CHARACTERIZATION OF AN IMIDAZOLEPYRUVIC ACID REDUCING SYSTEM FROM ESCHERICHIA COLI B [+)]

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The initial deamination of L-histidine by Escherichia coli B and the consecutive formation of imidazolepyruvic acid has recently been shown to be catalyzed by a transaminase and not by an L-amino acid oxidase (Wickramasinghe et al., 1967). The formation of imidazolelactic acid from imidazolepyruvic acid exhibits in vitro a cofactor requirement which can be fulfilled by a reduced pyridine nucleotide (Hedegaard et al., 1966), but the specificity of this aromatic α-keto acid reduction and thereby of the histidine degradation in E. coli remained unknown until now.

While the further metabolism of imidazolelactic acid by E. coli has not yet been confirmed, we present here results which show that the partially purified imidazolepyruvic acid reducing system from this microorganism (Cortese et al., 1968) differs markedly in its substrate specificities and affinities,

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cofactor requirement and pH optimum from the only other known aromatic α-keto acid reductase (Zannoni and Weber, 1966 ; Weber and Zannoni, 1966).

MATERIAL AND METHODS

NADPH and the imidazolepyruvic, α-ketobutyric and α-ketoglutaric acids are Calbiochem products. The 3-indolepyruvic, p-phenylpyruvic, α-ketocaproic, α-ketovaleric and imidazolelactic acids were obtained from Sigma, and pyruvic acid from Prolabo. We wish to thank Dr. Delcambe, C.P.E.M., Liège (Belgium) for his collaboration in the production of greater amounts of E. coli B (N° 54125) cell paste.

The purification of the imidazolepyruvic acid reducing system from crude sonicates of E. coli B has been described elsewhere (Cortese et al., 1968). In the present work was used an enzymatic preparation in which the imidazolepyruvic acid reducing activity was purified 330 fold. The aromatic α-keto acid reductase activity was determined by spectrophotometric measurements of the rate of disappearance of NADPH ($O.D._{340\ m\mu}$) in the presence of substrate. The enzymatic assays were carried out at room temperature directly in the cuvette. When the kinetics of the aromatic α-keto acid reduction as a function of the cofactor were studied, the composition of the assay was : 0.6 mg of protein, 5 µmoles of imidazolepyruvic acid, and 0.046 to 1 µmole of NADPH in 0.5 M phosphate buffer pH 7.2 (total volume 2.0 ml). When the kinetics of the aromatic α-keto acid reduction as a function of the substrate were studied, the composition of the assay was : 0.6 mg of protein, 0.74 µmoles of NADPH, and 5 to 50 µmoles of aromatic α-keto acid (imidazolepyruvic acid) in 0.5 M phosphate

buffer pH 7.2 (total volume 2 ml). The absence of NADPH oxidase in the enzymatic preparations made it possible to omit the cofactor from the control cuvette. Thus, the enzymatic reductions were initiated by the addition of NADPH to the assay cuvette and followed spectrophotometrically over a period of 2-5 min. In a similar way, the kinetics of the inhibition of the enzymatic activity were studied on one hand at increasing amounts (0 to 10 µmoles) of inhibitor (indolepyruvic acid) using phenylpyruvic acid as substrate (20 µmoles), and on the other hand at increasing amounts (5 to 50 µmoles) of substrate (phenylpyruvic acid) using 5 µmoles of inhibitor (indolepyruvic acid). A unit of enzyme has been defined as the amount of protein which permits the reduction of 1 µmole of imidazolepyruvic acid per min, and the specific activities are expressed as enzymatic units per mg of protein. Protein concentrations were determined by O.D. measurements at 260 mµ and 280 mµ. The Michaelis-Menten constants were determined from Lineweaver and Burck plots, while the inhibition constants were calculated according to Mahler and Cordes (1966).

RESULTS AND DISCUSSION

The kinetics of the inhibition of the imidazolepyruvic acid reducing system as a function of the cofactor are given in fig. 1. It is evident from these data that a marked inhibition of the enzymatic reduction (K_i of 4×10^{-4}) results from an excess of NADPH in the assay. Fig. 2 shows the kinetics of the reduction of the imidazolepyruvic acid in the presence of non-inhibitory concentrations of cofactor (NADPH). The affinity of the enzyme for imidazolepyruvic acid (K_m of 4.5×10^{-3}) is associated with a strong inhibition of the enzymatic reaction by excess of this substrate (K_i of 1.6×10^{-2}).

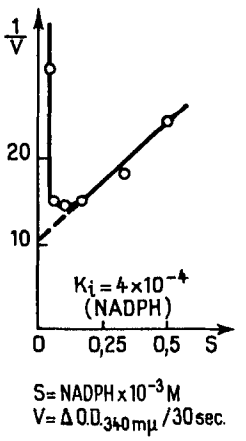

Fig. 1. Kinetics of the inhibition of the aromatic α-keto acid reduction as a function of cofactor.

