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Monoclonal antibody studies of creatine kinase

Antibody-binding sites in the *N*-terminal region of creatine kinase and effects of antibody on enzyme refolding

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(1) The binding sites of two monoclonal antibodies, CK-2A7 and CK-5H5, have been located to a 60-amino-acid sequence in the *N*-terminal region of creatine kinase (CK) by the use of chemical cleavage with formic acid (which cleaves proteins at Asp–Pro bonds) and cyanogen bromide (which cleaves at Met residues). (2) A simple method for preparing chemically-cleaved fragments of proteins for electrophoresis and Western blotting is described. (3) Binding studies with CK preparations from different animal species show that single amino acid changes at residues 39 or 82 prevent binding of CK-2A7 and CK-5H5 respectively. We suggest that Lys-39 and Glu-82 form parts of the binding sites on CK for the two monoclonal antibodies. The two sites lie in variable regions at each end of a highly-conserved sequence (residues 46 to 79) and are inaccessible to antibody in the native enzyme. (4) One of the antibodies, CK-2A7, inhibits the refolding of CK to native enzyme after denaturation by urea.

INTRODUCTION

Creatine kinase is well-characterized at the amino acid sequence level as a result of recombinant DNA sequencing, but its three-dimensional structure has not yet been published. In this respect, it is typical of a large and growing number of proteins. Physical studies indicate that the dimeric enzyme consists of two 'cigar-shaped' subunits lying side-by-side, while substrate analogue studies suggest that the catalytic site is formed by a narrow cleft in the surface of the enzyme (see review by Watts, 1973). Chemical modification studies have shown that reactive Trp, His, Arg and Lys residues are involved in enzymic activity (Cook *et al.*, 1981) and have associated the catalytic site with a highly-conserved amino acid sequence centred around Cys-282 (Watts, 1973). The enzyme undergoes a significant and essential conformational change on binding its substrates (Watts, 1973), a change which can be detected by its effects on antibody binding (Samuels, 1961). Under appropriate conditions, CK will refold after denaturation by urea or guanidine and will recover enzymic activity (Price & Stevens, 1982).

The advent of monoclonal antibodies which bind to unique sites on protein antigens enables a more precise immunochemical approach to enzyme structure and function. It should be possible to correlate specific amino acid sequences with functions such as catalytic activity, dimer formation and binding to intracellular structures (such as myofibrils in muscle; Turner *et al.*, 1973) by studying the effects of antibodies on these functions. Protein sequences accessible on the surface of native enzyme can be identified, and identification of binding sites which are exposed or destroyed during protein unfolding/refolding will provide information about these processes. With appropriate antibodies, conformational changes during catalysis could be investigated and a structural basis for differences between CK isoenzymes (Grossman, 1983) might be revealed. Monoclonal antibodies against CK have so far been applied to the study of structural differences in the B subunit between BB-CK and MB-CK isoenzymes (Jackson *et al.*, 1983), differences between oestrogen-induced protein and BB-CK (Kumar & Macario, 1985) and the site of proteinase K cleavage on the surface of native CK (Morris *et al.*, 1985).

The first step in the use of monoclonal antibodies as specific probes is to identify the corresponding antibody-binding site in the antigen. Monoclonal antibody-binding sites on lysozyme (Amit *et al.*, 1986) and neuraminidase (Colman *et al.*, 1987) have been characterized by X-ray diffraction of antigen–antibody complexes. More widely-used approaches include binding studies using naturally-occurring protein variants or mutants (e.g. Darsley & Rees, 1985) or using overlapping peptide fragments, generated either by chemical and proteolytic cleavage, or by direct synthesis (e.g. Wilson *et al.*, 1984). Individual amino acids, or short amino acid sequences, in the antigen which are necessary for antibody binding can often be identified by these methods, but they are limited by the availability of variants and by the fact that some antibodies will not bind to fragments of antigen. Monoclonal antibody binding can protect specific residues within and around the binding site from chemical modification (Burnens *et al.*, 1987) and can prevent access by other antibody molecules with contiguous binding sites, enabling the construction of a surface map (Tzartos *et al.*, 1981). This approach is particularly valuable for 'conformational' or 'assembled' determinants, which are formed by long-range interaction between distant parts of the amino acid sequence (Benjamin *et al.*, 1984).

In this report, we show that the binding sites on CK for two monoclonal antibodies can be located to a 60-

Abbreviations used: CK, creatine kinase; e.l.i.s.a., enzyme-linked immunosorbent assay.

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amino-acid sequence and that individual amino acids within this sequence which are essential for binding can be identified. It seems likely that these essential amino acid residues form part of the antibody-binding sites, which are not exposed on the surface of native CK. Potential application to structure/function studies is illustrated by the effects of one antibody on protein refolding. This antibody, CK-2A7, inhibits renaturation of urea-denatured CK, although the catalytic site for creatine binding lies in the *C*-terminal region of the molecule, far from the CK-2A7 binding site.

