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A monoclonal antibody to human brain-type creatine kinase

Increased avidity with mercaptans

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1. A monoclonal antibody (subclass immunoglobulin G1) has been raised against human brain-type creatine kinase (CK-BB). This antibody did not cross-react with either muscle-type creatine kinase (CK-MM) or heart-type creatine kinase (CK-MB). 2. The binding constant measured with native antibody was $6 \times 10^8\ M^{-1}$. In the presence of 2 mM-dithiothreitol this constant was some 40–50-fold greater. 3. Partial reduction and alkylation showed that the increased binding was due to a direct effect on the antibody and was associated with concomitant cleavage of the heavy–heavy interchain disulphide bonds. The binding constant measured with Fab' fragments produced from reduced and alkylated antibody was similar to that shown by the native, unreduced antibody. 4. The molecular weight of the complex found in the absence of mercaptans was consistent with one antibody and one CK-BB molecule, whereas the molecular weight estimated with reduced and alkylated antibody was consistent with a complex of two antibodies and two CK-BB molecules. 5. It is proposed that mercaptans increase the flexibility of the hinge region of the antibody molecule, allowing the formation of a higher-order complex with increased avidity for the CK-BB dimer

The enzyme creatine kinase (CK; EC 2.7.3.2) catalyses the reversible phosphorylation of creatine from ATP and has an important role in cellular energy metabolism (Watts, 1973). In vertebrates, cytoplasmic CK exists as three isoenzymes with the dimeric subunit structure MM, MB and BB. In adults, MM is the major isoenzyme of muscle and BB the major isoenzyme of brain, whereas MB only occurs in appreciable quantities in heart (Watts, 1973). There is, in addition, a mitochondrial isoenzyme that is distinct from cytoplasmic CK (Jacobs *et al.*, 1964).

Previous work has shown that the parental subunits of CK are immunologically distinct, although it is not clear whether or not a B subunit in a BB homodimer and a B subunit in an MB heterodimer are immunologically identical (Thompson *et al.*, 1980; Thompson, 1981). During the course of producing monoclonal antibodies for use in specific assays for CK isoenzymes we have noted an interesting property of one such monoclonal antibody (designated A2) in that binding to CK-BB is greatly increased by mercaptans. The purpose of the present study was to investigate the molecular basis of this effect.

Abbreviations used: BSA, bovine serum albumin; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; CK, creatine kinase; Ig, immunoglobulin.

Materials and methods

Materials

Tris, *N*-ethylmaleimide and papain (grade III, research grade) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. BSA was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Rabbit antisera to mouse IgG1, IgG2a, IgG2b, IgG3 and IgM were purchased from Miles Biochemicals, Slough, Bucks., U.K. All other chemicals were of AnalaR grade and were purchases from BDH Chemicals, Poole, Dorset, U.K.

CK-BB was purified from human brain as described previously (Thompson *et al.*, 1980) and CK-MM was purified from human skeletal muscle as described by Keutel *et al.* (1972). All tissues were obtained within 12 h *post mortem*. CK-MB was

produced by hybridizing CK-BB with CK-MM as described previously (Willson *et al.*, 1981).

CK-BB was iodinated by the method of Bolton & Hunter (1973) using *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl) propionate (The Radiochemical Centre, Amersham, Bucks., U.K.). Specific radioactivities were typically 2500–3000 d.p.m./ng.

Immunoadsorbent containing sheep anti-(mouse IgG) covalently coupled to cellulose was prepared as described previously (Soos & Siddle, 1982).

Preparation of monoclonal antibody

BALB/c mice (Bantin and Kingman, Hull, Yorks., U.K.) were hyperimmunized with CK-BB by a procedure recommended by Stähli *et al.* (1980). Fusion with P3-NS1/1-Ag4-1 myeloma cells, cloning at limiting dilution and growth of hybrids *in vivo* were carried out by using standard methods (Galfrè & Milstein, 1981). Antibody was purified from ascites fluid by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose ion-exchange chromatography (Parham *et al.*, 1982).

