SELECTIVE S-METHYLATION OF CYSTEINE IN PROTEINS AND PEPTIDES*

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Received October 12, 1970

Summary: Sulfhydryl groups in proteins and peptides may be quantitatively and selectively converted, under mild conditions, to the S-methyl derivatives by reaction with methyl *p*-nitrobenzenesulfonate. Amino acid analyses of reduced and methylated derivatives of eggwhite lysozyme and insulin B chain revealed, in each case, quantitative conversion of cysteine to S-methylcysteine. No additional amino acid modifications were detected.

Since the demonstration by Gross and Witkop (1) that cyanogen bromide selectively cleaves polypeptide chains at the carboxyl group of methionine residues, a number of investigators have focused their attention upon similarly induced cleavages at S-methylcysteine sites. The reactions of cyanogen bromide at methionine and S-methylcysteine residues do not proceed by the same mechanism, but extensive cleavage of the peptide chain at the *amino* group of the latter residue is observed under certain conditions (*cf* ref. 2 for a review and further references). Although a method of selective chemical cleavage directed toward cysteine residues would be of great utility in the sequential analysis of proteins, to date the reaction of cyanogen bromide at S-methylcysteine sites has not been applied to any great extent. One of the obstacles to the evaluation and use of this method has been the difficulty in obtaining a reagent which would specifically methylate cysteine

* This work was supported by Grant GB 8617 from the National Science Foundation.

‡ Recipient of a Research Career Development Award, United States Public Health Service, 5 K04 GM-11164-02.

without concomitant modification of other amino acid side chains. Recently, Nakagawa and Bender (3) reported the methylation of histidine-57 in *a*-chymotrypsin by the methyl ester of *p*-nitrobenzenesulfonate. The present communication describes general procedures whereby methyl *p*-nitrobenzenesulfonate may be employed as a methylating agent which is highly selective for cysteine residues in reduced and denatured proteins and peptides.

Materials and Methods: Eggwhite lysozyme (Grade I, L-6876) was purchased from Sigma and porcine insulin was the gift of Dr. Donald Steiner. Methyl *p*-nitrobenzenesulfonate was synthesized by Dr. Robert Silverstein as described earlier (4); the commercial product recently made available by Eastman was also employed in some experiments.

Protein concentrations were determined spectrophotometrically by a modification of the biuret reaction (5) and effluent fractions from column chromatographic procedures were monitored for protein content by measuring the absorbance at 280 nm. Amino acids were determined by automated ion exchange chromatography with a Bio Cal BC-200 analyzer. Protein samples were hydrolyzed *in vacuo* in 6 *N* HCl for 24 hours at 110°.

The reduction of proteins and peptides by 2-mercaptoethanol in 8 *M* urea was performed according to the general procedures of Crestfield, Moore, and Stein (6). Lysozyme (200 mg; 14.3 μmoles) and 7.22 g of urea were placed in a brown reagent bottle equipped with a magnetic stirring bar. Six ml of Tris buffer, pH 8.6 (5.23 g of Tris and 9 ml of 1.0 *N* HCl diluted to 30 ml with water), and 0.6 ml of EDTA solution (50 mg of Na_2EDTA per ml) were added under a barrier of nitrogen and the volume was adjusted to 15 ml with water. Upon complete dissolution of all the solutes, 100 μl of 2-mercaptoethanol were added and the bottle was tightly sealed under nitrogen. After 2 hours at room temperature, the bottle was placed in a water bath maintained at 40° on a combination heater-magnetic stirrer. The bottle was opened under the nitrogen barrier and 2 ml of a solution containing 434 mg (2 mmoles) of methyl *p*-nitrobenzenesulfonate in acetonitrile were added dropwise with

stirring. The small amount of precipitate which formed initially dissolved completely within 5 minutes. After stirring for 30 minutes at 40°, the contents of the bottle were dialyzed for 20 hours against 4 x 1 liter portions of H_2O. The dialyzed protein, freed of urea and reagents, was insoluble. The dialyzate was lyophilized and the residue (yield, 92%) was dissolved in 4 ml of 70% formic acid. A portion of this solution containing about 1 mg of protein was removed for amino acid analysis and the composition was compared to that determined for a sample of native lysozyme (Table I). The methods described above were also employed in the S-methylation of reduced porcine insulin, except that after completion of the reaction the mixture of methylated A and B chains was applied directly to a column (4 x 50 cm) of Sephadex G-75 in 50% acetic acid. Apparently, the methylated A chain precipitated on the column since a single peak of absorbance at 280 nm was observed and amino acid analysis of this material indicated it to be the S-methylated B chain (Table I). The yield of the methylated peptide was 84%.

