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Evidence for the participation of FAD in the alanine racemase (EC 5.1.1.1) reaction

OLIVARD, METZLER AND SNELL[1], as a result of their work on the non-enzymic racemization of alanine in the presence of pyridoxal and heavy metals, proposed that during the enzymic racemization the amino acid reacts with the pryidoxal derivative to form a Schiff base which is stabilized by the metal ion. This intermediate, by a simple rearrangement, would destroy the asymmetry of the α-carbon and racemization could occur. Although such an intermediate has not definitely been demonstrated in enzymic reactions, its existence appears to be highly probable. SNELL did not suggest a mechanism for the hydrogen transfer to yield the optically inactive intermediate. The experiments reported here are concerned with this mechanism and thus represent an extension of the earlier postulate.

In our laboratory, enzyme preparations from *Bacillus subtilis* have been purified by cell extraction, ammonium sulfate precipitation, ethanol fractionation, density-gradient sedimentation and density-gradient electrophoresis. It was observed during the density-gradient-sedimentation experiments, that the zone containing enzymic activity, in contrast to other zones, exhibited a green fluorescence. The enzyme preparation had been completely resolved from pyridoxal phosphate at this stage of purification and so it was of interest to investigate the source of the fluorescence. This fluorescence was similar to that of flavin compounds. Fluorometric determination[2] showed that riboflavin was associated with the enzyme.

TABLE I

DEMONSTRATION OF A SPECIFIC REQUIREMENT FOR FAD IN THE ALANINE RACEMASE REACTION

The activity was determined by incubating 4.5 μmoles pyridoxal phosphate, 33.5 μmoles glutathione and 400 moles of L-alanine with 0.02 ml enzyme solution containing 70–240 μg protein and 0.04 M phosphate buffer (pH 8.1) in a total volume of 5 ml. Where indicated, 4.0 μmoles of FMN or FAD were added to the assay. The reaction was stopped by immersing the tubes in boiling water for 5 min and, after cooling, the tubes were incubated for 2.5 h with 5 mg pig-kidney D-amino acid oxidase (EC 1.4.3.3). The pyruvate formed was determined colorimetrically as the 2,4-dinitrophenylhydrazone. The results given are averages of duplicate determinations along with the average deviations.

pH of treatment	*Specific activity* of starting materials*	*Specific activity of samples treated with acid ammonium sulfate*		
		Cofactors added to assay		
		None	*FMN*	*FAD*
2.8	153 ± 0	0	0	0
3.7	106 ± 3	84 ± 2	84 ± 2	108 ± 3
3.8	169 ± 0	133 ± 2	133 ± 1	169 ± 1
4.0	103 ± 3	88 ± 2	88 ± 2	106 ± 3
4.3	175 ± 0	125 ± 0	125 ± 0	175 ± 0

* μmoles D-alanine per mg protein per h.

