TECHNICAL NOTE

Simplified Method for Conjugating Macrocyclic Bifunctional Chelating Agents to Antibodies via 2-Iminothiolane

Michael J. McCall, Habibe Diril, and Claude F. Meares*

Chemistry Department, University of California, Davis, California 95616. Received February 8, 1990; Revised Manuscript Received March 13, 1990

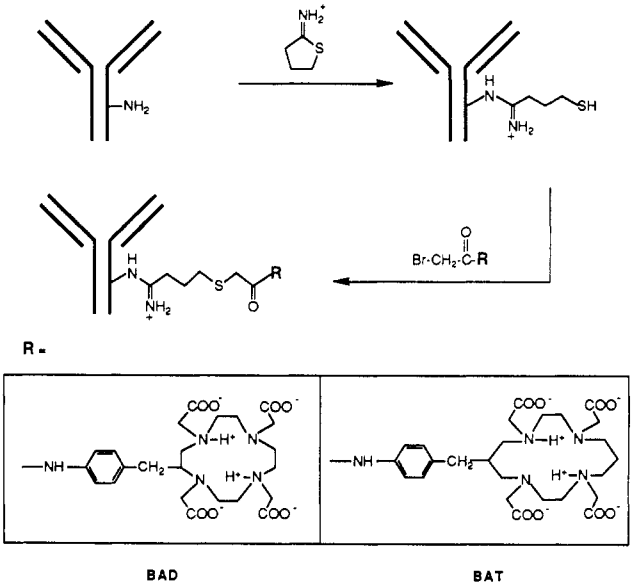
A one-step method for conjugating macrocyclic chelators to antibodies using the protein modification reagent 2-iminothiolane controls aggregation, maintains immunoreactivity, and produces consistent chelate/antibody ratios. Conjugation conditions have been investigated with the macrocyclic chelates 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid and 2-[*p*-(bromoacetamido)benzyl]-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid, with three different monoclonal antibodies. The bifunctional chelating agents are prepared by bromoacetylation of their amine precursors using a two-phase $H_2O/CHCl_3$ system, which improves product purity.

The attachment of metal ions to monoclonal antibodies (mAbs)[1] for medical applications demands extreme stability under physiological conditions, with no significant release of metal (*1–4*). Development of antibody–macrocyclic chelate conjugates for tumor localization and therapy has led to the following improvements.

In our earliest study of the conjugate of 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (BAT) with the mouse mAb Lym-1 it was observed that, rather than linking the macrocyclic bifunctional chelating agent directly to the antibody, it was necessary to employ a spacer group between the two moieties (*5*). Without this spacer, no practical uptake of radiometal (copper or cobalt) was observed when radiolabeling was attempted after conjugation. For use with short-lived radionuclides, postconjugation radiolabeling is an important feature. As shown in Scheme I, linkage was accomplished in a two-step, overnight procedure using Traut's reagent (*6*) 2-iminothiolane (2IT), which reacts with amino groups to produce mercaptobutyrimidyl groups, followed by alkylation of the mercapto sulfur with BAT. For Lym-1, this method led to variable amounts of protein aggregation via cross-linking. The degree of aggregation was difficult to control, ranging from 10% to >50%. Also, the use of ≈0.4 M 2-mercaptoethanol in the Traut procedure (to prevent oxidation of the mercaptobutyrimidyl groups, which could form disulfide cross-links) could possibly reduce antibody disulfide bonds, and it was necessary to remove the 2-mercaptoethanol prior to the addition of BAT.

2-Iminothiolane has also been used as the cross-linking reagent in the synthesis of antibody–toxin conjugates. Since the disulfide bond between the antibody and toxin proved to be unstable in vivo ($t_{1/2}$ = 6–8 h) (*7*), Carroll et al. (*8*) studied substituted 2IT's with a view to increasing the disulfide bond stability. In the latter study, the nascent mercaptobutyrimidyl groups reacted with the activated disulfide 5,5′-dithiobis(2-nitrobenzoic acid) to form a mixed disulfide. This work called our attention to the possibility that, since under mildly alkaline conditions bromoacetamide reagents react rapidly with sulfhydryl groups but only slowly with amino groups, the antibody, BAT, and 2IT solutions could be combined in a single reaction mixture. The method has been explored with three mAbs, Lym-1, 155H.7, and chimeric L6, and with either BAT or 2-[*p*-(bromoacetamido)benzyl]-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (BAD) as the chelator.

