STUDIES ON TRYPTOPHAN SYNTHETASE ENZYME ACTIVITY AND ITS REGULATION IN *PSEUDOMONAS AERUGINOSA*[1]

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Abstract: Tryptophan synthetase of *Pseudomonas aeruginosa* has a different optimum pH value in different buffer systems. In the phosphate buffer system, the optimum pH value was 7.4 and in tris, 7.9. The maximum reaction velocity was higher in phosphate buffer than in tris.

The reaction velocity was found to be proportional to incubation time over a period of twenty minutes, and to the protein concentration in a range of 0 to 600 μg per tube. The enzyme can carry on the reaction at a very low concentration of coenzyme pyridoxal phosphate (1×10^{-8} M). The Km for serine was 2.0×10^{-3} M and that for indole was 1.25×10^{-5} M. The concentration of serine (3.7×10^{-2} M) used in experiment had reached the saturation point while that of indole (2.5×10^{-4} M) had not.

The enzyme level of tryptophan synthetase was repressed by the addition of tryptophan, indole and anthranilate. At the same concentration (20 μg/ml), indole and anthranilate had a greater repression effect on tryptophan synthetase than tryptophan did.

The four analogues used in the experiment, 5-methyl tryptophan, 6-methyl tryptophan, indolepropionic acid and indoleacrylic acid, did not have any apparent effect on the growth of *Pseudomonas aeruginosa*. However, they showed repression on the enzyme level of tryptophan synthetase when they were added to the growth medium.

INTRODUCTION

The biosynthesis of tryptophan has been carefully studied in *Escherichia coli*, *Salmonella typhimurium* and *Neurospora crassa*, and partially studied in *Pseudomonas putida*. Tryptophan synthetase converts indole and serine to tryptophan, which is the last step in the tryptophan biosynthetic pathway (Crawford & Gunsalus, 1966) Tryptophan synthetase of *E. coli* consists of two protein subunits, A-subunit can convert indoleglycerol phosphate to indole and triose phosphate and B-subunit can convert indole plus serine to tryptophan. Tryptophan synthetase of *P. putida* and *N. crassa* can also carry on these three reactions. In *P. putida* it consists of two protein components (Enatsu *et al.*, 1968) while in *N. crassa* it only has one protein unit (Yanofsky, 1960). According to the studies of Doy (1964), indole can serve as a growth supplement for the tryptophan auxotroph of *P. aeruginosa* and this made us suppose that there were also three reactions carried on by tryptophan synthetase in this organism. However, whether the composition of tryptophan synthetase con-

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sists of one or two units, is not yet known.

The mechanism for the regulation of tryptophan synthetase is varied in different organisms, such as feedback repression in *E. coli* and *S. typhimurium* (Gibson *et al.*, 1969); substrate (indoleglycerol phosphate) induction in *P. putida* (Crawford *et al.*, 1966)and *N. crassa* (Turner *et al.*, 1968); and constant level in *Chromobacterium violaceum* (Wegman *et al.*, 1968). We have been interested in the regulation of tryptophan synthetase in *P. aeruginosa*, therefore we added tryptophan and its metabolites or analogues to growth cultures in order to study the enzyme level and growth effect so as to find out the regulation mechanism of tryptophan synthetase in *P. aeruginosa*.

MATERIALS AND METHODS

Strain: *Pseudomonas aeruginosa* strain 1, obtained from the Department of Microbiology, Oklahoma State University, was used for the studies.

Medium: Vogel and Bonner (1956) minimum medium was used, and 0.2% of filtrated glucose was added as a carbon source while culturing. If solid medium was desired, 1.5% of autoclaved agar was added. Other special chemicals in the medium were sterilized separately and added to the medium as the experiment required.

Growth Rate: Growth rate was calculated according to the equation $K=\log N_t - \log N_0/0.301\times T$ (Stanier *et al.*, 1970). N_0, N_t were the two subsequent measured cell numbers and T was the difference of time.

Crude Extract Preparation and Enzyme Assay: Cells were cultured in minimum medium and/or with indicated amount of supplements until the late log phase. The cells were cellected by centrifugation, washed once with 4°C, 0.1 M phosphate buffer pH 7.0, followed by centrifugation and resuspended in a small amount of 0.1 M phosphate buffer pH 7.8 at 4°C. The following procedures were carried out at 4°C. After disruption by ultrasonic oscillation with a Bronson sonifier, cell debris was removed by centrifugation at 30,000×g for 15 minutes. The enzyme activity was assayed as described by Smith and Yanofsky (1962) in phosphate buffer or in NaCl supplemented tris buffer. One unit of enzyme activity was determined as the consumption of 0.1 μmole indole in twenty minutes at 37°C. Protein concentration was determined by the method of Lowry *et al.* (1951).

