Volume 245, number 1,2, 219-222 FEB 06930 March 1989

Modification of a catalytically important residue of indoleglycerol-phosphate synthase from *Escherichia coli*

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Received 11 January 1989

The active-site residues of indoleglycerol-phosphate synthase from *Escherichia coli* were tentatively localized by comparing crystallographic data with the amino acid identities among the known indoleglycerol-phosphate synthase sequences. To test the validity of the resulting model of catalysis one of the residues in the presumptive active site, Lys 55, was changed to serine using oligonucleotide-directed mutagenesis. The specificity constant k_{cat}/K_m of the mutant is 3×10^4-times lower than that of the wild-type enzyme, due to a 60-fold decrease in k_{cat} and a 450-fold increase in K_m. This finding shows that Lys 55 is important for both catalysis and substrate binding.

Indoleglycerol-phosphate synthase; Catalytic mechanism; Site-directed mutagenesis; (*Escherichia coli*)

1. INTRODUCTION

The bifunctional enzyme phosphoribosylanthranilate isomerase:indoleglycerol-phosphate synthase (PRA isomerase:IGP synthase, EC 4.1.1.48) of *Escherichia coli* catalyzes two sequential steps in the biosynthesis of tryptophan (fig.1). Both biochemical and genetic studies have shown that the amino-terminal half of the protein catalyzes the IGP synthase reaction, whereas the carboxyl-terminal half is responsible for the PRA isomerase activity [1-3]. The crystal structure of the enzyme shows that the two activities reside on two well-separated domains [4]. Each domain has the folding pattern of triosephosphate isomerase [5], namely that of an 8-fold $\beta\alpha$ barrel.

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Abbreviations: CdRP, 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate; DTE, 1,4-dithiothreitol; IGP, indoleglycerol phosphate; PRA, *N*-(5'-phosphoribosyl-1-)anthranilate; PMSF, phenylmethylsulfonyl fluoride; rCdRP, reduced CdRP

More highly resolved structural information is necessary to propose a realistic mechanism for the catalytic process. However, the availability of 10 homologous amino acid sequences helps in identifying residues in the *E. coli* sequence that are likely to be involved in catalysis [4]. Here, one of these, Lys 55, has been chosen for analysis by oligonucleotide-directed mutagenesis.

2. MATERIALS AND METHODS

2.1. *Materials*

Oligonucleotides were synthesized automatically (Applied Biosystems 380B, phosphoramidite method) and purified by reverse-phase HPLC (Beckman HPLC, Waters μBondpak C-18 column). Restriction enzymes and DNA-modifying enzymes were from Boehringer, BRL and Pharmacia. Gel chromatography materials were from Pharmacia and radiochemicals from Amersham.

2.2. E. coli *strains, plasmids and phages*

E. coli W3310 *trpR ΔtrpEA* [6] harbouring the pWS1 plasmid [6] was used to produce both wild-type and mutant IGP synthase:PRA isomerase. *E. coli* W3310 *trpC*9800 recA^{56} Tc^r:Tn:10 [2], which is phenotypically *trpC*$^-$ *trpF*$^+$, was used to check for complementation in vivo. The strains BMH 71-18, BMH 71-18 mutS, MK 30-3 [7], and the phages M13mp9trpC(F) [8], M13mp9rev [7] were used for site-directed mutagenesis.

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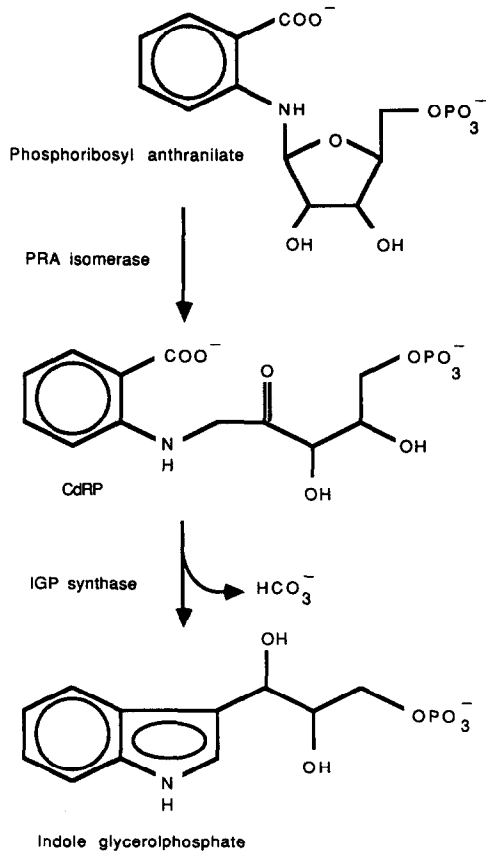

Fig.1. Metabolic reactions catalyzed by PRA isomerase and IGP synthase.