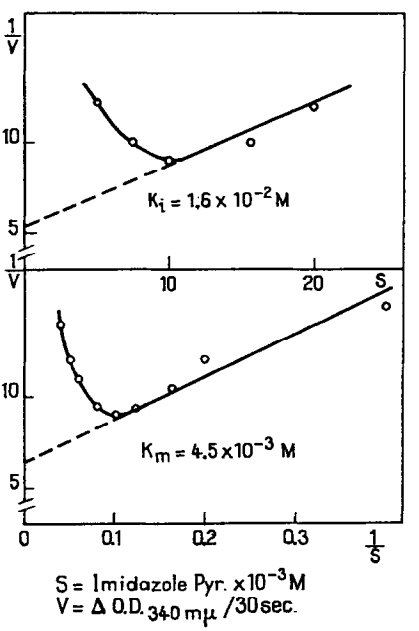

Fig. 2. Kinetics of the aromatic α-keto acid reduction as a function of substrate.

Table 1 summarizes the substrate and inhibitor specificities of several other aromatic α-keto acids for this

Table 1.
Substrate specificity and inhibition
of the imidazolepyruvic acid reducing system from E. coli.

Substrate	Maximal Activity	K_m	K_i
Imidazolepyruvic acid	100	4.5×10^{-3}	1.6×10^{-2}
Phenylpyruvic acid	110	7.1×10^{-3}	1.95×10^{-2}
p-Hydroxyphenylpyruvic acid	90	8.3×10^{-3}	1.95×10^{-2}
3-Indolepyruvic acid	5	-	1.46×10^{-3} x)

x) Inhibition by indolepyruvic acid tested with phenylpyruvic acid as substrate.

enzyme. It is clear from these results that not only imidazolepyruvic acid but also phenylpyruvic and p-hydroxyphenylpyruvic acids can serve as substrates, while this reductase is practically inactive with indolepyruvic acid. It is however noteworthy that this last aromatic α-keto acid is a potent inhibitor (K_i of 1.46×10^{-3}) of the enzyme and, as shown in fig. 3, this inhibition appears to be of an uncompetitive type (Mahler and Cordes, 1966). The complete lack of activity of this bacterial enzyme with pyruvic, α-ketoglutaric, α-ketocaproic, α-ketobutyric and α-ketovaleric acids excludes the presence of lactic dehydrogenase in this imidazolepyruvic acid reducing system. It should be further emphasized that the affinity of the enzyme for imidazolepyruvic acid is almost two fold higher than for any other aromatic α-keto acid tested. This last result is in good agreement with the higher inhibition of the enzymatic activity by excess of imidazolepyruvic acid than by any of the other substrates.

This imidazolepyruvic acid reducing system differs thus markedly from the only other so far well known aromatic

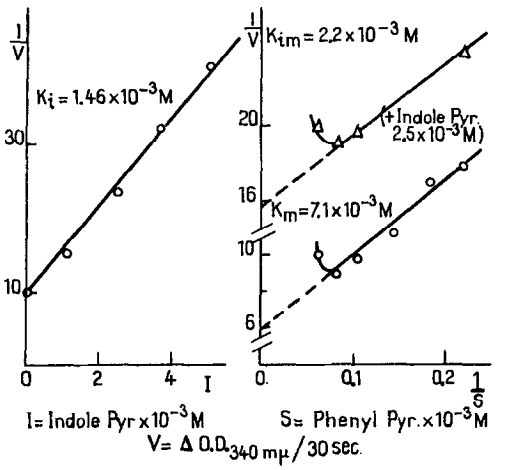

Fig. 3. Kinetics of the inhibition of the aromatic α-keto acid reduction by indolepyruvic acid.

α-keto acid reductase of mammalian origin (Zannoni and Weber, 1966). The latter enzyme has a cofactor requirement which is fulfilled by NADH and shows only slight activity with NADPH while, on the contrary, the imidazolepyruvic acid reducing enzyme is two times more active with NADPH than with NADH (Cortese et al., 1968). Furthermore, the mammalian aromatic α-keto acid reductase, which has a pH optimum between pH 6 and pH 7, is completely inactive with imidazolepyruvic acid while the bacterial enzyme described here exhibits a maximal affinity for this last substrate and has a sharp pH optimum at pH 7.2 (Cortese et al., 1968). The relative substrate specificities of phenylpyruvic and indolepyruvic acids emphasize further the important differences between these two enzymes.

As a result, it appears justified to consider the bacterial aromatic α-keto acid reducing system described here as a new enzyme which, despite its broad substrate specificity but because of its substrate and inhibitor affinities, we

suggest be named imidazolepyruvic acid reductase. The biological function of this new enzyme is however yet unclear.

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