Detection of Indoleglycerol Phosphate Accumulation: Indole was released by heating the indoleglycerol phosphate in the crude extract with diluted NaOH and detected as toluene extractable component (Dawson *et al.*, 1969; Turner *et al.*, 1968).

RESULTS

The Effect of Buffer and pH on Enzyme Activity: The activity of tryptophan synthetase was examined in both phosphate and tris buffer. Maximum activity was observed: pH 7.4 for phosphate buffer and pH 7.9 for tris. However the maximum reaction velocity in the latter was only 97% of that in the former (Fig. 1). This phenomenon, higher enzyme activity in phosphate buffer, was the same as that of purified tryptophan synthetase B-subunit of *E. coli* (Yanofsky, 1960). The figure also showed that the dome shape of enzyme activity in phosphate buffer was smoother than that in tris buffer. This suggested that the phosphate buffer was suitable for use in assaying enzyme activity.

The Effect of Incubation Time and Protein Concentration on Enzyme Activity: The reaction velocity was proportional to the incubation time over a period of twenty minutes (Fig. 2), and to the protein concentration in a range of 0 to 600 μg per tube (Fig. 3).

The Effect of Pyridoxal Phosphate Concentration on Enzyme Activity: At very low concentration of coenzyme pyridoxal phosphate (1×10^{-8}M), the enzyme had the reaction velocity of 0.32 units (Table 1). This suggested the concentration of coenzyme (2.5×10^{-5}M) used in the experiment was more than enough.

The Effect of Substrate Concentration on Enzyme Activity: The concentration of substrates serine and indole used in the experiment were 3.7×10^{-2}M and 2.5×10^{-4}M, and the reaction velocity was 0.64 units. At the constant concentration of indole (2.5×10^{-4}M), the Km for serine was 2.0×10^{-3}M, Vmax was 0.66 units (Fig. 4). At the constant concentration of serine (3.7×10^{-2}M), the Km for indole was 1.25×10^{-3}M, and Vmax was 4.0 units (Fig. 5). Obviouly, the concentration of serine used in experiment had reached the saturation point while that of indole had not. The reason why the unsaturation concentration of indole (2.5×10^{-4}M) was used, was that the absorbence of indole was proportional to the concentration of indole in a range of 0 to 2.5×10^{-4}M (Fig. 6).

The Effect of Tryptophan, Indole and Anthranilate on Cell Growth and Enzyme Level of Tryptophan Synthetase: Tryptophan showed feedback repression on tryptophan synthetase (Table 2). The repression increased as the concentration of added tryptophan increased. In *E. coli*, 100 μg/ml each of tyrosine and phenylalanine were added to the tryptophan-containing media in order to reach the maximum repression of the *trp* operon (Morse *et al.*, 1969). In *P. aeruginosa*, whether tyrosine and phenylalanine were added or not, the repression level was the same. Indole, just like tryptophan, when added to the culture repressed the enzyme level, but had no effect on cell growth. Anthranilate, on the contrary, had both effects: growth inhibition and enzyme level repression (Table 2). At the same concentration (20 μg/ml), indole and anthranilate showed greater repression effect on tryptophan synthetase than tryptophan did.

The Effect of Tryptophan Analogues on the cell Growth and the Enzyme Level of Tryptophan Synthetase: The growth inhibition of tryptophan analogues is usually effective for *E. coli* (Lester *et al.*, 1961) or carrot, tobacco cells (Widholm *et al.*, 1972) even in very low concentration. *P. putida* showed unusual resistance to tryptophan analogues (Maurer *et al.*, 1971). Cells of *P. aeruginosa* grew well in glucose minimum medium supplemented with 900 μg/ml of 5-methyl tryptophan or 800 μg/ml of indolepropionic acid or 500 μg/ml of indoleacrylic acid. Only 900 μg/ml of 6-methyl tryptophan showed slight inhibition on cell growth. The four analogues used in this experiment had no apparent effect on cell growth, but all of these four analogues had an effect on enzyme level (Table 3). This phenomenon is quite different from the inhibition of growth usually occurring as the enzyme level is repressed. It is the general assumption that analogues mimic the specific inhibitory or feedback effect of metabolites, however they are unable to replace the metabolites for building block synthesis, so growth ceases. According to this general assumption, the fact that growth was not inhibited while the enzyme level was inhibited, might be due to that in *P. aeruginosa* the amount of tryptophan synthesized under repressed condition was adequate for growth.

