THE REACTION OF N-BROMOSUCCINIMIDE WITH ENOLASE

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Ten tryptophan residues per one protein molecule were found to be present in the enolase from human and swine muscle.

In Tris buffer, N-bromosuccinimide (NBS) inactivated the enolases after oxidation of all 10 tryptophan residues. The presence of 2-phosphoglycerate (2-PGA) partially protected the activity, and in the presence of 2-PGA together with Mg^{2+} full protection was observed.

In phosphate buffer, only 6 tryptophan residues could be oxidized, but the enzyme was fully inactivated. 2-PGA made possible the oxidation of all 10 tryptophan residues, concomitant with full inactivation. In either case, Mg^{2+} had no effect.

The $K_m$ values and pH optima were the same for the native and partially NBS-modified enolases.

Although enolase has been studied for many years, there is little information available on the amino acid or peptide sequences involved in its active site (Wold, 1971). The enolase from yeast has been found to lose its activity upon photooxidation in the presence of methylene blue, with simultaneous destruction of 2 - 3 tryptophan and 8 histidine residues out of 5 and 14 residues, respectively, present in one molecule of the enzyme (Brake & Wold, 1960). The enzyme can be inactivated by the more specific photooxidation in the presence of Rose Bengal with the loss of a single residue of histidine (Westhead, 1965).

Experiments on carboxymethylation of yeast enolase led to the conclusion that methionine is a component of the active site (Brake & Wold, 1962). It is of interest that only the combination of Mg^{2+}, an activator of enolase, and substrate, 2-phosphoglycerate, was effective in protecting the enzyme against inactivation caused both by photooxidation and carboxymethylation.

The present work was undertaken to investigate the role of tryptophan residues in the catalytic properties of muscle enolase. It has been suggested that N-bromo-
succinimide\textsuperscript{1} is a highly specific reagent for modifying tryptophan residues in protein (Kirschbaum, 1971; Hirose \textit{et al.}, 1971; Liu & Dunlop, 1974); however, it attacks also other amino acid residues (Kronman \textit{et al.}, 1967; Williams, 1975).

\textbf{MATERIALS AND METHODS}

Enolase (2-phospho-d-glycerate hydratase, EC 4.2.1.11) from human muscle was purified by the procedure of Baranowski \& Wolna (1975), from swine muscle, by the method of Wolna \textit{et al.} (1971) and from rabbit muscle, according to Westhead (1966). \textit{N}-Bromosuccinimide (Schuchardt, München, G.F.R.) was twice recrystallized from acetic acid or deionized water. 2-Phosphoglycerate was obtained by the method described by Meyerhof \& Kiessling (1957). A commercial preparation from B.D.H. (Poole, England) was also used.

Enzyme concentration was calculated using the extinction coefficient of 0.9 at 280 nm as determined for rabbit muscle enolase solution (1 mg/ml; cf Wold, 1971).

The reaction with NBS was examined by adding increasing amounts of the reagent to a known amount of enolase, and measuring the destruction of tryptophan residues and changes in enzyme activity. Portions (5 - 10 \textmu l) of 1 mm-NBS were added successively to 2.5 ml of enolase (about 1 mg) in 0.1 m-Tris or 0.1 m-phosphate buffer. The mixture was cautiously stirred and the extinction at 280 nm was measured against a blank which contained only NBS and buffer. At each point of titration small samples (5 - 10 \textmu l) were withdrawn and the enzyme activity determined. The test mixture contained: 50 mm-imidazole buffer, pH 6.8, 3 mm-magnesium sulphate and 0.4 m-potassium chloride in a total volume of 3.0 ml. The reaction was started by the addition of 2-PGA to a final concentration of 1 mm, and was followed by measuring extinction at 240 nm at 25\textdegree C.

The total tryptophan content of the enzyme was estimated according to Spande \& Witkop (1967) by titration of the enzyme with NBS in 0.1 m-acetate buffer, pH 4.0, containing 4 m-urea. The number of tryptophan residues oxidized per mole of enzyme was calculated from the equation:

\[ n = \frac{-1.31 \times \Delta E_{280}}{5500 \times \text{molarity of enzyme}} \]

where \( n \) = number of tryptophan residues per mole of protein; \( \Delta E \) = decrease in extinction; 5500 = molar extinction coefficient at 280 nm for tryptophan; 1.31 = =empirical factor based upon oxidation of model tryptophan peptides by NBS (Williams, 1975).