MATERIALS AND METHODS

Materials

Peroxidase-labelled rabbit anti-(mouse Ig) antibody was obtained from DAKO, High Wycombe, Bucks., U.K. Ultra-pure urea (Schwartz–Mann, Rahway, NJ, U.S.A.) was used for enzyme refolding experiments. Cleavage reagents, proteases and protein A–Sepharose were obtained from Sigma and pre-stained protein markers from Gibco/BRL, Paisley, U.K. The peroxidase substrates, *o*-phenylenediamine and diaminobenzidine (Sigma) were handled as possible carcinogens, while cyanogen bromide (CNBr) was handled in a fume hood and destroyed with bleach.

MM-CK from chick breast muscle and BB-CK from chick heart were purified to homogeneity by the methods of Eppenberger *et al.* (1967). Partially-purified human MM-CK was a gift from Dr. James Milner-White (Glasgow University), rat MM-CK was from Dr. Graham Pay (CIBA/Geigy) and an electric organ membrane preparation from *Torpedo marmorata* containing an MM-form of CK was a gift from Dr. John Cavanagh and Professor Eric Barnard (MRC Centre, Cambridge, U.K.). Rabbit muscle CKs were obtained from Sigma.

Monoclonal antibody production

Balb/c mice were given 100 μg of chick BB-CK intraperitoneally in Freund's complete adjuvant, followed by 100 μg in incomplete adjuvant 3 weeks later, and boosted intravenously after a further 2 to 3 weeks. CK-2A7 and CK-5H5 were obtained from separate fusions (using NS0 and NS1 myeloma cells respectively) performed as described previously (Morris & Head, 1982). Hybridomas were screened by e.l.i.s.a. and cloned twice by limiting dilution. To determine IgG subclass, the anti-(mouse total Ig) second antibody in e.l.i.s.a. was replaced by subclass-specific second antibodies (Serotec, Banbury, U.K.). CK-2A7 is IgG1 and CK-5H5 is IgM. Both antibodies are CK-specific, binding only to a single CK band on Western blots from gels of total muscle cell proteins (not shown).

Affinity-purification of CK-2A7 immunoglobulin

Protein A-Sepharose was equilibrated with 100 mM-sodium phosphate, pH 8.0, at 4 °C. CK-2A7 ascites fluid was loaded on to the column after diluting 10-fold with the same buffer. After washing with 10 column vol. of the same buffer, IgG1 was eluted with sodium citrate, pH 6.0 (Ey *et al.*, 1978), and dialysed overnight against 100 mM-Tris/HCl, pH 7.5. The same volume of citrate buffer alone was also dialysed as a control in subsequent work with the IgG. SDS-gel electrophoresis revealed only two peptides at about 25 kDa and 50 kDa corresponding to IgG heavy and light chains (G. E. Morris, unpublished results).

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 to 50 vol.) of 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). Both enzymic and native immunological (radioimmunoassay) activity (80 to 90%) is lost after this treatment (Galatowicz & Morris, 1981). Renaturation is prevented by keeping the sample at 0 °C.

Digestions

CK (3 mg) was treated with a 30-fold molar excess (over Met) of CNBr (3.5 mg) in a final volume of 1 ml of 70% formic acid for 24 h at room temperature. Cleavage at Asp-Pro bonds by 75% formic acid (Sonderegger *et al.*, 1982) was performed at 37 °C for 24 h. Interestingly, no significant cleavage at these bonds was observed during CNBr treatment in formic acid at room temperature.

In each case, excess reagents, acids and salts, were removed on a Sephadex G-15 column (12 cm × 1 cm diameter) equilibrated with 8 M-urea/125 mM-Tris/HCl (pH 6.8)/0.1 mM-phenylmethanesulphonyl fluoride. The unretarded fraction, containing all fragments greater than 1–2000 M_r, was used directly for electrophoresis after addition of 1% SDS and 5% 2-mercaptoethanol and boiling for 2 min. The urea ensures that all peptides remain in solution and this sample preparation method was found to be superior to published methods involving dialysis and freeze-drying, in terms of yield, effectiveness, speed, convenience and safety (CNBr reaction mixtures never leave the fume hood).

For cleavage by protease V8, CK was first boiled for 2 min with 0.1% SDS in 100 mM-Tris/HCl, pH 7.6, to partially denature the protein. CK (10 mg) was then incubated with protease V8 (50 μg) at 37 °C for 1 to 10 min (the optimal time was determined by electrophoresis for each digestion). The reaction was terminated by incubation for a further 5 min with 1 mM-phenylmethanesulphonyl fluoride, followed by addition of SDS to 1% and 5% 2-mercaptoethanol and boiling for 2 min. After addition of 10% sucrose, this sample was used directly for electrophoresis.

