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Direct evidence for the participation of pyruvate in *N*-hydroxylation of lysine

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The contribution of pyruvate to the formation of N^6-acetyl-N^6-hydroxylysine by a cell-free system of *Aerobacter aerogenes* 62-1 involved in the production of the dihydroxamate siderophore, aerobactin, has been assessed by a study of the influence of its analogs as well as of inhibitors of thiamine pyrophosphate-dependent decarboxylation reactions. These studies have provided unequivocal evidence for pyruvate functioning not only as a source of reducing equivalents in the initial step of *N*-hydroxylation of lysine but also as a precursor of the acetyl moiety in the subsequent conversion of the *N*-hydroxy amino to its N^6-acetyl derivative.

N-Hydroxylation; Thiamine pyrophosphate; Pyruvate oxidation; Hydroxamate; Siderophore

1. INTRODUCTION

Many organisms, under conditions of iron limitation, have been shown to produce siderophores [1,2]. Aerobactin, a dihydroxamate siderophore comprised of 2 molecules of N^6-acetyl-N^6-hydroxylysine and 1 molecule of citrate, is produced by *Aerobacter aerogenes* 62-1 [3] and *E. coli* [4] under conditions of iron deprivation. Biosynthesis of this siderophore has been shown to involve: (i) hydroxylation of lysine to its N^6-hydroxy derivative; (ii) conversion of N^6-hydroxylysine to N^6-acetyl-N^6-hydroxylysine; and (iii) condensation of the latter with citrate to yield aerobactin [5,6]. Our laboratory has been successful in preparing a cell-free system of *A. aerogenes* 62-1 capable of catalysing all three transformations mentioned above [7,8]. A membrane component (P2) present in the cell-free system has been shown to contain enzymes that are capable of catalysing hydroxylation and acetylation reactions while the soluble component (S2) is characterized by the presence of enzymes involved in the production of aerobactin from its immediate precursors [9]. The stimulation of P2-mediated *N*-hydroxylation of lysine by pyruvate [8] and recent identification of the enzyme system, in P2, involved in pyruvate metabolism have been documented [10]. Current investigations which concern the inhibition of P2-mediated conversion of lysine to N^6-acetyl-N^6-hydroxylysine by inhibitors of thiamine pyrophosphate-dependent enzymes, provide direct evidence for the indispensibility of pyruvate metabolism to the initial two reactions in aerobactin synthesis.

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2. MATERIALS AND METHODS

(*E*)-4-(4-Chlorophenyl)-2-oxo-3-butenoic acid (cinnamylidene) [11] and methyl acetylphosphonate [12] were synthesized according to published procedures. The spectroscopic characteristics of these compounds were in agreement with those reported. Bromopyruvate was purchased from

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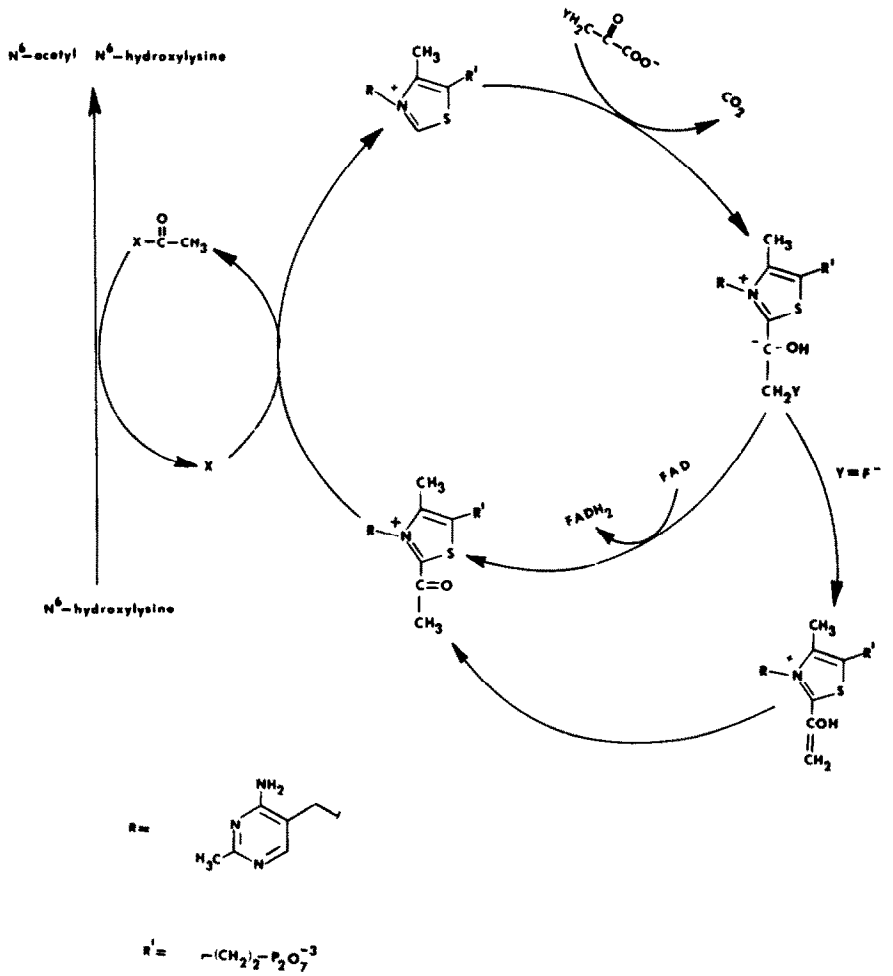
2.1. *Enzyme preparation*

