THE REQUIREMENT FOR FLAVINE ADENINE DINUCLEOTIDE IN THE FORMATION OF ACETOLACTATE BY SALMONELLA TYPHIMURIUM EXTRACTS[1]

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Acetohydroxy acid synthetase catalyzes the formation of acetolactate and acetohydroxybutyrate, the first and second intermediates in the biosynthesis of valine and isoleucine, respectively (Umbarger and Brown, 1958; Leavitt and Umbarger, 1961). Thiamine pyrophosphate (TPP) and Mg^{++} were early recognized as cofactors in both reactions. In addition, studies with extracts of Escherichia coli revealed the requirement for an unidentified factor which was present in boiled extracts (Leavitt, 1961, 1964). More recently, using Salmonella typhimurium extracts, it was observed that the unknown factor was also required for maximal sensitivity to endproduct inhibition by valine (Bauerle et al. 1964).

In this paper, the isolation of the factor from bakers yeast and its identification as flavine adenine dinucleotide (FAD) are described.

MATERIALS AND METHODS

An enzyme preparation, essentially resolved with respect to the requirement for the unknown factor, was obtained by Dr. R. O. Burns as a by-product during purification of threonine deaminase from an extract

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of S. typhimurium, strain ilva D-8, which had been grown so that the enzymes in the pathways to isoleucine and valine were derepressed (Freundlich et al. 1962). The enzyme was eluted from a DEAE-cellulose column when a KCl concentration of approximately 0.2M had been reached in a linear gradient in the presence of 0.1M potassium phosphate, pH 8.0.

Acetohydroxy acid synthetase activity was determined by measuring the amount of acetolactate formed in the assay described previously (Bauerle et al. 1964). Specific activity is expressed as μmoles of acetolactate formed per mg protein per hour. Protein was determined by the colorimetric method of Lowry et al. (1951).

Since activity was almost completely dependent upon the addition of boiled extract, yeast extract or suitable fractions prepared from them, it was possible to assay for the presence or absence of stimulatory activity at various stages in the isolation procedure.

TPP and FAD were obtained from Sigma Chemical Company. All other chemicals were of reagent grade. Bakers yeast was kindly supplied by Idun-Norex Fabrikker A.S., Oslo, Norway.

RESULTS

Preliminary observations made on boiled extracts of either S. typhimurium or "active dried" yeast indicated that the active component was in the fraction precipitating between 50 and 75% ethanol. The active fraction could be further purified by paper and column chromatographic methods. In each case, the stimulatory activity was accompanied by a yellow color, although the material so obtained was never pure enough to yield a useful spectrum.

In order to obtain a large enough quantity of the active material for additional purification, the 50-75% alcohol insoluble fraction obtained from a water extract (5 minute boiling in 0.75 volumes of water) of 41.7 gm of bakers yeast was applied to a DEAE-Sephadex A-25 column (21x2 cm). Elution was performed with 0.02M phosphate, pH 6.8

and sodium chloride, the concentration of which was increased stepwise: 0.1M (175 ml), 0.4M (190 ml), 0.9M (300 ml). Fractions of 5 ml were collected and read at 260 and 430 mμ. Material absorbing at 260 mμ was found in the fractions centered in tubes 25, 45, 65 and 80. The second of these bands exhibited a strong absorption at 430 mμ and stimulated acetolactate formation. Accordingly, the absorption spectrum of tube 45 was examined and was found to have maxima at 259, 370 and 445 mμ. Since the 250/260 and 280/260 ratios were similar to those of adenosine diphosphate, it appeared that the active fraction contained both flavine and adenine moieties.

Subsequently, a larger quantity of bakers yeast (4 kg) was treated according to the procedure employed by Siliprandi and Bianchi (1955) for the preparative separation of FAD from yeast. However, the final aqueous extract, after removal of the p-cresol, was chromatographed on DEAE-sephadex A-25 using 0.02M phosphate buffer and a gradient created by adding 1M sodium chloride through a 500 ml mixing chamber. After 175 ml were collected in 5 ml fractions, the sodium chloride in the reservoir was increased to 2 M. The O.D. at 260 mμ of each tube was determined and a sample containing 0.01 O.D. unit (O.D. units = optical density x volume) was tested for activity in the acetolactate synthetase assay. Stimulatory activity was confined to tubes 48 through 54. These tubes contained a strongly 260 mμ-absorbing component (peak V). The spectral characteristics of the material in peak V were identical to those of FAD (Table I).

