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Rapid degradation of FAD following lysis of Neurospora crassa cells: consequences for evaluation of flavin composition in vivo.

In an attempt to judge the possible participation of free flavins in blue light photoreceotion processes in Neurospora, we determined size and composition of intracellular flavin pools. To minimize artificial liberation of in vivo protein-bound (or associated) flavins during handling we worked with the cell wall-less mutant "slime" (FGSC #1118) which can be easily lysed by osmotic shock.

Cells were cultivated in a gyratory shaker (100 rpm) in darkness at 30° C in 250 ml Erlenmeyer flasks containing 50 ml Vogel's minimal medium (Vogel 1956 Microbial. Genet. Bull. 13: 42-43) with 10% sorbitol and 2% sucrose. Under these conditions maximal cell number is reached after 60 h; thus from the third day on, cultures are in the stationary phase. Cultures 2 to 9 d old were harvested (centrifugation 10 min/l90 g) and lysed with 4 to 8 ml cold double-distilled water. After centrifugation (Beckman Spinco L 50, 120 min/100,000 g), 2-4 ml of the resulting supernatant were run overnight at 5° C on a Sephadex G-50 medium column (1.55 cm, 90 cm length) in 50 mM KH_2PO_4 buffer at pH 7.0: fraction size was 1.8 ml. The protein content was monitored as A_{280} nm and also tested by the Lowry method. The elution profile of flavins was traced by measuring fluorescence emission at 525 nm upon excitation at 466 nm. Fractions containing flavoprotein or free flavin were

pooled separately. Analysis of individual flavins in both pools was performed by phenol-extraction (modified after Yagi, 1962. In Meth. Biochem. Anal. Vol. X, Glick ed.: 319-356). After saturation of samples with ammonium sulfate, flavins were extracted twice with phenol, thenre-extracted into a small volume of water and separated by TLC on Merck silica gel H type 60 with 135 mM Na_2HPO_4 as solvent. Flavin concentrations were determined after alkaline photolysis (Yagi 1962) as lumiflavin fluorescence.

From cell lysates of a cell culture of slime, bound-and free flavins were separated by Sephadex G-50 filtration. A representative fractionation pattern is shown where bound flavins appeared in fractions no. 21-29 and free flavins in fractions no. 58-70 (Fig. 1). The fluorescence of fractions 21-29 coincided with the protein peak and with the major absorbance maximum at 280 nm. A minor protein peak (peptides?) was observed in fractions 53-56 which appeared before the peak of free flavins; the latter was free of protein. The abosrobance at 280 nm in fractions 64-72 is likely due to nonproteinaceous components since there could be no more than 5 µg/ml protein in these fractions as determined by the Lowry method.

