ISOLATION AND PROPERTIES OF *ESCHERICHIA COLI* MUTANTS DEFECTIVE IN 2-KETO 3-DEOXY 6-PHOSPHOGLUCONATE ALDOLASE ACTIVITY

Pelin FAIK, H.L. KORNBERG and E. McEVOY-BOWE*

Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, England

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1. Introduction

Many microbial species [1] effect the entry of gluconate into the central routes of metabolism via the Entner-Doudoroff pathway [2]. The component enzymes of this route are: (i) gluconate kinase (GLK), which catalyses the phosphorylation of gluconate to 6-phosphogluconate; (ii) 6-phosphogluconate dehydrase (EDD), which catalyses the removal of the elements of water from its substrate to yield 2-keto 3-deoxy 6-phosphogluconate (KDPG); and (iii) KDPG aldolase (KGA), which effects the aldol cleavage of KDGP to glyceraldehyde 3-phosphate and pyruvate. This sequence of reactions is summarized in fig. 1.

Two main lines of evidence illustrate the role that this pathway plays in gluconate utilization by *Escherichia coli*. Although 6-phosphogluconate can be catabolized via the pentose-phosphate shunt, mutants devoid of 6-phosphogluconate dehydrogenase (GND) are impaired to only a slight extent in the rate of their growth on gluconate [3]; in contrast, GND^+ mutants that lack the dehydrase (EDD^-) grow very slowly on gluconate [4]. Moreover, whereas the specific activities of GND in *E. coli* extracts appear to be independent of the nature of the carbon source used for the growth of the organism, EDD is present in only trace amounts unless its synthesis is induced by exposure of *E. coli* to gluconate [5].

Peyru and Fraenkel [6] have located on the genome of *E. coli* the genes that specify the synthesis of GND (*gnd*) and EDD (*edd*): they are not closely linked, and are situated on the linkage map [7] at, respectively, 39 min (co-transducible with the *his* locus) and 35 min. Since EDD is a component enzyme of the Entner-Doudoroff pathway but GND is not, it is of interest to determine whether the genes that specify the synthesis of other enzymes of that pathway, and of the possible regulator genes that determine the inducibility of these enzymes, are linked to either of these markers. We therefore describe the isolation and properties of *E. coli* mutants devoid of KGA activity, and report that this marker (*kga*) is so closely linked to that specifying EDD activity as to be over 95% co-transducible.

2. Experimental

2.1. *Isolation of kga mutants*

KDPG is an intermediate in two types of catabolic route (fig. 1): both the catabolism of gluconate via the Entner-Doudoroff pathway, and the catabolism of hexuronic acids [8], involve the necessary activity of KGA. In order to isolate mutants specifically affected in *kga*, therefore, the starting organism chosen was the mutant DF1071, a derivative of an Hfr C strain of *E. coli* K12 that already lacked GND [9]. In this mutant, the catabolism of gluconate had perforce to involve KGA; moreover, this organism grew readily in media containing either glucuronate or galacturonate as sole carbon source.

The *gnd,kga*-mutants sought were isolated by treatment of DF1071 with ethylmethane sulphonate [10] and selection with penicillin [11] for clones that grew neither on gluconate nor on galacturonate, but that retained the ability to grow on glucose. (The composition of media, and the biochemical and genetic proced-

* Present address: Department of Biochemistry, University of Singapore Medical School, Singapore 3.

