IN VITRO COMPLEMENTATION IN THE TRYPTOPHAN SYNTHETASE SYSTEM OF NEUROSPORA

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When certain allelic mutants of Neurospora are mixed, an enzyme activity which was absent in the individually cultured mutants is restored. This phenomenon is generally called interallelic complementation (or as in the present communication, simply complementation). Such complementation may occur in vivo as the result of heterokaryon formation (Woodward et al., 1958; Fincham, 1959) or in vitro when certain mycelial extracts are mixed (Woodward, 1959).

The present report is concerned with in vitro complementation observed in the tryptophan synthetase system of Neurospora.

MATERIALS AND METHODS

Five mutants were used in the present study. Four of the mutants (td3, td101, td104 and td154) produce altered forms of tryptophan synthetase which can be detected as immunological cross reacting material (CRM) and which are incapable of catalyzing the formation of tryptophan from indole, at an appreciable rate. The other mutant (td48) forms no detectable amount of CRM (Lacy, 1959). Mutants, td3 and td104, form a heterokaryon which produce an active but abnormal enzyme (Suyama, 1962). The other mutants (td48, td101 and td154) do not complement with each other nor with either td3 or td104.

Mutant cultures were grown separately for 2 days at 30°C in Vogel's minimal medium containing 150 μg/ml L-tryptophan. Extracts were made as follows: Lyophilized mycelia were suspended in 15 parts (V/W) of buffer (A) (0.1M potassium phosphate buffer, pH 7.8, 0.01 M EDTA). The result-

ing suspension was stirred for 5 minutes in an ice-cold water bath and centrifuged at 30,000 xg for 20 minutes. The CRM activity in the supernatant was partially purified by fractionation with protamine sulfate followed by ammonium sulfate precipitation (Mohler and Suskind, 1960). The fraction precipitating at 0-45% of ammonium sulfate saturation was collected, dissolved in buffer (B) (0.01M potassium phosphate at pH 7.0, 10^{-5} M pyridoxal phosphate, 10^{-3} M EDTA and 2 x 10^{-3} M DL-serine) and passed through a Sephadex G-25 column previously balanced with the same buffer. The protein fraction was eluted from the column with the same buffer and was centrifuged to remove denatured protein.

Tryptophan synthetase activity was measured radioisotopically by the formation of tryptophan from indole as follows: After incubation of the 1 ml reaction mixture at 37°C for 60 minutes, the reaction was stopped by heating for 2 minutes in a boiling water bath. The reaction mixture was then extracted with 4 ml of toluene to remove free C^{14}-indole. To insure removal of C^{14}-indole, 0.1 ml of 0.005 M carrier indole was added to the reaction mixture and the extraction with toluene was repeated.

Radioactive tryptophan formed during the incubation was assayed as follows. To an appropriate aliquot (0.5 ml) of the toluene-extracted mixture was added 0.5 µmole of C^{12}-tryptophan to serve as a carrier. The tryptophan was then converted to indole by digestion with a 10-fold excess of tryptophanase (DeMoss, 1962). The indole formed was extracted into 4 ml toluene and radioactivity in 1 ml of the toluene was counted in a liquid scintillation spectrometer.

The complete reaction mixture for assay of tryptophan synthetase activity contained 50 µmoles phosphate buffer (pH 7.8), 0.1 µmole pyridoxal phosphate, 50 µmoles L-serine, 0.1 µmole C^{14}-indole (900,000 cpm/µmole) and extracts, in a final volume of 1 ml.

Mixing of extracts were made as follows. Two extracts were mixed in an ice-cold water bath and a volume of this mixture was diluted with an equal volume of buffer (A). For control, the individual extract was

first mixed with buffer (B) and a volume of the extract was further diluted with an equal volume of the buffer (A). These individually diluted extracts were also mixed.

CRM activity was determined according to the procedure of Suskind (1957). One unit of CRM is defined as that amount of material which will neutralize the antibody which would inhibit 1 μmole/hr (1 unit) of the reaction of wild type enzyme. Protein was assayed by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

The extracts of td3 and td104, singly and mixed with each other were treated with buffers as described in the method and their tryptophan synthetase activities were determined (Table I). The mixtures showed significantly higher activity than expected from the individual activities of the two extracts. The extracts mixed before dilution, however, showed a higher increase in activity than that of a mixture made after the extracts were individually diluted. Furthermore, the increase in the activity was proportional to the amount of td104 CRM added up to a ratio of 1:6 for td3: td104 (Figure 1). The maximum observed increase in activity corresponds to 0.0155 μmole tryptophan/hr per unit of td3 CRM and per 7 unts of td104 CRM.