TABLE I

Spectrophotometric properties of FAD and Peak V.

	$\frac{250}{260}$	$\frac{280}{260}$	$\frac{260}{450}$	Max	Min
FAD	0.72	0.51	3.42	264	238
Peak V	0.73	0.53	3.48	264	238

Chromatographic identity of the stimulatory factor and FAD. A sample of the material in Peak V was applied to Whatman No. 1 paper and chromatographed in 5% (w/v) disodium phosphate. A second sample was treated with 0.5 N hydrochloric acid for 5 min. at 100°C. Small samples of FAD and flavine mononucleotide (FMN) were chromatographed simultaneously, and the Rf values of the UV-absorbing areas were compared.

$$\text{Peak V} \quad \frac{\text{Rf hydrolyzed}}{\text{RF unhydrolyzed}} = 1.37$$

$$\text{Controls} \quad \frac{\text{Rf FMN}}{\text{Rf FAD}} = 1.35$$

Comparison of the Cofactor activity of FAD and Peak V. The isolated material and authentic FAD were also compared in an enzymic assay using varying amounts of each. As Table 2 shows, at low concentrations, the isolated material and FAD were equally effective but at a higher concentration it appeared that the isolated material was slightly inhibitory.

TABLE 2

Comparison of FAD and Peak V in the Stimulation of Acetolactate Formation.

Amount added O.D. Units	Moles acetolactate/mg protein/hr. FAD	Peak V
None	3.33	
0.014	13.70	11.45
1.4	16.25	18.85
5.6	18.10	11.45

Each tube contained in 1.0 ml: potassium phosphate pH = 8.0, 100 μmoles; sodium pyruvate, 40 μ moles; 0.10 mg enzyme protein; $MgCl_2$ 10 μmoles; TPP 80 μg and FAD or isolated material as indicated. Incubation, 20 min. at 37°C.

FAD as a Cofactor for acetohydroxy acid synthetase. Having ascertained that the cofactor active material in yeast extract was FAD, it was of interest to compare its activity with that of the other components in the complete system. This comparison is shown in Table 3.

TABLE 3

Cofactor Requirements of Acetohydroxy Acid Synthetase.

Omissions from complete system	μ Moles acetolactate/ mg protein/hr.
None	15.76
FAD, TPP, Mg^{++}	0.49
FAD	1.47
TPP	1.72
Mg^{++}	0.98
TPP, Mg^{++}	0.49

Each tube contained 2μg FAD, where indicated, in 1.0 ml. For other conditions, see Table 2.

The Km for FAD was found to be approximately 2.5×10^{-7}M. It may be of some interest that if all the components of the complete system except FAD were incubated at 37°C for 5 min., the maximal rate was achieved about 1 minute after FAD was added. Thereafter, the reaction proceeds at a steady rate for at least 20 minutes. The addition of TPP and Mg^{++} after preincubation in their absence have also been observed to yield maximal stimulation only after a lag (R. Bauerle, personal communication).

DISCUSSION

The role played by FAD in acetolactate formation is not immediately obvious since the overall reaction, $2\ CH_3 \cdot CO \cdot COOH \rightarrow CH_3 \cdot CO \cdot COH(CH_3) \cdot COOH + CO_2$, does not involve net hydrogen transfer. FAD could be pictured in a cyclic role transiently accepting hydrogen and electrons from an "active acetal" group and releasing them to reduce the resulting acetyl group at a later stage in the reaction. If this were the case, the formation of acetolactate might be quite similar in mechanism to pyruvate oxidation. It should be mentioned that pyruvic oxidase can be forced to form significant amounts of (racemic) acetolactate by using high concentrations of pyruvate (Juni and Heym, 1956). Another possibility that must be considered is that FAD may not participate directly in the catalysis, but may nevertheless be required for the enzyme to achieve a catalytically active state. An analogous paradox is the role of pyridoxal phosphate as a co-

factor for phosphorylase.

The FAD requirement for acetolactate formation is of further interest in view of the fact that the formation of tartronic semialdehyde by glyoxylate carboligase also requires FAD (H. L. Kornberg, personal communication, Gupta and Vennesland 1964). This reaction, which is also TPP-linked, is exactly analogous to acetolactate formation:

$$2\ CHO{\cdot}COOH \rightarrow CHO{\cdot}CH(OH){\cdot}COOH + CO_2$$

Undoubtedly the two reactions occur by quite similar mechanisms and the elucidation of the role of FAD for one reaction will provide an explanation of its role in the other.

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