Scheme I



[1]Abbreviations used are as follows: mAb, monoclonal antibody; BAT, 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; BAD, 2-[*p*-(bromoacetamido)benzyl]-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; 2IT, 2-iminothiolane; NO_2Bn, *p*-nitrobenzyl; NH_2Bn, *p*-aminobenzyl; DOTA, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; TETA, 1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid.

EXPERIMENTAL PROCEDURES

Reagents. Lym-1, an anti B cell lymphoma IgG_{2a} mAb (*9*), was obtained from Damon Biotech (Needham Heights,

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MA 02194; Encapcel murine mAb, lot # 3-171-860818). It was further purified by protein A affinity column chromatography prior to use. Chimeric (where the immunoglobulin constant domains, $C_{\gamma 2a}$ and C_κ, of the mouse mAb have been replaced by human $C_{\gamma 1}$ and C_κ domains) L6, a mAb against a carbohydrate antigen found at the surface of cells from human carcinomas of the lung, breast, colon, and ovary (*10*) (lot 88/42E), was obtained from Dr. I. Hellstrom, Oncogen, Seattle, WA. 155H.7, a murine antibody (IgG_{2a}) raised against a synthetic β-anomer of the Thomson–Friedenreich antigen (*11*) (lot 250588), was obtained from Dr. A. Noujaim, University of Alberta, Canada. Protein A on Sepharose-CL-4B and 2IT were purchased from Sigma Chemical Co. Cobalt-57 chloride was purchased from ICN (specific activity 7000 Ci/g). [1-^{14}C]Bromoacetic acid was purchased from Amersham (specific activity 0.383 Ci/g). Pure water (resistance 18 MΩ, NANOpure II, Barnstead, MA) was employed throughout. All glass labware was washed with a mixed acid solution and thoroughly rinsed with deionized, distilled water (*12*). All plastic labware was washed with 3 M HCl and thoroughly rinsed with deionized, distilled water. All other reagents were the purest commercially available.

Thin-Layer Chromatography. TLC was run on plastic-backed silica gel plates (EM Science) using a 10% (w/v) ammonium acetate/methanol (1:1 v/v) solution as the eluent. In this system, unchelated cobalt and conjugate remain at the origin while free chelates migrate to R_f 0.5–0.6.

High-Performance Liquid Chromatography. Gel-filtration HPLC of the immunoconjugates was performed at room temperature with Spherogel G3000SW (Altex). Protein molecular weight markers (Bio-Rad) were used to calibrate the column. The eluent was 0.1 M sodium phosphate buffer, pH 7.0, containing 0.025% NaN_3 by weight. The flow rate was 0.5 mL/min. The UV-absorbing fractions were detected at 280 nm.

Reversed-phase HPLC of BAT and BAD was performed at room temperature with a 10 × 250 mm C_{18} column (Alltech). A 20-min linear gradient, from 0.1 M sodium acetate, pH 7 (containing 1 mM EDTA), to 100% methanol, was used at a flow rate of 3.0 mL/min. The UV-absorbing fractions were detected at 254 nm.

Glycineamido-Bn-DOTA was purified by reversed-phase HPLC using a 21.4 × 250 mm C_{18} column (Dynamax). A 20-min linear gradient from 0.1 M ammonium acetate, pH 6, to 100% methanol was used at a flow rate of 12.5 mL/min, detected at 254 nm.

Ultraviolet Spectrophotometry. Optical density measurements at 280 nm were made on a Gilford Model 250 spectrophotometer using a 1 cm path length microcell. Antibody concentrations were determined with $E^{1\%}_{280}$ = 13.5 (*13*) and a molecular weight of 155K. Optical densities were measured on dilutions suitable to give absorbance readings of 0.1–1.0.

Radiation Counting. Gamma counting was done in a Beckman Model 310 counter with the appropriate energy windows set for ^{57}Co. TLC plates containing radiolabeled materials were visualized with an AMBIS Radioanalytic Imaging System. Beta counting was done in Aquasol, using a Beckman LS 6800 liquid-scintillation counter with the energy windows set for ^{14}C.

