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IMMUNOCHEMICAL STUDIES ON THE EVOLUTION OF TRYPTOPHANASE AND THE TWO SUBUNITS OF TRYPTOPHAN SYNTHETASE OF *ESCHERICHIA COLI* K 12

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SUMMARY : In order to test if the α and β_2 subunits of tryptophan synthetase and tryptophanase, three proteins involved in the metabolism of tryptophan in *Escherichia coli* K 12, have some common structural features reflecting an evolutionary filiation, an immunochemical comparison of these enzymes has been made using antibodies directed against either the native or the denatured β_2 protein. The lack of cross-reactivity observed in the case of the three proteins studied, even when in their denatured state, suggests that, despite their functional relationships, they probably do not derive from a common ancestor.

INTRODUCTION

During the last decades, lots of efforts have been invested in trying to understand the rules which have directed the evolution of proteins. In the case of proteins endowed with related functions, two conflicting views have prevailed : according to the hypothesis of "divergent" evolution, related proteins would have evolved from a common precursor which, through gene duplication and independent mutations on the two gene copies, would have evolved into differentiated proteins. Alternatively, the two related proteins could be the result of two independent series of evolutionary events each leading to a functional protein.

An interesting hypothesis, derived from the model of divergent evolution has been proposed by Horowitz (1) to account for the emergence of the enzymes involved in a metabolic pathway. According to Horowitz, a given enzyme on a biosynthetic pathway should always be derived by a series of mutations from the enzyme catalyzing the next step on the pathway. Though very attractive and based on apparently plausible assumptions, this model has not been submitted to extensive experimental verifications, mainly for lack of enough data on the amino acid sequences of enzymes involved in successive reactions.

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Recently, a rapid screening method has been developed to detect sequence homologies between proteins. This method is based on the immunological cross-reaction between denatured proteins, and this cross-reaction has been correlated with the existence of an homology between the amino acid sequences of the proteins. When used to compare the aspartokinases-homoserine dehydrogenases I and II from *E. coli* (2) it has clearly shown that these two enzymes are closely related. Similarly, the glyceraldehyde-3-phosphate dehydrogenases from several, evolutionary distant organisms have been shown by this method to exhibit strong homologies (3).

We have therefore undertaken, using this method, to test Horowitz's hypothesis on three enzymes involved in the metabolism of tryptophan in *E. coli* : the α and β_2 subunits of tryptophan synthetase, and tryptophanase. The α subunit of tryptophan synthetase is a monomeric protein, of molecular weight about 30,000 (4) whose amino acid sequence is known (5) and which catalyzes the following reaction (6):

$$\text{Indole glycerolphosphate} + H_2O \rightleftharpoons \text{Indole} + \text{3-phosphoglyceraldehyde} \quad \text{(I)}$$

The β_2 subunit is made of two identical polypeptide chains, of molecular weight 44,000 each (7). It requires for its function the presence of a coenzyme, pyridoxal-5'-phosphate, one molecule of which is bound to each chain (8). It catalyzes the following reaction (9):

$$\text{Indole} + \text{L-serine} \longrightarrow \text{L-tryptophan} + H_2O \quad \text{(II)}$$

Thus, it uses as one of its substrates indole, which is a product of the α chain.

Tryptophanase is made of four identical polypeptide chains of molecular weight 55,000 (10). Again each chain binds a molecule of pyridoxal-5'-phosphate (11). The reaction catalyzed is the following:

$$\text{L-tryptophan} + H_2O \rightleftharpoons \text{Indole} + \text{Pyruvate} + \text{Ammonia}$$

The substrate of what is thought to be the physiological reaction, L-tryptophan, is also the product of the β_2 subunit.

In addition to catalyzing successive reactions, α, β_2 and tryptophanase share one common feature : the three proteins have a specific binding site for indole and use indole as a substrate. Tryptophanase and α use it in their respective reverse reactions, while β_2 uses it in the "forward" reaction.

