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CHARACTERIZATION OF THE REACTIVITY OF SULPHYDRYL GROUPS IN TRYPTOPHANASE BY A DUAL-MONITORING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM WITH A SITE-DIRECTED FLUORESCENT REAGENT

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SUMMARY

Sulphydryl groups of *E. coli* tryptophanase (L-tryptphan indole lyase, E.C. 4.1.99.1) were made to react with a fluorescent maleimide derivative, N-(4-anilino-1-naphthyl)maleimide (ANM). By carefully controlling the reaction conditions it was possible to limit the extent of sulphydryl group modification. The modified enzyme was digested with (L-1-tosylamide-2-phenylethyl chloromethyl ketone)–trypsin. The fluorescent peptides obtained were analysed by reversed-phase high-performance liquid chromatography on a C_{18} column with a dual-monitoring system consisting of a UV and a fluorescence monitor connected in tandem. This was followed by the determination of the amino acid composition of the fluorescent peptides. Comparison of these results with the known, complete primary structure of tryptophanase from the K-12 strain of *E. coli* allowed the assignment of position 298 to the cysteine residue, which is more selectively modified by ANM under the conditions chosen and is involved in the maintenance of the catalytic activity.

INTRODUCTION

Tryptophanase from *E. coli* is a tetrameric enzyme, composed of four identical subunits, having a molecular weight of 55 000. The enzyme binds four pyridoxal 5'-phosphate (PLP) molecules per molecule of native tetrameric enzyme. Interaction of the enzyme with the cofactor, association–dissociation behaviour and catalytic activity are highly dependent on temperature, ionic environment and pH[1]. Chemical studies of tryptophanase have dealt with the role of several amino acids involved in regulating the catalytic activity, such as lysyl[2], sulphydryl[3,4], arginyl[5], histidyl[6], tryptophyl[11] and tyrosyl[7] residues.

In this work we have investigated further the role of cysteinyl residues in tryp-

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tophanase with the help of a fluorescent derivatizing reagent. Derivatization was followed by high-performance liquid chromatographic (HPLC) analysis of the peptides, produced by limited tryptic hydrolysis, and by the determination of their amino acid composition. The position in the polypeptide sequence of the most reactive cysteine residue was also assigned.

EXPERIMENTAL

Materials

E. coli strain B/1t7-A was kindly provided by E. E. Snell (Department of Biochemistry, Berkeley, CA, U.S.A.). Homogeneous tryptophanase was prepared by a modification of the procedure described by Kagamiyama *et al.*[2]. The apoenzyme was prepared by removing bound PLP, using DL-penicillamine, as described by Morino and Snell[8]. The enzyme preparations used in this work (specific activity: 45 units/mg protein) were homogeneous, as judged by polyacrylamide gel electrophoresis.

N-(4-Anilino-1-naphthyl)maleimide (ANM) and N-(7-dimethylamino-4-methylcoumarinyl)maleimide (DACM) were obtained from Nakarai Chemical (Kyoto, Japan). (L-1-Tosylamide-2-phenylethyl chloromethyl ketone)–trypsin (TPCK–trypsin) was prepared by the method of Wang and Carpenter[9].

Modification of tryptophanase with ANM

The enzyme was fully reduced prior to all experiments by incubation in 10 m*M* dithiothreitol (DTT) at 30°C for 30 min, followed by rapid gel filtration through a Sephadex G-50 column, equilibrated with 0.1 *M* potassium phosphate buffer (pH 7.7) containing 1 m*M* EDTA. After reaction with ANM, the modified enzyme was eluted through a Sephadex G-50 column, equilibrated with the buffer used for the subsequent tryptic digestion.

Tryptic digestion of the enzyme and amino acid analysis

Tryptophanase preparations (1 mg of protein each) were digested with TPCK–trypsin, as described elsewhere[10]. The amino acid composition was determined with a Kyowa Seimitsu (Tokyo, Japan) K-101-AS automatic amino acid analyser. Prior to the analysis, fractionated peptide samples were hydrolysed in 5.7 *M* hydrochloric acid at 110°C for 24 h.