Macrocycles. 6-(*p*-Nitrobenzyl)-1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (NO_2Bn-TETA) and 2-(*p*-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (NO_2Bn-DOTA) were prepared according to the method of Moi et al. (*2*, *5*). Reduction of each to the respective *p*-amino compound was accomplished in the following manner. NO_2Bn-DOTA (120 mg, 0.22 mmol) was dissolved in 30 mL of water and the pH was adjusted to 11.5 with 2 M sodium hydroxide. Ten percent palladium on carbon catalyst (25 mg) was added, and the reaction vessel was attached to an atmospheric-pressure hydrogenation apparatus, cooled in an ice bath, purged with N_2, and filled with H_2. The course of the reaction was monitored by observing the uptake of H_2 and noting the presence of a transient green color in the reaction mixture. When H_2 uptake had ceased (3–5 h), the reaction mixture was removed from the hydrogenation apparatus, the pH was adjusted to 6.8 with 1 M HCl, and the catalyst was removed by filtration through a double layer of Millipore GS membrane (0.22 μm). The filtrate, which was positive for amino groups by a fluorescamine test (*14*), was lyophilized. Proton NMR showed a shift of the aromatic aa′bb′ pattern from 7.5 and 8.2 ppm for NO_2Bn-DOTA to 6.8 and 7.1 ppm for NH_2Bn-DOTA. FAB mass spectrum: M + 1 peak at *m*/*e* 510 and M + Na peak at *m*/*e* 532 for the product, as expected. NO_2Bn-TETA was reduced in the same manner and characterized by a fluorescamine test; the same shift of the aromatic proton NMR as seen in the DOTA analogue and the FAB mass spectrum M + 1 peak of *m*/*e* 538 were as expected. NO_2Bn-TETA was reduced in the same manner and characterized by a fluorescamine test; the same shift of the aromatic proton NMR as seen in the DOTA analogue and the FAB mass spectrum M + 1 peak of *m*/*e* 538 were as expected. Each lyophilized residue was further tested for metal binding capacity by a ^{57}Co assay (*15*).

Conversion of the *p*-amino compounds to the *p*-bromoacetamido macrocycles BAT and BAD used a modification of Mukkala's method (*16*) rather than the one-solvent system used previously (*15*). The lyophilization residue containing NH_2Bn-TETA (ca. 0.22 mmol) was dissolved in 8.6 mL of water, and 231 μL (1.33 mmol) of *N*,*N*-diisopropylethylamine was added to adjust the pH to 8. Bromoacetyl bromide (159 μL, 1.82 mmol) was dissolved in 8.6 mL of $CHCl_3$. The chloroform and aqueous solutions were mixed and stirred vigorously for 10 min. A fluorescamine test on the aqueous layer indicated that amine groups were still present. The pH of the aqueous layer was adjusted to 8 with *N*,*N*-diisopropylethylamine (1.1 mL in 100-μL increments) and bromoacetyl bromide (two 100-μL portions) was added until the aqueous layer no longer tested positive for primary amine groups. A 2-mL portion of water was added, the chloroform layer was removed, and the aqueous layer was extracted with $CHCl_3$ (8 × 5 mL). The aqueous layer was acidified to pH 1 with 1 M HCl and again extracted with $CHCl_3$ until a test for alkylating groups using 4-(*p*-nitrobenzyl)pyridine (*17*) showed the absence of bromoacetic acid in the chloroform extracts. Following extraction, the aqueous solution's pH was adjusted to 6.4, and it was frozen in liquid N_2 and stored at −80 °C. FAB mass spectroscopy of the BAT solution gave the two expected M + 1 peaks of the product at 658 and 660 mass units, typical of a bromine (^{79}Br and ^{81}Br) containing compound.

The macrocyclic chelate BAD was synthesized from NH_2Bn-DOTA by the same procedure. FAB mass spectroscopy results for BAD were unsatisfactory due to interference by matrix peaks.

In order to characterize BAD, an aqueous solution of BAD was treated with NH_3(g) to form the stable amino derivative glycineamido-Bn-DOTA. The derivative was purified by C_{18} HPLC (see above) and lyophilized. It was positive for amino groups by fluorescamine test and

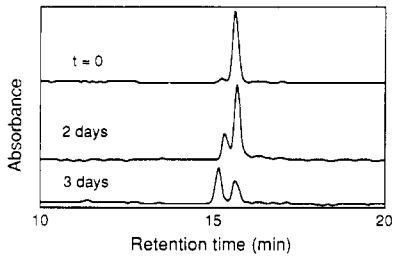

Figure 1. Reversed-phase HPLC of the macrocyclic chelate 2-[*p*-(bromoacetamido)benzyl]-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (BAD), showing decomposition in aqueous solution at pH 7.6, room temperature (20–22 °C).

negative for alkyl bromide with 4-(*p*-nitrobenzyl)pyridine. FAB mass spectroscopy showed the expected M + 1 peak at *m/e* 567. ^{1}H NMR (D_2O, pH 4.1, 300 MHz): 7.2–7.4 ppm (4 H, aromatic aa′bb′), 3.9 ppm (2 H, s, $NH_2CH_2C{=}O$), 2.6–3.8 ppm (27 H, broad multiplet, expected 25 H).