Binding assay

Antibody-containing supernatant (0.1 ml) was incubated with 0.1 ml of iodinated CK-BB (3 ng, 7500 d.p.m.) in 50 mM single-strength veronal buffer (SSV), pH 8.1, containing 5 g of NaCl/litre, 5 g of BSA/litre and 0.1 g of sodium azide/litre. After 3 h at room temperature, sheep anti-(mouse IgG) immunoadsorbent (0.25 mg in 0.05 ml of SSV) was added and incubated for a further hour. Samples were washed with 2 ml of ice-cold half-strength veronal buffer and centrifuged. The supernatants were removed and the pellet was counted for radioactivity in an NE 1600 gamma counter with a counting efficiency of 75%.

Immunoglobulin class determination

Chain class-specific and subclass-specific immunoadsorbents were prepared by incubating separately rabbit anti-(mouse IgG1), -(mouse IgG2a), -(mouse IgG2b), -(mouse IgG3) or -(mouse IgM) antisera with a sheep anti-(rabbit IgG) adsorbent as described previously (Soos & Siddle, 1982).

Antibody-containing supernatant (0.1 ml) was incubated with iodinated CK-BB label in SSV (0.1 ml) as for the binding assays except that bound label was separated from free using either sheep anti-(mouse IgG) immunoadsorbent or the chain-class-specific adsorbents prepared above.

Determination of binding constants and assessment of specificity

Antibody-containing fluid was diluted in SSV such that it half-maximally bound approx. 3 ng (7500 d.p.m.) of iodinated CK-BB. Conditions were as for the binding assay except that the first incubation was for 48 h at 4°C. This concentration of antibody was incubated under the same conditions with iodinated CK-BB and various concentrations of unlabelled CK-BB, CK-MB and CK-MM. Binding constants were calculated from the data for CK-BB by the method of Scatchard (1949). Specificity was assessed by the relative potency of unlabelled isoenzyme to inhibit binding of iodinated CK-BB to the antibody.

Reduction and alkylation

A solution of SSV without BSA was made 20 mM in DTT and 10 mM in EDTA. To 0.9 ml of antibody-containing fluid was added 0.1 ml of the above solution; this mixture was left for 2 h at room temperature. A saturated solution (0.1 ml) of *N*-ethylmaleimide was then added, mixed rapidly, and left for a further 1 h at room temperature. Excess *N*-ethylmaleimide was removed by gel filtration on a column (20 cm × 1.5 cm) of Sephadex G-25. The column was eluted with SSV containing no BSA or sodium azide, 1 ml fractions were collected and the absorbance at 280 nm measured. The first absorbance peak, representing protein, was pooled for further use.

Molecular-weight determination

The molecular weights of native antibody and reduced, alkylated antibody were measured by gel-filtration on a column (180 cm × 2 cm) of Sephadex G-200. Antibody supernatant or antibody supernatant that had been reduced and alkylated (1 ml of each) were separately eluted at a flow rate of 10 ml/h. The elution buffer was 50 mM-Tris/HCl, pH 8.1, containing 100 mM-NaCl and 0.1 g of sodium azide/litre. Fractions were collected every 15 min and 0.1 ml of each fraction was measured for binding activity in the absence and in the presence of 2 mM-DTT.

The molecular weights of the complexes formed between antibody and CK-BB were estimated by gel-filtration on a column (90 cm × 2 cm) of Sepharose 6B. Approx. 50000 d.p.m. of ^{125}I-labelled CK-BB was incubated with 0.3 ml of a limiting concentration of antibody (either native or reduced, alkylated) overnight at 4°C. These were separately eluted at a flow rate of 6 ml/h using the elution buffer described above. Alternate 1.5 ml fractions were collected and measured for radioactivity.

