THE TRYPTOPHANASE FROM *PROTEUS RETTGERI*: SULFHYDRYL GROUPS IN THE HOLO- AND APO-ENZYME PREPARATIONS

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1. Introduction

Tryptophanase is a pyridoxal phosphate (PLP) dependent enzyme which catalyzes a series of α,β-elimination and β-replacement reactions of L-tryptophan and some β-substituted amino acids [1–3]. The enzyme was first crystallized as the apoenzyme by Newton and Snell [4] from a constitutive mutant of *Escherichia coli*. Subsequently, we crystallized an inducible apotryptophanase from *Proteus rettgeri* [5,6]. Since these preparations of apotryptophanase are relatively unstable [7], many attempts have been made to crystallize the stable holoenzyme. We found that high concentrations of ammonium sulfate plus glycerol stabilize the enzyme against denaturation which occurs during the crystallization step and on long storage at a low temperature. We have subsequently prepared the stable crystalline holoenzyme by crystallizing the enzyme in the presence of glycerol and PLP [8]. This holoenzyme is much more stable, showing about 4 times the specific activity, than that reconstituted from the crystalline apoenzyme and PLP. Glycerol may protect the enzyme against changes in the native conformation of the protein that might be caused by long storage in the cold and by crystallization with ammonium sulfate [1].

To clarify the conformational differences in the crystalline holotryptophanase prepared in the presence of glycerol and the crystalline apoenzyme, as well as the holoenzyme reconstituted from the apoenzyme and PLP, the sulfhydryl groups of these enzyme preparations were investigated using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).

2. Materials and methods

Sephadex G-25 was purchased from Pharmacia. PLP was kindly provided by The Dainippon Pharmaceutical Company. DTNB was purchased from Nakarai Chemicals Ltd., Kyoto. All other chemicals used were commercial products.

The crystalline apotryptophanase was prepared from a cell extract of *Proteus rettgeri* grown in a tryptophan-supplemented medium as previously described [5]. Reconstitution of the apoenzyme with PLP was carried out as follows. The apoenzyme was dissolved in a minimum amount of 0.1 M potassium phosphate buffer, pH 8.0, containing 1 mM mercaptoethanol and 0.1 mM PLP, then it was dialyzed at 4°C for 48 hr against the same buffer. To remove excess free PLP, the dialyzed enzyme solution was passed through a column (1 × 10 cm) of Sephadex G-25 previously equilibrated with 0.1 M potassium phosphate buffer, pH 8.0, containing 1 mM mercaptoethanol. The crystalline holoenzyme was prepared in the presence of glycerol and PLP as previously described [8] and stored at 4°C in 0.1 M potassium phosphate buffer, pH 8.0, containing 60% saturated ammonium sulfate, 20% (v/v) glycerol, 10 mM mercaptoethanol and 0.1 mM PLP. Before use, the enzyme was dialyzed and freed from excess PLP by the procedure described above.

Tryptophanase activity was determined by measuring the amount of pyruvate formed from L-tryptophan under conditions described previously [5]. Protein determination was performed by measuring the absorbance at 280 nm as previously described [6].

Sulfhydryl groups of the enzyme were determined

with DTNB according to the procedure of Ellman [9]. The tryptophanase preparations were freed from mercaptoethanol by passing them through a column (1 X 10 cm) of Sephadex G-25 previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, containing 2 mM EDTA. The titration of sulfhydryl groups was started by the addition of 20 μl of 10 mM DTNB solution in 0.05 M potassium phosphate buffer, pH 7.5. The reaction was followed by measuring the absorbance at 412 nm with a Beckman DB-G spectrophotometer. The number of sulfhydryl groups which reacted was calculated from a molecular extinction coefficient (ϵ_{412} = 13 600 M^{-1} cm^{-1}) of the reduction product of DTNB [9]. The enzyme preparations were also titrated for their total sulfhydryl groups after the denaturation and reduction of disulfide linkages in the proteins. For this purpose, 16.0 mg of each enzyme preparation was incubated at pH 10.5 in 1.0 ml of a solution of 6 M guanidine hydrochloride, 50 mM EDTA and 50 mM dithiothreitol buffered with 0.1 M potassium bicarbonate. Incubation was carried out at 37°C for 2 hr. The mixture was then passed through a Sephadex G-25 column (1 X 10 cm) previously equilibrated at pH 5 with a solution containing 6 M guanidine hydrochloride, 20 mM potassium acetate and 50 mM EDTA. The resulting dithiothreitol free protein fraction was titrated with DTNB immediately after the separation.

3. Results and discussion

The crystalline holotryptophanase reacts slowly with DTNB, and the reaction stops when approx. 4 mol of sulfhydryl groups per mole of the enzyme are titrated (fig.1, A). However, enzyme activity is not decreased by this modification of the sulfhydryl groups (fig.1, B). The apoenzyme and the holoenzyme, which was reconstituted from the apoenzyme and PLP, react with DTNB more rapidly than does the crystalline holoenzyme. Approximately 8 mol of sulfhydryl groups per mol of the enzyme are titrated in these cases, and the activities decrease in parallel with the progress of the modification of the sulfhydryl groups (fig.1, A and B). The extent of inactivation is 86% with the apoenzyme and 60% with the reconstituted holoenzyme. The additional 4 free sulfhydryl groups, which are masked in the

