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Reaction of *N*-Acetylimidazole with L-Asparaginase

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SUMMARY

L-Asparaginase from *Escherichia coli* B was treated with *N*-acetylimidazole over a wide range of concentrations. The results indicate that the 44 tyrosyl residues of native asparaginase can be divided into three classes. The first group consists of 5–7 tyrosyl residues that react readily with the reagent and are not involved in the catalytic site. The second class consists of 10–12 aromatic residues that react less rapidly with acetylimidazole and whose modification results in a 70% decrease in enzyme activity. Full activity can be restored after deacetylation with hydroxylamine. The third class consists of the remaining tyrosyl groups that are unavailable for acetylation in the native enzyme.

All the tyrosyl residues could be acetylated in 8 M urea. Modification of 10 residues in urea did not interfere with subunit reassociation, while acetylation of 15–22 residues gave variable results. Modification of 30–44 residues interfered with reassociation of the subunits. Removal of the acetyl groups with hydroxylamine, however, did not allow the subunits to aggregate, because of other, irreversible reactions of acetylimidazole with the denatured protein.

INTRODUCTION

L-Asparaginase from *Escherichia coli* B inhibits the growth of tumors in mice (1) and is active against lymphoblastic leukemia in children (2). In spite of numerous reports dealing with this enzyme, the structure of the catalytic site and the nature of the forces through which the subunits associate are not completely understood. In an effort to elucidate these structural elements, we have undertaken a series of investigations on chemical modifications of the enzyme, using a wide range of concentrations of reagents. The present report presents the results obtained when the enzyme was treated with acetylimidazole in buffer and in 8 M urea.

MATERIALS AND METHODS

L-Asparaginase from *E. coli* B (lot C8067) was a product of Merck Sharp & Dohme and was supplied to us through the courtesy of the Cancer Chemotherapy Unit, National Cancer Institute. The lyophilized sample was dissolved in 0.05 M phosphate buffer, pH 7.5, and was dialyzed against 200 volumes of this buffer in order to remove the mannitol present as a preservative. The stock solution (14 mg/ml) was stored at 4°. After 1 week at this temperature the stock solution became cloudy, although the specific activity was not reduced significantly, and the material showed a monodisperse-homogeneous boundary in the ultracentrifuge. Freshly prepared solutions of the enzyme gave two bands on polyacrylamide gels, where 95% of the protein was the tetramer and the remaining 5% was the larger octamer.

N-Acetylimidazole was purchased from Cyclo Chemical Company and was stored over P_2O_5 in the cold. As soon as moisture appeared, the sample was dissolved in

benzene and dried over sodium sulfate. Urea (Fisher) was recrystallized from dilute ethanol and then dried under vacuum. Urea solutions were freshly prepared prior to use. Nessler's reagent was also purchased from Fisher.

Acetylation with *N*-acetylimidazole was carried out as follows. Asparaginase (14 mg/ml) in 0.01 M Tris buffer (pH 7.5) or in 8 M urea (pH 7.5) was treated with an acetone solution of acetylimidazole (40 mg/ml) to the desired molar excess. An aliquot of the mixture was removed, followed by another addition of the acetylating agent to the next higher concentration. This procedure was followed until the reagent was present in 100 M excess in buffer and 250 M excess in urea. The reaction mixtures were allowed to stand for at least 1 hr before they were dialyzed against several changes of 200 volumes of 0.05 M phosphate buffer, pH 7.5. The extent of *O*-acetylation was determined spectroscopically at 278 nm after treatment with 1 M hydroxylamine according to Simpson and Vallee (3).

The activities of asparaginase and acetylasparaginase were determined by the nesslerization method (4). In the case of the modified enzyme deacetylated with hydroxylamine, the excess hydroxylamine and the hydroxamate had to be removed by exhaustive dialysis before the assay could be performed.

Sedimentation velocity studies were carried out in a Spinco model E analytical ultracentrifuge according to the method described previously (5). Fluorescence spectra were taken with an Aminco-Bowman spectrophotofluorometer modified as described in an earlier publication (6). Absorption spectra were determined with a Cary 15 spectrophotometer, and difference spectra using tandem double cuvettes as previously described (5, 6).