These three proteins thus appeared functionally related and, if Horowitz's hypothesis were correct, they should bear in their primary structures some common features reflecting their evolutionary filiation. The present paper describes experiments aimed at detecting such common features

MATERIALS AND METHODS:

1. Proteins : Apotryptophanase (E.C. 4.1.99.1) from *E. coli* K 12 (strain 3000) was purified and cristallized as described by O. Raibaud and M.E. Goldberg (12). Its specific activity, measured as described by Suelter *et al.* (13) was 27 IU/mg at 25°C. Disc gel electrophoresis in the presence of 0.1% of sodium dodecyl sulphate revealed that no more than 1% of contaminants were present in the preparation.

The Apo-β_2 subunit of tryptophan synthetase (E.C. 4.2.1.20) was purified from *E. coli* (strain Δtrp ED102/F' trp ED102) as previously described by Högberg-Raibaud and Goldberg (14). The α-subunit of tryptophan synthetase was prepared from the same strain of *E. coli*, as described by Hatanaka *et al.* (6).

Aspartate semi-aldehyde dehydrogenase from *E. coli* K 12 was a generous gift from C. Hirth (Université Louis Pasteur, Strasbourg, France).

Yeast glyceraldehyde-3-phosphate dehydrogenase was obtained from Böehringer-Mannheim and bovine pancreatic ribonuclease A was purchased from Worthington.

2. Denaturation of proteins : The proteins were denatured and alkylated with N-ethylmaleimide or iodoacetic acid as described by Zakin *et al.* (2).

3. Antisera : Antibodies towards native and denatured β_2 subunit of tryptophan synthetase were obtained as described by Zakin *et al.* (2). Antisera used were samples pooled from three rabbits in each case.

4. Immunological assays : Passive hemagglutination and complement fixation were used as immunological assays.

Samples of each protein tested were solubilized in 0.1 ml of distilled water and 2 µl of 2 M sodium hydroxide, and the desired concentrations were obtained by adjusting the volumes with the appropriate buffer.

Passive hemagglutination experiments were carried out according to Herbert (5) using an antigen concentration of 20 µg/ml. Sheep erythrocytes were formalized, tanned and then coated with the antigen dissolved in 0.15 M phosphate buffer, pH 6.4. Antisera dilutions were performed in 0.15 M phosphate buffer, pH 7.2. The results were analyzed after 2-6 hr at room temperature.

For inhibition experiments, the inhibitor was incubated with the antiserum during 1 hr at room temperature and then antigen-coated sheep erythrocytes were added.

All samples were made at least in duplicate.

Complement fixation experiments were performed in isotonic veronal buffer containing 0.5 mM of $MgCl_2$, 0.15 mM of $CaCl_2$ and 0.1% of bovine serum albumine, according to Osler *et al.* (16). For each antigen concentration, two duplicates were made.

RESULTS :

1. Immunological comparison between the β_2 subunit of tryptophan synthetase and tryptophanase : Passive hemagglutination and complement fixation were the immunological methods used to search for a cross-reactivity between native β_2 and tryptophanase. Antibodies against native β_2 were obtained and no significant cross-reaction could be detected as shown in Tables I_A, I_B and II.

2. Immunological comparison between denatured β_2 and tryptophanase : Denatured proteins were obtained by alkylation of their sulfhydril groups in 6 M guanidine with either N-ethylmaleimide or iodoacetic acid. When

TABLE I_A - Passive haemagglutination titer of antiserum directed against native β_2.

	Antiserum against native β_2	Normal rabbit serum
Native β_2	1:20,000	1:9
Native tryptophanase	1:81	1:9

TABLE I_B - Inhibition of passive haemagglutination reaction between β_2 and the homologous antiserum by β_2 and tryptophanase.

Inhibitor	Inhibitor concentration (μM)	Haemagglutination titer
	0	1:20,000
β_2	1	1:81
Tryptophanase	2	1:20,000

N-ethylmaleimidated β_2 (NEM-β_2) was used as the immunogen the rabbits produced antibodies which, at an antiserum dilution of 1:500, gave 97% of complement fixation in the presence of 1.3 nmole of the homologous antigen

TABLE II - Complement fixation of the reaction of antiserum directed against native β_2 with native tryptophanase

Antigen tested	Antigen tested		Antiserum dilution	Complement fixation (%)
	nmole	μg		
β_2	5	0.16	1:1000	95
Tryptophanase	0.05 up to 260	0.004 up to 20	1:100	0

The antigen concentrations and percent complement fixation shown in the table correspond to the conditions where the maximum of complement fixation was observed at the antiserum dilution indicated.