HPLC analysis of BAD indicated a major peak at retention time 15.8 min and several minor peaks (total less than 10%). Upon lyophilization one of the minor peaks (retention time 15.2 min) increased, whereas the major peak decreased. Similarly the 15.2 min peak grew gradually when the sample was allowed to stand over a period of time (Figure 1). Both peaks tested positive for alkyl bromide with 4-(*p*-nitrobenzyl)pyridine, and both chelated ^{57}Co. To prevent this conversion, the sample should be frozen in liquid N_2 and stored at −80 °C. BAT behaved similarly.

[^{14}C]BAD was synthesized from NH_2Bn-DOTA by the procedures above except that [1-^{14}C]bromoacetyl chloride was substituted for bromoacetyl bromide used in the earlier preparation. The [1-^{14}C]bromoacetyl chloride was prepared by adding 1 mmol of bromoacetyl chloride to 2.00 mL of $CHCl_3$ containing approximately 246 μCi of [1-^{14}C]bromoacetic acid and allowing an exchange reaction to occur (12 days at room temperature in the dark) to form [1-^{14}C]bromoacetyl chloride. Aliquots of this compound were allowed to react with NH_2Bn-DOTA as above to give the product [^{14}C]BAD. HPLC analysis of the product also gave a major and minor peak as with the unlabeled compound; the separation was too small for efficient isolation. The [^{14}C]BAD stock solution was standardized by ^{57}Co assay.

Lym-1–2IT–BAT Conjugation. The Lym-1 antibody solution (15–20 mg/mL) was prepared for conjugation with a centrifuged gel-filtration column (*15*, *18*) with 0.1 M tetramethylammonium phosphate, pH 8, as the column buffer. To the collected effluent was added (in order) excess BAT in aqueous solution and freshly prepared 2IT in 50 mM triethanolamine hydrochloride, pH 8.7 (final approximate concentrations: Lym-1, 0.1 mM; BAT, 2 mM; 2IT, 1 mM). The pH of the solution was adjusted to 7.8 and the solution was incubated at 37 °C for 30 min. Excess BAT and 2IT were removed and the conjugate was placed in a 0.1 M tetramethylammonium acetate solution, pH 7, with a centrifuged gel-filtration column.

Antibody–2IT–BAD Conjugation. Conjugations using the macrocycle BAD were done as above, with the following exception. Following the 30-min incubation at 37 °C, 2-mercaptoethanol (1% v/v aqueous solution) was added in sufficient quantity to bring its final concentration to approximately 2 mM, and the solution was incubated at 37 °C for 10 min more to reduce the level of any alkylated methionine adduct side products. The excess 2-mercaptoethanol was removed in the final centrifuged gel-filtration column. Further experiments have shown that this 2-mercaptoethanol incubation was not necessary; the amount of readily dealkylated side products is quite small (see the Discussion Section).

Scheme II

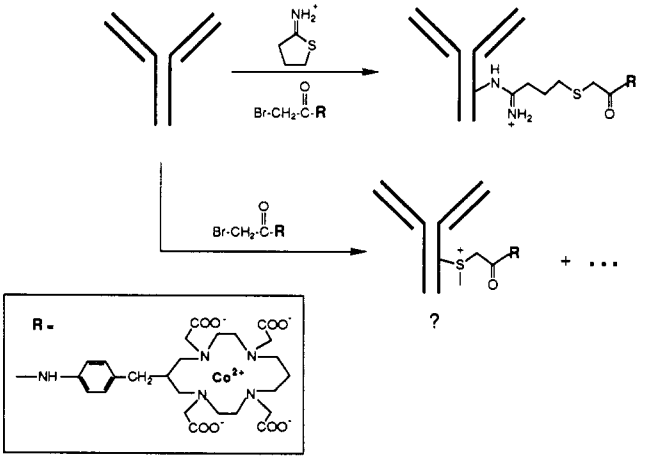

Table I. Lym-1–2IT–BAT Conjugation Reactions[a]

prep	chelates/mAb	% aggregates	% immunoreactivity
1	1.33	8.7	97
2	1.48	8.5	104
3	1.88	10	98
4	1.13	10	ND
avg ± SD	1.45 ± 0.32		

[a] Conditions: pH 7.8, 30 min, 37 °C, 2 mM BAT, 1 mM 2IT, 0.1 mM Lym-1.