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). Pre-stained marker proteins were also transferred to enable precise identification of antibody-binding bands. Markers are ovalbumin (43000), chymotrypsinogen (25700), lactoglobulin (18400), lysozyme/cytochrome *c* (13300), bovine trypsin inhibitor (6200) and insulin (3000).

The blots were blocked and incubated successively with monoclonal antibody, peroxidase-labelled second antibody and diaminobenzidine as described previously (Morris & Head, 1985).

RESULTS AND DISCUSSION

The binding sites on CK for two monoclonal antibodies are near Lys-39 and Glu-82

Chick and rabbit CK become effective competitors only after urea treatment in a competition e.l.i.s.a. with monoclonal antibodies CK-2A7 (Fig. 1) and CK-5H5 (results not shown). Most antibodies raised against native proteins are specific for the native conformation. Why are 2A7 and 5H5 specific for denatured CK? First of all, the BB-isoenzyme of CK was used as immunogen and may be susceptible to denaturation before, during or after injection with adjuvant (cf. Sams *et al.*, 1985). More importantly, in this e.l.i.s.a. method, which we also used for screening the original hybridoma culture supernatants, CK is extensively denatured when it binds to microtitre plates (Morris & Head, 1983), so that only antibodies which bind to denatured CK are detected. This is the first indication that we are dealing with 'local' antibody-binding sites rather than ones that require extensive tertiary folding.

CNBr cleaves proteins *C*-terminally to methionine residues, but Met–Thr bonds are often resistant (Croft, 1980). Using published CK sequences (Fig. 4), we can predict that CNBr cleavage will produce two main fragments of M_r 19900 (Met-29–Met-206) and 8700 (Met-272–Met-357) and these can be identified on the stained gel in Fig. 2. (All peptide M_rs are calculated from the amino acid sequence and rounded to the nearest 100). Both 2A7 and 5H5 bind only to the 19900 M_r fragment, Ala-30 to Met-206 (Fig. 3, lanes A5 and B5). Incomplete cleavage of Met–Ala-30 is, we believe, responsible for the minor fragment of about 24000 M_r to which both antibodies also bind (Figs. 2 and 3). The only deduction we require from this experiment is that the binding sites on CK for both antibodies lie between Ala-30 and Met-206. Interpretation of immunoblots is best restricted to major fragments which are prominent on stained gels, since minor bands might possibly be produced by irrelevant cleavages. However, the prominent 16000 M_r stained band in Fig. 2 presumably arises by cleavage of the resistant Met–Thr-179 bond, giving the fragment Ala-30 to Met-178, and both antibodies bind to it, as expected.

Formic acid cleaves proteins at Asp–Pro bonds, of which chick CK has two (Fig. 4) (Morris *et al.*, 1985). Partial digestion of chick M-CK by formic acid produces two pairs of peptides; 11000 plus 32000 and 14000 plus 29000 (Fig. 2). Fig. 3 shows that 2A7 and 5H5 bind to both small *N*-terminal fragments of M_r 11000 and 14000. Both antibody-binding sites on CK must therefore be *N*-terminal to Asp-89. Using all the data so far, we

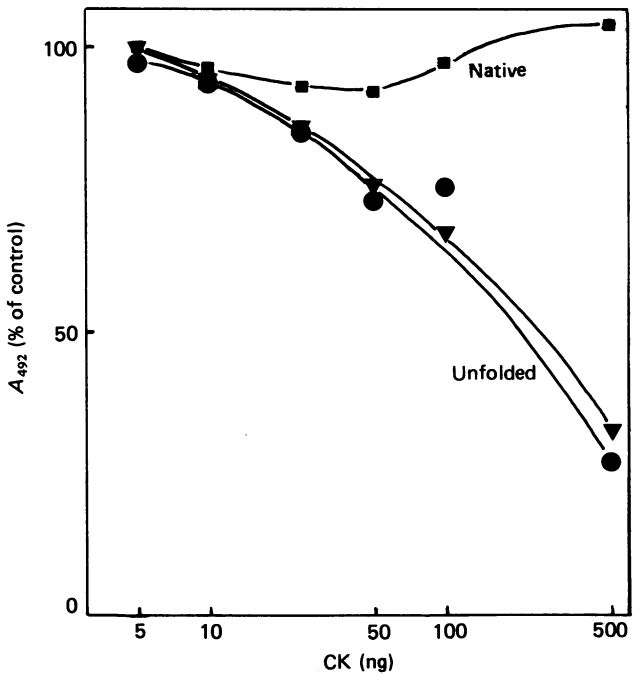

Fig. 1. Effect of urea unfolding of CK on binding to the monoclonal antibody CK-2A7, in a competition e.l.i.s.a. assay (Morris & Head, 1983)

Limiting amounts of antibody (1/500 dilution of culture supernatant) were pre-incubated with increasing amounts of native chick M-CK (squares), 8 M-urea-treated chick M-CK (circles) or urea-treated rabbit M-CK (triangles) at 4 °C for 1 h and then transferred to a microtitre plate coated with chick M-CK. Antibody bound to the plate was measured by the absorbance at 492 nm produced with peroxidase-labelled rabbit anti-(mouse Ig) and *o*-phenylenediamine (Morris & Head, 1983). Similar results were obtained with antibody 5H5.

