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THE MONOTHIOACETAL GROUP, A NEW CLEAVABLE CENTER FOR CROSS-LINKING REAGENTS

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Summary

A protein cross-linking reagent which contains a monothioacetal moiety is described. Cross-links generated using this reagent may be specifically cleaved by dilute mercuric ion at neutral pH.

Introduction

Recently, a number of bifunctional protein reagents which may be cleaved under specific conditions have been described (1). Most of these employ either a reducible disulfide residue (e.g. 2,3,4) or a periodate-sensitive vic-glycol group (5,6,7,8) as the breakable center. The use of these reagents coupled with two-dimensional electrophoretic analysis has simplified investigations into structural relationships of complex protein systems.

However, both the disulfide and glycol centers have certain disadvantages associated with their use (1). For example, in theory the disulfide group of a bridging reagent could undergo interchange with some errant thiol group in the sample, thereby creating new and possibly misleading cross-linked products. In addition, since intrachain disulfide bonds are not broken prior to first-dimensional electrophoretic separation, identification, in the second dimension, of the polypeptides of cross-linked complexes is sometimes very difficult.

The periodate treatment needed to break the bridge of glycolic centers will also remove sugar residues from glycoproteins, and, additionally, would not always be suitable during cross-linking investigations of protein-polynucleotide associations. Further, bridges in membrane protein systems may be refractory to periodate cleavage (9).

As an alternative, we have used a third type of cleavable moiety, the monothioacetal group, in a cross-linking reagent. This center is stable to

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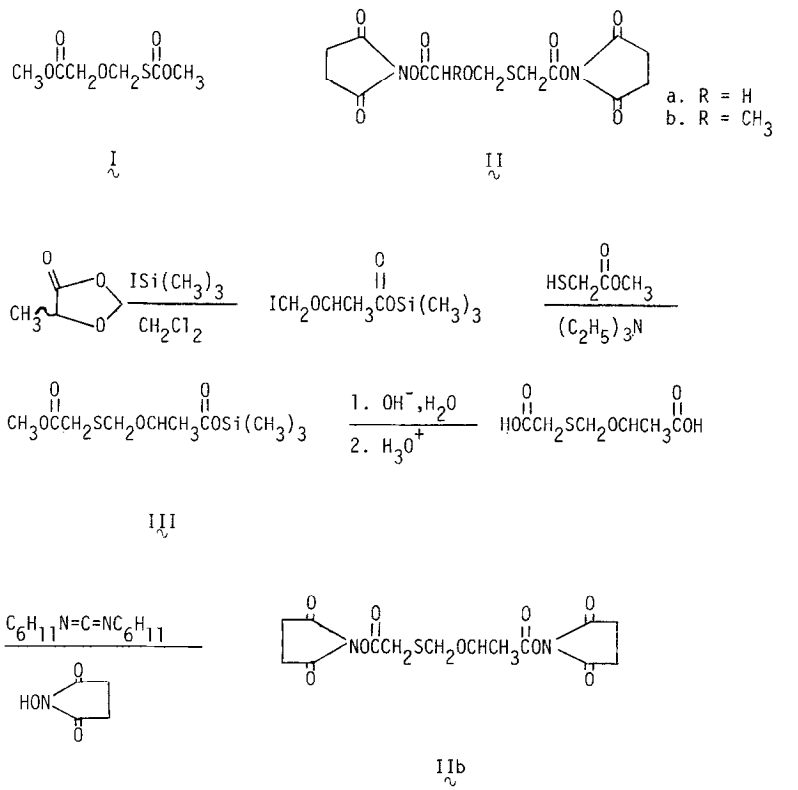

Figure 1. Synthetic Scheme

all except the extremes of acidity and basicity, and its hydrolysis is not catalyzed by ions normally found in biochemical preparations. However, monothioacetals are labile to aqueous mercuric chloride under mild neutral conditions (10). As an example, the diester I, originally made by Brehm and Levenson (11), when saponified and activated as the dissucinimidyl ester IIa, should be useful as a symmetrically bifunctional cross-linking agent bridging a maximum distance of 9 Å.

For ease of synthesis, we employed a more facile route to IIb than that employed by Brehm and Levenson to form the diester I. This scheme, which utilizes ester cleavage with iodotrimethylsilane (12), is shown in Figure 1.

This synthetic scheme generates a very useful diester intermediate, III, which has a methyl and a trimethylsilyl ester moiety at each end of the mole-

cule. Since these esters may be differentially converted to the respective acid residues, this intermediate provides a route to asymmetric cross-linking reagents with differing reactivity at each end of the molecule while retaining the mercury cleavable moiety.