Table II. Antibody–2IT–BAD Conjugation Reactions[a]

antibody	chelates/mAb	% aggregates	% immunoreactivity	pH
155H.7	2.4	6.4	86.7	7.0
	4.5	7.8	56.7	8.0
	2.3	3.6	46.7	9.0
chimer L6	2.4	2.5	96	7.0
	4.2	3.8	96.5	8.0
	2.7	1.8	90.6	9.0
Lym-1	2.1	10	86.4	7.0
	3.6	4.2	88.5	8.0
	2.2	2.9	101.4	9.0

[a] Conditions as in Table I, except for pH.

Parallel Lym-1–2IT–BAT–^{57}Co and Lym-1–BAT–^{57}Co Conjugations (Scheme II). These were performed at pH 8 by the above procedures on two samples of Lym-1, with BAT which had been radiolabeled with ^{57}Co prior to conjugation (21.8 μCi ^{57}Co/mol BAT). One solution contained 2IT while the other did not. Relative concentrations of Lym-1 and BAT were maintained by adding the appropriate amount of buffer (50 mM triethanolamine hydrochloride) to the second conjugation solution. Relative chelate to antibody ratios were determined from UV absorbances and radioactivities of the conjugate products.

Immunoreactivity Assay. Solid-phase radioimmunoassays for immunoreactivity on either ^{111}In- or ^{57}Co-labeled conjugates were done as reported previously (*19*) using ^{125}I-labeled antibody as the standard. Immunoreactivity values given in Tables I and II are relative to ^{125}I-labeled antibody.

RESULTS

Table I shows the results of four Lym-1–2IT–BAT conjugations using the new procedure, while Figure 2, parts

A and B, show typical "old and new" HPLC traces of the conjugate solutions with the decrease in aggregation clearly evident. The new method gives usable, consistent (±20%) chelate/antibody ratios and consistently low aggregation compared to the earlier method, while high immunoreactivity is maintained.

Exploring the scope of the method using a different macrocyclic chelate (BAD vs BAT), three different antibodies, and three different pH's gave the results shown in Table II. To test for reproducibility, five or six conjugations (7–10 mg each) of each of the three antibodies with BAD were done at the pH affording the best immunoreactivity. Chelate/antibody ratios and degree of aggregation were consistent and comparable to the values in Table II.

The results of three Lym-1–2IT–[^{14}C]BAD conjugations at pH 8.0 gave 5.6 ± 0.4 available chelates per antibody by ^{14}C analysis. However, ^{57}Co metal binding assay revealed that only 3.6 ± 0.2 chelates per antibody were still capable of radiolabel uptake, in excellent agreement with the corresponding data point in Table II.

For the [^{57}Co]BAT experiments, TLC analyses of the product conjugates showed direct attachment of [^{57}Co]BAT to Lym-1 (yielding Lym-1–BAT–^{57}Co) had occurred to a small but measurable extent (0.2 chelates/antibody) compared to indirect attachment (Lym-1–2IT–BAT–^{57}Co, 4.1 chelates/antibody). Challenging each conjugate with 2-mercaptoethanol (final concentration 10 mM) and incubating at 37 °C for 0.5 h showed by TLC that the directly alkylated Lym-1–BAT–^{57}Co conjugate was unstable, losing 30–40% of its radiolabel. There was only a minor loss of radiolabel (<2.5%) for Lym-1–2IT–BAT–^{57}Co under the same conditions, with no change on further incubation up to 1.5 h.

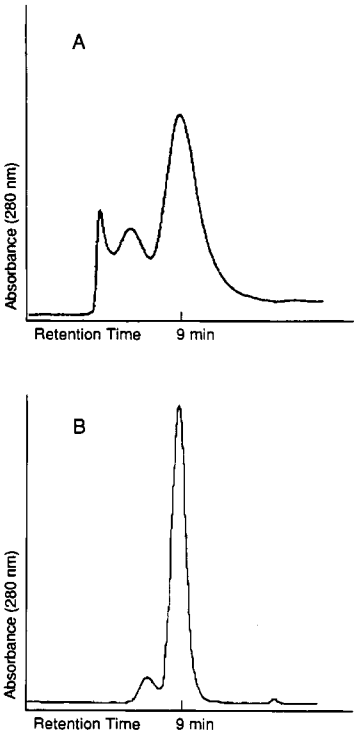

Figure 2. Gel filtration HPLC of Lym-1–2IT–BAT conjugates prepared (A) by the former method (*1*) and (B) by the one-step method. Here the immunoconjugate peak appears at a retention time of approximately 9 min, the peak preceding it contains the antibody dimer, and the peak preceding that in A consists of higher aggregates. The sample in A illustrates the variability of the former method, which could also yield results comparable to those of B.