Polyacrylamide-gel electrophoresis

This was performed using the buffers and stacking systems of Laemmli (1970) in the presence of 0.1% (w/v) SDS. Samples were loaded on to a 15 cm × 15 cm slab gel containing a linear 7.5 to 15% gradient of acrylamide and run at 30 V constant voltage overnight. Gels were stained with 0.05%

(w/v) Coomassie Blue in a 10% (v/v) acetic acid/50% (v/v) methanol solution. They were destained by washing in a 5% (v/v) acetic acid/10% (v/v) propan-2-ol solution followed by a 7% (v/v) acetic acid/10% (v/v) propan-2-ol solution.

Production of Fab' fragments from reduced, alkylated antibody

A solution of purified antibody (1 ml, 0.8 mg) was reduced and alkylated as described above. Fab' fragments were produced from this material using 0.04 mg of pre-activated papain as described by Parham *et al.* (1982).

Results

Characterization of the monoclonal antibody

The heavy-chain class of antibody A2 was determined by testing the ability of the antibody CK-BB complex to bind chain class-specific immunoadsorbents. This showed that antibody A2 was of the IgG1 subclass. Light-chain class was not established by this procedure.

Antibody specificity was determined by measuring the binding of ^{125}I-labelled CK-BB to the antibody in the presence of increasing concentrations of unlabelled CK-MB and CK-MM. It was found that neither CK-MB nor CK-MM showed significant cross-reactivity at the highest concentrations tested (Fig. 1). Although the CK-MB used in this study was produced by artificially hybridizing CK-BB with CK-MM, native CK-MB, partially purified from human heart muscle, also failed to cross-react with the monoclonal antibody (results not shown).

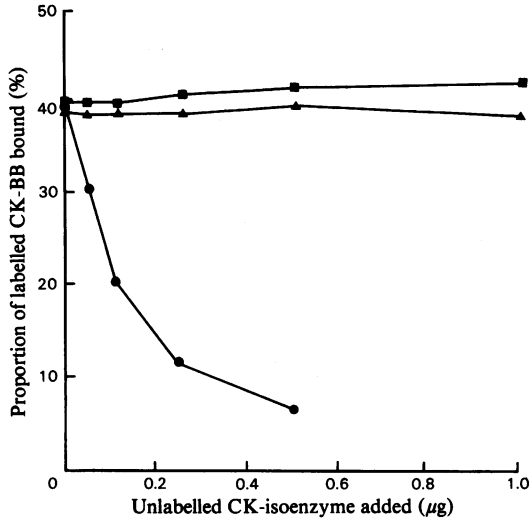

Fig. 1. *The cross-reactivity of the monoclonal antibody with CK isoenzymes*

The experiment was conducted as described in the Materials and methods section and shows the binding of iodinated CK-BB to the antibody of the presence of unlabelled CK-BB (●), unlabelled CK-MB (▲) or unlabelled CK-MM (■). Points are means of duplicates agreeing to within 5%.

Increased binding in the presence of mercaptans

During the course of characterization studies it was observed that the binding of the antibody to iodinated CK-BB was increased by the presence of mercaptans in the assay buffer. Initially, this was conveniently studied by measuring the dilution of antibody required to bind a fixed amount of CK-BB half-maximally. Typically, this value increased some 10-fold with 10 mM-2-mercaptoethanol and some 40–50-fold with 2 mM-DTT (Fig. 2). Maximal binding, under conditions of antibody excess, was not affected by such treatment (Fig. 2).

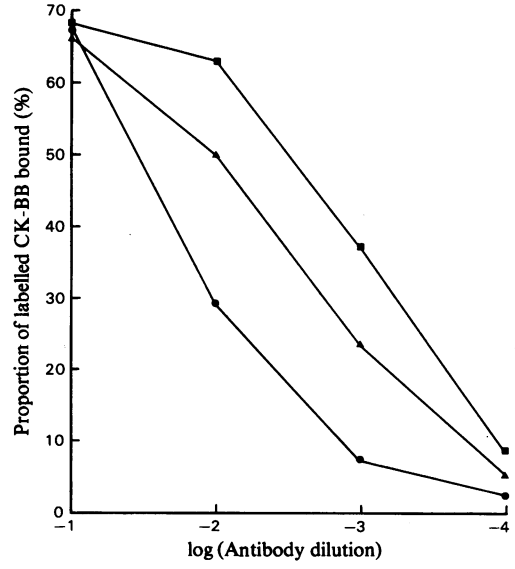

Fig. 2. *The effect of mercaptans on binding titre for the monoclonal antibody*

Incubations were conducted as for the binding assays: ●, with no addition; ▲, in the presence of 10 mM-2-mercaptoethanol; ■, in the presence of 2 mM-DTT. Points are the means of duplicates agreeing to within 5%; binding of label in the absence of monoclonal antibody A2 was 4%.