Detection of Indoleglycerol Phosphate Accumulation in Crude Extract: The

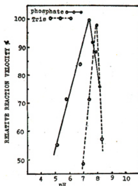

Fig. 1. Optimum pH for tryptophan synthetase enzyme activity. 1 ml reaction mixture contained 37 μmole DL-serine, 0.25 μmole indole, 0.025 μmole pyridoxal phosphate, 6.25 μmole buffer, and 0.46 mg protein extract. Relative reaction velocity was calculated as:

$$\frac{\text{reaction velocity at given pH value}}{\text{reaction velocity at phosphate buffer pH 7.4}}$$

Phosphate buffer pH 7.4 was prepared as 1 ml reaction mixture containing 6.25 μmole phosphate buffer pH 7.8.

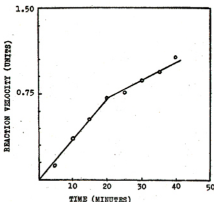

Fig. 2. Enzyme activity vs incubation time. 1 ml reaction mixture contained 37 μmole DL-serine, 0.25 μmole indole, 0.025 μmole pyridoxal phosphate, 6.25 μmole phosphate buffer pH 7.8, and 0.6 mg protein extract. Enzyme activity was determined after incubation at 37°C for five minutes intervals over a period of forty minutes without shaking.

Table 1. Pyridoxal phosphate concentration vs enzyme activity

pyridoxal phosphate concentration	reaction velocity (units)
1.0×10^{-8}M	0.32
2.5×10^{-7}M	0.62
2.5×10^{-5}M	0.63

1 ml reaction mixture contained 37 μmole DL-serine, 0.25 μmole indole, 6.25 μmole phosphate buffer pH 7.8, 0.61 mg protein extract, and indicated pyridoxal phosphate concentration.

Enzyme activity was determined after incubation at 37°C for 20 minutes without shaking.

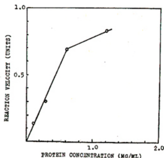

Fig. 3. Enzyme activity vs protein concentration. 1 ml reaction mixture contained 37 μmole DL-serine, 0.25 μmole indole, 0.025 μmole pyridoxal phosphate, 6.25 μmole phosphate buffer pH 7.8, and indicated protein concentration. Enzyme activity was determined after incubation at 37°C for 20 minutes without shaking.

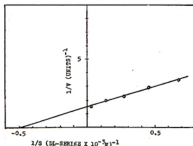

Fig. 4. Lineweaver-Burk plot of 1/v vs 1/s variable substrate, DL-serine. Indole concentration held constant at 2.5×10^{-4} M. 1 ml reaction mixture contained 0.25 μmole indole, 0.025 μmole pyridoxal phosphate, 6.25 μmole phosphate buffer pH 7.8, 0.41 mg protein extract, and indicated DL-serine concentration. Enzyme activity was determined after incubation at 37°C for 20 minutes without shaking.

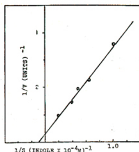

Fig. 5. Lineweaver-Burk plot of 1/v vs 1/s variable substrate, indole. DL-serine concentration held constant at 3.7×10^{-2} M. 1 ml reaction mixture contained 37 μmole DL-serine, 0.025 μmole pyridoxal phosphate, 6.25 μmole phosphate buffer pH 7.8, 0.41 mg protein extract, and indicated indole concentration. Enzyme activity was determined after incubation at 37°C for 20 minutes without shaking.

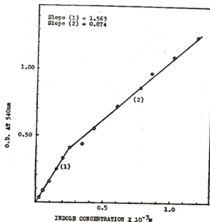

Fig. 6. Indole concentration standard curve. 1 ml enzyme blank reaction mixture contained 37 μmole DL-serine, 0.025 μmole pyridoxal phosphate, 6.25 μmole phosphate buffer pH 7.8, and indicated indole concentration. Indole concentration was determined by toluene extraction method after incubation of enzyme blank reaction mixture at 37°C for 20 minutes without shaking

Table 2. Effect of tryptophan, indole and anthranilate on cell growth and on the tryptophan synthetase enzyme level

growth medium	supplement with (μg/ml)	addition of *phe* & *tyr* (μg/ml)	relative enzyme level %	growth rate*
GMM	0	0	100	0.960
GMM	tryptophan 20	0	88.3	0.911
GMM	tryptophan 100	0	45.0	0.911
GMM	tryptophan 20	100+100	88.0	—
GMM	tryptophan 100	100+100	47.6	—
GMM	indole 20	0	64.0	1.000
GMM	anthranilate 20	0	66.0	0.824

Cells were cultured in glucose minimum medium and/or with indicated amount of supplements and incubated in shaking water bath, 128 cycles/min, at 37°C.
* Growth rate was calculated as generations/hour.