DISCUSSION

As shown in Scheme I, the 2IT reacted with the antibody to form mercaptobutyrimidyl groups, which were then alkylated by BAT. By keeping the BAT concentration high with respect to the nascent thiol groups, alkylation by BAT became the preferred reaction pathway rather than thiol oxidation to disulfides. Thus, the need for 2-mercaptoethanol to prevent oxidation was eliminated, and antibody aggregation was kept to a minimum. As shown in Table I, this single-step method takes less than 1 h to complete and gives consistent yields with relatively little aggregation of Lym-1. For unknown reasons, aggregate formation with Lym-1 is greater than with the other antibodies under similar conditions (Table II). The chelate/antibody ratios are similar for each antibody, but depend on the reaction pH, with the maximum yield occurring around pH 8 in each case. The degree of Lym-1 aggregation in each case is lower than that encountered with the previous two-step method (consistently ≤10% versus variable 10–50+%) and is lowest at pH 9. Changes in immunoreactivity with reaction pH are antibody dependent, with the best 155H.7 immunoreactivity seen with the pH 7 conjugate and the best Lym-1 immunoreactivity seen with the pH 9 conjugate. For chimeric L6, changing the reaction pH has only a minor effect on immunoreactivity.

To estimate the degree of direct attachment of BAT to Lym-1 and the efficiency of uptake of radiolabel by an attached versus a free chelate, a set of parallel Lym-1 + [^{57}Co]BAT experiments was conducted (Scheme II). One reaction contained 2IT and the other did not. No drastic differences in the rates of reaction were expected for [^{57}Co]BAT versus BAT since the location of change in the chelate's structure is distant from the point of attachment to the antibody and since the binding of Co^{2+} is accompanied by the loss of $2H^+$ (*20*).

As haloacetamides, BAT and BAD can attach to amine, sulfhydryl, imidazole, or thioether groups on amino acid side chains, with the sulfhydryl group being the most reactive (*21*). The relatively low pH of conjugation and the observed instability of the product Lym-1–BAT–^{57}Co led to the suggestion that, in the absence of 2IT, side chains of methionines were alkylated by [^{57}Co]BAT to form sulfonium adducts, which would be susceptible to hydrolysis (thus releasing the chelate). In the case of Lym-1–2IT–BAT–^{57}Co, the thioether linkage proved to be stable under the conditions employed. It is possible that a small number of methionine residues were alkylated during the preparation of Lym-1–2IT–BAT–^{57}Co, and the minor loss of radiolabel may reflect the loss of these chelates by hydrolysis.

The differences in the chelate/antibody ratios of the Lym-1–2IT–[^{14}C]BAD (total 5.6 chelates/mAb versus 3.6 chelates/mAb available to bind metal) suggest that even the 2IT linker does not make all antibody-bound chelating groups accessible for postconjugation radiolabeling. This was not unexpected, since previous work with backbone-substituted [^{14}C]EDTA's has shown that not all attached EDTA groups may be available for metal binding (*22*). The differences in the chelate/antibody ratios of the Lym-1–2IT–BAT seen in the parallel reactions (preradiolabeling, 4.1 chelates/mAb) versus the standard procedure (postradiolabeling, 1.45 chelates/mAb) are generally consistent with the [^{14}C]BAD results. While the observed differences might be caused by metal contamination during processing, such was not the case in ref 22, and we feel that steric hindrance of the protein-bound chelate is a more likely cause.

Finally, comparison of Table II values for Lym-1 + BAD at pH 8.0 with those of Table I for Lym-1 + BAT at pH 7.8 shows differences in chelate/antibody ratios. One possible reason for this may be that the attached BAD is more efficient in taking up the ^{57}Co used in the metal binding assay to determine chelate/antibody ratios. Our experience with the two macrocyclic chelates indicates that BAD is a good ligand for a variety of metals, while BAT appears to be limited to Cu and Co. The use of other linkers may improve accessibility of the bound macrocycles.

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