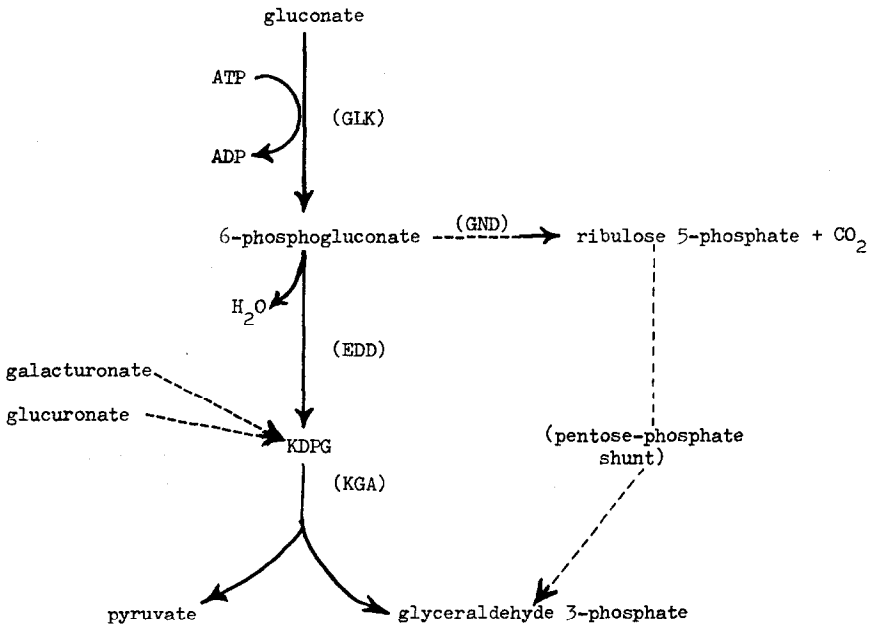

Fig. 1. Reactions of the Entner-Doudoroff pathway are indicated by bold arrows: related pathways mentioned in the text by broken arrows.

ures employed, have been previously described [12]). Of the mutants obtained with these characteristics, one designated DF1071-2B, was selected for further study.

3. Results and discussion

The above mutant grew at wild-type rates on glucose, glucose 6-phosphate, lactate, and on acetate: it had thus suffered no obvious lesions in either glycolytic or glucogenic pathways. However, it not only failed to grow on gluconate or on galacturonate, but the addition of either of these materials to cultures growing aerobically in nutrient broth resulted in a speedy cessation of growth. When cells, whose growth had been thus arrested, were harvested, suspended in a little water, and boiled, the solution obtained was rich in KDPG. Assay [13] of samples of such solutions indicated that, 3 hr after the addition of 5 mM gluconate, a culture of DF1071-2B (18 mg dry wt total) had accumulated 0.26 μmole of KDPG; this represents a KDPG concentration in the cell water of approx. 4 mM. The phosphorylated ketohexonic acid was also excreted as such into the medium [14]: in this experiment, the 18 mg dry wt of mutant cells had excreted over 18 μmoles of KDPG.

The absence, from DF1071-2B, of KGA activity was confirmed by direct spectrophotometric assay. For this purpose, the substrate KDPG (which is not commercially available), is readily prepared as follows: to 300 ml of a culture of DF1071-2B, grown overnight on double-strength "Oxoid" nutrient broth, with shaking at 37°, are added 6 ml of 0.5 M sodium gluconate and a further 300 ml of double-strength nutrient broth. After further shaking at 37° for 2 hr, the cells are harvested by centrifugation, washed once with 50 mM Tris-HCl pH 7.5, and are resuspended in 10 ml of this buffer. The thick suspension is cooled and the cells disrupted, by exposure for 1.5 min to the output of a MSE 100W ultrasonic disintegrator. Cell debris is removed by centrifugation at 20,000 *g* for 10 min; more prolonged (2–3 hr) centrifugation of the resultant suspension, at approx. 110,000 *g*, yields a clear solution with only low $NADH_2$ oxidase activity. To it (10 ml) is added 1 ml of a solution containing 30 μmoles of $MgCl_2$ and approx. 550 μmoles of 6-phosphogluconate, Na_3-salt. The mixture is incubated at 37°, samples (0.1 ml) being removed at 20 min inter-

