QUANTITATIVE ANALYSIS OF TRYPTOPHAN MODIFIED BY 2-HYDROXY-5-NITROBENZYL REAGENTS AFTER HYDROLYSIS WITH p-TOLUENESULFONIC ACID[1]

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SUMMARY: We wish to report that tryptophan modified with dimethyl (2-hydroxy-5-nitrobenzyl)-sulfonium bromide is stable to conditions of acid hydrolysis with 3 N p-toluenesulfonic acid. This technique of hydrolysis can thus be applied to quantitatively determine the number of tryptophans modified by 2-hydroxy-5-nitrobenzyl bromide or dimethyl (2-hydroxy-5-nitrobenzyl)-sulfonium bromide, reagents which selectively modify tryptophan. When hen lysozyme is treated with a 6 molar excess of dimethyl (2-hydroxy-5-nitrobenzyl)-sulfonium bromide at pH 6.0, approximately 1.1 tryptophan residues are modified with complete loss of enzymatic activity.

2-Hydroxy-5-nitrobenzyl bromide (HNB-Br)[3] was introduced several years ago as a selective modifying reagent for tryptophan (1,2). More recently, derivatives of HNB-Br, namely dimethyl-HNB-sulfonium salts, have been introduced which have the advantages of water solubility and greater stability in acidic aqueous solutions (3,4). Both the HNB-Br and the dimethyl-HNB-sulfonium salts have similar chemical reactivity towards tryptophan-containing compounds, and both yield identical products (4).

One of the difficulties encountered in studying the effects of tryptophan modification on the properties of various proteins

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[3]Abbreviations used: HNB, 2-hydroxy-5-nitrobenzyl.

is the quantitative determination of the number of tryptophan residues modified. Tryptophan is destroyed by hydrolysis with 6 N HCl, the usual method of choice for amino acid analysis (5). In a number of studies using HNB-reagents to modify proteins, spectrophotometric techniques have been used to estimate the number of HNB groups covalently attached (6-10). This technique cannot always be directly applied to determine the number of tryptophans modified, however, since disubstitution of tryptophan can accompany monosubstitution (11,12). Other investigators studying the effects of HNB reagents have used amino acid analyses for tryptophan after alkaline hydrolysis (1,3,13,14) and hydrolysis with 6 N HCl in the presence of thioglycolic acid (10), but these studies were either incompletely documented, or were characterized by unreliable quantitative results.

Recently, Liu and Chan have reported reliable amino acid analyses for tryptophan after hydrolysis with 3 N p-toluenesulfonic acid (15). We wish to report the applicability of this technique to the quantitative determination of tryptophan modified by HNB-reagents.

MATERIALS AND METHODS

Materials. Hen lysozyme, 3X crystallized, was obtained from Sigma Chemical Company. Dimethyl (2-hydroxy-5-nitrobenzyl)-sulfonium bromide and L-tryptophan were purchased from Nutritional Biochemicals Corporation. L-Arginine was a product of Matheson Coleman and Bell, and p-toluenesulfonic acid, sequanal grade, was purchased from Pierce Chemical Company.

Tryptophan Model Study. A stock solution of L-tryptophan (4.19 mM) and L-arginine (3.44 mM) was made up in distilled water. Ten ml of the stock solution was adjusted to pH 6.0 with 1 M NaOH, and an appropriate amount of solid dimethyl-HNB-sulfonium bromide

(either 0.7 or 3.0 equivalents, based on tryptophan) was added to initiate the reaction. The pH was maintained at 6.0 by manual addition of 1 M NaOH, and the reaction was allowed to proceed for 2.5 hours at room temperature. The solution containing the modified tryptophan was then stored at 4°C until use. Aliquots of the stock solution, the 0.7 modified solution[4], and the 3.0 modified solution (250 μl)[4] were withdrawn, diluted to 1.00 ml with 4 N p-toluenesulfonic acid, 0.27% in tryptamine, and subjected to the hydrolytic conditions of Liu and Chang (15) for various time periods. Amino acid analysis was performed on the short column (8 cm x 0.9 cm) of a Beckman Model 120 C amino acid analyzer.