Reduction and alkylation experiments

To determine whether the increased binding was caused by an effect of mercaptans on the antibody or the CK-BB label, reduction and alkylation experiments were carried out (Table 1). It was found that reduced and alkylated antibody bound an amount of iodinated CK-BB greater than that bound by the same amount of native antibody, but equivalent to that shown by native antibody in the

Table 1. *The effect of reduction and alkylation on the binding capacities of the monoclonal antibody and CK-BB*

Antibody A2 supernatant was diluted in SSV such that it bound only 40% of CK-BB label when tested in the standard binding assay. Of this solution 1 ml was reduced and alkylated and separated from excess *N*-ethylmaleimide by gel-filtration on Sephadex G-25. As a control, an identical dilution of the same batch of antibody was passed down the same G-25 column, but was not reduced and alkylated. This procedure was then repeated for ^{125}I-labelled CK-BB. The reactants were then incubated together, in every pairwise combination, both in the absence and in the presence of 2 mM-DTT as for the standard binding assay. Results are presented as means of duplicates agreeing to within 5%; non-specific binding was 5%.

	Proportion of label bound (%)	
	No DTT	2 mM-DTT
Control A2 + control CK-BB	27	63
Reduced and alkylated A2 + control CK-BB	70	69
Control A2 + reduced and alkylated CK-BB	14	22
Reduced and alkylated A2 + reduced and alkylated CK-BB	22	23

presence of 2 mM-DTT. Alkylation of the iodinated CK-BB label considerably decreased its binding to the antibody, possibly because alkylation of free thiol groups caused conformational changes in CK-BB. However, the binding of alkylated CK-BB was still increased when measured with either reduced, alkylated antibody, or with native antibody in the presence of 2 mM-DTT. The increased binding in the presence of mercaptans was therefore dependent on reduction of the antibody rather than the CK-BB antigen.

The molecular weight of native and reduced, alkylated antibody were measured under non-denaturing conditions by gel-filtration and it was found that both eluted at the same volume in the presence and in the absence of 2 mM-DTT (Fig. 3). On the other hand, when examined by SDS/polyacrylamide-gel electrophoresis, the reduced and alkylated antibody ran as free heavy and light chains (Fig. 4). Thus, the interchain disulphide bonds were cleaved by DTT but the heavy and light chains remained associated with normal stoichiometry under the mild conditions used.

Cleavage of interchain disulphide bonds as a function of dithiothreitol concentration: correlation with increased binding to CK-BB

By incubating the antibody with increasing concentrations of DTT, alkylating and electrophoresing on an SDS/polyacrylamide gel, the

