REVERSIBLE INACTIVATION OF AVIAN LYSOZYMES BY DIMETHYL (2-HYDROXY-5-NITROBENZYL)-SULFONIUM BROMIDE

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SUMMARY: The reaction of hen egg white lysozyme with a 4 molar excess of dimethyl (2-hydroxy-5-nitrobenzyl)-sulfonium bromide at pH 6.0 leads to total loss of enzymatic activity within 5 minutes. Upon standing, the inactivated enzyme spontaneously regains activity, leveling off at 60% of the original activity after 72 hours. Under the same conditions, turkey egg white lysozyme is reduced to less than 5% of its original activity within 5 minutes, then spontaneously reactivates to 85% of its original activity after 24 hours. Human lysozyme shows no dramatic loss of activity when treated under these conditions. The presence of the substrate, chitotetraose, prevents the initial inactivation of both hen and turkey enzymes.

2-Hydroxy-5-nitrobenzyl bromide (HNB-Br)[3] and the closely related dimethyl HNB-sulfonium salts have been used as selective, irreversible modifying agents for tryptophan in the absence of cysteine (1-4). We have recently reported on the complete inactivation of HEW lysozyme by dimethyl HNB-sulfonium bromide at pH 6.0, and were able to demonstrate that approximately 1.1 tryptophan residues were modified in the isolated preparation (5). Heinrich *et al.* have subsequently reported similar results on this enzyme (6).

In the face of all available evidence, when one finds total inactivation of an enzyme molecule accompanied by the modification of a single tryptophan by HNB reagents, the logical conclusion to

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[1]Taken in part (a) from the Senior Independent Study thesis of D.K.J., The College of Wooster, 1973, and (b) from the Senior Independent Study thesis of S.E.P., The College of Wooster, 1972.

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[3]Abbreviations used are: HNB, 2-hydroxy-5-nitrobenzyl; HEW, hen egg white; TEW, turkey egg white.

draw is that the tryptophan thus modified is the residue necessary for enzymatic activity. We wish to present evidence which might indicate otherwise in the case of avian lysozymes.

MATERIALS AND METHODS

Materials. HEW lysozyme, 3X crystallized, and heat-killed Micrococcus Lysodiekticus cells were obtained from Sigma Chemical Company. TEW lysozyme was prepared by published procedures (7), and gave a single band on disc electrophoresis at pH 4.5 (8). Human lysozyme was the generous gift of Dr. R. E. Canfield. Chitotetraose was prepared and isolated by published procedures (9). Dimethyl (2-hydroxy-5-nitrobenzyl)-sulfonium bromide was purchased from Nutritional Biochemicals Corporation, and all other chemicals were reagent grade.

Assay of Enzymatic Activity. Aliquots of native and modified lysozyme samples were assayed for enzymatic activity by measuring the rate of decrease in turbidity of a M. lysodiekticus suspension in 0.05 M sodium phosphate, 0.04 M KCl, pH 6.2, at 320 mμ.

Reaction of Lysozymes with Dimethyl HNB-sulfonium Bromide. Lysozyme (10 mg/ml) was dissolved in distilled water and the pH was adjusted to 6.0 with 5% HCl or 1 N NaOH. A drop of toluene was added to prevent microbial growth, and the appropriate amount of solid dimethyl HNB-sulfonium bromide (to give a 4 molar excess over lysozyme) was added to initiate the reaction. The mixture was stirred continuously at room temperature and the pH was maintained by manual addition of 1 N NaOH. Aliquots were withdrawn at the appropriate time intervals, diluted with 20 volumes of distilled water to quench the reaction, and assayed immediately for enzymatic activity as described above. The activity is expressed relative to a blank of the native lysozyme subjected to similar conditions and assayed at the same time intervals. For those

