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INACTIVATION OF ENOLASE WITH TETRANITROMETHANE *

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1. All 20 tyrosyl residues of enolase from human and pig muscle were nitrated with tetranitromethane in mild conditions; 2-phosphoglycerate and magnesium ions decreased the rate of reaction.
2. Inactivation took place in two steps: (a), nitration of 6–10 tyrosine residues of enolase decreased the activity by about 20%, without affecting $K_m$ value, molecular weight or pH dependence; (b) on nitration of 12 tyrosyl residues, inactivation was nearly complete, with concomitant profound changes in the enzyme properties. 2-Phosphoglycerate and magnesium ions present together protected significantly against inactivation.
3. The results suggest that the activity of enolase is dependent on intactness of tyrosyl residues.

Enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) catalyses conversion of 2-phosphoglycerate to phosphopyruvate, the second reaction of the glycolitic process in which a high-energy phosphate bond is generated. The enzyme has an absolute requirement for divalent cations (Wold, 1971).

Recently, several attempts have been made to elucidate the mechanism of the dehydration reaction (Mildvan et al., 1973; Failler et al., 1977). However, little is known about the active centre of enolase and the amino acids involved directly or indirectly in the catalytic process. Enolase isolated from yeast is inactivated by photooxidation or carboxymethylation (Wold, 1971), or treatment with diethylpyrocarbonate (George & Borders, 1979). Enolase isolated from human or pig muscle lost the activity completely after modification with $N$-bromosuccinimide (Wolna, 1977).

In the present paper, the effect of tetranitromethane on the properties of enolase from human and pig muscle is described. Tetrinitromethane is often

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used to convert free tyrosine residues of proteins into 3-nitrotyrosine (Riordan & Vallee, 1972; Bocharow et al., 1973; Alexander et al., 1977; Kawancki et al., 1977). The reaction is not specific for tyrosine residues since SH groups are also oxidized, but they are not involved in the catalytic activity of enolase (Oh et al., 1973).

MATERIALS AND METHODS

Enolase from human and pig muscle was purified by the procedure of Baranowski & Wolna (1975) and Wolna et al. (1971), respectively. Tetranitromethane was from Koch-Light Ltd (Colnbrook, England), 2-phosphoglycerate from B.D.H. (Poole, England). Enzyme concentration was determined as described by Wolna (1977).

The reaction of enolase with tetranitromethane was run as follows: to 2.5 ml of enolase solution (about 1 mg protein) dialysed previously against a suitable buffer (as described in the text) was added 20 µl of tetranitromethane solution in ethanol (final concentration 3.3 mm). The extent of nitration was determined spectrophotometrically by measuring absorbance of nitrotyrosyl residues at 428 nm in a Unicam SP-800 double-beam spectrophotometer, using the molar extinction coefficient $E_{428}^{\text{nm}} = 4100 \text{m}^{-1}\text{cm}^{-1}$ (Riordan & Vallee, 1972). During incubation 5 - 10 µl aliquots were withdrawn and diluted with 50 mm-imidazole buffer, pH 6.8, containing 3 mm-MgSO$_4$ and 0.4 m-KCl. The assay was started by the addition of 2-phosphoglycerate (final concentration 1 mm), and changes in absorbance at 240 nm at 25°C were followed.

Dilution of the modified enzyme in the assay medium stopped the reaction between enolase and tetranitromethane, and during about 2 h no further changes in the activity were observed. To control samples, 20 µl of ethanol without tetranitromethane was added.

Molecular weight of enolase before and after chemical modification was determined by chromatography on Sephadex G-150 using 0.05 m-Tris/HCl buffer, pH 7.2, for elution. The column was standardized with 1% solutions of native enolase, glyceraldehyde-3-phosphate dehydrogenase from pig muscle, and bovine serum albumin.

RESULTS AND DISCUSSION

When tetranitromethane was added to a mixture containing human enolase in 0.05 m-Tris/HCl buffer, pH 8.0, an increase in absorbance at 428 nm was observed. The nitration reaction was most rapid in Tris/HCl buffer containing 0.3 m-NaCl (Fig. 1). The rate of nitration was decreased on addition of the substrate, 2-phosphoglycerate, and even more so--of the substrate plus magnesium ions (Table 1). From the total increase in absorbance at 428 nm it was calculated that 1 mol of protein contained
Fig. 1. Time-course of nitration by tetranitromethane of tyrosyl residues of the human muscle enolase, in various buffer solutions. The reaction mixture contained, in a final volume of 2.5 ml, 10 μmol of enzyme and 3 μmol of tetranitromethane in: 0.05 M-Tris-HCl buffer, pH 8.0 (○); Tris/HCl buffer plus 0.3 M-NaCl (□); or 0.05 M-phosphate buffer, pH 8.0 (×). Nitrotyrosine content was calculated from absorbance of the reaction solution at 428 nm, as described in Materials and Methods.

about 20 tyrosyl residues. This value is in agreement with the results of amino acid analysis (unpublished). The same results were obtained also for the enolase isolated from pig muscle.

