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HETEROBIFUNCTIONAL CROSS-LINKING OF A MONOCLONAL ANTIBODY WITH 2-METHYL-N^1-BENZENESULFONYL-N^4-BROMOACETYLQUINONEDIIMIDE

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The Cyssor reagent, 2-methyl-N^1-benzenesulfonyl-N^4-bromoacetylquinonediimide, which will cleave a protein chain at Cys under acidic conditions, cross-linked unreduced and partially reduced antibody at pH 8.0. No cleavage of the antibody occurred suggesting that the Cyssor reagent may be useful with certain proteins as a heterobifunctional cross-linker.

We report the use of the Cyssor reagent (1,2,3) 2-methyl-N^1-benzenesulfonyl-N^4-bromoacetylquinonediimide [Scheme 1, *2*], for the attempted cleavage of a partially reduced monoclonal antibody. Partial reduction of an antibody generally breaks disulfide bridges in the hinge region of an antibody (4). This is a region often targeted by proteases for producing antibody fragments still capable of binding antigen. Since the Cyssor reagent is released from the fragment during cleavage [Scheme 1, *1* --> *6a* + *6b*], it was our intent to produce a new type of antibody fragment different from those produced by the classical pepsin (5) and papain (6) digestions. Instead of fragmentation, we observed cross-linking of the antibody by Cyssor reagent with both unreduced (control) as well as partially reduced (experimental) antibody.

MATERIALS AND METHODS

B6.2, a mouse anti human tumor monoclonal IgG1 (7,8), (hybridoma cells supplied by Dr. J. Schlom, N.C.I.) was purified by 50% $(NH_4)SO_4$ precipitation and cation exchange chromatography utilizing the Pharmacia FPLC system and a Mono S column (9). Purity was assessed on a Dupont GF250 gel permeation HPLC column and on 5-15% polyacrylamide discing SDS gels (10). Spectrophotometric measurements were used to quantitate antibody concentration using $\varepsilon = 1.89$ at 0.1% protein, 280 nm, based on amino acid analysis. The antibody, 1-3 mg/ml, in 0.1 M Na phosphate buffer, pH 8.0, 2 mM EDTA, was partially reduced by the addition of 0.05 M 2-mercaptoethanol followed by 45 min incubation at 37°C. Excess reductant was removed by G-25 Sephadex gel filtration on a 1 x 40 cm column loading 1-2 ml, collecting 1.5 ml/fraction.

ABBREVIATIONS

Cyssor, 2-methyl-N^1 benzenesulfonyl-N^4-bromoacetylquinonediimide; SDS, sodium dodecylsulfate.

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Sulfhydryl groups in the partially reduced antibody were quantitated with 2,2'-dithiodipyridine (Aldrithiol™-2 from Aldrich) using $\varepsilon = 7{,}600$ l/M at 343 nm (11,12).

The Cyssor reagent was purchased from the Sigma Chemical Company, lot 87C 0380, (structure confirmed by ^1H NMR in d_6Me_2SO) and dissolved (2.5 mg/ml) in Me_2SO (Pierce Chemical Company, Sequanal grade) prior to addition to the antibody.

Modification reactions were performed in 0.1 M Na phosphate buffer, pH 8.0, 2 mM EDTA at about 1.0 mg/ml antibody, using a 10 mol excess of Cyssor reagent, adding 10 µl/ml of antibody solution. Reactions were carried out overnight at 50°C.

Modified antibody samples were analyzed for inter- and intramolecular cross-linking under denaturing conditions on 5-15% polyacrylamide-SDS discing gels (10) under both reducing and nonreducing conditions using about 25 µg of protein per lane. The samples were boiled 3 min prior to electrophoresis.

Intermolecular cross-linking of antibody was determined for native protein by gel permeation on a Dupont GF250 HPLC column. The eluant was 0.2 M Na phosphate buffer, pH 7.0. Protein was monitored by absorption at 280 nm. The absorption peaks were quantitated with a Spectra-Physics 4200 integrator-recorder.

RESULTS

Antibody Partial Reduction

The reduction conditions for B6.2 monoclonal antibody generated about 10 mol of sulfhydryl/mol antibody.

Intermolecular Cross-Linking - Native Conditions

A gel permeation HPLC (native conditions) comparison of both unreduced (Fig. 1A) and partially reduced antibody (Fig. 1B) with the untreated control (Fig. 1C) indicated the presence of intermolecularly cross-linked antibody. Higher levels of interantibody cross-linking were evident for unreduced antibody (6.8%) than for the partially reduced antibody (2.5%). The Me_2SO treated control did not show even a trace of interantibody cross-linking. Clearly there was no evidence of cleavage of the antibody into Fc and either $F(ab')_2$ or Fab's. The large peak at 12 minutes in the chromatogram represents a contaminant present in the EDTA used in the reaction buffer. The tiny peak just beyond was seen only when Me_2SO was present.