Materials and Methods

A. Synthesis of IIb. A solution of 1.0 g (.01 mole) of dry, crude 5-methyl-1,3-dioxolan-4-one (13) in 10 ml dichloromethane was treated with 2.0 g (.01 mole) of iodotrimethylsilane. After one hour at room temperature, the mixture was cooled to 0°, and a mixture of 1.1 g (.01 mole) of methyl mercaptoacetate and 1.1 g (.01 mole) of triethylamine was added. The reaction was allowed to proceed for an additional hour, and then the solvent was removed in vacuo. The residue was shaken with .1 M Na_2CO_3 solution. The pH was maintained at a value of 11 by addition of 1 M NaOH solution. The resulting mixture was washed with several portions of ether. An additional portion of ether was added, the aqueous layer was made saturated in Na_2SO_4, and cold 1 M H_2SO_4 was added with stirring until the pH was at a value of 2.2. The ether layer was dried over Na_2SO_4 and stripped of solvent in vacuo. The residue was recrystallized from dichloromethane/ether to yield 602 mg (31%) of white crystals M.P. 93-95.NMR (acetone-d_6): δ 4.86, AB, 2H, C-OCH_2S; δ 4.37, q, J = 7Hz 1H, $CHCH_3$; δ 3.39, s, 2H, -C-CH_2-S-; δ 1.38, d, J = 7Hz, CH_3. This diacid was converted to the disuccinimidyl ester by dicyclohexyl carbodiimide condensation in tetrahydrofuran (7,14). NMR ($CDCl_3$): δ 2.81, s, 8H; δ 1.59, d, J = 7Hz, 3H.

Crosslinking. A 2 mg portion of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase in 1 ml of .01 M sodium phosphate pH 7.1 was diluted with 1 ml of .2 M triethanolamine hydrochloride pH 8.5. This solution was treated at room temperature with .75 mg IIb in 10 µl dimethylsulfoxide.

Cleavage in solution. The reaction mixture was dialyzed against .1 M Tris-HCl pH 7.0 and concentrated to half volume by dialysis against solid sucrose. This solution was diluted by an equal volume of 20% urea, 2% SDS*, and 2% β-mercaptoethanol and was heated slowly to 60°. The temperature was maintained for 30 minutes, and then the sample was dialyzed against .1 M sodium acetate - .1% SDS, pH 7.0. The mixture was dialyzed against another wash which also contained .03 M $HgCl_2$ for 30 minutes. The external buffer was then changed to .05 M Tris-HCl, pH 6.8 - .1% SDS and then to fresh buffer containing .01 M β-mercaptoethanol. The extent of cleavage was determined by electrophoresis on 4% 28:1 polyacrylamide gels using .1% SDS, and .2 M Tris-acetate pH 6.4 (Coomassie Blue staining).

Two Dimensional Electrophoresis. Following concentration and dissociation, the mixture was separated on a 5 mm 4% 28:1 polyacrylamide tubular gel, using .1% SDS - .2 M Tris-acetate pH 6.4 as buffer. The tube gel was soaked for 30 minutes in successive 25 ml washes, at pH 6.8, of .2 M sodium acetate - 1% SDS, .05 M sodium acetate - .01 M $HgCl_2$ - .1% SDS, .05 M Tris-HCl-1% SDS containing beads of BioRad "Chelex 100", and the same buffer containing .05 M β-mercaptoethanol. The gel was then embedded, using 1% buffered agarose, at the top of a 1 mm 7.5% slab gel made with the SDS stacking system of Laemmli (15). The protein was visualized using Coomassie Blue stain, and then the gel was dried.

