovalbumin (M_r 43 000), chymotrypsinogen (25 700), lactoglobulin (18 400), lysozyme/cytochrome *c* (13 300), bovine trypsin inhibitor (6 200) and insulin (3 000).

The blots were blocked and incubated successively with monoclonal antibody, peroxidase-labelled second antibody and diaminobenzidine as described previously (Morris & Head, 1985) or stained for protein with Amido Black (Bowen *et al.*, 1980).

RESULTS AND DISCUSSION

The 98 C-terminal amino acids of CK are sufficient for CK-ART binding and the last 30 amino acids are necessary

Fig. 1(a) shows the amino acid sequence of chick M-CK and the position of cysteine and methionine residues within it. One can calculate that cleavage at the four cysteine residues by NTCB will produce major fragments of M_r 8200, 8100, 12 500, 3400 and 10 600, labelled A, B, C, D and E in Fig. 1(b), with any higher- M_r fragments resulting from incomplete cleavage. Fig. 2 shows CK-ART binding to NTCB fragments on a Western blot (lanes 5 and 6) with prestained M_r markers (lanes 2 and 3). The result is consistent with a CK-ART binding site in the M_r 10 600 fragment (E in Figs. 1b and 2) with further bands due to incomplete cleavage at M_r 14 000 (10 600 + 3400 = DE) and higher, but the difference in M_r between fragment E and fragments A, B or C is hardly sufficient to be quite certain. Experiments with proteinase K, however, rule out the alternative possibilities. Proteinase K cleaves CK at a single Ala-Ala

Fig. 1. Chemical and proteolytic cleavage points in the amino acid sequence of chick M-CK

(a) Cysteine (C) and methionine (M) residues within the chick M-CK sequence (Kwiatkowski *et al.*, 1984) are shown, with the two CNBr-resistant Met-Thr bonds shown as '(MT)'. The Ala-Ala site for proteinase K cleavage (Lebherz *et al.*, 1986) is shown as '**'. (b) Schematic representation of CK cleavage by CNBr, NTCB, proteinase K and IBA. Numbers are $10^{-3} \times M_r$. NTCB fragments A-E are referred to in Fig. 2. Broken lines show regions in which cleavage points are too close together to generate large fragments.

bond indicated by asterisks in Fig. 1 (Morris *et al.*, 1985; Lebherz *et al.*, 1986). All NTCB fragments except fragment E are contained within the larger of the two proteinase K fragments (Fig. 1b), but this large proteinase K fragment on Western blots does not bind CK-ART (Fig. 3; Morris *et al.*, 1985). This confirms that it is the C-terminal NTCB fragment of M_r 10600 which binds CK-ART.

CNBr cleavage produces two main fragments of M_r 19900 (Met-29 to Met-206) and 8700 (Met-271 to Met-351) (Morris *et al.*, 1987) together with minor bands due either to incomplete cleavage at CNBr-sensitive methionines or to partial cleavage at CNBr-resistant Met-Thr bonds (Croft, 1980). The M_r 8700 fragment includes all of the C-terminal NTCB fragment except for the last 30 amino acids, which include four methionine residues, but CK-ART does not bind to it on Western blots nor does it bind to any other CNBr fragment (Fig. 2, lane 4). As a positive control, lane 1 shows an identical 'mirror-blot' to lane 4 stained with the antibody CK-JAC which binds to the M_r 19900 and related fragments.

The importance of this C-terminal 30-amino-acid sequence and its four methionine residues is confirmed by the effect of IBA, which cleaves at tryptophan residues. This produces a C-terminal M_r 12000 fragment which includes the whole of the NTCB fragment E (Fig. 1b) and would therefore be expected to bind CK-ART. Fig. 4, however, shows that CK-ART hardly binds to IBA-treated CK fragments at all (lane 1) compared with the control antibody, CK-JAC, on the 'mirror' blot (lane 6). The apparent inconsistency can be explained by the fact

that IBA also oxidizes methionine residues to methionine sulphoxide (Fontana *et al.*, 1983). This oxidation is reversible by prolonged incubation with high concentrations of thiols (Houghton & Li, 1983) and CK-ART can then bind to the M_r 12000 fragment and also to the incompletely-cleaved fragment of M_r 17200 (Fig. 4, lane 3) as predicted from sequence data (Fig. 1b). The CK-JAC binding site, in contrast, is evidently unaffected by methionine oxidation since CK-JAC binding is unchanged after incubation with thiols (cf. lanes 4 and 6).

