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OZONIZATION OF THE TRYPTOPHYL RESIDUE IN TRYPTOPHANASE

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Tryptophanase of *Escherichia coli* was inactivated by ozonization in aqueous solution in a time-dependent fashion following pseudo-first order kinetics. Upon ozonization of the apoenzyme, the absorption peak of the tryptophyl residue at 280 nm gradually decreased concomitant with an appearance of a new peak at 320 nm indicating conversion of the tryptophyl residue to N'-formylkynurenine. The spectrophotometric titration of the coenzyme binding to the enzyme protein at 430 nm indicated that the dissociation constant for the coenzyme was almost 100 times increased upon ozonization presumably by weakening the interaction between the coenzyme and the indole moiety of the tryptophyl residue in the enzyme protein.

Tryptophanase purified and crystallized from *Escherichia coli* B/lt7-A (1) has a molecular weight of 220,000 (2) and is composed of four identical subunits. Extensive studies by Snell and his coworkers revealed that the sulfhydryl (3) and arginyl (4,5) residues, in addition to the lysyl residue, are essential for the catalytic function and that the enzyme reaction proceeds through α- and β-elimination reactions in this order to yield indole, pyruvate, and ammonia (6). In this communication we wish to report that the tryptophyl residue of the enzyme is essential for the activity presumably contributing to the interaction between the coenzyme and the enzyme protein.

MATERIALS AND METHODS

Materials ——— *Escherichia coli* B/lt7-A strain was kindly provided from Dr. E.E. Snell. Crystalline tryptophanase was prepared from the bacterial cells by a modification of the procedure described by Kagamiyama *et al.* (1). The apoenzyme was prepared according to the method of Morino and Snell using DL-penicillamine (2). All amino acids were products of Kyowa Hakko Kogyo (Tokyo). All other chemicals were of reagent grade commercially available.

Assay of Enzyme Activity ——— The activity of tryptophanase was routinely determined as described by Newton and Snell (7) based on the

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colorimetric measurement of indole at 570 nm with *p*-dimethylaminobenzaldehyde. One unit of the enzyme was defined as the amount which produced one μmol of indole per min at 37°C.

Ozonization of the Enzyme ——— Ozonization was carried out according to the method of Kuroda *et al.* (8). Prior to ozonization, apotryptophanase was gel-filtered through a Sephadex G-50 column (1.0 x 20 cm), which had been equilibrated with 0.1 M potassium phosphate buffer, pH 7.7. The desalted enzyme preparation (0.6 mg of protein per ml) was treated at 0-4°C with a slow stream of ozone (0.1-0.2 μmol/min, 0.001-0.002% O_3/O_2; Ozonizer, Nihon Ozone Co., Ltd.). The concentration of ozone was estimated by titration with KI-$Na_2S_2O_3$.

Other Determinations ——— All spectrophotometric determinations were carried out in a Hitachi 124 automatic recording spectrophotometer. Measurements of absorption spectra and spectrophotometric titration were carried out in a Hitachi 323 automatic recording spectrophotometer. These two spectrophotometers were equipped with a constant temperature cell housing. Protein concentration was determined either by measurement of the optical density at 278 nm, taking $E_{1\,cm}^{1\%}$ as 7.95 (6) or by the method of Lowry *et al.* (9) using bovine serum albumin as a standard.

RESULTS

When apotryptophanase was subjected to ozonization at pH 7.7, a time-dependent inactivation occurred following pseudo-first order kinetics. When the enzyme was treated with oxygen instead of ozone under the same conditions, no appreciable inactivation was observed. In contrast to the apoenzyme, holotryptophanase was quite resistant to ozonization under the experimental conditions. These results are shown in Fig. 1.

The progress of the oxidation reaction was followed by monitoring the spectral changes of the enzyme protein due to both the decrease in the indole chromophore of the tryptophyl residue at 280 nm and the concomitant formation of the *O*-formylaminobenzoyl group which absorbed at 320 nm. The results shown in Fig. 2 indicated that the tryptophyl residue in tryptophanase was readily converted to N'-formylkynurenine upon ozonization.

