Localization of reactive tyrosine residues of baker's yeast transketolase

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Abstract Baker's yeast transketolase inactivated by tetranitromethane was digested with *Staphylococcus aureus* V8 protease. Four peptides absorbing at 360 nm were isolated by reverse-phase HPLC and sequenced. The modified tyrosines were identified as Tyr-184, Tyr-210 and Tyr-370.

Key words: Transketolase; Chemical modification; Tyrosine; Nitrotyrosine; Proteolysis

1. Introduction

The identity and role of the functional groups of TrK participating in catalysis and TPP binding can now be determined by means of site-directed mutagenesis, based on X-ray structural analysis of holo-TrK crystals [1–4]. However, this approach cannot be used to examine the residues forming the substrate-binding site because the spatial structure of the enzyme is changed drastically upon substrate binding [5] and no stable complex with a substrate analogue, suitable for X-ray analysis, is available. Thus, the use of mutagenesis to study these residues requires their prior identification in the enzyme structure by selective chemical modification.

Tyrosine residues have been shown, by chemical modification, to be necessary for the binding of keto substrates in the active site of TrK [6]. Detailed kinetic analysis of the functional consequences of the modification indicated that only one Tyr per active site is essential for the activity when three-carbon carbohydrates are used as keto substrates. For the conversion of large asymmetric six-carbon carbohydrates, at least two residues are required. In other words, there is a residue important for the binding of large substrates only, not small ones.

The present work was aimed at determining the number and location of the functionally important tyrosine residues in the polypeptide chain of TrK.

2. Materials and methods

2.1. Materials

Transketolase was isolated on an immunosorbent according to Tikhomirova and Kochetov [7], with modifications [8].

Staphylococcus aureus strain V8 protease was kindly provided by Dr. G.G. Tchastukhina (State Institute of Genetics and Selection of Micro-Organisms, Moscow).

Tetranitromethane was obtained from Sigma (USA), GdnCl from Merck (Germany), and 2-mercaptoethanol from Serva (Germany).

2.2. Enzyme activity assay

Enzyme activity was estimated from the rate of NADH oxidation in a coupled system with triosephosphate isomerase and glycerol-3-phosphate dehydrogenase [9].

2.3. Modification of transketolase

TrK (13 μM) was modified with 2.5 mM tetranitromethane in 100 mM Tris-HCl buffer, pH 8, in a total volume of 1 ml for about 40 min, and the reaction was quenched by the addition of a 10-fold excess of 2-mercaptoethanol. The residual activity of TrK was 10–15% of its initial value.

2.4. S. aureus V8 protease digestion and HPLC separation of peptides

The modified and native TrK was dialyzed overnight against 50 ml of 8 M urea containing 2% (by volume) 2-mercaptoethanol. The urea used had been purified by activated carbon and acid treatment [10]. Then 1% (by volume) 4-vinylpyridine was added. The samples were incubated with 4-vinylpyridine for 2 h and then dialyzed for 12–16 h against 50 mM potassium phosphate buffer, pH 7.8, with 2 mM EDTA and digested with V8 protease (1:50, w/w, protease/protein) for 8 h at 36°C. The protease was added in two equal portions: at zero time and in 4 h. The digestion was stopped by immersing the tube into boiling water for 1 min. The tubes were kept in liquid nitrogen until the samples were chromatographed.

The peptides were separated by HPLC on a 4.5×250 mm reverse-phase Nucleosil C-18 100 column equilibrated with a 0.1% water solution of trifluoroacetic acid using a Beckman model 344 HPLC chromatograph. A 0–60% acetonitrile concentration gradient in a 0.1% water solution of trifluoroacetic acid was used. The elution rate was 1.2 ml/min, and the gradient change time was 70 min. Absorbance was monitored simultaneously at 220 and 360 nm (the peak of nitrotyrosine absorbance is at 360 nm). Each of the 360-nm-absorbing peptides was dried, dissolved in 50–100 μl of 5 M guanidine hydrochloride and reinjected on an Ultraspher ODS column (4.6×250 mm, 5 μm). The separation was performed using a 15–75% linear gradient of acetonitrile for 60 min at a flow rate of 1.2 ml/min. The purified peptides were used for sequence determination.

2.5. Sequencing

The peptides were sequenced using an Applied Biosystems Model 816 (Knauer) gas-phase sequencer equipped with an online 120A PTH analyzer.

3. Results

The elution profile of the peptides derived from the nitroTrK digested with V8 protease is shown in Fig. 1. The number of absorbance peaks at 220 nm (lower curve) was about 70, in fair agreement with the presence of 68 carboxyl residues in the TrK amino acid sequence [11]. Only four peaks, marked as 1–4, also absorbed light at 360 nm (upper curve). The separation profile for native transketolase (not shown) was similar, but the upper curve had no peak.

The nitrated peptides were rechromatographed and used for sequencing. A series of chromatograms at the first seven cycles of degradation of peptide 3 is presented in Fig. 2. At the fourth cycle of the degradation two large peaks were observed. The smaller one had the elution time of PTH-tyrosine. The larger one was eluted between PTH-phenylalanine and PTH-isoleucine and could not be identified by comparison with the elution profile of a standard mixture of PTH-amino acids. It was found (not shown) that the latter peak coincided

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Abbreviations: TrK, transketolase; PTH, phenylthiohydantoin.