Table 3. Effect of tryptophan analogues on the tryptophan synthetase enzyme level

growth medium	analogues added (ug/ml)	other additional supplements (μg/ml)	relative enzyme level %	growth rate*
GMM	0	0	100	0.960
GMM	5-methyl tryptophan 100	0	78.5	0.977
GMM	5-methyl tryptophan 100	tryptophan 20	78.0	0.939
GMM	5-methyl tryptophan 100	tryptophan 100	35.7	0.938
GMM	5-methyl tryptophan 100	indole 20	53.3	0.962
GMM	6-methyl tryptophan 100	0	85.7	0.881
GMM	6-methyl tryptophan 100	tryptophan 20	72.7	0.914
GMM	6-methyl tryptophan 100	tryptophan 100	34.6	0.955
GMM	6-methyl tryptophan 100	indole 20	48.5	0.938
GMM	indolepropionic acid 20	0	57.9	0.936
GMM	indoleacrylic acid 20	0	43.0	0.917

Cells were cultured in glucose minimum medium and/or with indicated amount of analogues and incubated in shaking water bath, 128 cycles/min, at 37°C.
* Growth rate was calculated as generations/hour

concentration of indoleglycerol phosphate accumulated in cell free extract was nil whether the media contained such supplements as indole, anthranilate, indolepropionic acid and indoleacrylic acid or not.

DISCUSSION

Enzymes in the biosynthetic pathway are usually regulated by feedback inhibi-

tion or repression. The regulation of tryptophan synthetase in *E. coli* is an example. Maximum repression of *trp* operon in *E. coli* was obtained by addition of tyrosine and phenylalanine to the media to suppress the formation of tryptophanase and chorismate dimutase; these two enzymes can interfere with assays of low levels of the *trp* operon-specified enzymes. However, these two amino acids did not affect feedback repression of tryptophan on tryptophan synthetase in *P. aeruginosa* (Table 2). This may be due to the fact that the chorismate mutase in *P. aeruginosa* is not repressed by phenylalanine and tyrosine, like that in *Bacillus subtilis* (Gibson *et al.*, 1969).

That indole could serve as the growth supplement for the growth of the tryptophan auxotroph (Doy, 1964) and reverse the growth inhibition of 6-methyl tryptophan (Table 3) suggested that the repression of indole was due to the conversion of indole to tryptophan. However, that anthranilate showed growth inhibition and repression suggested that the repression of anthranilate might not be due to tryptophan. Gunsalus (1967) found tryptophan synthetase was induced by its substrate, indoleglycerol phosphate, and proposed the pseudofeedback repression. This led us to consider the repression mechanism of anthranilate on tryptophan synthetase from another point of view.

Lester *et al.* (1961) suggested that the formation of tryptophan synthetase in *E. coli* was stimulated by the presence of anthranilate or 3-methyl anthranilate in the medium. This effect could be attributed to the inhibition of the conversion of 1-(0-carboxyl-amino)-deoxyribulose-5-phosphate to indole-3-glycerol phosphate, presumably causing a reduction in the concentration of endogenous tryptophan. The formation of tryptophan synthetase in *N. crassa* was also stimulated by the presence of anthranilate (Turner *et al.*, 1968), but this effect was quite different from that in *E. coli*, and contributed to the accumulation of indole-3-glycerol phosphate. That the presence of anthranilate repressed the enzyme level of tryptophan synthetase in *P. aeruginosa* and did not affect the level of indole-3-glycerol phosphate suggested that anthranilate repressed tryptophan synthetase might be due to the decrease of indoleglycerol phosphate. If a *trp* A^- mutant should be isolated, we would be better able to get more information about the level of indoleglycerol phosphate and trace the relationship between indoleglycerol phosphate and tryptophan synthetase.

Indoleacrylic acid caused accumulation of indoleglycerol phosphate and subsequently stimulated tryptophan synthetase in *N. crassa*, but other indole derivatives did not (Turner *et al.*, 1968). The results of this experiment show that indoleacrylic acid did not cause any accumulation of indoleglycerol phosphate but repressed enzyme level in *P. aeruginosa*, and that indolepropionic acid (a derivative of indole) had the same effect on *P. aeruginosa* as indoleacrylic acid. These phenomena suggest that the analogues can have specific effects on different organisms. So the use of different strains may not be reliable for determining the regulation mechanism of other strains. The truth will only be found by further studies.

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