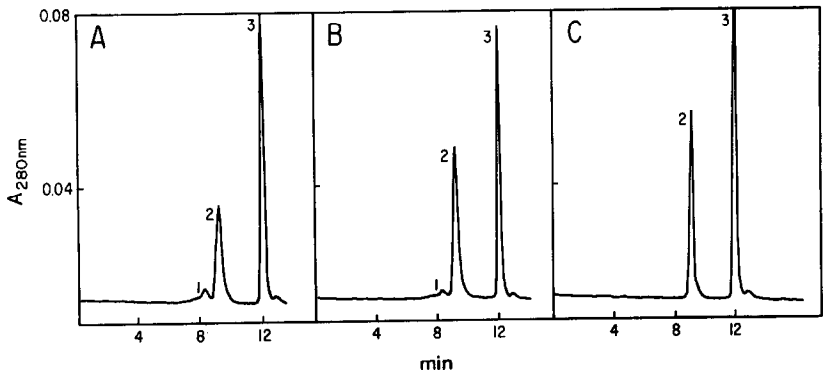
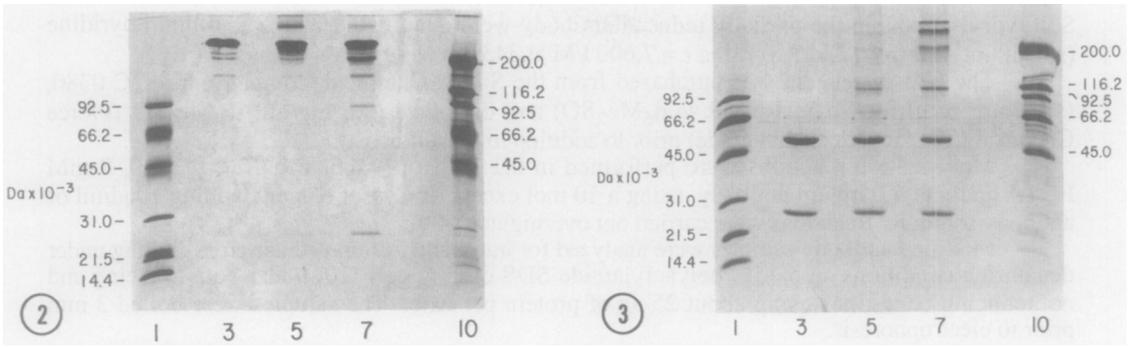


FIGURE 1. A - Size exclusion HPLC of unreduced antibody (10µg) with Me_2SO (5%) and Cyssor reagent at a 10 mol excess. Peak 1, antibody intermolecularly cross-linked; peak 2, monomeric antibody; peak 3, buffer contaminant. Other chromatography conditions as described in the Materials and Methods. B - Partially reduced antibody (10µg) with Me_2SO and a 10 mol excess of Cyssor reagent. C - Unreduced antibody control (10µg) with Me_2SO (5%) and no Cyssor reagent. No intermolecularly cross-linked antibody present.



FIGURES 2 and 3

SDS 5-15% polyacylamide gel electrophosesis done under non-reducing conditions; Figure 2, or reducing conditions; Figure 3. Lanes 1 and 10, low and high molecular mass standards which were supplied by Bio-Rad and consisted of lysozyme, 14,400 Da; soybean trypsin inhibitor, 21,500 Da; carbonic anhydrase, 31,000 Da; ovalbumin, 45,000 Da; bovine serum albumin, 66,200 Da; phosphorylase B, 92,500 Da; for low molecular mass and the latter three plus B-galactosidase, 116,250 Da; and myosin, 200,000 Da for high molecular mass; lane 3, unreduced antibody (25μg), treated with 5% Me_2SO, no Cyssor; lane 5, unreduced antibody (25μg) treated with 5% Me_2SO and a 10 mol excess of Cyssor; lane 7, partially reduced antibody (25μg), treated with 5% Me_2SO and a 10 mol excess of Cyssor. Sample preparation and electrophoresis conditions described in the Materials and Methods.

Inter and Intramolecular Cross-Linking Under Denaturing Conditions

SDS gel electrophoresis under nonreducing conditions did not reveal any evidence of interantibody cross-linking for antibody treated with Me_2SO only (Fig. 2, Lane 3). Interantibody cross-linking was, however, clearly evident for the unreduced antibody treated with the Cyssor reagent, (Fig. 2 Lane 5). Somewhat less interantibody cross-linking was evident for the partially reduced antibody treated with the Cyssor reagent (Fig. 2, Lane 7).