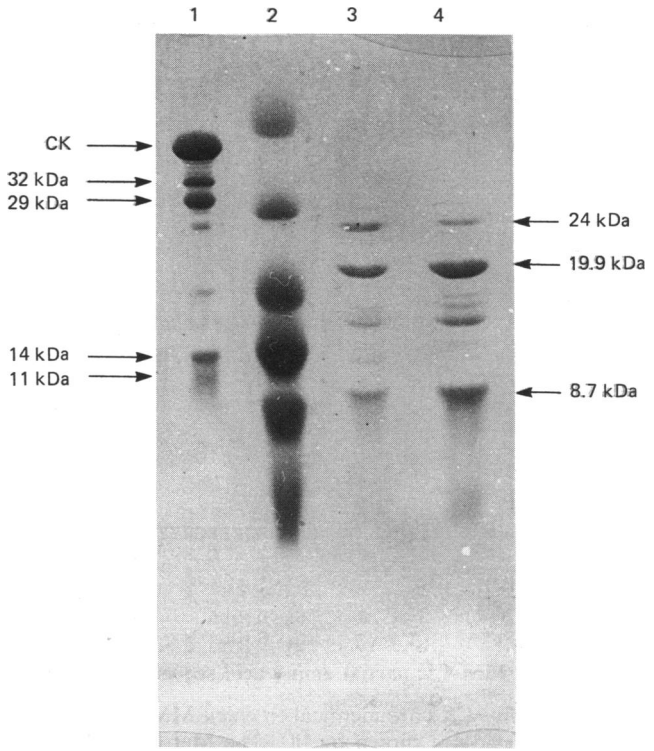

Fig. 2. Digestion of chick MM-CK with formic acid or cyanogen bromide

The 15% SDS/polyacrylamide gel was stained with Coomassie Brilliant Blue R250. Lane 1: Formic acid treatment (M_r of the four major fragments are shown $\times 10^{-3}$). Lane 2: M_r markers (see the Materials and methods section for six sizes). Lanes 3 and 4: Cyanogen bromide treatment (lane 3 shows the three main fragments, while lane 4 was loaded with twice as much sample to reveal the minor bands).

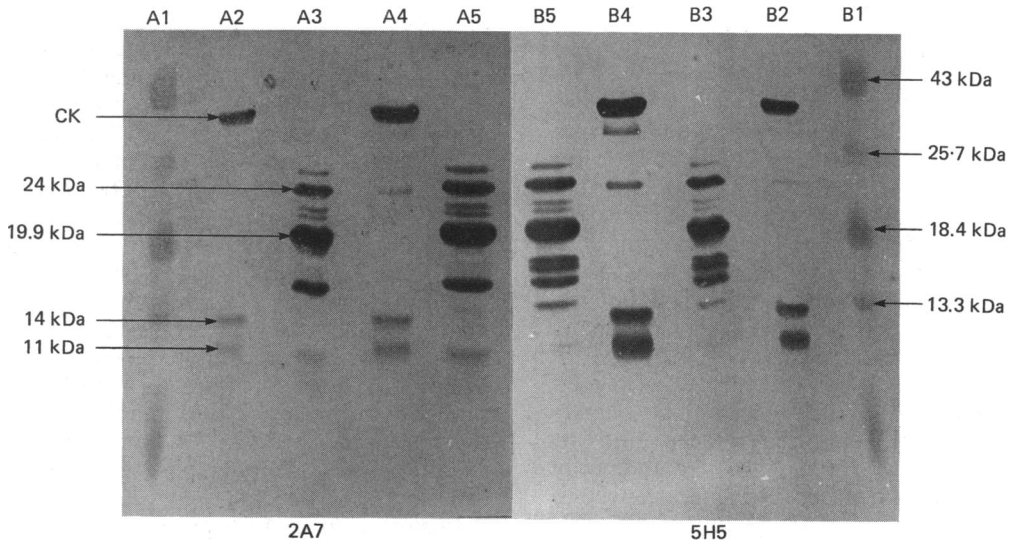

Fig. 3. Monoclonal antibody binding to chemical cleavage fragments on Western blots

