Production of a novel tryptophan analog, β-1-indazole-L-alanine with tryptophan synthase of *Escherichia coli*

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The tryptophan synthase $\alpha_2\beta_2$ complex from *Escherichia coli* has been found to catalyze the β-replacement reaction of L-serine with indazole, an indole analog which has a nitrogen atom at the 2-position (pyrazole ring). The reaction product was isolated and identified as β-indazolealanine by mass spectrometric, elemental and NMR analyses. Careful assignment of ^1H- and ^{13}C-signals with several NMR techniques revealed that the β-carbon of the product alanine moiety was bound to the 1-N-position of the indazole ring. This is the first example of the β-replacement reaction catalyzed by tryptophan synthase occurring at any other position than the 3-position of indole analogs.

Tryptophan synthase *β-1-Indazole-L-alanine* *Enzymatic synthesis*

1. INTRODUCTION

Tryptophan synthase (EC 4.2.1.20) is a unique pyridoxal 5′-phosphate enzyme with a multiple function and occurs in various bacteria, yeasts, fungi and plants. The $\alpha_2\beta_2$ complex of tryptophan synthase of *Escherichia coli* catalyzes not only the physiological β-replacement reaction involving L-serine and indole-3-glycerol phosphate, but also several other reactions [1]. Both the separate subunits alone catalyze their own specific reactions as well. Recently, we reported that *S*-substituted cysteines and *Se*-substituted selenocysteines could be synthesized effectively by the β-replacement reaction of serine and its derivatives with thiols and selenols, respectively [2,3]. In an effort to synthesize new tryptophan analogs, we have surveyed a wide variety of indole analogs for substituent donors in the β-replacement reaction by tryptophan synthase and found that indazole, a pyrazolyl analog of indole, can serve as an efficient substrate [4]. We here describe the isolation of the product from L-serine and indazole and the structural analysis of a new tryptophan analog, β-1-indazole-L-alanine. The structure is entirely unexpected in view of the fact that all the tryptophan analogs so far prepared chemically or enzymatically are confined to β-3-indolealanine derivatives such as 5-hydroxytryptophan [5].

2. MATERIALS AND METHODS

Crystalline $\alpha_2\beta_2$ complex of tryptophan synthase was obtained from cell-free extracts of *E. coli* (trpR$^-$ΔtrpED102/F′ΔtrpED102) [6]. L-Serine was the product of Ajinomoto, Tokyo. The other chemicals were analytical grade reagents.

The β-replacement reaction of L-serine with indazole was carried out at 37°C in the dark in a

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reaction mixture (1.0 ml) containing 100 μmol Tris-HCl buffer (pH 7.8), 40 μmol L-serine, 50 μmol indazole, which was dissolved in methanol beforehand, 180 μmol NaCl, 50 nmol pyridoxal 5'-phosphate and 400 units of tryptophan synthase. Sodium azide (3 mM) was added as an antibacterial agent for prolonged incubation. The formation of β-1-indazolealanine was monitored by a method similar to the spectrophotometric measurement of tryptophan formation [7]. Since β-1-indazolealanine and indazole exhibited different absorption spectra with the maximum difference at 303 nm, the reaction could easily be followed by reading the absorbance change at 303 nm. The production of 0.1 μmol β-1-indazolealanine corresponds to the absorbance change of 0.252. L-Serine was determined fluorometrically with *o*-phthalaldehyde by the method of Benson and Hale [8]. NMR spectra were taken on a Jeol JNM-FC200 spectrometer. The conditions for measurements of nuclear Overhauser effect (NOE) in ^{1}H-NMR were optimized with 4% isovanillin in degassed $CDCl_3$. Mass spectrum was taken on a Jeol JMS-DX300 spectrometer.

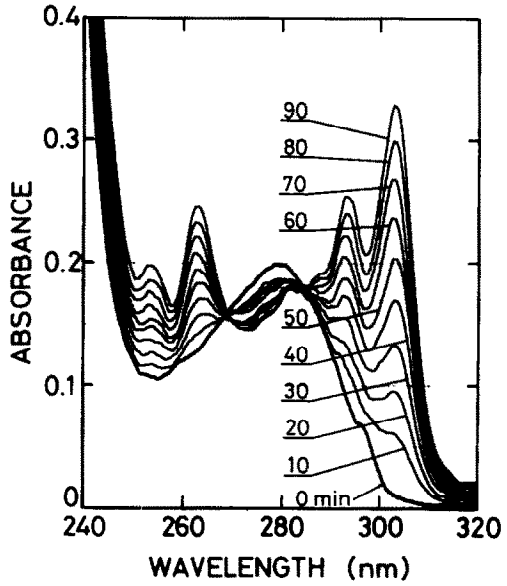


Fig.1. Spectrophotometric monitoring of the β-replacement reaction of L-serine (40 mM) with indazole (0.2 mM) catalyzed by the $\alpha_2\beta_2$complex of tryptophan synthase (190 units). Other conditions were as described in the text. The enzyme was replaced by water in the reference cell. Spectra were recorded at 10 min intervals.