A. aerogenes 62-1 was grown in an iron-deficient medium and the cell-free extract prepared as in [7,8]. The pellet component, P2, served as the source of enzyme in these studies.

2.2. *Assay procedure*

A typical assay mixture, total volume 10 ml, consisted of pyruvate (1 mM), lysine (1 mM) and potassium phosphate (50 mM), pH 7.0. In some experiments, pyruvate was replaced by acetaldehyde. Reaction mixtures were incubated at 37°C for 1–2 h. In inhibition experiments, the desired compound was included in the assay at the concentrations indicated.

The following analytical procedures were employed: changes in the concentration of pyruvate and its analogs were monitored by HPLC using a 7.8 × 300 mm Aminex HPX-87H column (Biorad) with 4 mM H_2SO_4 (pH 2.1) serving as eluant [12]. Experimental conditions were: flow rate, 0.5 ml/min; ambient temperature and detection by absorbance at 210 nm. The determination of N^6-hydroxylysine was performed by the iodine oxidation procedure [14] and quantitation of N^6-acetyl-N^6-hydroxylysine was achieved by the colorimetric method employing bismercaptoacetate-Fe(III) reagent [15].



Scheme 1.

3. RESULTS AND DISCUSSION

Previus studies in this laboratory have provided evidence for the participation of a pyruvate oxidase in P2-mediated conversion of pyruvate to acetate [10]. In the current investigations, analysis of assay mixtures containing pyruvate and lysine for N^6-hydroxylysine by the iodine oxidation procedure [14] and for N^6-acetyl-N^6-hydroxylysine by the use of bismercaptoacetate-Fe(III) reagent [15] revealed a 1 : 1 stoichiometry between the two products. Furthermore, P2 was found to be capable of converting N^6-hydroxylysine to its N^6-acetyl derivative in the presence of pyruvate. These observations would appear to be compatible with pyruvate serving as a source of acetyl moiety in the process of P2-mediated conversion of lysine to N^6-acetyl-N^6-hydroxylysine. This view was further substantiated by the observation that acetaldehyde was as effective as pyruvate in stimulating P2-catalyzed conversion of lysine to N^6-acetyl-N^6-hydroxylysine. Other aldehydes, e.g. formaldehyde or propionaldehyde, had no effect on these P2-mediated reactions.

Since enzyme-catalyzed oxidative decarboxylation of pyruvate in many instances is thiamine pyrophosphate-dependent (TPP) (scheme 1), studies were undertaken to assess the influence of analogs of pyruvate and other compounds on the ability of P2 to metabolize pyruvate as well as to promote the formation of N^6-acetyl-N^6-hydroxylysine. Advantage was taken of the recent reports that cinnamylidene [11] and methyl acetylphosphonate [12] function as inhibitors of TPP-dependent decarboxylation of pyruvate due to their ability to form covalent adducts with the cofactor. Cinnamylidene at such low concentrations as 0.2 mM in the assay effected complete inhibition of metabolism of pyruvate by P2. Methyl acetylphosphonate at 2 mM exerted moderate inhibition of this process. These results are shown in fig.1. The greater potency of the cinnamylidene to inhibit the utilization of pyruvate by P2 relative to methyl acetylphosphonate may be a reflection of its ability to form a more stable adduct with TPP and/or its overall more hydrophobic structure than the latter compound. Fluoro- and bromopyruvate were not metabolized by P2. Furthermore, each of these compounds was found to inhibit the metabolism of pyruvate by P2.