RESULTS

Asparaginase in buffer was treated with acetylimidazole in molar excesses ranging from 20- to 100-fold. The percentage of the 44 tyrosyl residues that were acetylated and the corresponding loss in catalytic activity obtained when the concentration of acetylimidazole was increased are shown in Fig. 1. When the acetylating agent was present in 20 M excess, between 5 and 7 tyrosyl residues (11–15%) were acetylated, none of which was involved in the catalytically active site. Increasing the acetylimidazole to a 50-fold excess resulted in acetylation of a total of 15–17 residues (34–38%), accompanied by a 70% loss of catalytic activity. Further increases in the concentration of acetylimidazole did not affect the number of acetyltyrosines formed, nor was the activity decreased further.

The acetylasparaginases were examined in

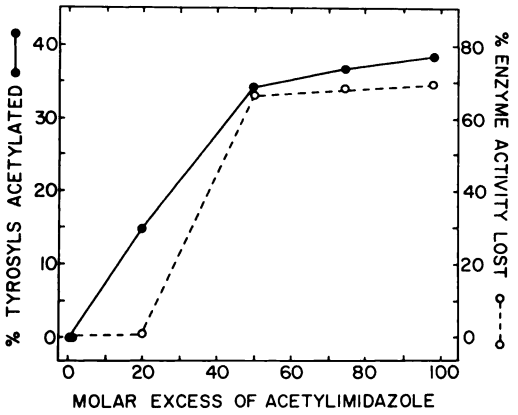

FIG. 1. *Percentage of 44 tyrosyl residues acetylated and of catalytic activity lost after treating asparaginase with various concentrations of acetylimidazole*

The enzyme, in 0.01 M Tris buffer, pH 7.5, was treated with the molar excess of reagent shown, and the reaction was allowed to proceed for at least 1 hr before the excess reagent was removed by dialysis.

TABLE 1

Number of tyrosyl residues acetylated in 8 M urea, and effect on reconstitution after removal of urea

Excess acetylimidazole	Tyrosyls acetylated	State of association
M		
20	10–12	Tetramer
50	15–22	50 ± 20% tetramer 50 ± 20% slower sedimenting
100	30–35	100% slower
250	44	100% slower component

the ultracentrifuge, and all samples were found to have a homogeneous, monodisperse boundary with a sedimentation coefficient of 7.4 S, indicating that the 20 tyrosyl residues that were acetylated were not involved in holding the subunits together.

The fluorescence spectra of all samples showed an emission maximum at 317 nm, identical with the spectrum of the native, unmodified enzyme. Therefore the single tryptophyl residue in each subunit was still located in a very hydrophobic region. The intensity of this emission band as a function of the amount of light absorbed at 278 nm also remained constant.

The acetyl groups were removed by treating the acetylasparaginase samples with 1 M hydroxylamine for 10 min, after which the samples were exhaustively dialyzed. All the samples regained full activity after deacetylation.

Reactions in 8 M Urea. Asparaginase was dissociated in 8 M urea and then treated with increasing amounts of acetylimidazole. The number of tyrosyl residues that were modified and the effect on reconstitution of the subunits are summarized in Table 1. The lowest concentration of the acetylating agent modified 10–12 tyrosyl residues and did not affect the reassociation of subunits. The results obtained when 15–22 residues were acetylated were quite variable. Some samples showed 50% of the sedimenting material to be a tetramer, and the other 50% to be a more slowly sedimenting, homogeneous boundary with a sedimentation coefficient of 3.2 S. In other samples, treated with the same amount of acetylimidazole, the ratios of these two species varied. The acetylation of 30–44 tyrosyl residues prevented formation of any tetramer, and the acetylated subunit gave the sedimentation pattern shown in Fig. 2. The observed sedimentation coefficient (3.2 S) was considerably larger than the value of 1.5 S observed for the monomer in urea (6) or the succinylated subunit (7).

The fluorescence spectra of the samples obtained at molar ratios of 50:1 and 250:1 are compared with the native enzyme in Fig. 3. The 50:1 sample consisted of 50% each of the 7.4 S and 3.2 S species and had its emission maximum at wavelengths longer than 317 nm. The 250:1 sample, which contained 100% of the 3.2 S material, had a single emission maximum at 335 nm. The