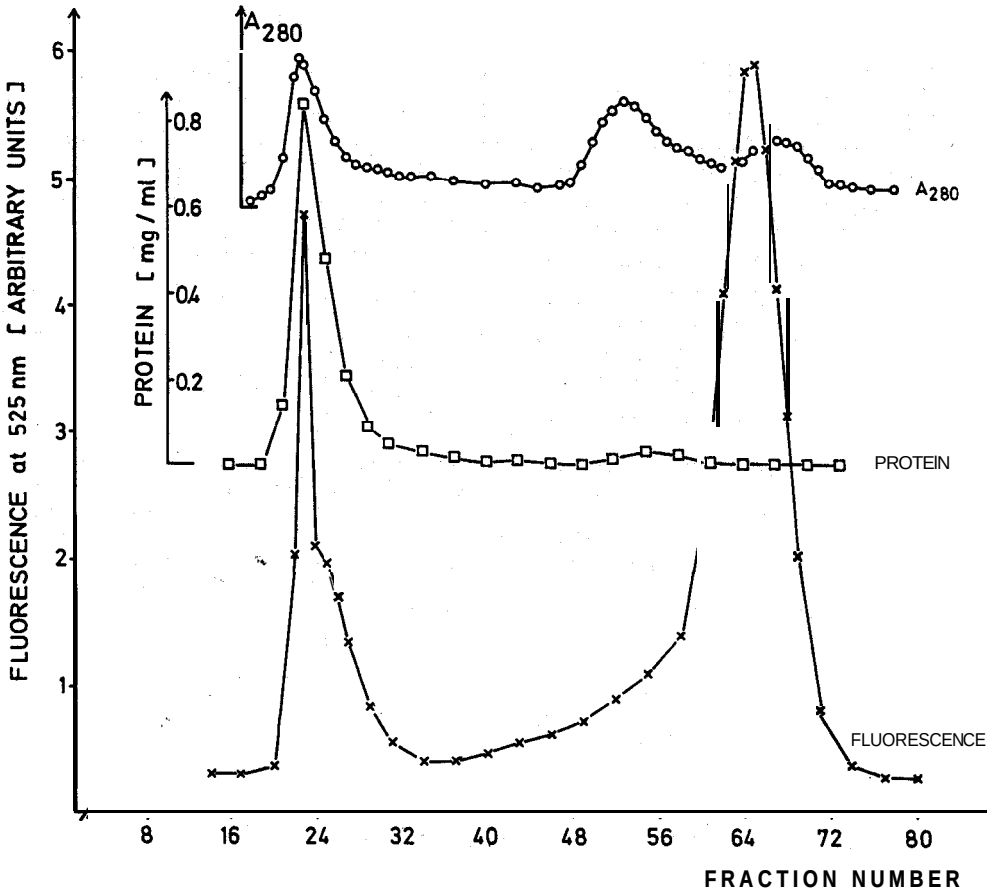

Figure 1.-- 100 ml of 2 d old culture were harvested, lysed osmotically in 4 ml of ice-cold double-distilled water and centrifuged (2 h/100,000 g). 2 ml of the resulting supernatant (6.5 mg of protein) were used for separation of free and bound flavins of Sephadex G-50. Fraction size: 1.8 ml o-o protein monitored by UV-detector, A280 in fraction 23 was 0.18 (pathlength 0.5 cm); ε-ε protein content (Lowry-method); x-x flavin fluorescence at 525 nm.

TLC analysis of free flavins and the flavoprotein fractions after gel filtration of lysates of 2 to 9 d cultures revealed high concentrations of flavin mononucleotide (FMN) and riboflavin (RF) in the free flavin fraction (60 to 40% and 25 to 55% of total flavin present in the fraction, respectively) and about 10% flavin adenine dinucleotide (FAD). In the bound flavin fraction, ll-28% FAD was detected and around 55% and 25% of FMN and RF respectively. 1.0 to 3.3 nmoles of total flavin were put onto the gel.

The unexpectedly low concentration of free FAD in both fractions could have been due to degradation during processing. Adding exogenous FAD to a cell lysate of a 5 d culture and exposing this mixture to our experimental conditions showed fast degradation of the added FAD. Conditions were: a) leaving the mixture for maximally 5 set after addition of FAD at C and heating to 80° C. This resulted in a decrease in the FAD content from 89% of total flavin to 63%; b) incubation of mixtures for 10 min at 5° C before heating: this resulted in a decrease in the FAD concentration to 28%; c) after gel filtration at

5° C overnight on Sephadex G-50 (i.e. under the same conditions as in the experiments mentioned earlier) only 25% of total flavin in the eluate was detected as FAD; the amount of FMN increased correspondingly (Table I).

TABLE I

Degradation of exogenous FAD in a cell lysate

Sample	Treatment	nmoles			%		
		RF	FMN	FAD	RF	FMN	FAD
Controls:							
(1) Lysate		0.8	1.7	1.0	23	48	29
(2) $FAD_{exog.}$		0.8	1.5	39.0	2	4	94
calculated flavin content of mixture		1.6	3.2	40.0	4*	7*	89*
(1) plus (2)	5 sec at RT	4.1	11.7	27.1	10	27	63
(1) plus (2)	10 min at 5°C	7.9	26.1	13.3	17	55	28
(1) plus (2)	G-50 at 5°C overnight	1.9	26.6	9.3	5	70	25

* These numbers result from summarizing the absolute nmoles of flavins in lysate plus $FAD_{exog.}$ (=44.8 nmoles), 1.6 nmoles of which are RF, 3.2 and 40.0 FMN and FAD, respectively.

250 ml 5 d old culture were lysed in 8 ml double-distilled water, pottered by band and centrifuged (20 min/50,000 gf. 2 ml portions of the supernatant were mixed in the cold with 700 µl TLC-purified FAD and treated with conditions a) b) or c) described in the text. Controls: 1) Flavins extracted from 2 ml supernatant with 18 ml of hot water and 2) 700 µl TLC-purified FAD made up to 20 ml with hot water and taken through the entire phenol-extraction procedure.

TABLE II

Heat-lability of FAD-degradation

	Flavin content in nmoles/sample				
	RF	FMN	FU+	FAD	total
Control: FAD added	0.2	1.0	6.0	17.3	24.5
Heat treated sample (15 min at 75°C)	0.5	1.7	1.0	12.9	16.1
Sample without heat-treatment	1.9	8.0	1.1	6.4	17.4

+unidentified flavin component of commercial FAD-preparation

100 ml of 4 d old culture were divided, harvested and the cells lysed with 20 ml double-distilled water for 15 min at room temperature ok at 75° c. The heat-treated sample was cooled to 23° C, 500 µl of unpurified FAD, added to each sample and to a third vial containing 25 ml water. After 15 min at 23° C samples and control were heated for 15 min at 75° C. After cooling and centrifugation of the crude lysates for 20 min/50,000 g, flavins were extracted from the supernatants and the contra1 by the phenol method and separated. The difference of total flavins in the FAD control compared with the heat treated and untreated samples is probably due to loss of flavins during pelleting of membranes and organelles after incubation.

The rapid degradation of FAD in the cell lysate has not been shown to be heat-labile and is likely to be enzyme-catalysed (Table II}. These results can be explained by phosphatase(s) splitting off the adenosine group of FAD. The enzyme(s) is reacting fast even at low temperature and within the shortest possible handling time of a few seconds or minutes. Enzyme activities of this kind must be taken into account before making statements on the composition of intracellular flavin pools in Neurospora or other organisms. Data obtained after standard extraction procedures can be perturbed by enzymatic degradation of FAD and/or FMN after decompartmentation following lysis of the cells and may not represent in vivo conditions. We are investigating the possibilities of specfic inhibition of the FAD degrading reaction(s).

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