Methylmaleic Anhydride as a Reversible Blocking Agent During Specific Arginine Modification

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Received January 20, 1971

SUMMARY: A method for specific and irreversible modification of arginine in proteins has been devised using a sequential treatment with methylmaleic anhydride and trimeric 2,3-butanedione at pH 8.0. The amino groups blocked by the methylmaleylation are liberated by dialysis at pH 3.5 according to Dixon and Perham. This technique was applied to bovine plasma albumin and bovine pancreatic ribonuclease A to yield proteins with 93 and 75% of their arginines modified. Amino groups were quantitatively recovered. When a parallel procedure was applied to a mixture of standard amino acids 97% of the arginine was derivatized while recoveries of other amino acids were 100 $\pm$ 4%.

Recent studies have shown crystalline trimeric 2,3-butanedione (TB) to be an effective reagent for arginine modification near neutral pH (1,2). A side reaction, however, which occurs with amino groups becomes relatively more important at alkaline pH despite the more rapid reaction of guanidinium groups. In addition, study of the reagent's specificity on side chains of amino acids was previously clouded by amino group reactivity (2). The purpose of the present communication is to furnish direct information on the specificity of this arginine reagent and describe a method of modification that is both irreversible and specific.

Experimental and Results: A stirred solution of Beckman Amino Acid Standard Mixture (5.0 ml) containing 2.5 μmoles/ml of each of 17 amino acids was adjusted to pH 8.0 with 5.0 N NaOH. Citraconic anhydride (Aldrich Chemical Company) was added in three 25 μl portions while maintaining pH by base addition. These conditions were found adequate to quantitatively block the amino

groups in the mixture as determined by assay with 2,4,6-trinitrobenzene sulfonic acid (TNBS) according to Habeeb (4). After acylation was complete and the pH had stablized, 1 ml of the mixture was treated with an equal volume of a freshly prepared 0.4 M solution of TB in 1 M pH 8.0 phosphate buffer. The mixture was protected from light and incubated for 24 hr at 25°. The mixture

Table I: Complete Analyses of Amino Acid Mixture and RNase Serially Modified with Methylmaleic Anhydride and TB.

	Amino Acid Mixture (μmoles)		RNase (Residues)	
Amino Acid	Control	Modified	Control	Modified
Aspartic Acid	0.206	0.206	15.1	15.3
Threonine	0.206	0.209	9.1	9.4
Serine	0.200	0.201	12.9	13.4
Glutamic Acid	0.206	0.210	12.5	12.6
Proline	0.199	0.204	4.2	4.0
Glycine	0.199	0.192	3.5	3.5
Alanine	0.203	0.200	12.0	11.9
Valine	0.198	0.201	8.9	8.8
Cystine (half)	0.201	0.209	6.6	6.6
Methionine	0.197	0.200	3.6	3.5
Isoleucine	0.196	0.201	2.3	2.3
Leucine	0.198	0.204	2.4	2.2
Tyrosine	0.200	0.206	5.3	4.6[a]
Phenylalanine	0.203	0.206	3.0	3.0
Lysine	0.205	0.210	10.6	10.2
Histidine	0.209	0.201[b]	3.9	3.9
Arginine	0.204	0.006	4.3	1.3[c]

[a] A decreased recovery of tyrosine is characteristic of the procedure unless phenol is added to the 6 N HCl as a protecting agent prior to hydrolysis (2).

[b] Integration of histidine was based on the descending limb of the peak to avoid a contribution from product Z.

[c] Corrected for regeneration of arginine during acid hydrolysis by dividing the observed loss by 0.88.

was then treated with an equal volume of 0.5 M HCl and 3 ml of pH 2.2 citrate sample dilution buffer (5). Incubation of this acid dilution for 24 hr (protected from light) was followed by amino acid analysis. As a control an identical 1 ml aliquot of the citraconylated amino acids was carried through the same procedure omitting TB. Table I shows that the sole amino acid altered was arginine and it was 97% modified. Three new peaks appeared in the basic region of the chromatogram (Fig. 1b) corresponding to products Y,Z and Z_o previously described (2). Since product Z appeared under the leading edge of histidine, in a separate experiment arginine was substituted for the mixture