The lower molecular mass bands observed in Fig. 2, Lane 7, were attributed to the partial reduction of the antibody rather than Cyssor induced cleavage. Had such cleavage occurred it should have been evident also on the sample electrophoresed under reducing conditions (Fig. 3, Lane 7) and clearly was not.

SDS-polyacrylamide gel electrophoresis under reducing conditions did not reveal any evidence of intraantibody or interantibody cross-linking for the unreduced antibody treated with Me_2SO only (Fig. 3, Lane 3). Intramolecular cross-linking and traces of intermolecular cross-linking were evident for unreduced antibody treated with the Cyssor reagent (Fig. 3, Lane 5). Finally, partially reduced antibody treated with Cyssor reagent clearly showed evidence of significant interantibody as well as intrantibody cross-linking.(Fig. 3, Lane 7).

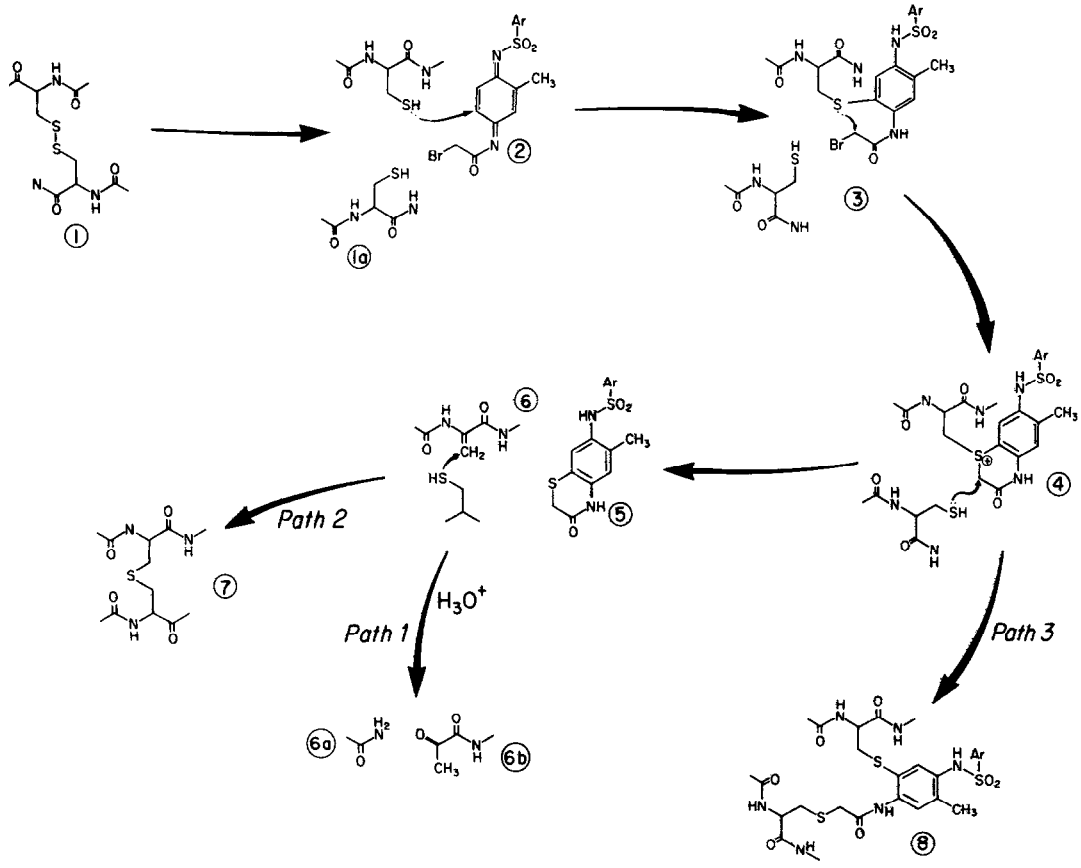
Other than the expected heavy and light chain protein bands of 50,000 and 25,000 Da, no other bands of less than 50,000 Da were evident . Cyssor induced cleavage did not appear to have occurred.

Ovalbumin, which has free sulfhydryl groups, was cross-linked by the Cyssor reagent under the conditions described in the Materials and Methods. A diffuse stained band at about 90,000 Da was seen on SDS gels (data not shown). Also, ovalbumin was readily cleaved by the same lot of Cyssor reagent under acidic conditions after 80°C incubation as described in (1).