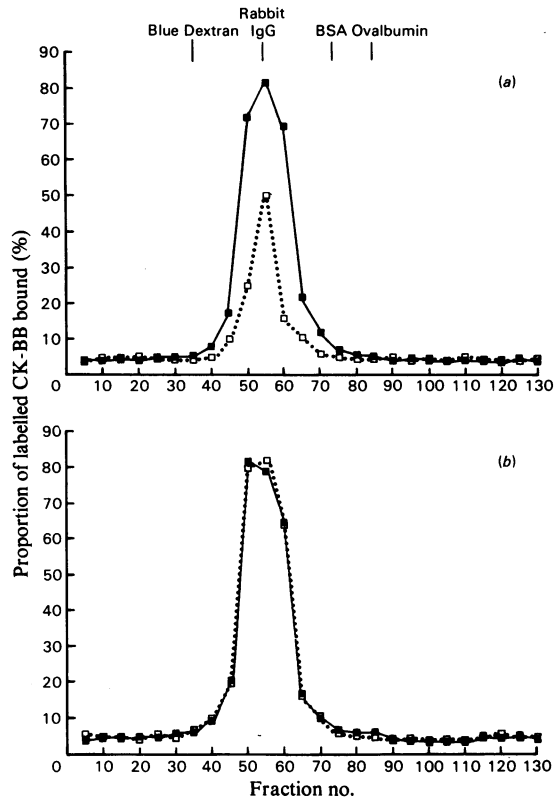

Fig. 3. *Gel-filtration of unmodified or reduced and alkylated antibody*

The experiment was conducted as described in the Materials and methods section. Of every fifth fraction 0.1 ml was tested for the ability to bind iodinated CK-BB in the absence (□) or in the presence (■) of 2 mM-DTT. (*a*) Native, unmodified antibody; (*b*) reduced and alkylated antibody. The column was calibrated with Blue Dextran, rabbit IgG (M_r 156000), bovine serum albumin (M_r 68000) and ovalbumin (M_r 42000).

concentration ranges over which the heavy–heavy and the heavy–light interchain disulphide bonds were cleaved could be estimated (Fig. 4). It was found that the heavy–light interchain disulphide bonds began to be cleaved at a concentration of 0.14 mM-DTT as judged by the appearance of free light chain on the gel. In contrast, free heavy chains did not appear until 0.4 mM-DTT.

Other bands also appeared on the gel over the concentration ranges studied (bands i, ii and iii in Fig. 4). These probably represented intermediate fragments composed of two heavy chains and one light chain, two heavy chains only and one heavy and one light chain. To confirm these assignments the bands were therefore cut out from the gel, heated

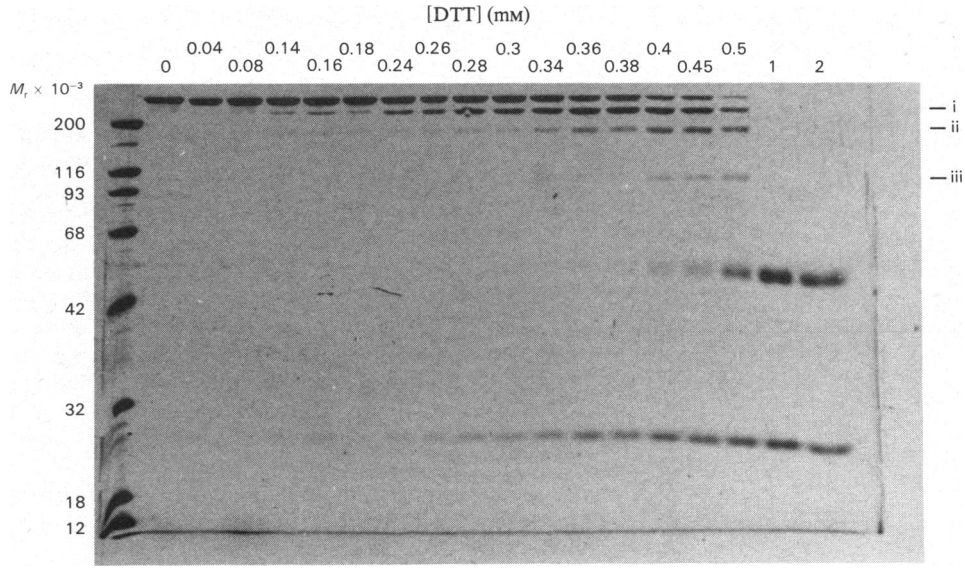

Fig. 4. *Cleavage of interchain disulphide bonds by dithiothreitol*

Samples of purified antibody A2 (16μg) were incubated with increasing concentrations of DTT in 50mM-Tris/HCl buffer, pH8.1 (total volume 0.04ml). After 2h at room temperature samples were alkylated with 0.02ml of a saturated solution of *N*-ethylmaleimide and 10μg of each analysed by SDS/polyacrylamide-gel electrophoresis in the absence of mercaptans. Bands (i), (ii) and (iii) represent intermediate fragments referred to in the text.

in SDS sample buffer containing 10mM-DTT and run on a 12% polyacrylamide gel containing 0.1% SDS. This showed that bands (i) and (iii) possessed both heavy and light chains, whereas band (ii) possessed only heavy chains (results not shown).