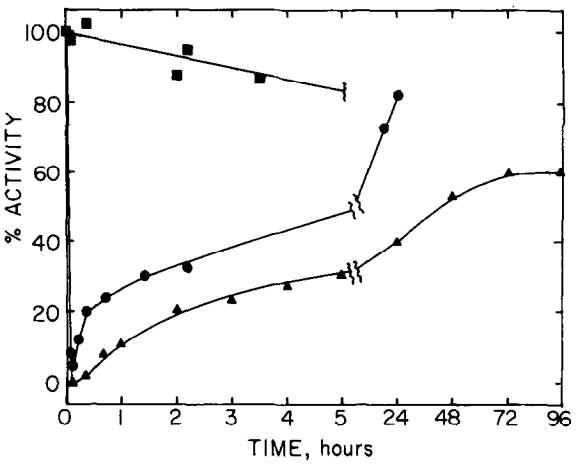

Figure 1. The effect of a 4 molar excess of dimethyl HNB-sulfonium bromide on the enzymatic activity of various lysozymes: (■), human lysozyme; (●), turkey egg white lysozyme; (▲), hen egg white lysozyme. See text for details.

experiments where the effects of chitotetraose was of interest, it was included in the original reaction mixture at a concentration of 10 mg/ml.

RESULTS AND DISCUSSION

Figure 1 indicates the effects of a 4 molar excess of dimethyl HNB-sulfonium bromide on various lysozymes at pH 6.0. It can be seen that both HEW and TEW lysozymes undergo a very rapid inactivation, the HEW having 0% and the TEW having $<5\%$ activity after five minutes. Both avian enzymes then show a gradual spontaneous reactivation, with the TEW lysozyme being the more rapidly reactivated. It attains 83% of its original activity after 24 hours, while the HEW lysozyme regains 40% activity after 24 hours and levels off at 60% activity after 72 hours.

The results with both avian lysozymes are in sharp contrast to those obtained with human lysozyme. The latter shows very little inactivation by dimethyl HNB-sulfonium bromide, retaining 86% of its original activity after 3.6 hours.

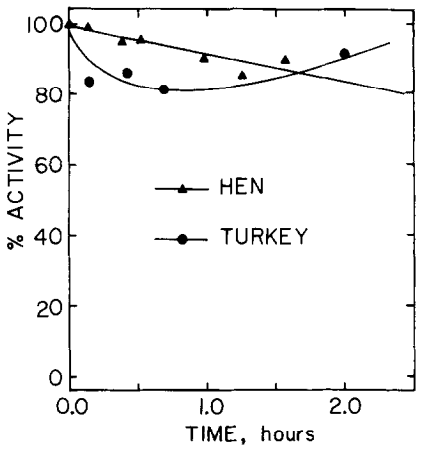

Figure 2. The effect of a 4 molar excess of dimethyl HNB-sulfonium bromide on the enzymatic activities of hen egg white and turkey egg white lysozymes (10 mg/ml) in the presence of chitotetraose (10 mg/ml).

Figure 2 demonstrates that chitotetraose, which is known to bind to the active site of HEW lysozyme (10), and presumably does the same for TEW lysozyme, protects these enzymes from inactivation. Since both these enzymes have 6 tryptophan residues in identical positions, and since tryptophan is known to be involved in the active site of HEW lysozyme (11), it would appear logical to conclude that the substrate prevents the modification of an essential tryptophan. Tryptophan-62 is an appealing choice, since it is replaced by tyrosine in the equivalent position of human lysozyme (12), and the study reported here shows that the activity of human lysozyme is affected very little by dimethyl HNB-sulfonium bromide under conditions where the avian lysozymes are rapidly inactivated.

However, all reports on the reaction of HNB reagents with tryptophan would indicated that the reaction is irreversible (1-4). HEW lysozyme, which has been reacted for 2 hours with dimethyl HNB-sulfonium bromide has been separated into four major components by ion exchange chromatography (D. K. Jorkasky and C. L. Borders, Jr., unpublished results), all of which demonstrate appreciable enzymatic activity. Three of these com-

ponents have modified tryptophan, and the fourth appears to be native lysozyme. All this seems to point to the fact that the initial inactivation of avian lysozymes is due to the reversible modification of some residue other than tryptophan. Barman has reported the incorporation of an "acid-labile" HNB group into HEW lysozyme by treatment with HNB bromide, but makes no mention of any effects on enzymatic activity (13). We have extended the study of the reactivation of HEW lysozyme to pH's 3.0 and 10.0 after initial inactivation at pH 6.0. It was found that the reactivation profiles at pH 3.0 and pH 6.0 were very similar, while at pH 10.0 the reactivation proceeds at about half the rate as the other two pH's. We are presently investigating the identity of the essential labile residue.

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