HPLC analysis

HPLC analysis of peptides was carried out with a Spectra-Physics (San Jose, CA, U.S.A.) SP 8700 solvent delivery system, using a C_{18} reversed-phase column (250 × 8 mm I.D.). The column, prepared from 5 μm Develosil (surface area 330 m^2/g) (Nomura Chemical, Seto, Japan) by a modification of the method of Tanaka *et al.*[10], was kindly provided by Dr. Nobuo Tanaka (Kyoto Institute of Technology, Kyoto, Japan). Peptides were detected with a Toyo Soda (Yamaguchi, Japan) UV-8 absorbance monitor and a Shimadzu (Kyoto, Japan) RF-530 fluorescence monitor, connected in tandem. The gradient system was composed of acetonitrile (solvent A) and 5 m*M* potassium phosphate buffer (pH 7.4) (solvent B). Elution was performed by linearly increasing the concentration of solvent A from 0% to 80% in 80 min. The flow-rate was 1.0 ml/min. The fluorescent peaks thus obtained were further fraction-

ated using a gradient system composed of acetonitrile (solvent A) and 0.1% of heptafluorobutyric acid (HFBA) (solvent B). The concentration of solvent A was increased linearly from 1% to 80% in 80 min. The flow-rate was 0.7 ml/min.

Other determinations

All spectrophotometric determinations were carried out in a Hitachi 220 or a Hitachi 124 spectrophotometer (Tokyo, Japan). Tryptophanase concentration was determined using the molar absorptivity, $E_{1\,cm}^{1\%} = 7.95$ at 278 nm[12].

Enzyme assay

Enzyme activity was measured as described by Suelter *et al.*[13], using S-*o*-nitrophenylcysteine as substrate. Prior to enzyme assay, pre-incubation of the apoenzyme was always carried out in 0.12 *M* potassium phosphate buffer (pH 7.8)–3 m*M* DTT–10% (v/v) glycerol–60 μM PLP at 37°C for 10 min. After cooling the incubate to 30°C, 5 m*M* S-*o*-nitrophenyl cysteine (140 μl) was added to 2 ml of the solution containing the reconstituted enzyme and the absorbance change was monitored at 370 nm. One enzyme unit was defined as the amount catalysing the formation of 1 μmol of the product per minute under the conditions of the assay.

RESULTS

Modification of sulphydryl groups of tryptophanase with ANM

ANM is an N-maleimide derivative that becomes fluorescent on reaction with a sulphydryl group-containing substance (Fig. 1). A stable adduct is formed immediately on reaction with an SH group, and no additional treatment is needed to convert it into a more stable product, as in the case of modification with DACM.

The apoenzyme form of tryptophanase was made to react with ANM at 30°C (pH 7.7) for 75 s, the reaction being stopped by the addition of 2 μl of 10 m*M* DTT. The modified enzyme was digested with TPCK–trypsin at 37°C for 12 h, as described under Experimental. The tryptic digests were fractionated by reversed-phase HPLC on the C_{18} column as described. Peptides were monitored by their absorbance at 220 nm and by their fluorescence ($\lambda_{ex} = 355$ nm, $\lambda_{em} = 448$ nm). As shown in Fig. 2, two main fluorescent peaks, I and II, were obtained.

Peak I was further purified by reversed-phase HPLC with a linear gradient of 0.1% HFBA in acetonitrile as the eluent. As HFBA is more hydrophobic than TFA, a higher recovery of the modified peptides is expected. In this instance, peak I was separated into three peaks, Ia, Ib and Ic.

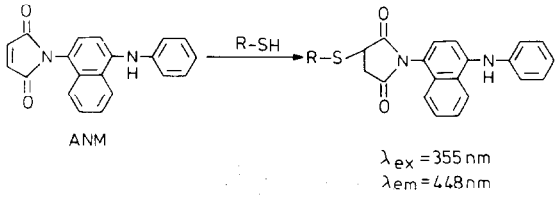

Fig. 1. Reaction scheme of ANM with sulphydryl group-containing compounds.

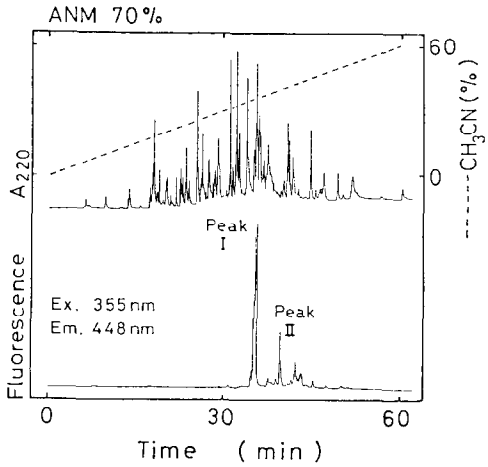

Fig. 2. HPLC patterns of trypsin digests of ANM-labelled enzyme. Column: Develosil (5 μm) (250 × 8 mm I.D.). The sample was a tryptic digest (33 μg) of ANM-modified apoenzyme (final residual activity 70%). The gradient system was composed of solvent A (acetonitrile) and solvent B (5 m*M* potassium phosphate buffer, pH 7.4). Elution was performed by linearly increasing the concentration of solvent A from 0% to 80% in 80 min. Temperature, 35°C; flow-rate, 1 ml/min. Peptide detection was carried out by monitoring the absorbance at 220 nm (top pattern) and the fluorescence at 448 nm (λ_{ex} = 355 nm) (bottom pattern).