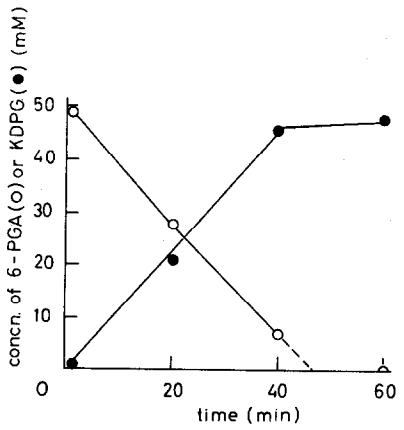

Fig. 2. Conversion of 6-phosphogluconate (○) to KDPG (●) by an extract of DF1071-2B, an *E. coli* mutant devoid of KGA activity. For details, see text.

vals to assess the progress of the reaction. Such samples are pipetted into 0.9 ml of water and are boiled for 5 min; the 6-phosphogluconate content is assayed spectrophotometrically with commercial GND and NADP at 340 nm, whilst that of KDPG formed can be readily determined [13] at the same wavelength with commercial lactate dehydrogenase, $NADH_2$, and extracts of any strain of *E. coli* (centrifuged at high speed to reduce $NADH_2$ oxidase activity) as source of KGA. The typical progress plot, given in fig. 2, shows that there is a quantitative and stoicheiometric transformation of the added 6-phosphogluconate to KDPG: although extracts of KGA^+-cells also remove 6-phosphogluconate under these conditions, no such accumulation of KDPG occurs. The data of fig. 2 thus also provide evidence for the absence of KGA from the mutant DF1071-2B. The KDPG formed can be easily isolated as the barium salt and purified in good yield [15].

When the HfrC-strain DF1071-2B, which is sensitive to streptomycin, was crossed with the F^- recipient K2.1t, (*his*, *arg*, *thr*, *leu*, *pps*, *str*, i.e. which requires histidine, arginine, threonine and leucine for growth, is devoid of PEP-synthase activity, and is resistant to streptomycin, [12]), and streptomycin-resistant recombinants were selected that had lost specific amino acid requirements, KGA^--clones were detected only among recombinants that had lost their requirement for histidine. As expected from the known [6] close linkage of *his* and *gnd*, all the HIS^+ recombinants tested were also GND^-.

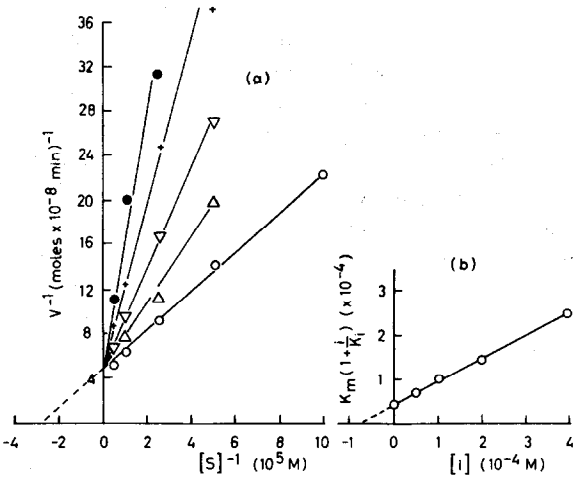

Fig. 3. Competitive inhibition of the 6-phosphogluconate dehydrogenase activity of *E. coli*, strain DF10. (a) The rate of $NADPH_2$ production from NADP by extracts incubated with various concentrations of 6-phosphogluconate either alone (○) or in the presence of (△) 50 μM, (▽) 0.1 mM, (+) 0.2 mM, and (●) 0.4 mM KDPG, was measured spectrophotometrically at 340 nm. (b) The K_I of the inhibition of GND by KDPG was calculated from the data of (a).