The antibody, CK-2A7, was used for the first mirror-image blot, while CK-5H5 was used for the identical second blot. Lanes are: A1, B1, prestained protein markers; A2, A4, B2, B4, formic-acid treated chick MM-CK; A3, A5, B3, B5, cyanogen bromide-treated CK. Lanes 4 and 5 were loaded with twice as much sample as lanes 2 and 3 to show up the minor fragments more clearly. Arrows indicate undigested CK (40000), the main antibody-binding CNBr-fragment (19900) and the two antibody-binding formic acid fragments (14000 and 11000) (cf. stained gel in Fig. 2). The minor high M_r bands in lanes A4 and B4 do not correspond to the large Asp–Pro cleavage fragments in Fig. 2 and probably result from spurious cleavages in the *C*-terminal region. The two smallest M_r markers (6200 and 3000) were not separated on this gel.

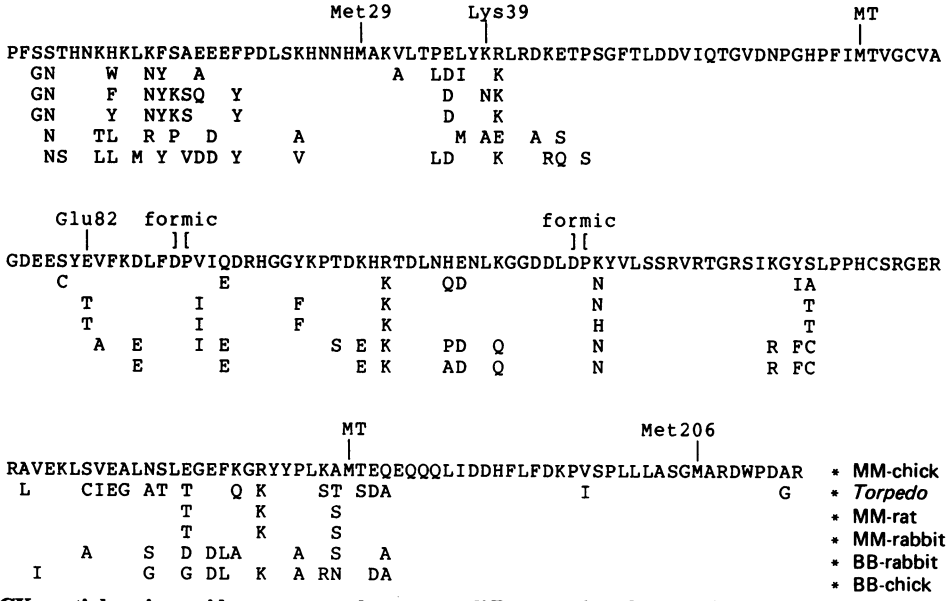

Fig. 4. Chick MM-CK partial amino acid sequence and sequence differences in other species and isoenzymes

The other five CKs are identical to chick MM-CK except where differences are shown. The two Asp–Pro (DP) bonds cleaved by formic acid are shown by '][', The Met residues at 29 and 206, which are cleaved by CNBr to give the main 19900 M_r fragment, are indicated. The two CNBr-resistant Met–Thr bonds, which give rise to minor CNBr fragments, are shown by 'MT'. Sources of the sequences were chick MM (Kwiatkowski *et al.*, 1984), *Torpedo marmorata* (Giraudat *et al.*, 1984), rat MM (Benfield *et al.*, 1984), rabbit MM (Putney *et al.*, 1984), rabbit BB (Pickering *et al.*, 1985) and chick BB (Hossle *et al.*, 1987).

can localize both binding sites to a 60-amino-acid sequence between Ala-30 and Asp-89.

The specificities of 2A7 and 5H5 are strikingly different and the molecular basis for their differences becomes apparent when the sequences are examined. Both direct binding (Table 1) and competition experiments (Fig. 1) show that 2A7 recognizes chick M-CK and B-CK, rabbit M-CK and torpedo CK, but not rat M-CK or rabbit B-CK. In contrast, 5H5 recognizes chick M-CK and B-CK, torpedo CK and rabbit B-CK, but not rat or rabbit M-

Table 1. Effect of amino acid changes at Lys-39 and Glu-82 on antibody binding to creatine kinase in e.l.i.s.a.

Microtitre wells were coated with each CK at 5 μg/ml. All results are A_{492} readings and are proportional to the amount to monoclonal antibody bound. For CK-5H5, (*a*) and (*b*) are two separate experiments.