TABLE I

AMINO ACID COMPOSITION OF ANM-LABELLED PEPTIDES Ia, Ib AND Ic

The amino acid ratio was calculated based on the value for arginine = 1.0. The numbers in parentheses indicate the amino acid composition of the peptide from Thr-296 to Arg-317. Tryptophan and cysteine could not be detected by the method used.

Component	*Peak Ia*	*Peak Ib*	*Peak Ic*
Asx	0.5 (0)	0.7 (0)	0.5 (0)
Thr	2.1 (2)	2.2 (2)	2.1 (2)
Ser	0.2 (0)	0.0 (0)	0.0 (0)
Glx	4.7 (4)	4.8 (4)	4.8 (4)
Pro	1.1 (1)	1.1 (1)	1.1 (1)
Gly	6.7 (5)	6.7 (5)	6.7 (5)
Ala	1.6 (1)	2.0 (1)	1.6 (1)
Val	2.2 (2)	2.4 (2)	2.3 (2)
Met	1.1 (1)	1.2 (1)	1.0 (1)
Ile	0.2 (0)	0.0 (0)	0.0 (0)
Leu	2.2 (2)	2.9 (2)	2.6 (2)
Tyr	1.3 (1)	0.9 (1)	1.4 (1)
Phe	1.2 (1)	1.0 (1)	1.0 (1)
Lys	0.2 (0)	0.0 (0)	0.0 (0)
His	0.0 (0)	0.0 (0)	0.0 (0)
Arg	1.0 (1)	1.0 (1)	1.0 (1)
Trp	— (0)	— (0)	— (0)
Cys	— (1)	— (1)	— (1)

296 298
Thr-Leu-Cys-Val-Val-Gln-Glu-Gly-Phe-Pro-Thr-Tyr-Gly-Gly-

317
Leu-Glu-Gly-Gly-Ala-Met-Glu-Arg

Fig. 3. A peptide possibly corresponding to peak I. Cys-298 is the one considered to be essential for the activity.

Each peptide was subjected to acid hydrolysis and amino acid analysis. The results are shown in Table I. It can be seen that, within experimental error, all three peaks have the same amino acid composition and can therefore be considered to be due to the same peptide. By comparison with the known primary structure of tryptophanase from the K-12 strain[14], this peptide may be presumed to be the tryptic peptide from Thr-296 to Arg-317 in the amino acid sequence (Fig. 3). If this assumption is true, Cys-298 is the target of the ANM modification. A similar analysis was carried out on peak II, obtained by HPLC with the phosphate buffer–acetonitrile eluent. When rechromatographed with 0.1% HFBA in acetonitrile, peak II was separated into two fluorescent peaks, IIa and IIb. Each peak was hydrolysed with HCl and subjected to amino acid analysis. As shown in Table II, the peptide in peak IIa had an amino acid composition similar to that of the peptide from Met-128 to Arg-151, present in the sequence of tryptophanase from the K-12 strain. Similarly, the amino acid composition of peak IIb was consistent with the peptide from Ile-335

TABLE II

AMINO ACID COMPOSITION OF ANM-LABELLED PEPTIDES IIa AND IIb

The composition ratio was calculated based on the value for arginine = 1.0. The numbers in parentheses indicate the amino acid composition of peptide from Met-128 to Arg-151 for peak IIa and of peptide from Ile-335 to Lys-366 for peak IIb. Tryptophan and cysteine could not be detected by this method.

Component	*Peak IIa*	*Peak IIb*
Asx	3.2 (2)	3.1 (2)
Thr	2.1 (3)	1.3 (0)
Ser	1.3 (1)	1.6 (0)
Glx	2.1 (0)	4.0 (6)
Pro	1.0 (0)	1.1 (0)
Gly	3.0 (3)	4.7 (5)
Ala	1.8 (0)	3.5 (5)
Val	1.6 (1)	4.6 (5)
Met	0.6 (1)	0.7 (0)
Ile	0.9 (1)	1.8 (2)
Leu	0.8 (1)	2.4 (2)
Tyr	1.1 (1)	ND*(1)
Phe	1.6 (2)	ND*(1)
Lys	0.5 (0)	1.5 (1)
His	0.5 (1)	0.0 (1)
Arg	1.0 (1)	1.0 (0)
Trp	— (0)	— (0)
Cys	— (1)	— (1)

* Not detectable because of low signal-to-noise ratio.

to Lys-366, derived from the same sequence. However, some unexpected amino acids were also detected. The reason for this discrepancy is not known at present. The only possibilities envisaged are insufficient tryptic digestion or incomplete peptide purification.