3. RESULTS AND DISCUSSION

When indazole was incubated at 37°C for 2 h with the $\alpha_2\beta_2$ complex of tryptophan synthase in the presence of L-serine, the formation of a new amino acid which reacted with ninhydrin was observed upon thin layer chromatography on a silica gel 60 F_{254} plate (Merck) with *n*-butanol: acetic acid: water (4:1:1) as a solvent. As incubation time was prolonged, the color intensity of ninhydrin spot of the new amino acid ($R_f = 0.51$) increased concomitantly with the decrease in that of L-serine ($R_f = 0.11$), indicating that the reaction progressed further. To determine quantitatively the rate of β-replacement of L-serine with indazole, the absorption spectrum of the reaction mixture was taken at appropriate time intervals (fig.1) because the spectrum change was expected to occur as in the case of β-replacement of L-serine with indole by the $\alpha_2\beta_2$ complex of tryptophan synthase [7]. The maximum change in absorption was observed at 303 nm with a molar difference absorption coefficient ($\Delta\epsilon$) of 2520. The initial rate of the β-replacement reaction with indazole determined by this spectrophotometric method was about 1/350 of the rate of tryptophan synthesis and the apparent K_m value for indazole was 0.52 mM. When the β-replacement reaction with indazole was examined in various buffers, the highest reaction rate (about 1.7-fold higher than the rate at pH 7.5) was observed at around pH 10, which was in contrast to the optimum pH for the reaction of tryptophan synthesis (pH 7.5). Whether the unusually high optimum pH for the reaction with indazole is explained by the solubility or electronic properties of indazole as a substituent substrate remains to be examined.

To isolate and identify the reaction product from L-serine and indazole, a large scale reaction was carried out at 37°C in 50 ml of the reaction mixture. Although indazole, added as a solid, was not completely dissolved initially, it became soluble as the reaction proceeded and a white precipitate, the reaction product, soon appeared. After incubation for 64 h, 1.77 mmol L-serine was consumed and 1.75 mmol of the product (β-1-indazole-L-alanine, see below) was formed with an overall yield of 87.5% based on L-serine. After the residual indazole was extracted from the reaction

mixture with toluene, the aqueous layer was treated with 2% trichloroacetic acid to precipitate the enzyme protein. The supernatant solution was applied on a Dowex 50X8 (H^+ form) column (2.2 × 12 cm). After the column was washed with 120 ml of 0.2 M NH_4Cl to elute the remaining L-serine followed by washing with excess water, the product was eluted with 0.2 M NH_4OH. The eluate was evaporated to dryness at 40°C under reduced pressure. The residue was dissolved in a small volume of hot water and crystallized from water/ethanol: yield, 0.328 g (80%) white granules; m.p. 210–212°C (dec.); UV λ_{max} (in water, pH 6.8) 253 nm (ϵ 3930), 291 (ϵ 4130), 303 (ϵ 2950); mass spectrum, *m/e* 205 (relative intensity, 10) (M^+), 160 (1) ($M - CO_2H$), 131 (100) ($M - CH(NH_2)CO_2H$); IR (KBr) 1610 cm^{-1}, 1670, 2000–3100, and 3400. Analysis – calcid for $C_{10}H_{13}O_3N_3$ as monohydrate: C, 53.81; H, 5.87; N, 18.82. Found: C, 53.37; H, 5.83; N, 18.75. These analytical data suggest that the isolated compound is β-indazolealanine.