In order to investigate further the relationship of the *gnd* and *kga* markers, one of the recombinants (designated K2.1.8.24) was purified by repeated isolation of single colonies. This organism had the properties of the female parent, in so far as it was PPS^- and streptomycin-resistant, and of the male parent, in so far as it grew neither on gluconate nor on galacturonate, and its growth on nutrient broth was arrested by the addition of either of these materials: as found with DF1071-2B, KDPG accumulated in both intracellular water and in the growth medium. Moreover, whereas the KGA activity [13] of extracts of K2.1t sufficed to form more than 0.5 μmole of pyruvate from KDPG/min/mg of protein, similar extracts of K2.1.8.24 contained less than 1% of this activity.

KGA^+ recombinants were obtained from genetic crosses between K2.1.8.24 and two Hfr strains of *E. coli*. Strain AT2571 injects its genome in the order *o...trp...pps...his...*: analysis by period interruption of conjugation showed that KGA^+ recombinants appeared approx. 4 min after the entry of the *pps* allele. Strain KL96, on the other hand, injects its genome in the order *o...his, gnd...pps...trp...*: recombinants capable

of growth on galacturonate (and hence KGA^+) appeared approx. 4 min before entry of the *pps*-marker. Surprisingly, although the *his* (and hence the *gnd*) markers are among the earliest to be transferred by KL96, no colonies appeared in a selection for gluconate$^+$ galacturonate$^-$ (i.e. GND^+, KGA^-) until colonies selected for KGA^+ were already fully grown. When these tardy gluconate$^+$ colonies were isolated and tested, it was found that their growth in gluconate media was very poor (mean doubling time approx. 8 hr); that their growth in nutrient broth was arrested by the addition of gluconate, with consequent accumulation of KDPG in cells and medium; and that cell-free extracts were GND^+ and KGA^-. It was thus apparent that, despite the restoration of 6-phosphogluconate dehydrogenase activity, the formation and accumulation of KDPG from gluconate severely restricts the utilization of gluconate via the pentose-phosphate shunt. This explanation is supported by the properties of mutants devoid both of EDD and KGA activities. Such mutants grow on gluconate, since KDPG is not formed from that substrate [14]; however, the addition of galacturonate speedily arrests their growth.

KDPG and 6-phosphogluconate are of sufficiently similar structure to compete for the active sites on GND: the measured K_i for KDPG (7.5×10^{-5} M; fig. 3) is sufficiently low to compete effectively with the normal substrate, 6-phosphogluconate ($K_m = 4 \times 10^{-5}$; cf. [16]). This effect is the counterpart of the competition of KDPG and 6-phosphogluconate for the KDPG-aldolase, described by Pouysségur and Stoeber [14], though in that example both the K_m of the enzyme for KDPG (2×10^{-4} M) and the K_i for 6-phosphogluconate (8×10^{-4} M) are ten times higher.

The location of the *kga* marker on the *E. coli* genome was established more precisely by means of phage-mediated transduction. For this purpose, phage P1-kc was grown [12] on the *E. coli* mutant DF10, which is known to lack EDD activity [4] but grows on galacturonate and is thus KGA^+. Infection of strain K2.1.24 (one of the GND^+, EDD^+, KGA^- recombinants obtained from the cross of KL96 and K2.1.8.24) and selection on agar plates containing galacturonate (for KGA^+) yielded 107 transductants; tests with nutrient broth:gluconate:tetrazolium media [4] and direct assay of EDD activity in extracts of clones grown on nutrient broth + 5 mM gluconate, showed that 105 of these were EDD^-. This close linkage of *edd* and *kga* was confirmed by transducing phage P1-kc, grown on strain DF10 (*edd*), into the orginal mutant DF1071-2B (*gnd, kga*): again, KGA^+ transductants were selected on galacturonate. Of 1080 such KGA^+ transductants tested, 1036 were EDD^- whereas only 42 were EDD^+: however, all 1080 transductants had retained the GND^- phenotype. These results therefore confirm both that *gnd* and *edd* are not sufficiently closely linked to be co-transducible [4] and that two of the enzymes of the Entner-Doudoroff pathway, the dehydrase and the aldolase, are specified by genes that are over 95% co-transducible.

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