DISCUSSION

As ANM forms a stable fluorescent derivative immediately on reaction with SH groups, tryptic digestion could be readily conducted after a gel filtration step instead of dialysis.

The solvent gradient used for further purification of the fluorescent peptides was 0.1% HFBA in acetonitrile instead of the more commonly used 0.1% TFA in acetonitrile. As HFBA is more hydrophobic than TFA, the yield of the modified peptides was expected to be higher with this system.

In summary, Cys-298, Cys-148 and Cys-352 (numbered according to the K-12 strain tryptophanase sequence) were found to be modified by ANM. Of these, Cys-298 was the most extensively labelled with ANM. Preliminary data also show that Cys-298 was less extensively labelled with ANM when the reaction was carried out on the enzyme that had been previously modified with bromopyruvate, an active-site affinity reagent for sulphydryl groups (data not shown). This indicates that Cys-298 is the essential SH group susceptible to modification at the active site.

The amino acid analysis of peaks IIa and IIb (Table II) gave a composition similar to that of the peptides containing Cys-148 and Cys-352, respectively, as determined from the amino acid sequence of the K-12 strain enzyme. However, as lysine and arginine were also detected, it is possible that these peptides were not extensively digested by trypsin. From the partial amino acid sequence obtained by Kagamiyama *et al.*[15] for the enzyme isolated from the B strain of *E. coli*, it appears that Cys-148 is located in a so far undetermined region of the polypeptide chain. It is possible that in this instance the sequence of the peptide containing Cys-148 is different from the corresponding sequences of the enzyme from the K-12 strain. The accessibility of Cys-148 and Cys-352 to ANM indicates that it is likely that these residues are exposed at the outside of the protein molecule but not directly involved in the catalytic activity.

If it is assumed that the Cys-298 residue is the only sulphydryl group that is essential for the enzymic activity and that its reaction with ANM yields a totally inactive molecule, the number of reacted Cys-298 residues can be determined from the residual activity of the chemically modified enzyme. Using this assumption, it could also be calculated that the recovery of the Cys-298-containing peptide was 35% in the experiments reported above. This value is much higher than that obtained when a similar experiment was carried out with the other fluorescent reagent, DACM (data not shown).

Under the conditions of the second HPLC separation (Fig. 4), peak I yielded three peaks when chromatographed with a gradient 0.1% HFBA in acetonitrile. Such a phenomenon has been reported in other instances. For example, it has been reported that three peaks were obtained with a peptide containing an Asn–Gly bond, owing to β-transition occurring during the limited proteolysis step and to the possible formation of cyclic imido intermediates[16]. In addition, it is known that proline-con-

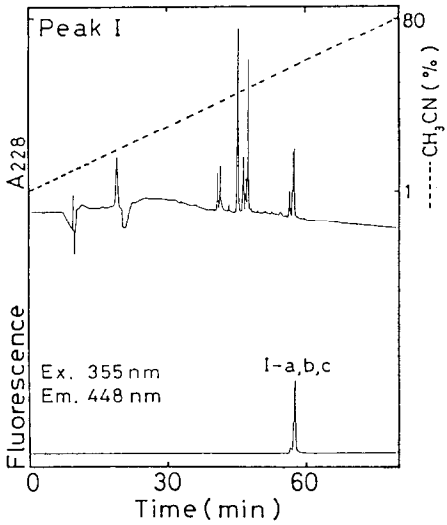

Fig. 4. HPLC patterns of ANM-labelled peptides. The sample was peak I of Fig. 2 (the starting amount of the enzyme was 1.3 mg). The solvent system was composed of solvent A (acetonitrile) and solvent B (0.1% HFBA). Elution was performed by linearly increasing the concentration of solvent A from 1% to 80% in 80 min. Other parameters as in Fig. 2.

taining peptides give two peaks owing to cis–trans isomerization[17]. In fact, peptide I (from Thr-296 to Arg-317) contains proline. It has no Asn–Gly bond, but the possibility cannot be excluded that the ANM molecule present at the modified cysteine residue could be involved in the appearance of multiple peaks.

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