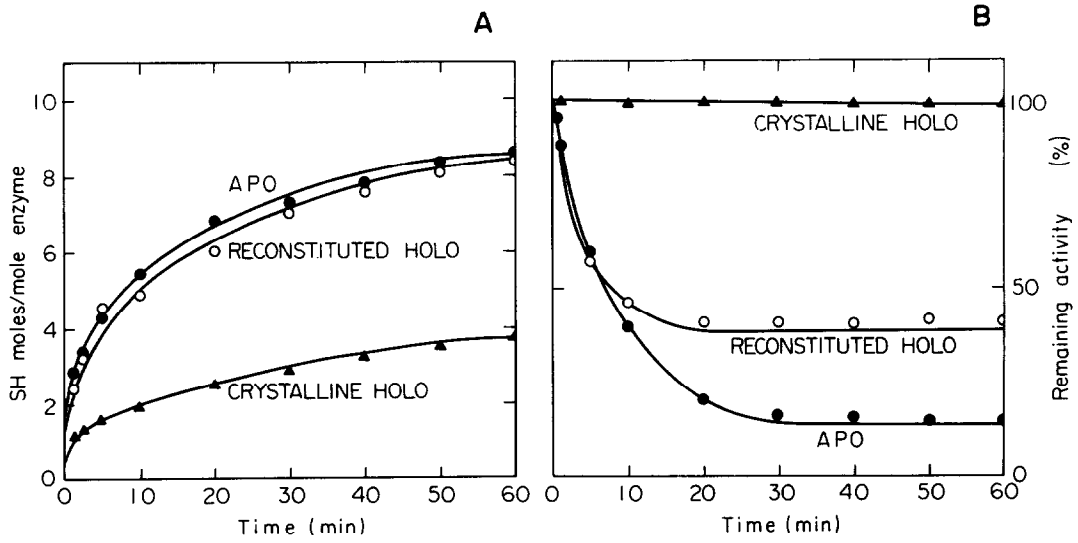

Fig.1. The rate and extent of the reaction of the sulfhydryl groups of tryptophanases with DTNB (A); and the effect of DTNB on enzyme activity (B). APO, crystalline apoenzyme; Crystalline Holo, crystalline holoenzyme prepared in the presence of glycerol; Reconstituted Holo, holoenzyme reconstituted from the apoenzyme. Reaction conditions are described in the text. In fig.1A, each reaction mixture contained 1.36 mg per ml of enzyme. In fig.1B, after the incubation of each enzyme with DTNB, five μl of the reaction mixture was diluted and the enzyme activity was assayed.

crystalline holoenzyme and do not react with DTNB, seem to be much more essential* for tryptophanase activity.

When the crystalline holoenzyme, apoenzyme and reconstituted holoenzyme are denatured and reduced with 6 M guanidine hydrochloride and dithiothreitol, all react rapidly with DTNB and 24 mol of sulfhydryl groups are titrated per mol of the enzyme. This shows that 20 and 16 sulfhydryl groups are masked in the holoenzyme and apoenzyme, respectively, and that these sulfhydryl groups are newly exposed by treating the enzymes with guanidine hydrochloride and dithiothreitol. The total number of sulfhydryl groups are consistent with the previous results of amino acid analysis of the enzyme carried out after performic acid oxidation [10].

The tryptophanase of *Proteus rettgeri* has been shown [10] to be composed of four identical subunits with mol. wts of 55 000. One mol of PLP is bound per subunit. Amino acid analysis showed that each subunit contains 6 half-cystine residues. The present study shows that, of these 6 half-cystine residues, one free sulfhydryl group is titrated by DTNB with the crystalline holoenzyme; and that two sulfhydryl groups are titrated with the apoenzyme and the reconstituted holoenzyme. Only in the latter case, does the enzyme activity decrease parallel with the titration. These results suggest that the essential sulfhydryl group of the enzyme is in a protective environment in the glycerol-stabilized crystalline holoenzyme preparation and that it is not modified by DTNB. Conformational change in the enzyme protein may occur during the crystallization step of the apoenzyme. By this change, the sulfhydryl group is exposed in the apoenzyme preparation and is easily modified with DTNB. The conformational change may be irreversible and is not reversed by reconstitution of the apoenzyme with PLP.

Further investigation of the conformational differences in these tryptophanase preparations from *Proteus rettgeri* is in progress in our laboratory.

* Since the enzyme activity is not fully inhibited by the modification, it is not clear whether the effect of modification is limited to interference with a specific catalytic or substrate-binding step. The inhibition might be due to a conformational change at the active center or to the presence of a bulky group at the active center.

References

[1] Newton, W. A., Morino, Y. and Snell, E. E. (1965) J. Biol. Chem. 240, 1211–1218.
[2] Hoch, S. O. and DeMoss, R. D. (1973) J. Bacteriol. 114, 341–350.
[3] Yoshida, H., Kumagai, H. and Yamada, H. (1974) Agr. Biol. Chem. 38, 463–464.
[4] Newton, W. A. and Snell, E. E. (1964) Proc. Natl. Acad. Sci. USA 51, 382–389.
[5] Yoshida, H., Utagawa, T., Kumagai, H. and Yamada, H. (1974) Agr. Biol. Chem. 38, 2065–2072.
[6] Yoshida, H., Kumagai, H. and Yamada, H. (1974) Agr. Biol. Chem. 38, 2073–2079.
[7] Raibaud, O. and Goldberg, M. E. (1973) J. Biol. Chem. 248, 3451–3455.
[8] Yoshida, H., Kumagai, H. and Yamada, H. (1974) FEBS Lett. 48, 56–59.
[9] Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[10] Yoshida, H., Kumagai, H., Yamada, H. and Matsubara, H. Biochim. Biophys. Acta, in press.