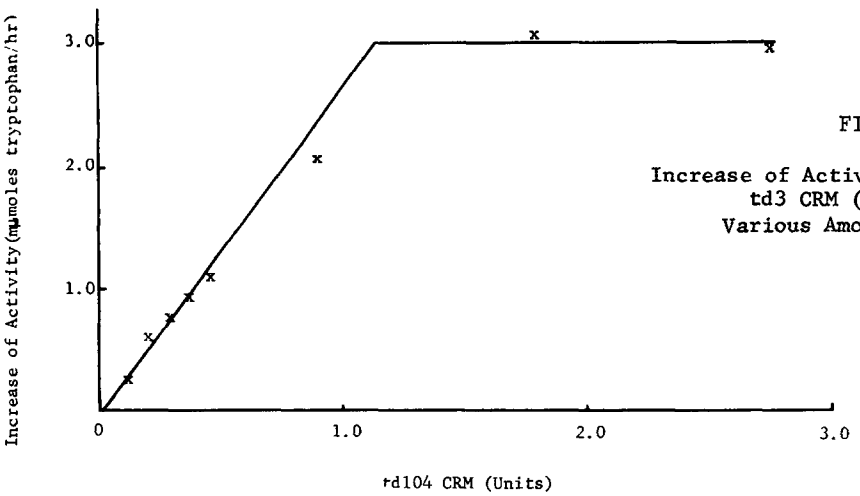

FIGURE I

Increase of Activity in Mixtures of td3 CRM (0.2 unit) with Various Amounts of td104 CRM

TABLE I

TRYPTOPHAN SYNTHETASE ACTIVITIES OF PARTIALLY PURIFIED EXTRACTS FROM TD3 AND TD104, AND MIXTURES OF THESE EXTRACTS

	Protein (mg)		CRM (unit)		Tryptophan Formed (μmole/hr)	Increase (μmole/hr)
	(I)	(II)	(I)	(II)		
td3 (I)	0.36	--	0.71	--	0.0028	
td104 (II)	--	0.30	--	1.0	0.00053	
Mixture (a)	0.36	0.30	0.71	1.0	0.0086	0.0053
Mixture (b)	0.36	0.30	0.71	1.0	0.0054	0.0021

Mixture (a): A mixture of I and II was diluted with buffer (A).

Mixture (b): I and II were individually diluted with the same buffer and mixed.

Since it is known that td3 and td104 are *in vivo* complementing mutants, it is of interest to determine whether the increase in activity is specific to mutant pairs which can complement *in vivo*. Furthermore, it should be demonstrated that the increase is not due to non-specific protein stimulation. For these purposes, two CRM forming mutants (td101 and td154) and a CRM^- mutant (td48) were selected. Extracts of these were combined and tested for increase in activity.

As can be seen in Table II, extracts from td48 and td154 showed no significant stimulation when combined with td3 or td104. On the other hand, a mixture of td3 and td101 CRMs exhibited a significant increase in activity over that expected from activities of individual extracts. Furthermore, the increase per unit of td101 CRM was much higher than that of td104 CRM. These results demonstrate that the stimulatory

TABLE II

ACTIVITIES OF INDIVIDUAL EXTRACTS AND MIXTURES* OF EXTRACTS FROM VARIOUS MUTANTS

CRM I			CRM II			μmoles Tryptophan/hr		
Mutant	CRM Units	(Protein) (mg)	Mutant	CRM Units	(Protein) (mg)	Observed Activity	Expected Activity	Increase in Activity
td3	0.71	(0.36)	td48	0	(0.90)	0.0028	0.0028**	--
td104	0.50	(0.15)	td48	0	(0.90)	0.00030	0.00027	--
td48	0	(0.90)	--			<0.00005	--	
td101	1.00	(0.83)	--			0.00010	--	
td154	0.38	(0.50)	--			0.018	--	
td101	0.50	(0.43)	td154	0.19	(0.25)	0.0095	0.0095	--
td3	0.71	(0.36)	td101	1.0	(0.83)	0.0144	0.0029	0.0115
td3	0.71	(0.36)	td154	0.38	(0.50)	0.021	0.021	--
td104	1.00	(0.30)	td101	0.50	(0.42)	0.00025	0.00050	--
td104	1.00	(0.30)	td154	0.19	(0.25)	0.0093	0.0093	--

*Mixtures made before dilution.

**See Table I for expected activities of td3 and td104 CRMs.

effect was specific to certain pairs of CRMs, and not due to a non-specific protein interaction. It is of interest that td101 and td3 showed this stimulation *in vitro*, since no complementation between them has been demonstrated *in vivo*.

It has been shown earlier (Suyama, 1962) that the tryptophan synthetase formed by the td3-td104 heterokaryon is different from the wild type enzyme in Km values for indole and pyridoxal phosphate and also in heat-decay characteristics. Such results together with the present observations can be interpreted as evidence that interallelic complementation involves an interaction between two defective mutant proteins to yield a hybrid or an active aggregate (see Partridge, 1960). The question whether the stimulation of activity observed in the present experiments results from a direct association between CRMs, remains to be answered.

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