Taken together, these results show that the last 98 amino acids of CK are sufficient for CK-ART binding and that some of the last 30 amino acids are necessary.

The CK-ART epitope is formed by protein folding

CK is actually 'nicked' rather than cleaved by proteinase K since the two fragments remain together until separated by agents such as urea or SDS (Williamson *et al.*, 1977).

Fig. 5 shows competition e.l.i.s.a. experiments in which increasing amounts of competitor in solution compete with a fixed amount of CK bound to a microtitre plate for a limiting amount of CK-ART monoclonal antibody. Proteinase K treatment of CK does not reduce CK-ART binding (Fig. 5b; in this particular experiment, the treatment appears to increase slightly its effectiveness as a competitor), which shows that the CK-ART epitope must remain intact, even though enzymic activity is completely lost (Table 1; Williamson *et al.*, 1977).

Treatment of intact CK with 8 M-urea also destroys enzymic activity but has little effect on CK-ART binding

Fig. 2. Binding of CK-ART to NTCB and CNBr cleavage fragments of chick M-CK

Parts of 'mirror-blots' are shown, so that lanes 1 and 4 are blots of the same lane on the 15% polyacrylamide gel. Similarly, lanes 2 and 3 are marker proteins derived from a single gel lane. Lanes, with approximate loadings, are: 1 and 4, CNBr-treated CK (20 μ g); 2 and 3, prestained M_r markers as indicated; 5 and 6, NTCB-treated CK (20 μ g and 5 μ g). The lettering, A-E, of NTCB fragments is explained in Fig. 1(b) and in the text. Lanes 3-6 were developed with CK-ART antibody and the 'mirror-blot' (lanes 1 and 2) with a control antibody, CK-JAC, both at 1:100 dilution of culture supernatant.

Fig. 3. CK-ART does not bind to the large proteinase K fragment of chick M-CK

'Mirror-blots' of the same gel (one has been laterally reversed during photographic processing) were either (a) stained for protein with Amido Black or (b) developed with a 1:100 dilution of CK-ART culture supernatant. The 10% polyacrylamide gel was loaded with 0.2 μ g (lane 1) or 2 μ g (lane 3) of chick MM-CK and with 2 μ g of CK treated for 30 min with proteinase K (lane 2) as described under 'Materials and methods'. CK-ART fails to bind significantly to the M_r 38000 proteinase K fragment

Fig. 4. CK-ART binding to IBA fragments of chick M-CK and the effect of thiol reduction

'Mirror-blots' are shown of a 15% polyacrylamide gel loaded with 8 μ g of IBA-treated CK, before and after incubation with 700 mM-dithioerythritol (DTE) for 24 h to reduce methionine sulfoxide back to methionine. The blots were developed with CK-ART or with control antibody, CK-JAC, as indicated, each at 1:200 dilution of culture supernatant. CK-ART binds only to undigested CK before reduction (lane 1) but two CK-ART-binding fragments are revealed after reduction (lane 3), indicated by M_r values of 17200 and 12000 calculated from the sequence data in Fig. 1. M_r values of prestained protein markers (lanes 2 and 5) are also shown, though the smallest markers (M_r 6000 and 3000) are barely detectable on these blots. In contrast, control antibody, CK-JAC, binds to the largest IBA fragment (cf. Fig. 1b) and this binding is unaffected by methionine oxidation and reduction (lanes 4 and 6).

(Fig. 5a), which suggests that the CK-ART epitope either has no secondary structure or has a structure which renatures very readily (Morris & Head, 1983). The second possibility exists because the urea concentration has to be reduced by dilution at 0 $^{\circ}$ C in order to allow subsequent antibody-antigen interaction. In fact, any conditions which allow the protein-protein interactions required for antibody binding will also allow intermolecular interactions within the CK antigen and partial refolding of some CK secondary, and higher-order, structures.