As Snell and his coworkers elucidated (6,10), tryptophanase exhibits a marked absorption maximum at about 510 nm upon addition of either L-alanine or L-ethionine. They attributed this chromophore to an intermediate formed by the α-proton elimination reaction. In an attempt to elucidate the role of the tryptophyl residue in the tryptophanase reaction, the ab-

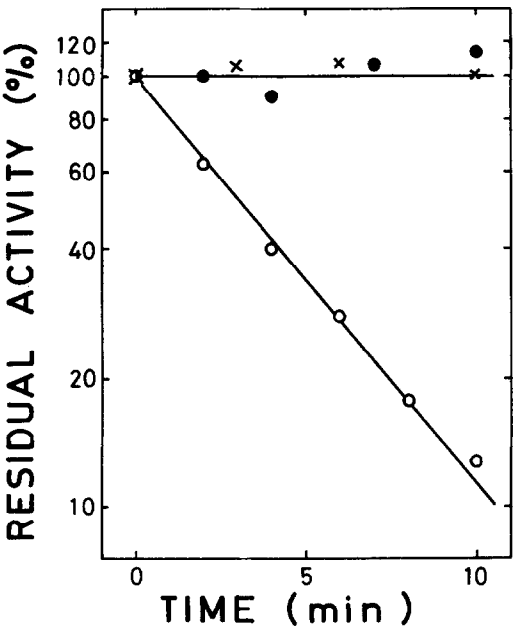

Fig. 1. Inactivation of tryptophanase by ozonization. Tryptophanase (1.8 mg of protein) was ozonized at 0°C as described in MATERIALS AND METHODS in 3 ml of 0.1 M potassium phosphate buffer, pH 7.7 at an ozone concentration of 0.2 μmol/min. Small aliquots were withdrawn at designated time intervals and the residual activity was assayed under the standard assay conditions (7). —o—, ozonized apotryptophanase; —●—, ozonized holotryptophanase; —x—, oxygen-bubbled apoenzyme.

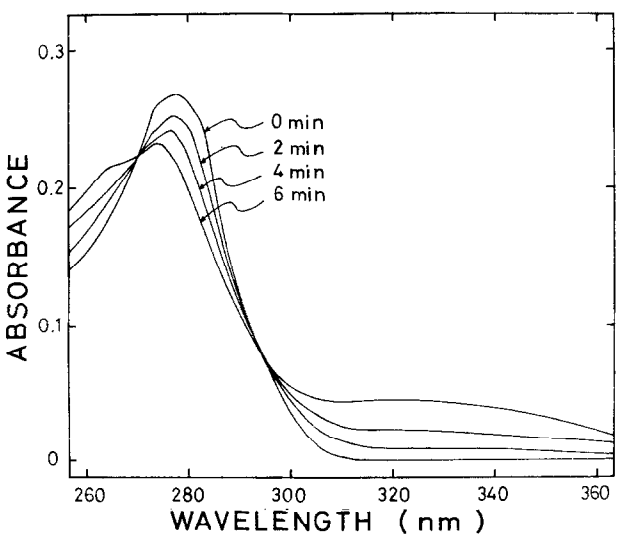

Fig. 2. Changes in the ultraviolet absorption spectrum of apotryptophanase during ozonization. The ozonization of tryptophanase was carried out at 0°C as described in MATERIALS AND METHODS. The reaction mixture contained apotryptophanase (2.5 mg of protein) and 0.1 M potassium phosphate buffer, pH 7.7 in a total volume of 6 ml. At designated time intervals the absorption spectra were measured at 25°C.

sorption spectrum of the ozonized tryptophanase was measured upon addition of ethionine after incubation of the enzyme with a large excess of pyridoxal phosphate. Although the 510-band was detected upon addition of DL-ethionine, further examination of the data revealed that the intensity of the 510-band was proportional to the amount of the remaining unmodified (active) enzyme species, and not due to the ozonized enzyme preparation (data not shown). The result is compatible with the possibility that the tryptophyl residue in the enzyme is essential for the reaction steps prior to the β-elimination reaction.

The apparent dissociation constant (*Kd*) for pyridoxal phosphate was next determined before and after the ozonization by spectrophotometric titration of the Schiff base formation at 430 nm. For the experiments, the sample cuvette contained either 0.54 mg protein of the native or 1.9 mg protein of ozonized apoenzyme in 0.1 M potassium phosphate buffer, pH 7.7 in a total volume of 2.5 ml. After incubation of the enzyme solution for 20 min, the absorption change at 430 nm was measured against the reference solution which contained pyridoxal phosphate at the same concentration as that in the sample cuvette. From the Scatchard type plots (11), 0.8 mol of pyridoxal phosphate was estimated to bind to mol of both the native and ozonized enzyme subunits. Furthermore, the *Kd* value of the native enzyme for the coenzyme was estimated to be 0.18 μM, whereas that of the ozonized enzyme preparation having 46% residual activity was increased to 16.8 μM. These results are shown in Fig. 3.