The dependence of enolase activity on nitration of tyrosyl residues with tetranitromethane is shown in Fig. 2. In Tris/HCl buffer, two kinds of tyrosyl residues could be distinguished: the reaction with tetranitromethane of the first 10 residues caused only an about 20% loss in the enzyme activity. Nitration of further tyrosyl residues led to a nearly complete in-

Table 1

Effect of 2-phosphoglycerate and Mg$^{2+}$ on nitration of tyrosine residues in enolase from human muscle

The enzyme, 1 mg, was nitrated with 3 μmol of tetranitromethane in 2.5 ml of 0.05 M-Tris/HCl buffer, pH 8.0, containing where indicated 1 mM-2-phosphoglycerate with or without 3 mM-magnesium sulphate. The results are expressed as nitrotyrosine residues per molecule of enolase.

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<th>Time (min)</th>
<th>Tris/HCl buffer</th>
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<td>30</td>
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<td>180</td>
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activation of the enzyme. Inactivation of the enzyme in the second phase was lessened on addition of the substrate. Magnesium ions as a sole addition did not change the course of inactivation, but when they were added together with the substrate, enolase retained about 50% of its original activity even when all tyrosyl residues were modified.

When enolase was nitrated in phosphate buffer, the rate of inactivation followed a different course (Fig. 2B). Nitrination of the first two tyrosyl residues had but little effect on the enzymatic activity. Further nitration led to marked inactivation, total on modification of 20 tyrosyl residues per mol of protein. The combined protective effect of the substrate and magnesium ions was more pronounced than in Tris/HCl buffer. The inactivation process was essentially monophasic, and after modification of all 20 tyrosyl residues the activity was decreased only by 40%.

Nitrination of 6 tyrosyl residues of the enolase from human muscle, despite the reduction of the activity by 30%, did not change its pH dependence, showing a sharp maximum at pH 6.8. On nitrination of 12 tyrosyl residues, the pH-dependence curve was flattened and lowered, showing a broad maximum in the pH range from 6 to 8 (Fig. 3).

Chromatography on Sephadex G-150 demonstrated that, in the presence of tetranitromethane, enolase aggregated to tetramers with molecular weight of 180 000 (not shown). This kind of aggregation of protein molecules in the presence of tetranitromethane was also observed by Shifrin & Solis (1972).

Nitrination of either 6 or 12 tyrosyl residues in enolase did not change its affinity to the substrate since the $K_m$ value was the same as for the native enzyme, i.e. $3 \times 10^{-4}$ M.

All 20 tyrosyl residues present in the molecule of enolase from human or pig muscle are accessible to nitration, but the extent of nitration and
Fig. 3. pH-dependence of native enolase (○) and enolase modified by nitrilation of 6 (●) or 12 (×) tyrosyl residues per mol of protein. The conditions were the same as for Fig. 2A.

loss of the activity depend on the kind of buffer used and the presence of the substrate and magnesium ions. It seems that, for total activity of enolase, intactness of all tyrosyl residues is required.

REFERENCES


INAKTYWACJA ENOŁAZY TETRANITROMETANEM

Streszczenie

1. Wszystkich 20 reszt tyrozynowych obecnych w enolazie ludzkiej i wieprzowej ulega nitrowaniu działaniem tetrinitrometanu w łagodnych warunkach; obecność 2-fosfogliceryianu i jonów magnezu obniża szybkość reakcji.
2. Inaktywacja enzymu przebiegala w dwóch etapach: a) nitrowanie 6 - 10 reszt tyrozynowych enolazy obniżało aktywność o ok. 20% bez powodowania zmian w stałej Michaelisa, ciężarze cząsteczkowym i zależności aktywności enzymu od pH; b) po nitrowaniu 12 reszt tyrozynowych inaktywacja była prawie całkowita, jednocześnie zmianie ulegały właściwości enzymu. 2-Fosfogliceryjan i jony magnezu obecne jednocześnie w mieszaninie reakcyjnej powodowały znaczną ochronę przed inaktywacją.
3. Z przytoczonych wyników można wnioskować, że dla pełnej aktywności katalitycznej enolazy wydzielenie z mięśni ludzkich i wieprzowych, reszty tyrozynowe muszą być zachowane w stanie nieznaczonym.

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