# *N*-(5-Phosphoribosyl)anthranilate Isomerase–Indoleglycerol-phosphate Synthase. 1. A Substrate Analogue Binds to Two Different Binding Sites on the Bifunctional Enzyme from *Escherichia coli*†

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ABSTRACT: The binding of a fluorescent substrate analogue to a bifunctional enzyme of the tryptophan biosynthesis pathway has been studied at equilibrium by equilibrium dialysis, steady-state kinetics, fluorescence titration, and difference spectroscopy. The substrate analogue was obtained by reducing 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CdRP) with sodium borohydride. The reduced CdRP (rCdRP) has been identified with *N*-(5-phosphoribityl)anthranilate. Equilibrium dialysis in 0.1 M Tris buffer, pH 7.5, 20 °C, gives two different binding sites for rCdRP on the monomeric, bifunctional PRA isomerase–InGP synthase from *Escherichia coli*. $K_{d,1}$ equals 0.2 $\mu$M; $K_{d,2}$ equals 12.5 $\mu$M. These measurements agree with the values of the dissociation constants obtained from fast-reaction kinetics [Cohn, W., Kirschner, K., & Paul, C. (1979) *Biochemistry* (following paper in this issue)]. Steady-state inhibition constants show that the high-affinity binding site is identical with the active site of indoleglycerol-phosphate (InGP) synthase. This activity is also competitively inhibited by indolepropanol phosphate (IPP), a product analogue of InGP. The absorption and fluorescence spectra of rCdRP change upon binding to each of the two different binding sites. Fluorometric titrations confirm the value of the dissociation constant of the tight enzyme–ligand complex. Upon replacement of Tris buffer with 0.1 M phosphate buffer, pH 7.6, 20 °C, the values of $K_M$ for CdRP and $K_i$ for rCdRP, InGP, and IPP increase approximately 40-fold, whereas the dissociation constant of the loose enzyme–ligand complex is affected only moderately.

(Phosphoribosyl)anthranilate (PRA)[1] isomerase and indoleglycerol-phosphate (InGP) synthase catalyze the two metabolic steps of tryptophan biosynthesis preceding the tryptophan synthase reactions (Creighton & Yanofsky, 1970; Creighton, 1970). As seen in Scheme I, PRA isomerase catalyzes the Amadori rearrangement of PRA to CdRP. InGP synthase is responsible for catalyzing the ring closure of CdRP to InGP.

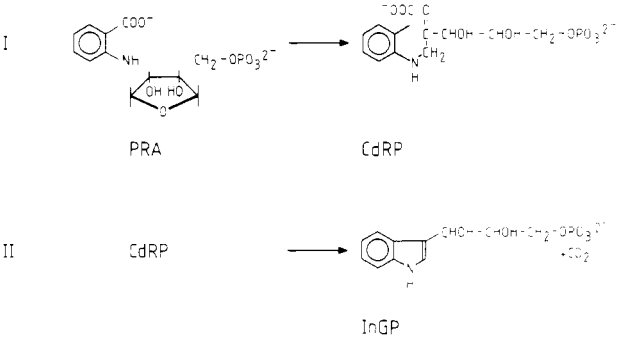
In all organisms studied, the same reaction sequence leads to conversion of PRA to InGP. However, the structural organization of the two enzymes differs widely (Crawford, 1975). In *Bacillus subtilis* (Hoch et al., 1969; Hoch & Crawford, 1973) and *Pseudomonas putida* (Enatsu & Crawford, 1968), the two reactions are catalyzed by separate enzymes. In *Brevibacterium flavum* (Sugimoto & Shiio, 1977), the enzymes form a loose multienzyme complex. By contrast, only a single protein is responsible for the catalysis of both reactions in *Escherichia coli* (Creighton & Yanofsky, 1970; Creighton, 1970).

Multifunctional enzymes have only recently been recognized as a subclass of the category of complex enzymes [for reviews see Kirschner & Bisswanger (1976), Stark (1976), Pauckert et al. (1976), and Gaertner & Cole (1977)]. It is of general interest what functional advantages arise from having several active sites engaged in catalyzing sequential metabolic steps on a single, multifunctional enzyme molecule (Welch, 1977; Gaertner, 1978).