DISCUSSION

In spite of a considerable number of reports describing the essential requirement of the tryptophyl residue in various enzymes, little information is available as to how the tryptophyl residue is involved in the enzyme function. In the present work is described that the tryptophyl residue in tryptophanase is essential for the activity and that the indole

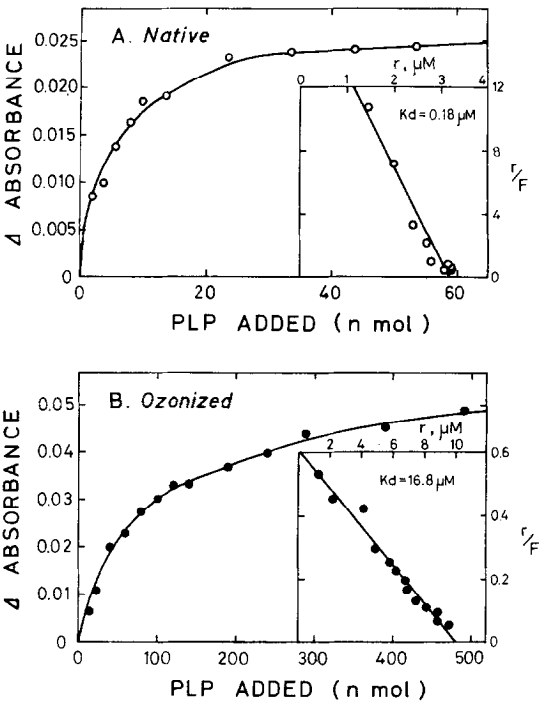

Fig. 3. Determination of *Kd* values for pyridoxal phosphate. The values for pyridoxal phosphate of the native and ozonized apotryptophanase preparations were determined by spectrophotometric titration at 430 nm by a modification of the method of Kazarinoff and Snell (4). The *inset* shows Scatchard type plots of the data. A, native apotryptophanase (0.54 mg of protein); B, ozonized apotryptophanase (1.9 mg of protein; residual activity, 46%). The *Kd* values were calculated from the slope of the lines. r, concentration of pyridoxal phosphate bound to the enzyme; F, concentration of unbound pyridoxal phosphate. Total volume, 2.5 ml.

moiety of the amino acid residue is critical for binding of pyridoxal phosphate. Our preliminary experiments indicated that one of two tryptophyl residues per subunit of the enzyme was oxidized to N'-formylkynurenine concomitant with the inactivation and that no amino acid other than tryptophan was modified to an appreciable extent as examined by amino acid analysis. As far as tested, no appreciable change in the quaternary structure of the enzyme occurred upon ozonization, as examined by molecular sieve chromatography and disc gel electrophoresis. The results of the spectrophotometric titration experiments strongly suggest that the indole moiety of the tryptophyl residue locates in a close vicinity of

pyridoxal phosphate. This possibility is compatible with the observation of Isom and DeMoss that excitation of holotryptophanase from *Bacillus alvei* at 280 nm yielded two distinct peaks, one at 350 nm due to tryptophan fluorescence and one at 500 nm reflecting protein bound pyridoxal phosphate excited by way of an energy transfer from tryptophan (12).

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REFERENCES

1. Kagamiyama, H., Wada, H., Matsubara, H., and Snell, E.E. (1972) J. Biol. Chem. 247, 1571-1575
2. Morino, Y. and Snell, E.E. (1967) J. Biol. Chem. 242, 5591-5601
3. Morino, Y. and Snell, E.E. (1967) J. Biol. Chem. 242, 5602-5610
4. Kazarinoff, M.N. and Snell, E.E. (1977) J. Biol. Chem. 252, 7598-7602
5. Kazarinoff, M.N. and Snell, E.E. (1977) Fed. Proc. 36, 2890
6. Morino, Y. and Snell, E.E. (1967) J. Biol. Chem. 242, 2800-2809
7. Newton, W.A. and Snell, E.E. (1962) Proc. Natl. Acad. Sci. U.S. 48, 1431-1439
8. Kuroda, M., Sakiyama, F., and Narita, K. (1975) J. Biochem. 78, 641-651
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
10. Watanabe, T. and Snell, E.E. (1977) J. Biochem. 82, 733-745
11. Scatchard, G., Coleman, J.S., and Shen, A.L. (1957) J. Am. Chem. Soc. 79, 12-20
12. Isom, H.C. and DeMoss, R.D. (1975) Biochemistry 14,4291-4297