For calculation, the following molecular weights were used: for human and swine muscle enolases, 90 000 (Baranowski \& Wolna, 1975; Wolna \textit{et al.}, 1971) and for rabbit muscle enolase, 82 000 (Wold, 1971).

The total tryptophan content was also determined from extinction at 280 and 288 nm of the enzyme at pH 6.5 in 6.0 m-guanidine hydrochloride, according to Edelhoch (1967).

\footnote{Abbreviations used: NBS, \textit{N}-bromosuccinimide; 2-PGA, 2-phosphoglycerate.}
Unicam SP-800 recording spectrophotometer or Unicam SP-500 spectrophotometer were used.

RESULTS AND DISCUSSION

 Determination of tryptophan content. Since the tryptophan content in enolases isolated from human and swine muscle is not known, the number of tryptophan residues per molecule of enzyme was determined. The enolase from rabbit muscle, which is known to contain 10 tryptophan residues (Holt & Wold, 1961), served as a control.

Using the Edelhoch’s procedure it was found that one molecule of enolase, irrespective of its origin (human, swine or rabbit), contains 10 tryptophan residues. The same content of tryptophan was calculated from the NBS titration in 0.1 M-acetate buffer, pH 4.0, in the presence of 4 M-urea. Titration of the enzyme from human muscle is shown in Fig. 1. The enzymes from swine and rabbit muscle gave the same profile.

![Graph](image)

Fig. 1. Titration of tryptophan residues of human muscle enolase with N-bromosuccinimide. Enzyme concentration 4 mM. Buffer: 0.1 M-acetate, pH 4.0 + 4 M-urea (□); 0.1 M-Tris, pH 7.2 (■); 0.1 M-phosphate, pH 6.5: alone (○); with 10 mM-Mg²⁺ (▲); with 1 mM-2-PGA (●); with both 10 mM-Mg²⁺ and 1 mM-2-PGA (△).

In 0.1 M-Tris buffer (without urea added) in the pH range from 6.0 to 7.5, all 10 tryptophan residues of the enzyme could be oxidized. Addition of Mg²⁺ which is an activator of enolase, or substrate, 2-PGA, did not change the results of titration.

However, when the titration of human muscle enolase with NBS was performed in 0.1 M-phosphate buffer in the range of pH 6.0 to 7.5, only 6 tryptophan residues were detected. Further addition of NBS resulted sometimes in formation of turbidity which interfered with determination of tryptophan. Also in the presence of Mg²⁺,
only 6 tryptophan residues were oxidized, but protein did not precipitate on further addition of NBS. The presence of 2-PGA or 2-PGA + Mg²⁺ led to oxidation of all 10 tryptophan residues, but the consumption of NBS was about twice as high as in Tris buffer (Fig. 1). Similar results were obtained for the enolases from swine and rabbit muscle.

As control, the titration of free tryptophan with NBS was performed in both buffers and in the absence and presence of magnesium ions, 2-PGA, or Mg²⁺ and 2-PGA together. No differences were found in the course of titration curve.

*The effect of NBS on the activity of enolase from human, swine and rabbit muscles.* Simultaneously with the NBS titration in Tris and phosphate buffers, the enolase activity was examined.

The oxidation in Tris buffer (Fig. 2) of the first two tryptophan residues had little effect on the activity. Further oxidation resulted in an almost linear inactivation, and on oxidation of 10 tryptophan residues the enzymes were fully inactivated. Human enolase appeared to be somewhat more resistant to the action of NBS than the rabbit and swine muscle enolases. Mg²⁺ did not protect the enzymes against inactivation. In the presence of 1 mM-2-PGA, the inactivation was less pronounced, and after modification of 10 tryptophan residues the rabbit and swine enolases retained about 40% of their activity, and human enolase, 60%. The protecting effect of 2-PGA was enhanced by Mg²⁺ so that the enzyme was fully active as shown for human enolase in Fig. 3, and for the swine enzyme in Table 1.

The action of NBS on enolase activity in phosphate buffer was different from that in Tris buffer. However, the presence of 2-PGA protected the enolase against NBS inactivation in phosphate buffer (Fig. 4), similarly as it did in Tris buffer.

![Fig. 2. Change in the activity of enolases from human, swine and rabbit muscle, caused by oxidation of tryptophan residues by N-bromosuccinimide. The oxidation was performed in 0.1 M-Tris buffer, pH 7.2, and the activity was determined in 0.05 M-imidazole buffer (for details see Methods).](image-url)
Fig. 3. Protection of the human muscle enolase by 2-phosphoglycerate (2-PGA) and 2-PGA + Mg^{2+}, against inactivation by N-bromosuccinimide. The enzyme, 0.5 mg/ml, was titrated with NBS in Tris buffer, pH 7.2, alone (∆); with 1 mM-2-PGA (○); and with 1 mM-2-PGA + 10 mM-Mg^{2+} (●). The activity was determined in imidazole buffer. For details see Methods.