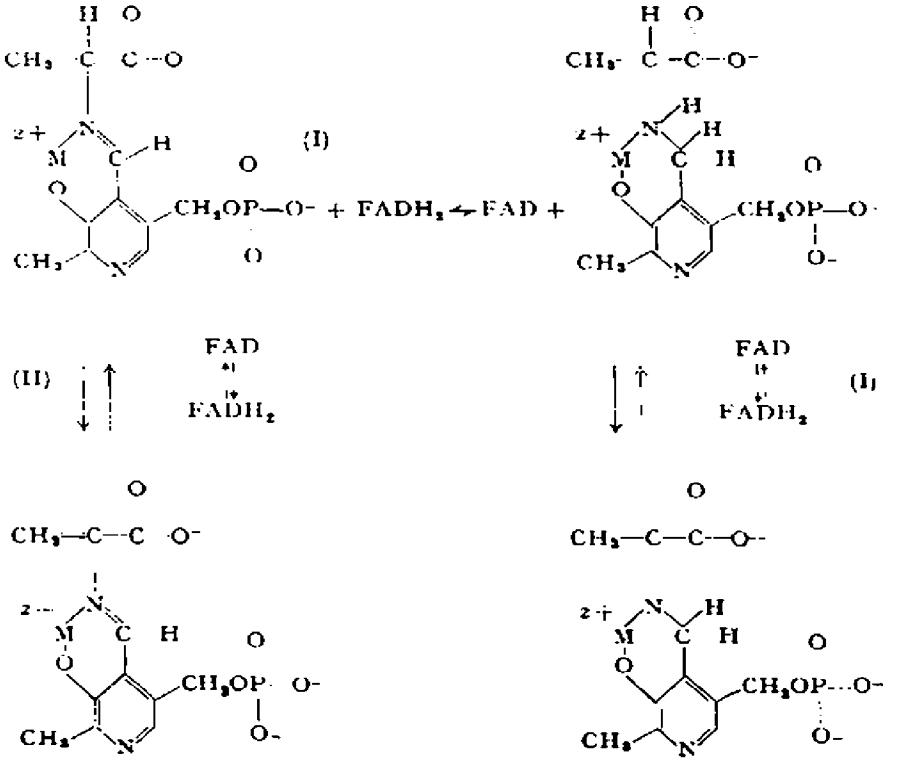
In an attempt to resolve the enzyme from the cofactor, ammonium sulfate was added to 50% saturation and the pH adjusted to the desired value by the addition of 1 N HCl. Precipitates were separated by centrifugation, dissolved in 0.05 M

phosphate buffer (pH 8.1) and dialyzed against the same buffer for 4 h. Alanine racemase activity was measured by an assay developed in this laboratory.

In several experiments 20–30% resolution was obtained but the enzyme could not be fully resolved by this procedure due to its instability at low pH. FAD, but not FMN, NAD, NADH nor NADP, restored the activity. Results of typical experiments are shown in Table I. The observation cannot be explained by a partial resolution of the D-amino acid oxidase employed in the assay system from its known cofactor, FAD, since when known amounts of D-alanine are incubated with the oxidase system in the absence of any added FAD, quantitative oxidation is obtained. Furthermore the FAD is not serving as a coenzyme to activate a contaminating oxidase or dehydrogenase since no pyruvate is formed in the absence of D-amino acid oxidase.

Determinations performed on unresolved enzyme and enzyme resolved at pH 4.3 showed that a 36% loss of riboflavin had occurred. This was accompanied by a 29% loss in enzymic activity, which suggests that the loss in activity was due to the resolution of a flavin coenzyme from the enzyme. Addition of FAD to enzyme preparations at this stage of purification without acid ammonium sulfate treatment caused no stimulation of the activity.

These new results suggest that FAD could function in the reaction as a specific acceptor for the α-hydrogen which must be exchanged during the racemization. The following equations give two plausible mechanisms for the action of FAD in the racemization.



Both schemes include the Schiff base intermediate formed by the reaction of alanine with pyridoxal phosphate as postulated by Snell. Mechanism I involves a hydrogen transfer similar to that which occurs in transamination reactions. If this scheme is operative, the optically inactive intermediate must be stable since cleavage would produce pyridoxamine phosphate and pyruvic acid which do not participate in the reaction. Mechanism II would involve α,β unsaturation and FAD is known to participate in such reactions. Further experiments are in progress to distinguish between these two mechanisms.

FAD, or other hydrogen-accepting coenzymes, may participate in a similar manner in the enzymic racemization of other amino acids. GLASER[3] found large losses of enzymic activity on acid ammonium sulfate precipitation of glutamate racemase (EC 5.1.1.3) which were not restored by pyridoxal phosphate and could have resulted from the loss of a hydrogen acceptor. Also TANOKA, KATO AND KINOSHITA[4] have suggested that the glutamate racemase from *Lactobacillus fermenti* is a flavoprotein.

A similar mechanism could be invoked to explain the hydrogen transfer in other enzymic reactions requiring pyridoxal phosphate. The hydrogen transfer in enzymic transamination could occur by such a flavin-linked reaction. It is of interest that COHEN[5] has observed a fluorescence typical of flavoproteins to be present in transaminase preparations and diamine oxidase (EC 1.4.3.6) has been found to require both pyridoxal phosphate and FAD as coenzymes[6].

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