2.3. *Oligonucleotide-directed mutagenesis*

Site-directed mutagenesis was carried out with the gapped duplex strategy [7]. 5'-Phosphorylation was performed according to [9]. Mutants were screened by dot-blot hybridization [9], restriction analysis and sequencing [10]. A fragment containing the mutated region was isolated from the replicative form of the recombinant M13mp9 phage, and used to substitute the corresponding region of the *trpC(F)* gene in pWS1 expression vector [6].

2.4. *Purification of IGP synthase:PRA isomerase*

Mutant IGP synthase:PRA isomerase was purified as in [11] with the following modifications: *E. coli* W3310 *trpR ΔtrpEA* [6] harbouring pWS1 [6] was grown for 16 h in LB medium containing 30 mg/l chloramphenicol [12] with good aeration. 310 g cells were harvested from 35 l of culture by centrifugation, washed with 50 mM Tris-Cl (pH 8.5) and opened in 50 mM Tris-Cl (pH 8.5), 5 mM EDTA, 1 mM DTE, 0.1 mM PMSF and 5 mM serine, using a Sonicator cell disruptor (model W 375) with two pulses of duration 2 min each at maximum amplitude. The homogenate was cleared by centrifugation and DNA was precipitated with protamine sulfate solution (10 ml of 5% solution per g DNA). The supernatant (310 ml containing 8.7 g protein) was applied to a column of Q-Sepharose fast flow (Pharmacia, 1100 ml gel volume), which was equilibrated with 50 mM Tris-Cl (pH 8.0), 5 mM EDTA, 1 mM DTE and 0.1 mM PMSF. Protein was eluted with a linear gradient of 50–350 mM NaCl in the above buffer (2700 ml total gradient volume). PRA isomerase activity appeared at 150 mM NaCl. Active fractions were dialysed vs 20 mM potassium phosphate, pH 7.5 (final volume 190 ml, 440 mg protein), and pumped onto a column of DEAE-Sepharose fast flow (Pharmacia, 800 ml gel volume). A linear gradient of 20–300 mM potassium phosphate (pH 7.5) was applied. PRA isomerase activity appeared at 180 mM potassium phosphate. Active fractions were concentrated either by ultrafiltration (Amicon PM-10 membrane) or precipitation with ammonium sulfate at 70% saturation, and loaded in two batches (1.6 ml each) onto a column of Sephacryl S-200 (Pharmacia, 140 ml gel volume) equilibrated with 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 1 mM DTE. PRA isomerase activity appeared at 78 ml elution volume.

2.5. *Enzyme assays*

IGP synthase and PRA isomerase activities were measured as described [11,13].

3. RESULTS

The amino acid sequence alignment of IGP synthase from several organisms shows that identical amino acids are clustered within the carboxyl-terminal halves of the β-strands and the following loops [4]. The homology is particularly pronounced in the first β-strand–loop junction where Glu 53, Lys 55, Ala 57, Pro 59, Ser 60 and Gly 62 are 100% conserved. Moreover, the substrate analogue 5-iodo-rCdRP binds to this highly conserved region [4]. In contrast, both the amino-terminal halves of the β-strands and the external α-helices are significantly less conserved. These observations suggest that the active site of IGP synthase lies at the carboxyl end of the eight parallel β-strands and that the first β-strand including the following loop plays an important role.

No cofactors or metal ions have been identified as essential for catalysis [14]. Moreover, no covalent enzyme-substrate complexes have been observed [15]. A kinetic mechanism for IGP synthase has been established but the chemical step that requires a protonated amino acid side chain with an apparent pK_a value of about 7 has not been resolved into intermediates [15]. Conserved histidine residues, which might explain the pH dependence of the chemical step, are absent from the active site.