Results and Discussion: The procedures described herein for the reduction and methylation of proteins are similar to those developed earlier (6) for the conversion of cysteine residues to the S-carboxymethyl derivatives. Alkylation is carried out at pH 8 to 8.5 in 8 *M* urea or 6 *M* guanidine-HCl; attempts to methylate reduced lysozyme and insulin in 50% acetic acid were unsuccessful. In the present studies, the ratio of methyl *p*-nitrobenzenesulfonate to the thiol from the mercaptoethanol employed in the reduction was 1.4. Addition of a solution of the reagent in acetonitrile to the reduced protein in 8 *M* urea was attended by heavy precipitation at ambient temperature and this was overcome by performing the methylation at 40°. Reduced and methylated lysozyme precipitated upon dialysis of the reaction mixture against water or a number of salt solutions buffered at neutral pH. The insolubility of the methylated product poses no problem when its size is such that urea and reagents may be removed by dialysis. With peptides, however, it may be necessary to employ gel filtration for this purpose and solvent systems

Table I

AMINO ACID COMPOSITIONS OF THE S-METHYL DERIVATIVES OF LYSOZYME AND INSULIN B CHAIN

Amino Acid	Number of Residues				
	Lysozyme		S-Methyl lysozyme	S-Methyl B chain	
	Expected[a]	Found[b]	Found[b]	Expected[c]	Found[b]
Aspartic Acid	21	20.7	20.9	1	1.1
Threonine	7	6.4	6.3	1	0.8
Serine	10	9.2	9.3	1	0.7
Glutamic Acid	5	5.1	5.0	3	2.9
Proline	2	2.0	1.9	1	0.9
S-Methylcysteine[d]	0	0.0	8.0	2	1.9
Glycine	12	11.8	11.9	3	3.0
Alanine	12	12.0	12.0	2	2.0
1/2 Cysteine	8	7.2	0.0	0	0.0
Valine	6	5.8	5.8	3	3.1
Methionine	2	1.8	1.9	0	0.0
Isoleucine	6	5.9	6.0	0	0.0
Leucine	8	7.8	8.1	4	3.9
Tyrosine	3	2.9	2.8	2	1.9
Phenylalanine	3	3.1	3.0	3	3.1
Lysine	6	6.0	5.9	1	1.1
Histidine	1	1.1	1.0	2	1.9
Arginine	11	10.8	11.1	1	1.0

[a] Based upon the analysis of Canfield and Liu (8).

[b] Amino acids liberated by hydrolysis *in vacuo*, 6 *N* HCl, 110°, 24 hours. No correction was made for decomposition of labile amino acids. Tryptophan was not determined.

[c] Based upon the analysis of Brown, Sanger and Kitai (9).

[d] The constant for S-methylcysteine was 90% of the value for alanine.

must be selected in which the S-methyl peptide is soluble. Gel filtration in 50% acetic acid of reduced and methylated insulin yielded only the B chain product; methylated A chain presumably precipitated on the column. Dissolution of S-methyl lysozyme was achieved in 50% acetic acid, 70% formic acid, or, at neutral pH, by citraconylation (7).

Amino acid analyses of native lysozyme, the S-methyl derivative, and S-methyl B chain from porcine insulin are presented in Table I. The only modification observed under the conditions described for the methylation is the conversion of cysteine to S-methylcysteine. Lysine, histidine, methionine, tyrosine, and serine were all recovered in the expected proportions relative to the other amino acids. S-methylcysteine is quite stable under the conditions of acid hydrolysis and emerges from the long column of the analyzer at a position immediately following proline. With the amino acid analytical system employed, alanine gave a constant of 160 H x W units per μmole and the value obtained for S-methylcysteine was 145, similar to that of methionine.

One of the major difficulties in the preparation of S-methylated proteins has been the lack of specificity of methylating reagents such as methyl iodide. The results of this investigation indicate that the cysteine residues in reduced and denatured polypeptides may be selectively converted to S-methylcysteine by reaction with methyl *p*-nitrobenzenesulfonate. These findings are of interest not only with respect to the fact that they provide another means for the irreversible blockage of sulfhydryl groups in polypeptides, but also because the S-methylated proteins are themselves of potential importance both in sequence analysis and in the relationship of structure to function in sulfhydryl enzymes. It has been mentioned above that S-methylcysteine residues are target sites for the cleavage of peptide chains by cyanogen bromide (2) and, with the use of the methylating procedures described in this communication, this method may be more generally applied in the future to the sequential analysis of proteins. In addition, it may be possible to convert sulfhydryl enzymes which do not contain methionine to derivatives in which the active site cysteine is replaced by a serine residue. This is in fact what takes place, under certain conditions, during the reaction of S-methylcysteine sites with cyanogen bromide (2, 10). The problem then becomes one of selectively methylating only the active site cysteine and minimizing the cleavage reaction. It

would be interesting to determine the effect of replacing the catalytically essential sulfhydryl group of an enzyme with a hydroxyl group; the reverse has been achieved with the serine proteinase subtilisin (11). Further studies of both the cleavage and replacement mechanisms are currently in progress.

Acknowledgements

The author is deeply indebted to Dr. Ferenc J. Kézdy for many stimulating discussions during the course of this work. Thanks are also due to Dr. Robert Silverstein who synthesized the methylating reagent and to Mr. Daniel P. Mass (supported by a Biomedical Sciences Support Grant awarded to the University of Chicago) who assisted in the experimentation.

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