The critical result is that 95% of CK-ART binding is lost when proteinase K treatment of CK is followed by

('38K') (cf. lanes 2 and 3 in each blot), although the proteinase K-treated sample does contain some undigested CK (less than 0.2 μ g or 10% of the original amount: cf. lanes 1 and 2 on b).

Fig. 5. Dissociation of the two fragments of proteinase K-nicked CK by 8 M-urea results in loss of CK-ART binding, though 'nicked' CK itself still binds the antibody

Chick MM-CK was treated with proteinase K as described under 'Materials and methods'. (a) Proteinase K-treated CK (○) or untreated CK (□) were each treated with 8 M-urea as described and diluted to 10 µg/ml with e.l.i.s.a. incubation buffer containing 0.5 mg of bovine serum albumin/ml. Native chick MM-CK was used as a control (●). Serial dilutions were preincubated with CK-ART culture supernatant (1:500 final concn.) for 1 h at 0 °C before transfer to a second microtitre plate coated with chick MM-CK (5 µg/ml) for competition e.l.i.s.a. Each point is the average of duplicate microtitre wells. (b) A separate experiment in which untreated (●) and proteinase K-treated (○) chick MM-CK were diluted to 2 µg/ml and serial dilutions were used for competition e.l.i.s.a. as in (a).

Table 1. Effect of proteinase K digestion on chick MM-CK enzymic activity

Creatine kinase (1 mg/ml) was treated with proteinase K (0.3 µg/ml) in 10 mM-magnesium acetate/100 mM-sodium acetate/10 mM-Tris/acetate, pH 9.0 (Williamson *et al.*, 1977) at 30 °C for the times indicated. The reactions were terminated by addition of PMSF (0.1 mM) and incubation for a further 5 min. The samples were then diluted immediately in CK assay buffer [100 mM-Tris/acetate (pH 6.8)/100 mM-magnesium acetate] containing bovine serum albumin (1 mg/ml) and assayed for CK activity (Morris & Cole, 1972). Results are expressed as a percentage of zero-time incubations and are the average of triplicate (Expt. 1) or duplicate (Expt. 2) assays. Incubation of CK in pH 9.0 buffer without proteinase K had no significant effect on CK activity. Abbreviation: n.d., not determined.

Digestion time (min)	CK activity (% of control)	
	Expt. 1	Expt. 2
0	100	100
2	85	84
10	65	61
30	n.d.	0

dissociation of the fragments with 8 M-urea (Fig. 5a). This shows that a contribution from each of the two proteinase K fragments is necessary to form the CK-ART epitope. The same result is obtained when electrostatic forces are used instead of 8 M-urea to disrupt CK structure. When CK binds to a plastic microtitre plate, enzyme activity is lost (Morris & Head, 1983),

presumably as a result of electrostatic forces on protein conformation. As with urea treatment, however, the effect on CK conformation is insufficient to prevent CK-ART binding (Fig. 6; Morris & Head, 1983), but 90 % of CK-ART binding is lost when proteinase K-treated CK binds to plastic (Fig. 6). CK structure is evidently weakened when the enzyme is 'nicked' at Ala-328, so that the epitope is more easily disrupted.

Together, these experiments show that the epitope either is a simple amino acid sequence which spans the proteinase K cleavage site or is formed by long-distance interactions involving parts of the CK sequence on both proteinase K fragments. The former possibility is ruled out by the fact that the CK-ART antibody is specific for avian M-CK subunit and does not bind to rat, mouse, rabbit, human, or *Torpedo* CKs (Morris & Head, 1983, and unpublished results). Chick, rat and mouse M-CK sequences (see Fig. 7) are identical for 15 amino acids on either side of the Ala-Ala bond at which proteinase K cleavage occurs, so a simple 'structureless' sequence in this region could not be chick-specific. Furthermore, the M_r 8700 CNBr fragment (Fig. 1) includes all of this region around Ala-328 and does not bind CK-ART (Fig. 2).