		A_{492}				
	Enzyme ...	Chick M	Rabbit M	*Torpedo*	Rabbit B	Rat M
	Position 39 ...	Lys	Lys	Lys	Ala	Asn
Antibody	Position 82 ...	Glu	Thr	Glu	Glu	Thr
CK-2A7		> 2.000	> 2.000	> 2.000	0.025	0.046
CK-5H5		(*a*) 0.58	0.08	–	0.61	0.06
		(*b*) 1.38	–	0.72	1.18	0.03

CK (Table 1). Replacement of Lys-39 by either Asn or Ala inhibited 2A7 binding by 98% and replacement of Glu-82 by Thr inhibited 5H5 binding by 87–97% (Table 1). Examination of CK sequences (Fig. 4) shows that only single amino acid changes at positions 39 and 82 respectively can produce these specificities. The amino acid changes which prevent antibody binding do involve major charge or structure differences (Lys to Asn or Ala for 2A7, and Glu to Thr for 5H5) and so would be expected to have strong local effects, even though they do not appear to have sufficient effect on overall conformation to affect enzyme activity drastically.

We therefore suggest that Lys-39 is involved in forming the 2A7 binding site on CK and that Glu-82 is involved in the 5H5 binding site. The observation that 5H5 binds to some CNBr fragments to which 2A7 does not bind (Fig. 3, lanes A5 and B5) shows that the two binding sites are separate and do not overlap. It is not necessary for the argument to identify these fragments, but it seems likely that some of them arise from some cleavage at the resistant Met–Thr bond at Met-69 (Fig. 4).

Proteolytic cleavage can also be useful for mapping antibody-binding sites, even though the cleavage points and peptide fragments cannot be identified from the amino acid sequence. Further evidence for separation of 2A7 and 5H5 binding sites comes from Western blots of staphylococcal V8 protease digests of MM-CK shown in Fig. 5. In order to align the bands exactly, the digest was loaded as a continuous band on the slab gel and the blot was cut into strips for incubation with each antibody. The monoclonal antibody, CK-ART (lane 1), is a marker for the *C*-terminal region of chick MM-CK (Morris *et al.*, 1985). The patterns for 5H5 and 2A7 (lanes 5 and 6 respectively) are clearly different. Two monoclonal antibodies which bind to the central region of CK (G. E. Morris & L. C. Frost, unpublished results) are included (lanes 2 and 3), since, as expected, they share some bands with both CK-ART and 2A7/5H5 antibodies. Lane 4 is a control monoclonal antibody which does not bind to CK on blots. There are no fragments which bind both 2A7 and ART, which is consistent with the two binding sites being at extreme ends of the CK sequence. The protease seems to have cut between the 2A7 and 5H5 sites, leaving the 2A7 site on the small *N*-terminal fragments visible as a broad band near the bottom of lane 6.

Since CK must be unfolded for antibody binding and since a short, 60-amino-acid sequence is sufficient for binding, it is clear that both monoclonal antibodies

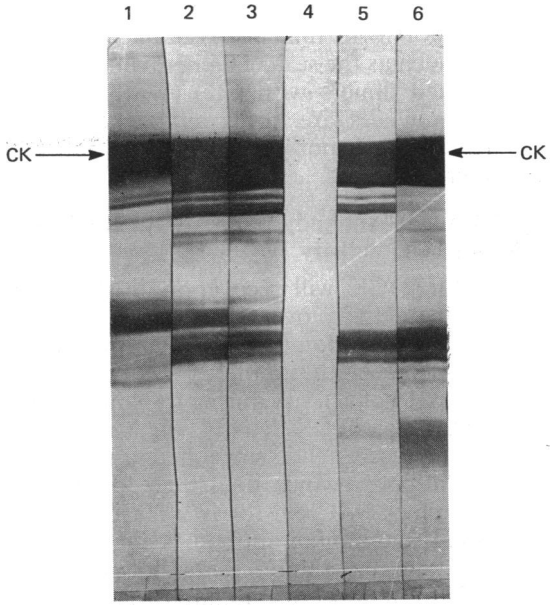

Fig. 5. Monoclonal antibody binding to cleavage fragments produced by staphylococcal V8 protease

V8-cleavage products of chick MM-CK were separated using the whole width of an 11% SDS/polyacrylamide gel and the Western blot was cut into strips. Each strip was incubated with a monoclonal antibody culture supernatant as follows, with dilutions: lane 1, CK-ART, 1:50; lane 2, CK-JIL, 1:30; lane 3, CK-JAC, 1:50; lane 4, control antibody, 1:10; lane 5, CK-5H5, 1:20; lane 6, CK-2A7, 1:20. Arrows indicate undigested CK.

recognize a 'local' sequence or conformation of amino acids rather than distant sequences brought together on the surface of the native molecule by protein folding ('conformational determinant'). Some authors (Benjamin *et al.*, 1984) have used the terms 'single segment' and 'assembled' determinants and these describe the distinctions we are making here better than the earlier terms, 'sequential' and 'conformational'. Lys-39 and Glu-82 may be part of the antibody-binding sites, interacting directly with 2A7 and 5H5 respectively. Less likely, perhaps, these amino acids could be essential for maintaining the local conformation of an adjacent group of amino acids which actually interact with the antibody. The interacting amino acids must at least be close to Lys-

39 and Glu-82 however, since the two binding sites are separable by both V8 and CNBr cleavages.