TABLE III - Complement fixation of the reaction of antiserum directed against denatured (alkylated by N-ethylmaleimide) β_2 with denatured proteins.

Antigen tested	Antigen added		Antiserum dilution	Complement fixation (%)
	nmole	µg		
(*) NEM-β_2	1.3	0.02	1:500	97
NEM-tryptophanase	52	1	1:100	92
	52	1	1:200	50
	8	0.16	1:400	8
NEM-aspartate semi-aldehyde-dehydrogenase	752	10	1:100	95
	752	10	1:200	79
	752	10	1:400	28
NEM-glyceraldehyde--3P-dehydrogenase	79	1	1:100	90
	79	1	1:200	51
	13	0.16	1:400	11
CM-(*) β_2	952	15	1:500	84
CM-tryptophanase	1039	20	1:50	38
CM-α	2857	30	1:50	40
CM-aspartate-semi-aldehyde-dehydrogenase	1504	20	1:50	34
CM-ribonuclease A	4082	20	1:50	12

(*) NEM and CM refer to N-ethylmaleimide and carboxymethyl modified proteins.

The antigen concentrations and percent complement fixation shown in the table correspond to the conditions where the maximum of complement fixation was observed at the antiserum dilution indicated.

(NEM-β_2). As shown in Table III, NEM-tryptophanase also reacted significantly with the same antiserum. However, several other unrelated proteins, alkylated with NEM, showed a similar or even higher degree of reactivity (Table III).

The results suggested that the antiserum directed against β_2 alkylated with N-ethylmaleimide contains some antibodies recognizing the alkylating group. To test this possibility, iodoacetate-carboxymethylated tryptophanase, aspartate semi-aldehyde dehydrogenase and ribonuclease A were reacted with anti-NEM-β_2 antiserum. All the carboxymethylated proteins showed a similar very low reactivity with the antiserum directed against the N-ethylmaleimide modified β_2. This indicates that indeed the antiserum analyzed contains antibodies directed against the alkylating groups but also that there is no detectable cross-reactivity between tryptophanase and the β_2 subunit of tryptophan synthetase in their denatured state.

3. Immunological comparison between the denatured α and β_2 subunits of tryptophan synthetase : Complement fixation experiments were used to test the possibility of an immunological cross-reactivity between the two components of tryptophan synthetase of *E. coli*, when denatured. Only the carboxymethylated α-subunit was assayed since the antiserum used, as reported above, reacts unspecifically with NEM-modified proteins.

As indicated in Table III carboxymethylated α showed some reactivity with the antiserum directed against NEM-β_2, since 2857 nmoles of CM-α fixed about 40% complement at a 1:50 dilution of the antiserum, but a similar reactivity is obtained with the unrelated proteins used as controls.

This result shows that there is no significant cross-reactivity between the two subunits of tryptophan synthetase when in their denatured state.

DISCUSSION : The results reported in the present work clearly demonstrate that the denatured α and β chain of the tryptophan synthetase from *E. coli* K 12 do not give rise to an immunological cross-reaction. This can be interpreted, according to Zakin *et al.* (2), as indicating that the two proteins do not exhibit homologies strong enough to allow for the presence of common antigenic determinants in their unfolded state. Thus, though catalysing two successive steps in the biosynthesis of tryptophan, and though both of them carry a binding site for the same ligand (indole) these two proteins do not appear to be closely related to a common evolutionary ancestor.