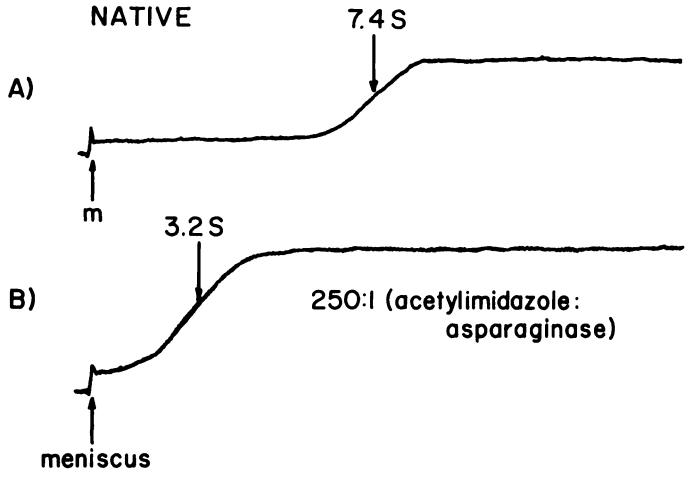

FIG. 2. *Sedimentation velocity ultracentrifugation of asparaginase and acetylasparaginase in 0.05 M phosphate buffer, pH 7.5*

A. Unmodified asparaginase was denatured in 8 M urea and then dialyzed against phosphate buffer. B. Asparaginase was acetylated with a 250 M excess of acetylimidazole in 8 M urea, followed by dialysis against buffer. The direction of sedimentation is from left to right. The tracings were made 50 min after rotor acceleration was started. Speed, 60,000 rpm at 20°. The monochromator was at 280 nm for these ultraviolet absorption scans.

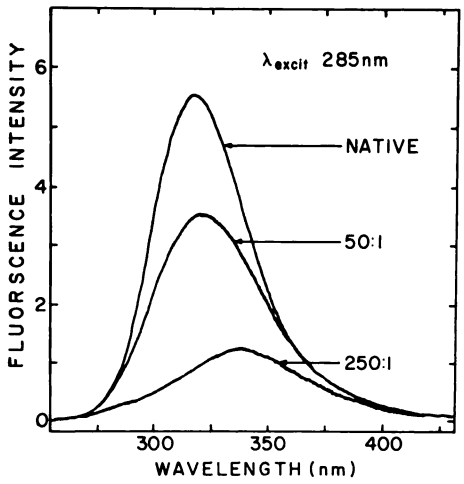

FIG. 3. *Fluorescence spectra of native asparaginase and of asparaginase treated with a 50 M excess of acetylimidazole in 8 M urea (50:1) or a 250 M excess of reagent in 8 M urea (250:1)*

The urea and excess reagent were removed by dialysis.

dissociated subunit obtained from urea or by succinylation showed two maxima: one at 340 nm, due to tryptophan in an aqueous environment, and the other at 303 nm, assigned to tyrosine. The latter was absent from this sample, since acetyltyrosine is nonfluorescent (7). Removal of the acetyl group with hydroxylamine resulted in the appearance of tyrosine fluorescence (303 nm).

Deacetylation of all samples with hydroxylamine did not result in reassociation of the subunits, indicating that acetylimidazole reacted irreversibly with the enzyme in urea.

DISCUSSION

The 20 "free" tyrosyl residues that can be acetylated with acetylimidazole may be compared with the 14 tyrosyl residues that ionize with a normal pK value (8). This difference is somewhat greater than the 10% variation obtained in the determination of acetyl groups by the method of Simpson and Vallee (3). It is clear, however, that the first 10–15% of the tyrosyl residues acetylated are not involved in catalytic activity and that among the next most reactive residues are some involved in the active site. These results agree with the report by Liu and Handschumacher (9) that 25% of the tyrosyl residues could be nitrated with tetranitromethane, which results in a 70% loss of enzyme activity. The complete reversibility of the inhibition lends further support to the role of tyrosyl residues in the active site.

The involvement of tyrosyl residues in subunit interactions is more ambiguous, since acetylimidazole reacts irreversibly with the enzyme in 8 M urea. In addition to the reaction with the phenolic hydroxyl group, acetylimidazole can acetylate ε-amino groups of lysine, which are not essential for catalytic activity but are involved in subunit interactions (5). Acetylation of this amino group would disrupt any electrostatic interactions between the positively charged lysine and some of the carboxylate groups.

We have demonstrated above that there are three classes of tyrosyl residues in native asparaginase. Five to seven residues react readily with acetylimidazole and are not involved in the active site. The next 10 residues react less readily with the reagent, but among these is at least 1 residue that participates in the enzymatic site. The rest of the 44 tyrosines are not available for reaction with acetylimidazole until the protein is dissociated by 8 M urea. Because of the irreversible reaction of acetylimidazole with groups other than tyrosine in the denatured protein, it has not been possible to define the role of tyrosine residues in subunit interactions.

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