PRA isomerase–InGP synthase from *E. coli* is one of the few simple representatives of multifunctional enzymes because it is a monomer. The enzyme is the product of the *trpC* gene within the *trp* operon of *E. coli* (Crawford, 1975). Biochemical and genetic studies have shown that the N-terminal portion of the polypeptide chain is responsible for InGP synthase activity whereas the C-terminal portion apparently generates the active site for PRA isomerase (Smith, 1967; Yanofsky et al., 1971). This evidence is consistent with the notion that the polypeptide chain is folded into two functional domains, each bearing a particular active site (Kirschner & Bisswanger, 1976). Kinetic studies of the two consecutive reaction steps have shown that CdRP accumulates to a steady-state level during catalysis of the overall reaction of PRA to InGP (Creighton, 1970). By this indirect evidence the two different active sites appear not to overlap.

The ease of purifying large amounts of PRA isomerase–InGP synthase makes this enzyme an attractive choice for a detailed examination of possible interactions between the

Scheme I

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[1] Abbreviations used: PRA isomerase–InGP synthase, *N*-(5-phosphoribosyl)anthranilate isomerase–indole-3-glycerol-phosphate synthase (EC 4.1.1.48); PRA, *N*-(5-phosphoribosyl)anthranilate; InGP, indoleglycerol phosphate; IPP, indolepropanol phosphate; CdRP, 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate; rCdRP, reduced CdRP; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate.

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different active sites by the more direct experimental approach of ligand binding studies. Unfortunately, PRA is a very labile compound (Creighton, 1968) and CdRP cannot be purified easily (Doy, 1966). We have found that reduction of CdRP with sodium borohydride leads to a stable compound (reduced CdRP, rCdRP) that binds specifically to two different sites on the PRA isomerase–InGP synthase from *E. coli*. In preparation for studies of the mechanism of binding of rCdRP with rapid-reaction techniques (Cohn et al., 1979), this work describes the determination of the number of binding sites and the dependence of the two enzyme–ligand dissociation constants on the nature of the buffer ion. The changes of absorbance and fluorescence spectra of rCdRP observed upon its binding to the enzyme are useful spectroscopic signals for equilibrium and kinetic studies.

## Experimental Procedures

*Materials.* Crystalline dicyclohexylammonium salts of InGP and indolepropanol phosphate (IPP) were obtained as described previously (Kirschner et al., 1975).

*Chemical Syntheses.* rCdRP. The reaction mixture of 1 mmol of anthranilic acid and 1 mmol of sodium ribose 5-phosphate in 2 mL of 50% ethanol was adjusted to pH 5.0 (measured after 20-fold dilution with water). After 4 h at 25 °C, the synthesis of CdRP (Creighton & Yanofsky, 1970) was stopped by addition of 4 mL of water. Three portions each of 0.2 mL of a fresh solution of 5 M sodium borohydride in 0.05 M NaOH were added slowly with intense stirring. After 1 h, the pH value of the solution was adjusted with HCl to 4.8 and excess anthranilic acid was extracted with ethyl acetate. The pH was readjusted to 8.0 with $NH_4OH$, and the solution was applied directly to the top of a column (2.5 × 50 cm) of DEAE-cellulose (Whatman DE 52) equilibrated with 0.05 M ammonium bicarbonate, pH 8.0. The product should not be exposed to light from this step onward. The product was eluted with 1 L of a linear gradient from 0.05 to 1.00 M ammonium bicarbonate, concentrated in vacuo, and rechromatographed as described. rCdRP was detected by its blue fluorescence (excitation at 254 nm) and was found to be pure by thin-layer chromatography on silica plates [$R_f$ = 0.43 with concentrated $NH_3$–water–ethanol (10:20:55 v/v)]. The extinction coefficient of rCdRP at 327 nm (3.43 $mM^{-1}$ $cm^{-1}$) was calculated from the concentration as determined by phosphate analysis (King, 1932). $^3H$-Labeled rCdRP was synthesized on a small scale by the above procedure using sodium [$^3H$]borohydride. The specific activity of the product was $1.2 \times 10^8$ cpm/μmol. It is particularly important to use freshly chromatographed material for equilibrium dialysis. The controls for determining the radiochemical purity of the labeled ligand are described under Equilibrium Dialysis. Partially decomposed preparations have an increased absorption at 278 nm. Thus, the value for $E_{252}/E_{278}$, which equals 25 for the pure compound, is a sensitive measure of purity.