These intermediates, as well as the unreduced antibody, ran anomalously relative to the molecular-weight markers. The reason for this is unknown but it was not seen on gel-filtration where the unreduced antibody eluted at the same volume as rabbit IgG (Fig. 3).

The concentration range of DTT over which increased binding to labelled CK-BB occurred (Fig. 5*a*) was directly compared with the range over which cleavage of heavy–light and heavy–heavy disulphide bonds occurred (Fig. 5*b*). Quantification of disulphide bond cleavage (Fig. 5*b*) was achieved by densitometry of the gel shown in Fig. 4. It was found that increased binding to CK-BB closely followed the cleavage of the heavy–heavy interchain bonds as measured by the combined appearance of free heavy chains and heavy–light half molecules.

Mouse IgG1 antibodies have three closely spaced heavy–heavy interchain disulphide bonds connecting the two Cγ2 domains (Svasti & Milstein, 1972). Since the appearance of free heavy chains on the gel required the cleavage of all three interchain bonds, the experiment also suggested that the increased binding to CK-BB depended on the cleavage of all three bonds rather than one or two.

Determination of avidity of native and reduced antibodies

Apparent binding constants for (*a*) native, (*b*) reduced and (*c*) reduced and alkylated antibody as well as Fab′ fragments from the latter were determined from the gradients of Scatchard plots derived from binding data (Table 2). These plots were linear within experimental error. The results showed that the apparent binding constants of reduced antibody and reduced alkylated antibody were similar, and were some 40–50-fold greater than that of native antibody (Table 2). In contrast, Fab′ fragments produced from reduced, alkylated antibody had an apparent binding constant even lower than that of native antibody. This result, taken with the observation that the increase in CK-BB binding correlated with the cleavage of the heavy–heavy interchain disulphide bonds, suggested a role for antibody bivalence in the phenomenon rather than a direct effect of DTT on the antibody binding site.

Nature of the antibody CK-BB complex

The molecular weight of the complexes between ^{125}I-labelled CK-BB and native and or reduced and alkylated antibody were estimated by gel-filtration. At a concentration of antibody that bound 40% of the CK-BB label, the molecular weight of the native antibody CK-BB complex was approx. 250000 (Fig. 6). Assuming a molecular weight for CK-BB of