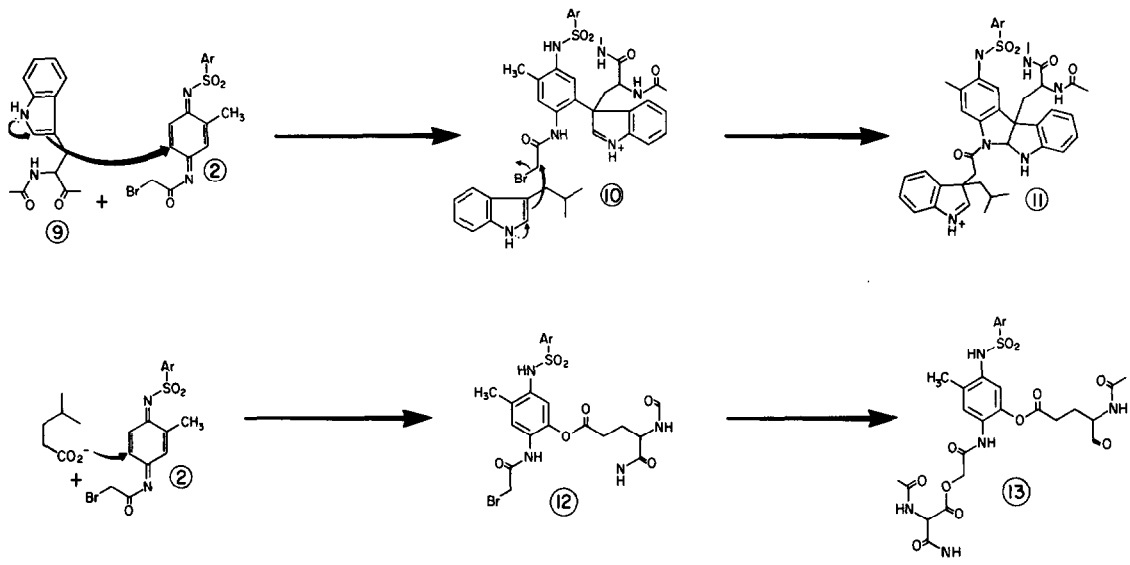
DISCUSSION

Partial reduction of a mouse monoclonal IgG has been shown to reduce preferentially the disulfide bridges in the hinge region making them available for chemical modification by sulfhydryl modifying reagents (6, 13). Our objective was to see if the cysteine sites afforded by the reduction of the cystine S-S bonds could be used as cleavage points on the antibody structure [Scheme 1, *3* --> *7* or *3* --> *6a* + *6b*]. Since the Cyssor reagent is a bis alkylating agent it is not surprising to find recross-linking of the reduced cystines rather than cleavage because of their close proximity [Scheme 1, *3* --> *7* or *3* --> *8*]. The nature of the cross-linking interaction remains obscure however, since there is little change in the amino acid composition of the antibody (data not shown).

Dehydroalanine residues [Scheme 1, *6*], intermediates in the fragmentation process, can be formed from interaction of the Cyssor reagent with cysteine and this dehydroalanine residue can interact subsequently with another cysteine to form lanthionine cross-links [Scheme 1, *6* --> *7*]. Alternately, intermediate 4 can be intercepted by a cysteine residue to yield Cyssor cross-linked cysteines [Scheme 1, *3* --> *4* --> *8*]. Thus, on amino acid analysis we anticipated either the formation of carboxymethylcysteine (from *8*), lanthionine (from *7*) or at least the loss of cysteine



SCHEME 1



SCHEME 2

residues (from *6*) in the amino acid analysis. None of these were observed. Appparently there is sufficient interaction of the Cyssor reagent with other functions of the antibody chains to preclude these paths. Suggested alternatives are that the cross-linking is taking place between residues for which amino acid analysis is not sensitive or that the conditions of hydrolysis release the linker from the attachment sites (or both). Residues which are likely candidates are tryptophan or aspartic and glutamic acids. Tryptophan is known to interact with the Cyssor reagent through quinoneimine addition to the indole ß position [Scheme 2, *9* --> *10* --> *11*] and alkylation of the indole residue with the bromoacetyl function is also known. Alternatively, aspartic and glutamic residues can act as nucleophiles for additions to the quinoneimine portion of the reagent to give phenol esters and carboxyl groups can as well displace the bromine of the bromoacetyl residue [Scheme 2, *12* --> *13* --> *14*]. The ester links would hydrolyze easily under the acidic digestion of the protein yet would still give cross-links stable to reduction. Such cross-linking interactions seem dubious in a competition against SH groups but specific domain associations of the Cyssor reagent may enhance this chemistry.

While we report on Cyssor cross-linking at pH 8.0 in this study, we have observed cross-linking activity from pH 4.0-8.0 with the levels dropping with the pH for both unreduced and partially reduced antibody. Me_2SO induced precipitation of the antibody precluded HPLC analyses of the pH 4.0 samples. No Cyssor induced antibody cleavage was observed under any of the experimental conditions described in this study. The cross-linking activity of the Cyssor reagent at neutral pH under mild conditions gives protein chemists another path for cross-linking.

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