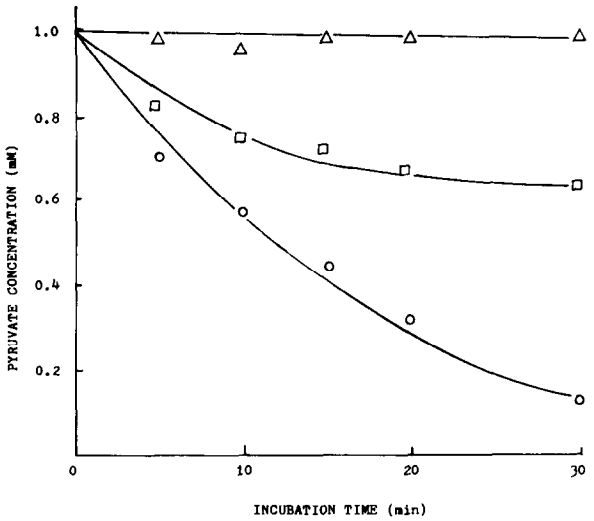


Fig.1. Effect of inhibitors of thiamine pyrophosphate-dependent enzymes on pyruvate metabolism. Typical assay mixture consisted of: 50 mM phosphate (pH 7.0), lysine (1 mM), pyruvate (1 mM) and P2 (0.2 ml) in a final volume of 2.0 ml. (○—○) No inhibitor, (△—△) cinnamylidene (0.2 mM), (□—□) methyl acetylphosphonate (2.0 mM).

The influence of the above-mentioned compounds on the ability of P2 to catalyse *N*-hydroxylation of lysine in the presence of pyruvate or acetaldehyde was examined. The results are presented in table 1. Inclusion of cinnamylidene (0.2 mM) in the assay resulted in a complete inhibition of the ability of P2 to catalyse *N*-hydroxylation of lysine. Similar inhibition by cinnamylidene was also observed in experiments with pyruvate replaced by acetaldehyde. Methyl acetylphosphonate exerted a moderate inhibition of P2-mediated conversion of lysine to its *N*-hydroxy derivative. Both fluoro- and bromopyruvate inhibited P2-mediated *N*-hydroxylation of lysine regardless of the presence or absence of pyruvate in the assay. Furthermore, when N^6-hydroxylysine replaced lysine in the assay, both compounds failed to serve as acetyl donors. While the inhibition of P2-mediated conversion of lysine to N^6-acetyl-N^6-hydroxylysine by bromopyruvate may be attributable to its function as an active-site oriented inhibitor of the enzyme(s) involved in the reactions, the observations with fluoropyruvate are particularly noteworthy. While the inhibition of P2-mediated oxidation of pyruvate by

Table 1

Influence of inhibitors of pyruvate metabolism on lysine: *N*-hydroxylation

Compound added	N^6-Hydroxylysine production (nmol)
None	19
Pyruvate	100
Bromopyruvate	20
Fluoropyruvate	19
Pyruvate + bromopyruvate	20
Pyruvate + fluoropyruvate	21
Pyruvate + iodoacetamide	20
Pyruvate + methyl acetylphosphonate	80
Pyruvate + cinnamylidene	15
Acetaldehyde	120
Acetaldehyde + cinnamylidene	20

Assay mixture, in a final volume of 10 ml, contained lysine (1 mM), phosphate (50 mM), pH 7.0, and the compounds (1 mM) with the exception of cinnamylidene (0.2 mM)

fluoropyruvate could be due to its ability to serve as a potent competitive inhibitor, its adduct with TPP, subsequent to its decarboxylation, could be expected to yield, via elimination, an enol intermediate which would undergo tautomerism to acetyl-TPP (scheme 1) [16]. Thus, one would have anticipated fluoropyruvate to serve as an acetyl donor in P2-catalyzed conversion of N^6-hydroxylysine to its N^6-acetyl derivative. Such inability of fluoropyruvate to serve as an acetyl donor in P2-catalyzed conversion of N^6-hydroxylysine to its N^6-acetyl derivative. Such inability of fluoropyruvate to serve as an acetyl donor may be a reflection of a slow rate of tautomerism of enol intermediate to acetyl-TPP and/or the ability of the enol intermediate to inactivate the enzyme(s) involved by covalent modification of its functional groups essential for catalytic function. These observations on the inhibitory action of cinnamylidene and pyruvate analogs demonstrate that pyruvate not only serves as a source of acetyl moiety but also plays a vital role in the *N*-hydroxylation of lysine, the initial step in P2-mediated conversion of lysine to N^6-acetyl-N^6-hydroxylysine. In conclusion, the findings in this study constitute the first direct evidence for the indispensibility of pyruvate metabolism to the production of N^6-acetyl-N^6-hydroxylysine and hence raise the possibility of pyruvate functioning as a co-substrate in *N*-hydroxylation of primary amines.

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