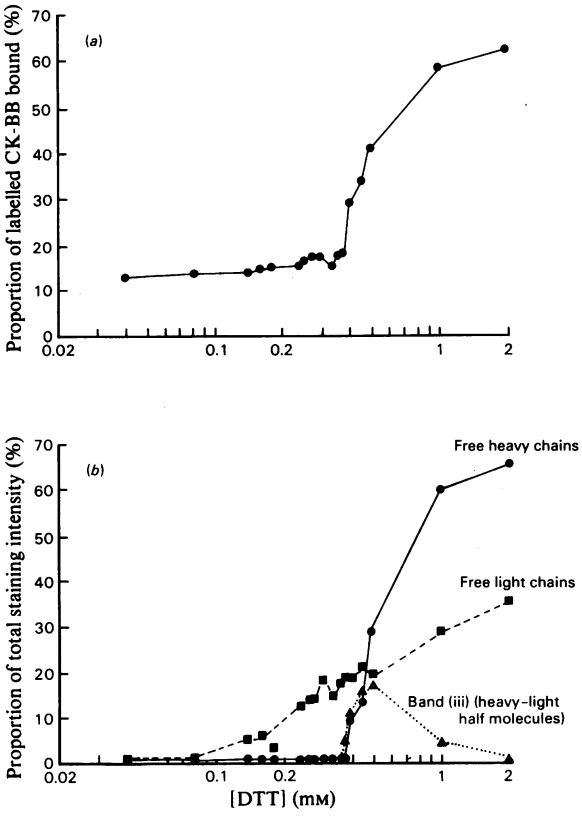

Fig. 5. *Comparison of increased CK-BB binding with disulphide bond cleavage of the monoclonal antibody*
The remaining 6 μg of the samples described in Fig. 4 were diluted 10^3-fold into SSV and 0.1 ml of this dilution was measured by binding activity in the normal manner. The gel shown in Fig. 4 was scanned at 540 nm using a Joyce Loebl Chromoscan 3 gel scanner. The rate of appearance of free heavy, free light and heavy–light intermediates were plotted by expressing the staining intensity for a given band as a percentage of the total staining intensity for all the bands on a given track (*b*).

Table 2. *Summary of Scatchard-plot data*

	K_a (M^{-1})
Native antibody	6×10^8
Antibody + 2 mM-DTT	3×10^{10}
Reduced and alkylated antibody	2×10^{10}
Fab' fragments produced from reduced and alkylated antibody	2×10^8

80000 (Watts, 1973), this was equivalent to one CK-BB molecule and one antibody molecule. When the experiment was repeated using reduced and alkylated antibody the complex eluted at the higher molecular weight of 440000 (Fig. 6). This was approximately equivalent to a complex containing two antibodies and two CK-BB molecules.

Discussion

The IgG1 monoclonal antibody described in the present paper was raised against CK-BB and showed negligible cross-reactivity with either CK-MB or CK-MM (Fig. 1). This failure to cross-react with CK-MB is surprising since the B subunit represents one-half of the CK-MB molecule. Nevertheless there are reports of differences in immunoreactivity between the B subunit in CK-BB and CK-MB. Thus, in a radioimmunoassay for CK-BB using polyclonal sheep antisera, we have found that CK-MB immunoreactivity shows non-parallel dilution characteristics compared with CK-BB immunoreactivity (Thompson *et al.*, 1980). In addition, an autoantibody to CK-BB has been described that failed to cross-react with CK-MB (Urdal & Kierulf, 1981).

Two possible structural explanations can be suggested for this lack of cross-reactivity. First, the epitope to which the antibody binds may be one which, although exposed in CK-BB, is inaccessible in CK-MB. A second possibility is that the antibody binds to an epitope at the bridging region between the two subunits of the CK-BB dimer.

The effect of mercaptans was greatly to increase the apparent affinity of the monoclonal antibody for CK-BB (Table 2). This has been shown to be due to a direct effect on the antibody rather than the antigen (Table 1). The appearance of increased binding to CK-BB was closely correlated with the concomitant cleavage of the heavy–heavy interchain disulphide bonds (Figs. 4 and 5). Conversely, no increased affinity was seen with Fab' fragments produced from reduced and alkylated antibody (Table 2). These results indicate that the increase in apparent affinity caused by mercaptans was due to heavy–heavy interchain bond cleavage and not to a direct effect on the antibody combining site. In the absence of DTT, at a concentration that half-maximally bound the CK-BB label, the antibody predominantly bound monovalently (Fig. 6). It should be emphasized, however, that this does not preclude the formation of higher-molecular-weight complexes at different antibody and antigen concentrations, merely that at the concentrations used such high-molecular-weight complexes are not favoured. In the presence of DTT, the predominant species is a complex containing two antibodies and two CK-BB dimers (Fig. 6). It is possible, in view of the large increase in binding constant shown in Table 2, that this is a true cyclic complex in which every antigen-binding arm of the antibody binds one epitope on a CK-BB dimer. Such a complex would presumably be more stable than the monovalent complex formed by the unreduced antibody.