Fig. 4. Protection of the human muscle enolase by 2-phosphoglycerate against inactivation by N-bromosuccinimide, in 0.1 M-phosphate buffer, pH 6.5. The enzyme, 0.5 mg/ml, was titrated with NBS in phosphate buffer alone (∆); with 10 mM-Mg^{2+} (▲); with 1 mM-2-PGA (○); with both 1 mM-2-PGA and 10 mM-Mg^{2+} (●). For details see Methods.

### Table 1

**Effect of Mg^{2+} and 2-phosphoglycerate on the activity of swine muscle enolase during titration with N-bromosuccinimide**

The enzyme at a concentration of 0.5 mg/ml in Tris or phosphate buffer containing the indicated additions was titrated with NBS, then the activity was assayed in imidazole buffer. For details see Methods. The residual activity was calculated as percent of activity of the native enzyme.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Tris buffer</th>
<th>Phosphate buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trp oxidized</td>
<td>residual activity (%)</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Mg^{2+}, 10 mm</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>2-PGA, 1 mm</td>
<td>6</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>2-PGA, 1 mm + Mg^{2+}, 10 mm</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>
When in the former buffer 6 tryptophan residues were modified, the enzyme activity was inhibited by only 20%. 2-PGA enabled modification of all 10 tryptophan residues, concomitant with full inactivation of the enzyme. The addition of Mg^{2+} did not change the effect of 2-PGA.

The effect of tryptophan oxidation on $K_m$ and pH optimum. The enzyme samples used in these experiments were partially modified in Tris buffer. Only 6 tryptophan residues per molecule were oxidized and the residual activity was about 50% that for the native enolases. The Michaelis constants for 2-PGA and pH optima were the same for the native and partially modified enolases from human and swine muscle; $K_m$ was 30 $\mu$M, and pH optimum 6.8.

Concluding remarks

The results of the presented experiments can be summarized as follows. NBS oxidized all 10 tryptophan residues of the molecule of enolase in Tris buffer, but only 6 in phosphate buffer; in both cases, the oxidation caused a loss of the enzyme activity. When the NBS treatment was carried out in the presence of 2-PGA, the extent of inactivation was lower. The enzyme was fully protected against NBS-inactivation by 2-PGA and Mg^{2+} together (in Tris buffer). Mg^{2+} alone did not protect the enzyme. Partial NBS-oxidation of enolase did not change the $K_m$ and pH optimum profile.

At the present time it is difficult to explain all the presented effects. It seems, however, that tryptophan residues in enolase are not directly involved in the catalytic process. They may be essential for the maintenance of suitable ordered structure in the neighbourhood of the enzyme active site, and in the area where, in the presence of substrate, additional two magnesium ions have been bound (Brewer, 1976).

The higher inhibition of enolase activity by NBS in phosphate buffer than in Tris buffer could be due to conformational changes in the enzyme molecule caused by phosphate (Wang & Himoe, 1974).

REFERENCES

Wpływ imidu kwasu N-bromobursztynowego na enolazę

Streszczenie

W enolazie otrzymanej z mięśni ludzkich i wieprzowych stwierdzono obecność 10 reszt tryptofanowych w cząsteczce białka.

W buforze Tris, imid kwazu N-bromobursztynowego hamuje całkowicie aktywność enolazy po utlenieniu wszystkich 10 reszt tryptofanowych. Obecność 2-fosfogliceryianu chroni przed inaktywacją enzym ludzki w 60%, a wieprzowy w 40%. Jony magnezowe nie zabezpieczają enzymu przed inaktywacją, natomiast wspólnie z 2-fosfogliceryianem chronią enzym całkowicie.

W buforze fosforanowym imid kwazu N-bromobursztynowego utlenia tylko 6 reszt tryptofanowych enolazy z jednoczesną utratą aktywności. W obecności 2-fosfogliceryianu utlenianiu ulega 10 reszt tryptofanowych, aktywność enzymatyczna nie jest jednak zachowana.

Wartości stałej Michaelisa i zależność aktywności enzymu od pH dla enolazy rodzimej i po częściowej modyfikacji były takie same.

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