The chemical structure of the product, particularly the mode of binding of side chain alanyl moiety to the indazole ring, was studied by ^{1}H-NMR analysis. In addition to proton signals due to the alanyl moiety (at 3.65 ppm, 1H, triplet, α-C-H, and at 4.63 ppm, 2H, doublets of doublet, β-CH_2), 5 proton signals were observed in the aromatic region (fig.2A). By comparing with the ^{1}H-NMR spectrum of indazole (not shown), the singlet signal at 8.08 ppm was found to be due to the C-3-H, 2 doublet signals at 7.56 and 7.80 ppm were due to either of the 2 protons at C-4 and C-7 positions, and 2 triplet signals at 7.22 and 7.48 ppm were due to either of the 2 protons at C-5 and C-6 positions. Since the compound, β-indazolealanine, clearly showed the signal due to the proton at C-3 position, it was suggested that the alanyl moiety was bound to either of the 2 nitrogen atoms of indazole ring, not to the C-3 carbon atom as in tryptophan. We further examined the ^{13}C-NMR spectrum (fig.2B). Thus, 2 signals at 124.6 and 140.9 ppm were assigned to the quaternary carbons at the 3a and 7a positions, and others to methyne carbons at the 3, 4, 5, 6 and 7 positions based on the results obtained by ORD (off-resonance decoupling) and INEPT (insensitive nuclei enhanced by polarization transfer) techniques on ^{13}C-NMR. These 7 carbon atoms in the indazole ring showed almost identical signals with those of authentic indazole, indicating that the ring carbons of β-indazolealanine were little affected by the alanyl moiety. The conclusive assignment of the ^{1}H- and ^{13}C-NMR signals derived from the indazole ring was

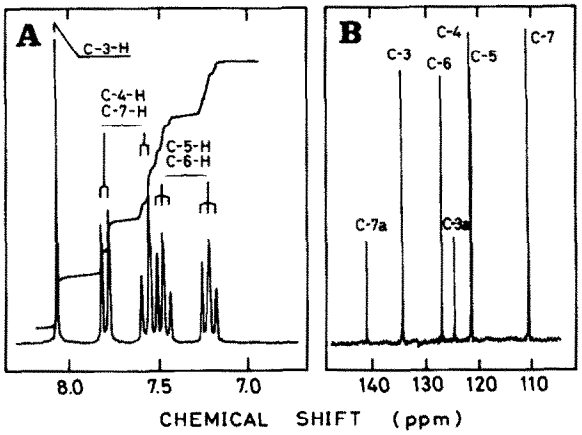
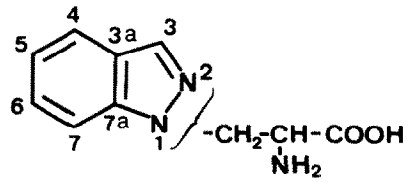


Fig.2. ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of the product from L-serine and indazole. ^{1}H- and ^{13}C-NMR spectra were taken at 2% in D_2O/NaOD (0.07 N) and dimethyl sulfoxide-d_6/$CDCl_3$ (4:1), respectively, with sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 as an internal standard.

Table 1

Assignment of ^{1}H- and ^{13}C-NMR signals derived from the indazole ring of reaction product

Position	Chemical shift (ppm)	
	^{1}H-NMR spectrum	^{13}C-NMR spectrum
3	8.08 s	134.3 d
4	7.80 d	121.6 d
5	7.22 t	121.4 d
6	7.48 t	127.0 d
7	7.56 d	110.3 d
3a		124.6 s
7a		140.9 s



Coupling: s, singlet; d, doublet; t, triplet. Coupling of ^{13}C-NMR signals was determined by ORD analysis

made possible by the selective decoupling method which, by irradiation at each proton signal, revealed the correspondence of each carbon atom to 1 of 5 aromatic protons (table 1). In addition, when the methylene protons on the β-carbon of alanyl moiety were irradiated to measure the NOE in ^{1}H-NMR, the integration value of the C-7-H proton signal (7.56 ppm) increased 15–17%, whereas that of the C-3-H proton signal (8.08 ppm) did not change essentially. This finding indicates that the β-carbon of alanyl moiety is attached to the 1-N-position of indazolyl moiety.

The product, β-1-indazolealanine, was quantitatively decomposed to indazole, pyruvic acid and ammonia by the α,β-elimination reaction catalyzed by tryptophanase (EC 4.1.99.1) purified from *E. coli* B/1t7A. Since tryptophanase acts exclusively on the L-isomer of tryptophan [9], β-1-indazolealanine is probably the L-isomer: its specific optical rotation ($[\alpha]_D^{24}$, 2% in 0.1 N NaOH) was determined to be +6.6° with a Perkin-Elmer 241 recording polarimeter.

Although the enzymatic synthesis of indazolyl analog of tryptophan was briefly mentioned in a previous report [10] dealing with the efficiency of indole analogs as substrates for *E. coli* tryptophan synthase, the chemical structure of the product, which was named 'tryptazan', was not established but interpreted as identical with that of β-3-indazole-DL-alanine synthesized chemically from isatin [11]. Our present report provides a distinct chemical structure of the new enzymatically produced tryptophan analog, β-1-indazole-L-alanine, whose physiological activity is currently under investigation.

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