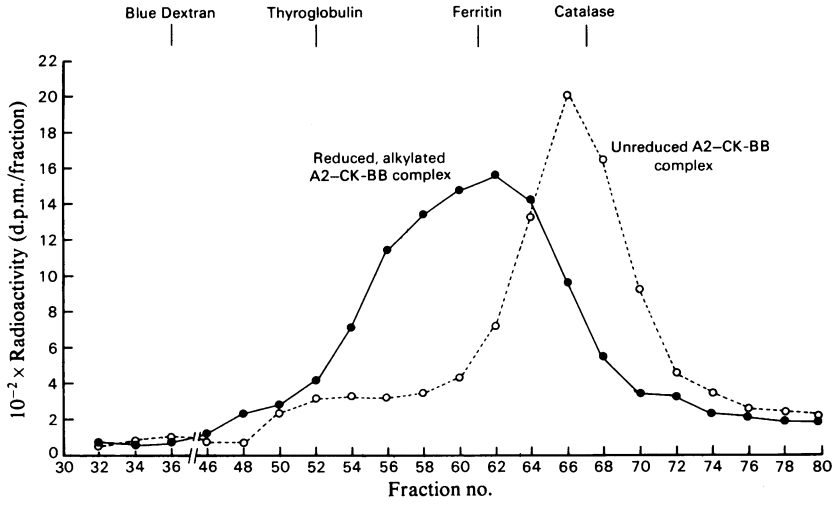

Fig. 6. *Gel filtration of the CK-BB–antibody complex for native antibody and reduced and alkylated antibody*
The experiment was conducted as described in the Materials and methods section. Every second fraction was counted for radioactivity. The column was calibrated with Blue Dextran, thyroglobulin (M_r 670000), ferritin (M_r 440000) and catalase (M_r 210000) (elution positions of these shown by arrows).

The effect on IgG antibodies of reduction under mild conditions has been investigated by several groups (Venyaminov *et al.*, 1976; Chou & Cathou, 1977). It has been found that cleavage of the interchain disulphide bonds by mercaptans under non-denaturing conditions does not cause the dissociation of the heavy and light chains but does impart a greater flexibility to the antibody molecule. Studies on a human IgG1 myeloma protein suggest that, although the Cγ3 domains of the Fc portion of the molecule interact strongly by non-covalent forces, there is no such interaction between the two Cγ2 domains. In the intact IgG molecule, therefore, the Cγ2 domains are only connected by the covalent heavy–heavy interchain disulphide bonds (Ellerson *et al.*, 1976). Assuming this also applies to mouse IgG1, the increased flexibility of the antibody after reduction may allow the two Cγ2 domains to adopt a more exposed conformation. The effect of DTT may therefore be to increase the length of the antigen-binding portion of the antibody and allow the formation of high-avidity cyclic complexes at a much lower antibody concentration than would normally be the case.

A previous report of the effects of mercaptans on antibody binding has been made by Aarden *et al.* (1977). They noted a 6-fold increase in binding when a polyclonal antisera to double stranded DNA was reduced. They suggested that mercaptans produced this effect both by reducing the heavy–light interchain bond, causing an increase in intrinsic antibody affinity, and by reducing the heavy–heavy interchain bonds, which would allow bivalent binding to the DNA molecule. Neither explanation is correct in the present case. First, the intrinsic antibody affinity is not affected by reduction and alkylation (Table 2) and secondly, bivalent binding to CK-BB after reduction would not explain the increased molecular weight of the reduced antibody–CK-BB complex (Fig. 6).

It has previously been reported that mixtures of two or more monoclonal antibodies directed against different epitopes may show an avidity for antigen much greater than that of the individual antibodies (Ehrlich *et al.*, 1982; Parham *et al.*, 1982). Holmes & Parham (1983) have recently explained such enhancement with two monclonal antibodies directed against HLA-A2 antigen in terms of the formation of stable tetrameric cyclic complexes between two antigen and two antibody molecules. However, this was only seen when two monoclonal antibodies directed against different epitopes were used. The present work would appear to be the first reported case of a similar effect with a single monoclonal antibody forming either low-affinity or high-avidity complexes depending on the conditions of the assay.

The mercaptan effect described here has the practical consequence of directly increasing the avidity of at least some monoclonal antibodies used in assays for CK isoenzymes and may represent a general phenomenon applicable in assays for other biological macromolecules containing two or more identical epitopes.

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