To reinforce this conclusion, it must be shown directly that the smaller proteinase K fragment cannot bind CK-ART. Although this fragment is clearly visible on stained gels (Morris *et al.*, 1985), it is difficult to detect on Western blots, possibly because it does not bind well to nitrocellulose. Proteinase K-treated CK was therefore applied to a Sephadex G-50 column in the presence of 8 M-urea. Fig. 8 shows that no immunological activity was eluted in the low- M_r region and all residual CK-ART binding activity after proteinase K treatment (7 % of the original) is found in the excluded volume,

containing intact CK and the large fragment. Together with the results in Fig. 3, this shows that residual undigested CK can account for all CK-ART binding activity remaining after proteinase K treatment.

CK-ART antibody appears to recognize a conformational or assembled determinant which is reformed very

Fig. 6. Loss of CK-ART binding when proteinase K-nicked CK binds to plastic

Proteinase K-treated CK (○) and untreated CK (●) were each diluted to 5 µg/ml with phosphate-buffered saline. Serial dilutions were used to coat the wells of a plastic microtitre plate. After incubation with CK-ART culture supernatant (final concn. 1:500) for 1 h, bound antibody was detected with peroxidase-labelled rabbit anti-(mouse Ig) antibody and *o*-phenylenediamine as substrate. Each point is the average of duplicate wells.

Fig. 8. CK-ART binding to proteinase K fragments after separation by gel filtration in 8 M-urea

Chick MM-CK (2.45 mg) was treated with proteinase K as described in the Materials and methods section; 8 M-urea was added to a final concn. of 5.3 M and the whole sample (0.3 ml) was applied to a column (50 cm × 1 cm) containing Sephadex G-50 (fine) equilibrated with 8 M urea/10 mM-sodium phosphate, pH 8.0. The column had previously been calibrated and blocked using 1 mg each of bovine serum albumin (excluded volume, V_e) and insulin (Ins). The continuous line shows the A_{280} peak of the insulin marker. Column fractions (1.1 ml) were diluted and used to coat microtitre plates for an e.l.i.s.a. assay of CK-ART binding (1:500 dilution of culture supernatant). A standard curve (see inset) was generated on the same e.l.i.s.a. plate using serial dilutions of chick MM-CK and the CK-ART binding activity of each fraction (■) was calculated from this. The equivalent of 0.19 mg of MM-CK was recovered, 7% of the original sample applied.

Fig. 7. Amino acid sequence of the C-terminal region of creatine kinases

The sequence of the C-terminal NTCB fragment of chick M-CK is shown, together with the differences in sequence of M-CKs and of B-CKs (BB-) from various species. The sequence is highly conserved; most amino acid changes with potential structural significance occur within the two boxes. The only three chick M-CK-specific residues are indicated by diamonds and the proteinase K cleavage site is shown by an arrow. Horizontal bars indicate the two shortest sequences (40 and 44 amino acids) which contain the proteinase K cleavage site, a chick-specific residue and a methionine residue. Sources of the sequences were: chick MM, Kwiatkowski *et al.* (1984); *Torpedo marmorata*, Giraudat *et al.* (1984); dog MM, Roman *et al.* (1985); human MM, Perryman *et al.* (1986); mouse MM, Buskin *et al.* (1985); rat MM, Benfield *et al.* (1984); rabbit MM, Putney *et al.* (1984); rabbit BB, Pickering *et al.* (1985); rat BB, Benfield *et al.* (1985); human BB, Villarreal-Levy *et al.* (1987); dog BB, Billadello *et al.* (1986); chick BB, Hossle *et al.* (1987).