The 2A7 and 5H5 binding sites on CK are separated by a highly-conserved sequence common to all M- and B-isoenzymes (residues 48–79, Fig. 4). Such a sequence is likely to be essential for enzyme function and two functions common to all isoenzymes are substrate binding and dimerization. The creatine binding site is known to involve the *C*-terminal region of the molecule around Cys-282 (Watts, 1973), but this does not exclude co-involvement of *N*-terminal regions as a result of protein folding. Dimers form freely between different CK subunits (Eppenberger *et al.*, 1967), so one might expect the interaction site(s) to be specific and highly conserved. It is interesting to note that, although arginine kinase from lobster has a very similar creatine binding site sequence to CK, residues 48–79 have little similarity to those in CK (Regnouf *et al.*, 1981) and the lobster enzyme does not form dimers. Arginine kinases from some species, such as the sea cucumber, will form dimers (including mixed dimers with CK) (Watts, 1973), but amino acid sequences are not available for these. At present, therefore, it is not possible to rule out involvement in either function.

Binding of CK-2A7 antibody to urea-treated CK inhibits renaturation of CK activity

Urea-denatured CK will recover more than half its original enzymic activity over a period of 30 min after removal of urea by dilution (Fig. 7). Fig. 6(*b*) shows that increasing amounts of the antibody 2A7 progressively inhibit the recovery of activity during refolding, but there is no direct inhibition of CK activity (Fig. 6*a*). This is not a general effect of ascites fluid since there is no inhibition by CK-JOE antibody, which does bind to both native and denatured chick M-CK (Morris & Head, 1983) (Fig. 6). Ascites fluids containing antibodies against non-CK antigens also failed to inhibit (data not shown). The failure of 2A7 to inhibit after boiling (Fig. 6*b*) suggests that protein is responsible and purification of IgG using protein A-Sepharose confirms that antibody is responsible (Fig. 7). Fig. 7 also shows the effect of 2A7 antibody on the kinetics of renaturation. The initial rate of CK reactivation is reduced to 30% of the control rate in the presence of antibody (approx. 3.5-fold molar excess). This experiment has not yet been performed with antibody 5H5.

The nature of protein refolding after denaturation is still under active investigation (Kim & Baldwin, 1982). It is thought to consist of several distinct phases, the individual importance of which may depend on the size and complexity of the protein. Small proteins, like RNAase A, refold biphasically over periods of milliseconds or seconds, depending on whether isomerization at proline residues has occurred (Kim & Baldwin, 1982). CK, however, is a large enzyme likely to consist of several domains and renaturation of activity occurs over several minutes (Fig. 7; Price & Stevens, 1982; Zhou & Tsou, 1986). It would seem certain that some refolding, at least of secondary structure, occurs before activity recovers as a result of a 'final' conformational change (Watts, 1973; Price & Stevens, 1982; Zhou & Tsou, 1986). Intermolecular binding of 2A7 antibody to CK will inevitably be slower than the fastest of the intramolecular interactions which occur during CK refolding, so it is clear that antibodies against 'denatured

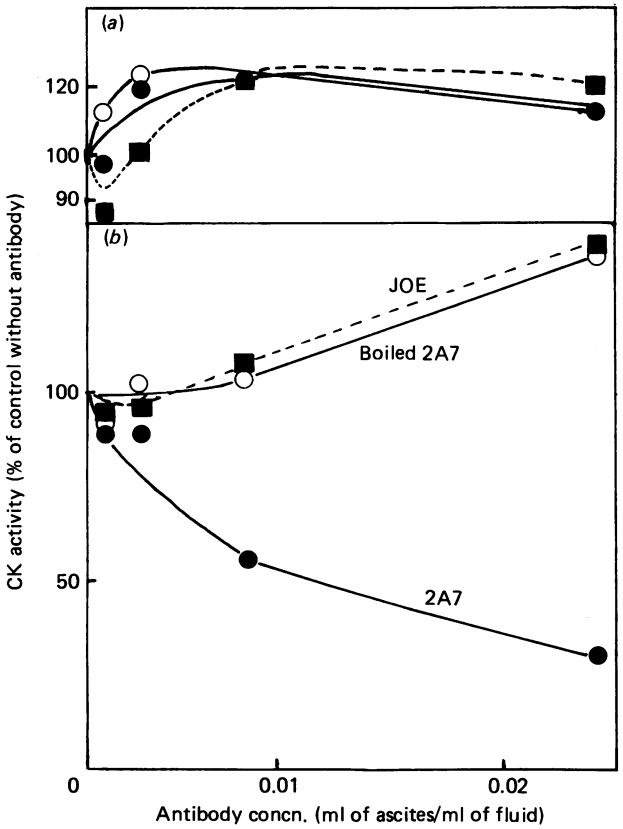

Fig. 6. Effect of antibodies on (*a*) CK activity and (*b*) CK refolding.