More suprising is the lack of immunochemical homology between tryptophanase and the β_2 subunit, even when unfolded. Indeed in addition to having binding sites for three common ligand (L-serine, indole and pyridoxal-5'-phosphate) the two proteins have very similar catalytic mechanisms (9, 17). Furthermore, they both require the same activating ions and both catalyze a series of β-replacement and α,β-elimination

reactions like the deamination of L-serine or L-cysteine, the condensation of L-serine to various nucleophilic reagents, and so on... These two enzymes thus appear, on a functional basis to be extremely closely related. Furthermore, the distance separating the structural genes coding for the β chain and for tryptophanase is about one half of the bacterial chromosome, a relative position which is often observed for couples of enzymes likely to have evolved one from the other through gene duplication (18). One would therefore have expected to find close structural homologies between tryptophanase and the β_2 subunit of tryptophan synthetase. Yet, the immunochemical investigations reported here failed to detect any homology between these two proteins either native or unfolded. This observation corroborates the finding that the peptides around the lysyl residue to which pyridoxal-phosphate binds do not show sequence homologies in these two proteins (8, 19).

The results reported in this paper suggest that tryptophanase and the β_2 protein might be a striking example of convergent evolution, and that for these two enzymes as well as for the α subunit of tryptophan synthetase, the evolutionary model of Horowitz does not seem to apply.

However, the validity of these two important conclusions is strongly dependant on the sensitivity of our immunological methods to detect sequence homologies. Indeed, though it has been shown that known sequence homologies between various glyceraldehyde-3-phosphate dehydrogenase (3) can be revealed by an immunological cross-reactivity between the denatured proteins, no attempt has been made to correlate the extents of cross-reactivity and sequence homologies. Furthermore, the minimal sequence homologies required to give rise to a detectable cross-reactivity in the immunological tests used has not yet been determined. Therefore, it will be of considerable interest to compare the amino acid sequences, which should be available in a near future, of the three proteins studied in the present paper, and thus verify the validity of conclusions based at present solely on the immunochemical test.

REFERENCES :

1. Horowitz, N.H. (1945) Proc. Natl. Acad. Sci. U.S., 31, 153-157.
2. Zakin, M.M., Garel, J.R., Dautry-Varsat, A., Cohen, G.N., and Boulot, G. (1978) Biochemistry 17, 4318-4323.
3. Zakin, M.M., Hirth, C., Garel, J.R., and Cohen, G.N., Manuscript submitted for publication.
4. Henning, U., Helinsky, D.R., Chao, F.C., and Yanofsky, C. (1962) J. Biol. Chem., 237, 1523-1530.
5. Guest, J.R., Drapeau, G.R., Carlton, B.C., and Yanofsky, C. (1967) J. Biol. Chem., 242, 5442-5446.
6. Hatanaka, M., White, E.A., Horibata, K., and Crawford, I.P. (1962) Arch. Biochem. Biophys., 97, 596-606.

7. Hathaway, G.M., Kida, S., and Crawford, I.P. (1969) Biochemistry, 8, 989-997.
8. Fluri, R., Jackson, L.E., Lee, W.E., and Crawford, I.P. (1971) J. Biol. Chem., 246, 6620-6624.
9. Miles, E.W., Hatanaka, M., and Crawford, I.P. (1968) Biochemistry 7, 2742-2753.
10. London, J., and Goldberg, M.E. (1972) J. Biol. Chem., 247, 1566-1570.
11. Newton, W.A., Morino, Y., and Snell, E.E. (1965) J. Biol. Chem. 240, 1211-1218.
12. Raibaud, O., and Goldberg, M.E. (1976) J. Biol. Chem., 251, 2814-2819.
13. Suelter, C.H., Wang, J., and Snell, E.E. (1976) FEBS Lett., 66, 230-232.
14. Högberg-Raibaud, A., and Goldberg, M.E. (1977) Biochemistry, 16, 4014-4020.
15. Herbert, W.J. (1967) Handbook of Experimental Immunology (D.M. Weir, ed.), p. 270, Blackwell, Oxford.
16. Osler, A., Srauss, J.H., and Mayer, M. (1952) Am. J. Syph. Gonorrhea, Vener. Dis., 36, 140-153.
17. Morino, Y., and Snell, E.E. (1967) J. Biol. Chem., 242, 5591-5601.
18. Zipkas, D., and Riley, M. (1975) Proc. Natl. Acad. Sci. U.S., 72, 1354-1358.
19. Kagamiyama, H., Morino, Y., and Snell, E.E. (1970) J. Biol. Chem., 245, 2819-2824.