*abbreviation; SDS - sodium dodecylsulfate

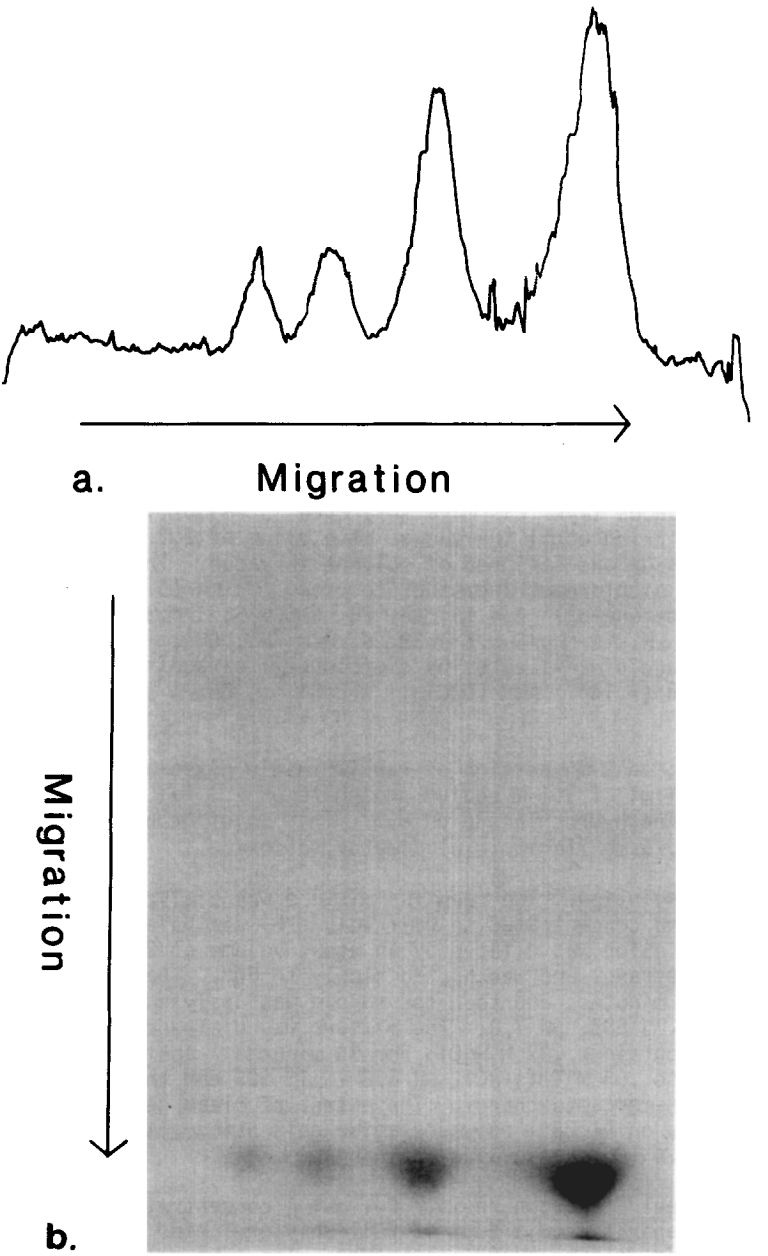

Figure 2 a. Glyceraldehyde-3-phosphate dehydrogenase cross-linked with IIb and separated on a 4% 5 mm polyacrylamide-sodium dodecylsulfate gel. [Densitometric trace (560 nm).]

b. Pattern on a 1 mm thick 7.5% polyacrylamide-sodium dodecyl sulfate slab after a gel identical to a was treated with mercuric ion and imbedded at the top of the slab.

Discussion

When rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, a tetrameric protein, was treated with IIb, the expected four band cross-linked pattern (16) was observed upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These bands correspond in apparent molecular weight to the chemically cross-linked tetramer, trimer, dimer and unmodified monomer species. Dialysis of the cross-linked sample against 30 mM $HgCl_2$ in sodium acetate solution followed by a second dialysis against 10 mM mercaptoethanol resulted in material migrating as a single band coincident with the protein monomer. The removal of the excess mercuric ion (in this case by dialysis against a thiol containing buffer) is essential since mercuric ion itself can cross-link thiol containing subunits.

This reagent may also be used in a two dimensional analysis. Here, the cross-linked material is first separated by electrophoresis in a tube gel. The gel is extruded, treated with mercuric ion to cleave the cross-links, and is then applied to the top of a slab gel. The material, with cross-links broken, runs to its characteristic monomer position in the second dimension of the slab gel.

Figure 2 shows the two dimensional SDS-PAGE pattern for glyceraldehyde-3-phosphate dehydrogenase after treatment with IIb. In Figure 2b, sufficient mercuric ion was added to cleave the cross-links. Were the cross-links not cleaved, the various cross-linked products would run identically in both dimensions and form a diagonal in the two dimensional gel. When the cross-links are cleaved, however, each of the various cross-linked products runs as the monomer in the second dimension, clearly demonstrating that monomers were cross-linked to form dimers, trimers and tetramers.

This simple result shows the utility of this approach to the synthesis of cleavable cross-linking reagents.

Acknowledgements

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