Modification of Lysozyme. Lysozyme (14 μmole) was dissolved in distilled water to a concentration of 10 mg/ml. The pH was adjusted to 6.0 with 1.0 M NaOH, and solid dimethyl-HNB-sulfonium bromide (56 μmole) was added. The reaction was allowed to proceed at room temperature for 2.5 hours; then an additional 28 μmole of solid dimethyl-HNB-sulfonium bromide was added. Aliquots were withdrawn periodically from the reaction mixture and analyzed for enzymatic activity against Micrococcus lysodiekticus (16). After 4 hours reaction, the lysozyme was separated from dimethyl-HNB-sulfonium bromide by-products by gel filtration on a column (2.5 cm x 45 cm) of Sephadex G-25-40 equilibrated with 10% acetic acid, and lyophilized. Amino acid analysis of native and modified lysozymes was performed on a Beckman Model 120 C amino acid analyzer (17) after hydrolysis with 3 N p-toluenesulfonic acid (15).

RESULTS AND DISCUSSION

The results of the amino acid analysis of the various trypto-

[4]These solutions containing modified tryptophan will subsequently be referred to as the 0.7 solution (dimethyl-HNB-sulfonium bromide: trp = 0.7) and the 3.0 solution (dimethyl-HNB-sulfonium bromide: trp = 3.0).

phan and modified-tryptophan solutions are shown in Figure 1.

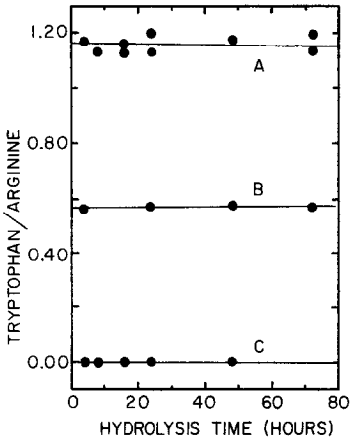

Figure 1. Amino acid analysis of tryptophan modified by dimethyl-HNB-sulfonium bromide after hydrolysis with 3 N p-toluenesulfonic acid. A stock solution containing tryptophan (4.19 mM) and arginine (3.44 mM) was modified as described in the text with: (A) no dimethyl-HNB-sulfonium bromide; (B) 0.7 equivalents (based on tryptophan) of dimethyl-HNB-sulfonium bromide; and (c) 3.0 equivalents of dimethyl-HNB-sulfonium bromide.

This study not only indicates that tryptophan is relatively stable to hydrolysis with 3 N p-toluenesulfonic acid, as has been reported (15), but that HNB-labelled tryptophan does not regenerate tryptophan under the conditions of hydrolysis with 3 N p-toluenesulfonic acid. This is the case with the 1:1 HNB:tryptophan adduct, which is most certainly the species present in the 0.7 solution (4), and most likely with the 2:1 HNB:tryptophan adduct, as seen from analysis of the 3.0 solution in which tryptophan is probably present as a mixture of 1:1 and 2:1 adducts (4). The arginine served as a convenient inert marker in these studies and facilitated quantitative calculations.

The results of the modification of lysozyme are shown in Table 1. These results seem to indicate that the procedure des-

TABLE 1

Amino Acid Analyses of Native and Modified Lysozymes. Values given are the average of 24, 48 and 72-hour hydrolyses, unless otherwise indicated. Values are based on 12.0 alanine for the acidic and neutral amino acids, and 11.0 arginine for the basic amino acids.