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with that of PTH-NO_2-tyrosine and could be thus identified as a PTH-derivative of nitrotyrosine.

The sequence chromatograms for the other three peptides (not shown) were similar. All the four peptides were identified within the primer structure of transketolase: 1, Val-369–Glu-375 (VYNQLPE); 2, Asp-368–Leu-373 (DVYNQL...); 3, Ile-181–Gln-187 (IAIYDDN...); 4, Ala-209–Glu-213 (AYGWE).

Therefore, three tyrosine residues in TrK accessible to nitration are Y184, Y210 and Y370.

4. Discussion

Theoretically, 26 tyrosine, 17 histidine and 6 tryptophan residues of transketolase can be nitrated under the action of TNM. However, only three tyrosine residues were found to react with TNM under the conditions chosen. This fact agrees with previous kinetic data on the specificity of the modification and the nature and amount of the essential residues involved [8].

A comparison of the primary structures of TrK from different sources showed that two of the three Tyr residues are located in relatively conserved regions. Tyr-184 was found in *Escherichia coli* and *Hansenula polymorpha* TrK and in yeast TrK 1 and 2; Tyr-210 was found in *E. coli* TrK and yeast Trk 1 and 2 [12]. According to the X-ray data for holoenzyme, Tyr-184 is situated in the vicinity of the co-factor-binding site [1], presumably, in the substrate-binding site.

Correlation of the extent of Tyr modification and enzyme activity by the method of Tsou revealed different involvement of Tyr residues in catalysis with small and large substrates [6]. It was shown that only one Tyr residue is involved in the conversion of small substrates, whereas two residues are involved in the conversion of large substrates. Clearly, at least two of the three Tyr residues, reacting with tetranitromethane, can be critical for activity and interaction with keto substrates.

The data presented in this paper can be used as a guide for site-directed mutagenesis of Tyr residues in order to gain insight into their roles in the active site of TrK.

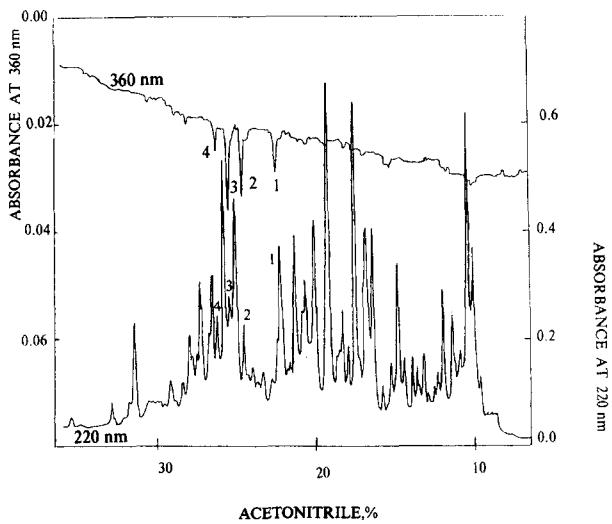

Fig. 1. Reverse-phase HPLC separation of nitrotransketolase digest. The effluent was monitored at both 360 (upper curve) and 220 nm (lower curve). The peaks isolated for further work are numbered.

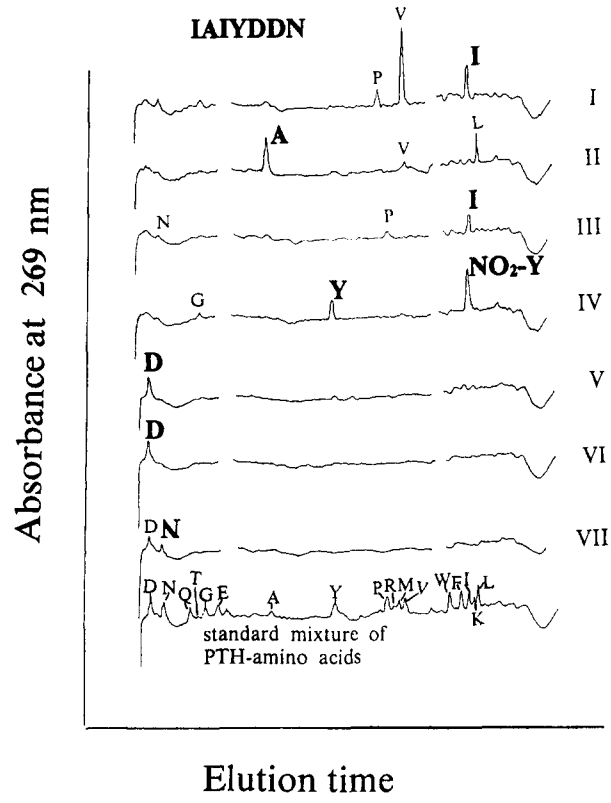

Fig. 2. The first seven sequencing cycles of peptide 3. The lower sequence curve is the elution profile of a standard mixture of PTH-amino acids. Amino acids composing sequence IAIYDDN, which coincides with the region of transketolase Ile-181–Gln-187, are denoted by bold letters.

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