For refolding from urea (*b*), 27 μl of 8 M-urea/100 mM-2-mercaptoethanol/100 mM-Tris/HCl, pH 7.5, was added to 3 μl of chick MM-CK (6 mg/ml) and incubated at 25 °C for 30 min. In (*a*), procedures were identical except that urea was omitted. To 10 μl of control of urea-treated CK, 240 μl of antibody in ice-cold 100 mM-Tris/HCl, pH 7.5, was added (2A7 ascites fluid ●——●; 2A7 ascites boiled for 1 min at 1/40 dilution ○——○; CK-JOE ascites fluid ■----■) at dilutions between 1/40 and 1/1080 and portions were taken immediately for CK assay. The antibody–antigen mixtures (final CK concn. 24 μg/ml, final urea concn. 0.3 M) were then incubated at 25 °C for 30 min, cooled in iced-water and portions were taken again for CK assay. In (*a*), which shows direct effects of antibody on CK activity, the results of the second CK assay were normalized to controls without antibody (100%) and plotted against antibody dilution. In (*b*), which shows effects of antibody on renaturation of CK activity, the increase in activity between the first and the second CK assays was normalized to that of the control without antibody (100%). This procedure is necessary since about 20% of the original CK activity remained after urea treatment and about 70% was recovered during renaturation in the absence of antibody.

CK' or 'urea-unfolded CK' are binding to a protein with significant secondary structure, at least, and not to a simple sequence of amino acids. The monoclonal antibody, CK-2A7, is presumed to bind to this inactive, partially-restructured CK, though the antibody-binding site itself may or may not have significant structure. The presence of antibody clearly slows down the recovery of enzyme activity (Fig. 7), possibly as a result of steric hindrance. Control experiments show that this is a result

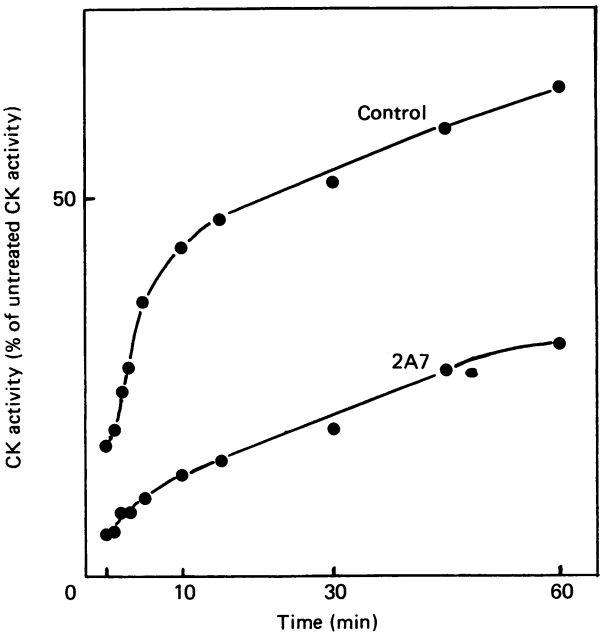

Fig. 7. Refolding kinetics in the presence of purified antibody

Chick MM-CK (600 μg/ml) was incubated at 25 °C for 30 min in 100 mM-Tris/HCl, pH 7.5/100 mM-2-mercaptoethanol/9 M-urea/0.05% bovine serum albumin. The reaction volume was 80 μl in an Eppendorf centrifuge tube. The unfolded CK (5 μl) was then incubated for 30 min at 0 °C with 120 μl of affinity-purified 2A7 antibody which had been dialysed against 100 mM-Tris/HCl, pH 7.5, (final concns.: CK, 24 μg/ml; antibody, 175 μg/ml) before transfer to 25 °C for refolding. For the control refolding curve, the 2A7 antibody was replaced by 100 mM-sodium citrate, pH 6.0, dialysed at the same time against the same solution of 100 mM-Tris/HCl, pH 7.5, as the 2A7 antibody (see the Materials and methods section). Portions were removed at various times during refolding and assayed for CK activity (Morris & Cole, 1972). Results are expressed as a percentage of the activity of untreated CK.

of specific antigen–antibody interaction (Fig. 6). Further experiments are necessary to determine whether the antibody interferes with the 'final' conformational change or whether it slows down an earlier step sufficiently for it to become rate-limiting. Monoclonal antibodies with known conformational requirements for binding would be of significant value in studies of protein folding mechanisms.

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