Fig. 9. Evidence for an intermediate conformational state in CK refolding

The diagrams are schematic only and are not intended to suggest actual conformations.

rapidly during refolding by an interaction involving distant amino-acid sequences on each side of Ala-Ala-328, the proteinase K cleavage site. It must be emphasized that the overall extent of refolding in this 'urea-treated' CK is very limited. Not only is the protein enzymically inactive, it is also unable to bind most antibodies against native CK and several antibody-binding sites are exposed which are inaccessible in the native enzyme (Morris *et al.*, 1987). It follows, therefore, that CK-ART is recognizing a distinct intermediate in the refolding process, as illustrated diagrammatically in Fig. 9. It may be that amino acid residues from both proteinase K fragments make contact with the antibody or that one of the regions is required to impose a structure on the other sequence which CK-ART recognizes. At least one of these two regions must have a chick MM-CK-specific amino acid sequence and this is most likely to include several chick-specific amino acid residues which actually make contact with the antibody in order to account for the clear-cut species specificity.

Only three amino acids in Fig. 7 are unique to chick CK (Lys-300, His-312 and Asn-369) and only the two boxed sequences contain chick-specific sequences, so that one or other (or possibly both) of these sequences is likely to form part of the CK-ART epitope. Interaction with a sequence on the other side of the proteinase K cleavage site to create the epitope would involve an

intervening sequence containing a minimum of 40–45 amino acids (Fig. 7), although folding of a much larger region could be necessary to stabilize the epitope. Strong intermolecular interactions here would account for both the rapid refolding of this region and the failure of the two fragments after proteinase K treatment to separate. The driving force for the folding may, of course, lie in interactions between the highly-conserved sequences in this region.

Further evidence for an intermediate in CK refolding

Antibodies can accelerate refolding of the epitopes they recognize (Chavez & Benjamin, 1978). The faster refolding of the C-terminal region containing the ART epitope might therefore be a consequence of the presence of CK-ART antibody and might not occur in its absence. Experiments with trypsin, however, suggest that a folding intermediate with its C-terminal region more similar to the native state than the N-terminal region is formed rapidly even in the absence of antibody.

Native CK is resistant to trypsin (Williamson *et al.*, 1977; Price & Stevens, 1982; Fig. 10d). Urea-treated CK (containing the 'intermediate') gives a ladder of Coomassie Blue-stained fragments (Fig. 10a), all of which bind CK-ART (Fig. 10b) but none of which bind N-terminal marker antibodies, 2A7, 5H5 or 6F2 (Fig. 10c), indicating that trypsin is preferentially digesting the

Fig. 10. Effects of trypsin on native and urea-treated CK

Chick muscle CK (25 mg/ml) was added to solid urea to give a final urea concn. of 9 M and incubated at 37 °C for 30 min. It was cooled in ice/water and ice-cold 50 mM-Tris/HCl, pH 7.6, was added to give a final CK concn. of 0.75 mg/ml in 0.45 M-urea. Trypsin was added (final concn. 0.7 µg/ml in *a-d*; 6.25 µg/ml in *e* and *f*) and incubated at 37 °C, taking samples into PMSF (final concn. 1 mM) at various times to stop the digestion. SDS and 2-mercaptoethanol were added to 1% and 5% respectively and samples were boiled for 2 min before electrophoresis on 15% (*a-d*) or 10% (*e,f*) gels and Western blotting. The procedure was identical for native CK (*d*) except that urea was omitted. (*a*)–(*d*): lane 1, 5 min incubation with trypsin; lane 2, 15 min; lane 3, 30 min. (*e*), (*f*): lane 1, 0.5 min incubation with trypsin; lane 2, 1 min; lane 3, 5 min. The pairs of blots (*b*)/(*c*) and (*e*)/(*f*) are identical 'mirror-blots' from the same gel (one of each pair has been printed 'reversed' during photographic processing). The Coomassie Blue stained gel (*a*) is also the same gel as (*b*) and (*c*). The blots were developed with the

N-terminal region. Very little, if any, can have been lost from the *C*-terminus in these fragments since sequences near this end are necessary for CK-ART binding (Figs. 3 and 4). The smallest CK fragment which binds CK-ART has M_r 20000 (Figs. 10*b* and 10*e*). Since Arg and Lys residues are uniformly distributed in CK (Fig. 1*b*), the trypsin resistance of the *C*-terminal region is probably due to its refolding to a conformation resembling the native state.