Fig.2 presents a plausible chemical mechanism for the cyclization reaction catalyzed by IGP synthase. There are two completely conserved residues sufficiently close to the reacting atoms of CdRP for catalytic assistance: Lys 55 and Arg 186. One out of several models views Arg 186 as an ion-pair partner of the substrate's carboxyl group, and Lys 55 as proton donor interacting with the substrate's carbonyl group. In order to check the importance of Lys 55 in catalysis, its side chain was eliminated by replacing Lys 55 with Ser, using site-directed mutagenesis [7]. The mutant gene was expressed in *E. coli* [17], the corresponding protein purified to homogeneity, and its catalytic properties analyzed kinetically. Table 1 lists the steady-state parameters of both the wild-type and mutant enzymes. The K_m value of the PRA isomerase reaction is the same for both wild-type and mutant enzymes. It is not clear whether the 2-fold difference in k_{cat} is due to the mutation in the adjacent domain or to partial inactivation. Nevertheless, it appears that the integrity of the PRA isomerase domain is not affected significantly by the mutation. In contrast, k_{cat} of IGP synthase is decreased by a factor of 60, and K_m is increased by a factor of 450, resulting in a 3×10^4-fold decrease in k_{cat}/K_m compared to the wild-type enzyme.

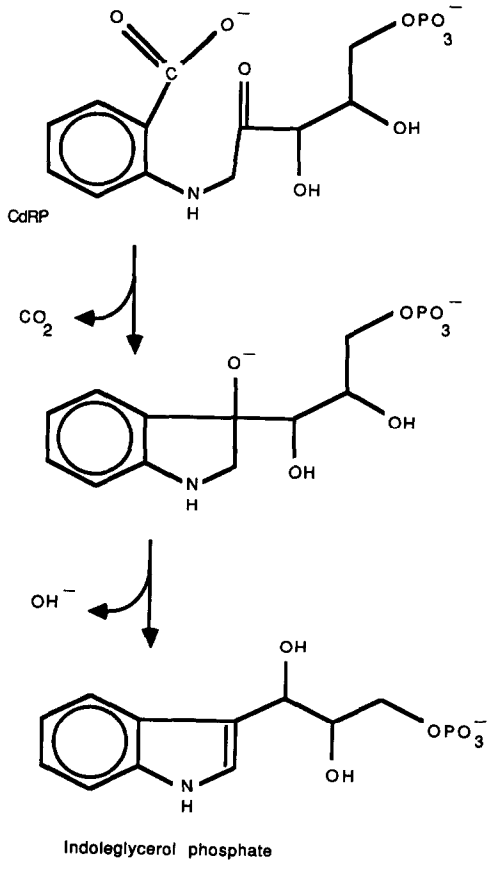

Fig.2. Proposed chemical catalysis of IGP synthase. In a modified version of Rawn's proposal [16], a cyclic intermediate is formed by concerted decarboxylation and carbon-to-carbon bond formation. This intermediate is converted to the indole ring formally by release of OH^-.

Table 1

Steady-state parameters of IGP synthase:PRA isomerase wild-type and K55S mutant

Reaction	Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat} ($\mu M^{-1} \cdot s^{-1}$)
PRA isomerase	wild-type	38.9	7.1	5.5
	K55S mutant	22.1	7.1	3.1
IGP synthase	wild-type	7.2	1.2	6.0
	K55S mutant	0.12	540	2.2×10^{-4}

The PRA isomerase reaction was measured in 50 mM Tris-Cl (pH 7.5), 4 mM $MgCl_2$, 2 mM DTE, at 25°C [13]. IGP synthase activities were measured in 100 mM Tric-Cl (pH 7.8), 5 mM EDTA, 1 mM DTE, at 37°C [11]. K_m and k_{cat} were determined using the direct linear plot method [19]

4. DISCUSSION

Assuming that the structure of IGP synthase has not been altered apart from the change of Lys 55 to Ser, these results show that the side chain of Lys 55 stabilizes both the enzyme-substrate complex and the enzyme-transition state complex to a similar extent. The relatively small decrease in k_{cat} indicates that Lys 55 stabilizes the transition state somewhat more than the ground state of the enzyme-bound substrate [18]. Although Lys 55 is clearly important in catalysis, it is not essential. It is not clear whether Lys 55 acts as a general acid or as a partner for electrostatic interactions stabilizing the lone electron pair of the intermediate in fig.2. In the former, the intrinsic pK_a value of the ϵ-amino group of Lys 55 would have to be decreased by 3 units.

The replacement of other conserved residues is currently being undertaken in order to gain further insight into the catalytic mechanism of IGP synthase.

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