Amino Acid	Native	Modified	Literature (21)
Asp	21.4	21.9	21
Thr	7.0[a]	6.8[a]	7
Ser	10.2[a]	9.7[a]	10
Glu	5.4	5.2	5
Pro	2.6	2.3	2
Gly	12.0	12.4	12
Ala	12.0	12.0	12
Cys/2	6.2[a]	6.9[a]	8
Val	5.9[b]	6.0[b]	6
Met	2.0	1.9	2
Ile	5.6[b]	5.9[b]	6
Leu	8.2	8.3	8
Tyr	3.0	3.1	3
Phe	3.0	3.1	3
Lys	6.0	5.8	6
His	0.9	0.9	1
Arg	11.0	11.0	11
Trp	5.6[c]	4.5[c]	6

[a]Value taken from 24-hour hydrolysate.

[b]Value taken from 72-hour hydrolysate.

[c]Value obtained by extrapolation to zero hydrolysis time.

cribed here is valid for proteins as well as for free amino acids. They indicate that approximately 1.1 tryptophan residues are modified, and that no other amino acid is affected by the reagent.

Enzymatic analysis of the modified lysozyme shows that the modification of approximately one tryptophan residue by dimethyl-HNB-sulfonium bromide results in the complete loss of enzymatic activity. This is in line with the observations of others that tryptophan plays an important role in the catalytic activity of this enzyme (18-20). It is interesting to compare the results of

this study with those of Bewley and Li (6), who report that when hen lysozyme is treated with the closely related reagent HNB-bromide, two tryptophan residues can be modified without any loss of enzymatic activity. We are currently investigating the identity of the essential tryptophan in hen lysozyme modified by dimethyl-HNB-sulfonium bromide.

REFERENCES

1. Koshland, D. E., Jr., Karkhanis, Y. D., and Latham, H. G., J. Am. Chem. Soc. 86, 1448 (1964).
2. Horton, H. R., and Koshland, D. E., Jr., J. Am. Chem. Soc. 87, 1126 (1965).
3. Horton, H. R., and Tucker, W. P., J. Biol. Chem. 245, 3397 (1970).
4. Tucker, W. P., Wang, J., and Horton, H. R., Arch. Biochem. Biophys. 144, 730 (1971).
5. Moore, S., and Stein, W. H., in S. P. Colowick and N. O. Kaplan (editors), Methods in Enzymology, Vol. VI, Academic Press, New York, 1960, p. 819.
6. Bewley, T. A., and Li, C. H., Nature 206, 624 (1965).
7. Dopheide, T. A. A., and Jones. W. M., J. Biol. Chem. 243, 3906 (1968).
8. Terao, T., and Ukita, T., Biochim. Biophys. Acta 181, 347 (1969).
9. Poulos, T. L., and Price, P. A., J. Biol. Chem. 246, 4041 (1971).
10. Naik, V. R., and Horton, H. R., Biochem. Biophys. Res. Comm. 44, 44 (1971).
11. Loudon, G. M., Portsmouth, D., Lukton, A., and Koshland, D. E., Jr., J. Am. Chem. Soc. 91, 2792 (1969).
12. Robinson, G. W., J. Biol. Chem. 245, 4832 (1970).
13. Chan, T.-L., and Schellenberg, K. A., J. Biol. Chem. 243, 6284 (1968).
14. Barman, T. E., Biochim, Biophys. Acta 258, 297 (1972).
15. Liu, T.-Y., and Chang, Y. H., J. Biol. Chem. 246, 2842 (1971).
16. Fawcett, R. L., Limbird, T. J., Oliver, S. A., and Borders, C. L., Jr., Can. J. Biochem. 49, 816 (1971).
17. Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem. 30, 1190 (1958).
18. Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R., Proc. Roy. Soc. (London) B167, 378 (1967).
19. Raftery, M. A., and Dahlquist, F. W., Fortschritte der Chemie organischer Naturstoff 27, 340 (1969).
20. Schechter, Y., Burstein, Y., and Patchornik, A., Biochemistry 11, 653 (1972).
21. Canfield, R. E., J. Biol. Chem. 238, 2698 (1963).