Even after very brief treatments (30 s) with higher concentrations of trypsin, no large fragments ($M_r > 23000$) which bind *N*-terminal marker antibodies can be detected (Fig. 10*f*). This suggests that trypsin resistance of the M_r 20000 *C*-terminal region is acquired very rapidly after dilution of urea. Smaller *N*-terminal fragments do appear transiently, as would be expected, but seem to be rapidly digested further by trypsin (Fig. 10*f*).

In these experiments trypsin is used as the conformational probe rather than antibody, so there is no possibility of antibody-induced folding accounting for the results, which are consistent with the model shown in Fig. 9. Similar results have been obtained with chymotrypsin (not shown).

Conclusions

The antibody CK-ART binds equally well to native and urea-denatured chick MM-CK (Morris & Head, 1983). This shows that the CK-ART epitope on native CK is accessible to antibody and therefore exposed on the surface of the native protein. This could either mean that the epitope is a stretch of 'random-coil' sequence on the protein surface or that the local conformation of the epitope is reformed much more rapidly after removal of urea than the overall conformation and enzymic activity of the protein. The results presented here show that the latter alternative is correct and demonstrate the possibility of mapping certain kinds of conformational surface epitope by chemical and proteolytic cleavage methods.

The evidence for an intermediate in CK refolding in which the *C*-terminal region is closer to the native state is summarized schematically in Fig. 9. We suggest that after removal of urea by dilution at 0 °C some local refolding occurs throughout the CK sequence to produce a structure akin to the 'collapsed' state (Goldberg, 1985) and that sufficient further refolding occurs in the *C*-terminal region to form the CK-ART epitope and to protect this region specifically against digestion by trypsin and chymotrypsin. Preferential refolding of *C*-terminal regions has also been observed in the refolding of ribonuclease and serum albumin (Chavez & Benjamin, 1978). At this intermediate stage, CK remains enzymically inactive and will still bind the monoclonal antibodies CK-2A7 and CK-5H5, which bind to local sequences in the *N*-terminal region of denatured CK (Morris *et al.*, 1987). Subsequently, at higher temperatures, a further slow transition to the native state occurs and enzyme activity is regained in parallel with loss of CK-2A7 and CK-5H5 binding (Morris *et al.*, 1987).

The existence of an apparent intermediate may be a

following dilutions of monoclonal antibody culture supernatants: (*b*), (*d*) and (*e*), 1:100 CK-ART; (*c*), 1:200 CK-6F2; (*f*), 1:100 CK-2A7.

simple consequence of differential folding rates and does not imply that *N*-terminal folding is dependent on previous *C*-terminal folding. The *C*-terminal conformation in the intermediate state may not be identical to that of native chick M-CK. Indeed, experiments with a monoclonal antibody against human BB-CK (Nguyen thi Man, A. J. Cartwright & G. E. Morris, unpublished work) suggest that an extreme *C*-terminal sequence may still be accessible to antibody at this stage but hidden in native BB-CK dimer.

Although Price & Stevens (1982) found no evidence from fluorescence measurements for an intermediate in CK refolding, the present studies are not inconsistent with their results. Formation of an intermediate, or 'collapsed state', may be complete within a few seconds of removal of the denaturant (Goldberg, 1985). Grossman *et al.* (1986) measured intrinsic fluorescence during refolding of monkey BB-CK and concluded that most of the native subunit structure was re-established 3 s after denaturant removal when their measurements began. Price & Stevens (1982) also studied susceptibility to trypsin during refolding. They found no evidence for either a trypsin-stable, enzymically inactive intermediate or a trypsin-sensitive, enzymically active intermediate. Their results are consistent, however, with the intermediate we propose which is still both trypsin-sensitive and enzymically inactive (Fig. 9).

It is significant that the partially denatured enzymically inactive state which results when CK binds to plastic (Fig. 6; Morris & Head, 1983; Morris *et al.*, 1987) corresponds closely in terms of monoclonal antibody accessibility to that of the proposed intermediate or 'collapsed state'. This suggests that the driving forces for this initial refolding are stronger and less easily reversed than those for the subsequent slower renaturation to the native state.

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