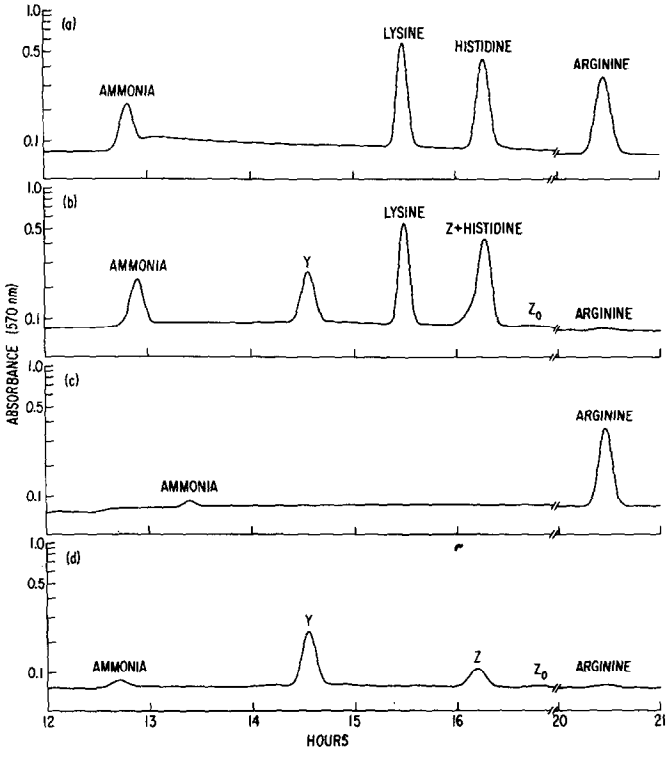

Fig. 1. Chromatography showing basic region of amino acid samples treated with TB and corresponding control samples. All samples were treated with methylmaleic anhydride at pH 8.0, diluted with an equal volume of 1.0 M, pH 8.0 phosphate buffer (with or without 0.4 M TB) and incubated at 25° for 24 hr. Acyl groups were removed by incubation with HCl-pH 2.2 citrate buffer for 24 hr (25°). Aliquots containing 0.2 μmole of each amino acid were applied to the 130 cm chromatography column used for the Technicon 22 hr separation. Frame (a): Mixture of standard amino acids treated in the absence of TB. Frame (b): Same as (a) but including TB. Frame (c): Arginine after treatment not including TB. Frame (d): Same as (c) but including TB.

of amino acids and the entire procedure was repeated. Both the conversion yield of arginine (97%) and the location of these products in the chromatogram were verified (Fig. 1d). The products Y,Z and Z_o appeared in the ratio of 16:6:1 and their combined recovery accounted for 96% of the modified arginine. These derivatives had been shown previously to be resistant to reversal (2).

To evaluate the procedure on proteins 45 mg of bovine pancreatic ribonuclease A (RNase) or bovine plasma albumin (BPA) was dissolved in 3.0 ml of water containing 41 mg of sodium acetate. (The acetate guards against acid denaturation of the BPA during the initial addition of the anhydride to the otherwise unbuffered solution.) Citraconylation was effected by adding five 25 µl aliquots of methylmaleic anhydride to the stirred solution of protein while maintaining the pH at 8.0 with 5.0 N NaOH. A 3 ml portion of the resulting solution was treated with an equal volume of freshly prepared 0.4 M TB in 1 M, pH 8 phosphate buffer. The sample was incubated for 24 hr as for the amino acid mixture. Deacylation was accomplished by dialyzing the modified protein (protected from light) against 0.1 M, pH 3.5 ammonium formate buffer (3) for 24 hr at 25°. Transient precipitation occurred during the initial stages of dialysis, but after transfer to 0.1 M, pH 7.0 sodium phosphate buffer the proteins returned to solution. TNBS analysis showed that the acylation

Table II: Extent of Arginine Modification of Proteins at pH 8.0 and 7.0[a]

Protein	pH (25°, 24 hr)	% Arginine Modified[b]
RNase	8.0	75 (69)
	7.0	50
BPA	8.0	93 (91)
	7.0	79
	7.0 (6 hr)	38

a Each sample was reversibly blocked with citraconic anhydride before treatment with 0.2 M TB. Proteins were deacylated at pH 3.5, 25°. Recoveries of amino groups in modified samples were 98% or better as measured by TNBS.

b Arginine recoveries were determined by Sakaguchi analysis as described earlier (2) except for those values in parenthesis which were determined by amino acid chromatography. All data are corrected for 12% release of arginine from derivatives during acid hydrolysis.

and deacylation steps were quantitative. In a similar procedure arginine was modified at pH 7.0 while the acylation and deacylation procedures were essentially unchanged. Table II shows that while more arginines are blocked at pH 8.0, extensive modification still occurs at pH 7.

Discussion: The blocking of amino groups of a mixture of standard amino acids with methylmaleic anhydride followed by arginine modification with crystalline trimeric 2,3-butanedione at pH 8.0 and subsequent liberation of amino groups at pH 3.5 reveals a specific and essentially quantitative modification of arginine. Chromatograms shown in Figure 1 reveal that the products (Y, Z and Z_o) are identical to those reported earlier using this reagent under different conditions (2).

When RNase and BPA were serially modified with methylmaleic anhydride followed by TB, arginine modification was found to be extensive while amino groups were fully and reversibly protected. Citraconic anhydride has also been shown by Gibbons and Perham (6) to acylate hydroxyl groups of serine and threonine. The esters, however, are cleaved under the same mildly acidic conditions used for liberating amino groups (6). These observations are pertinent here because TB modification of hydroxy amino acids clearly does not occur after a mixture of amino acids is reacted with the anhydride. The data, however, do not rule out some modification of hydroxyl groups in extensively modified proteins not protected by the acylation. Proteins treated serially with methylmaleic anhydride and TB at pH 8.0 or 7.0 suffer little or no discoloration.

Several features of methylmaleic anhydride bode well for its use as a protecting agent in specific arginine modification: (A) It is quantitative with regard to acylation and deacylation steps. (B) Intermolecular cross-linking is rendered unlikely in view of the repulsive charges introduced by the methylmaleation. (C) Finally, the reversible blocking procedure may be used to obtain direct information on effects of buffers on specificity and product identity.

Acknowledgement: The competent assistance of Mrs. Judy Seitz in performing the amino acid analyses is gratefully acknowledged. This research was supported by Grant GB-17985 from the National Science Foundation.

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