*N*-Ribitylanthranilate was synthesized by reduction of the product described by Lingens et al. (1957) as D-ribosylanthranilide with sodium borohydride. A total of 0.81 g (3 mmol) of ribosylanthranilide was suspended in 40 mL of 0.1 M triethanolamine hydrochloride buffer, pH 8.6, and reduced by addition of 0.1 mL of 5 M $NaBH_4$ in 0.05 M NaOH. The suspension became clear immediately and was stirred for 60 min at room temperature. After acidification with 1 M HCl to pH 4.5, the product was extracted with ethyl acetate and finally recrystallized twice from water: yield, 178 mg (22% of theory) of long white needles; mp 186–188 °C. Anal. Calcd for $C_{12}H_{17}O_6N$ (269.3): C, 53.13; H, 6.27; N, 5.18; O, 35.42. Found: C, 53.08; H, 6.07; N, 5.33; O, 35.19. The assignment of structure is supported by the fact that the product has a melting point differing from that of authentic ribosylanthranilide [mp 121 °C (Doy & Gibson, 1958); mp 127–128 °C (Lingens et al., 1957)].

*Phosphatase Action on rCdRP.* A total of 13 μmol of the compound was dissolved in 1 mL of 0.05 M Tris-HCl buffer, pH 8.0, and incubated with 0.8 unit of alkaline phosphatase for 16 h at room temperature. The reaction mixture was analyzed by thin-layer chromatography. The original material had been quantitatively converted to a compound which had the same mobility as authentic *N*-ribitylanthranilic acid with three solvent systems on silica gel plates: (a) methanol–water–25% $NH_4OH$ (7:2:1), $R_f$ = 0.83; (b) methanol–acetone–chloroform–25% $NH_4OH$ (4.2:2.5:1.7:1.7), $R_f$ = 0.48; (c) 1-butanol–glacial acetic acid–water (5:4:1), $R_f$ = 0.60.

*Enzyme Assays.* The enzyme was assayed routinely by following the progress of the InGP synthase reaction at 280 nm (Creighton & Yanofsky, 1970) in 0.1 M Tris-HCl buffer, pH 7.8, containing 0.1 mg of bovine serum albumin per mL at 37 °C. The reaction was initiated by adding CdRP to a final concentration of 0.15 mM. One unit of synthase activity is defined as the amount required for the conversion of 1 μmol of CdRP to InGP in 1 min.

For accurate determination of the inhibition constant of rCdRP and $K_M$ for CdRP, the assay was conducted with a Durrum-Gibson stopped-flow apparatus equipped with a modified fluorescence cell. The production of InGP was measured by fluorescence at $\lambda > 320$ nm (excitation at 297 nm). Three to ten progress curves were stored and averaged by an on-line data acquisition system involving a Datalab 905 transient recorder and a PDP 11-40 computer. Initial velocities were calculated from $[CdRP]_0$ and the best fit values of $K_M$ and $V_{max}$ as obtained by a nonlinear least-squares fit of the averaged progress curves to the integrated Michaelis–Menten equation (Fernley, 1974).

*Protein Determination.* The extinction coefficient of purified enzyme was calculated from the protein dry weight determined by Hunter's (1966) method. $E_{278nm}^{0.1\%} = 0.84$ $cm^2$ $mg^{-1}$, $E_{278}/E_{250} = 2.8$, and $E_{280}/E_{260} = 2.0$. Aliquots of the standard enzyme solution were analyzed with the Folin phenol reagent (Hartree, 1972). A total of 1.08 mg of PRA isomerase–InGP synthase gives rise to the same absorbance change as 1 mg of crystalline bovine serum albumin.

*Bacterial Strain and Growth Conditions. E. coli W 3110 trpR⁻ cysB⁻Δtrp LD 102/F'col VB cysB⁺Δtrp LD 102* was obtained from Dr. C. Yanofsky and was grown on minimal medium supplemented with 20 μg of L-tryptophan per mL as described by Adachi et al. (1974). The yield was 800 g of wet cell paste per 100 L.

*Enzyme Purification.* All operations were performed at 0–4 °C. All buffer solutions were supplemented with 5 mM EDTA and 0.2 mM dithioerythritol. The preparation of cell-free crude extract and the chromatography on DEAE-cellulose (or DEAE-Sephacel) were identical with steps 1 and 2 described for the purification of the α subunit of tryptophan synthase from *E. coli* (Kirschner et al., 1975). The enzyme was eluted with 10 L of a linear gradient from 70 to 350 mM NaCl in 0.05 M Tris-HCl buffer, pH 7.5, and appeared at approximately 0.17 M NaCl.

*Step 3.* Active fractions of the preceding step were pooled (1.2 L), carefully adjusted to pH 7.0 by addition of 5 M acetic acid, and diluted with 1.2 L of distilled water. The resulting solution was pumped directly on to a column (5 × 45 cm) of DEAE-Sepharose CL-6B that was previously equilibrated with

50 mM potassium phosphate buffer, pH 7.0. After washing with 1 L of the same buffer, the enzyme was eluted with a linear concentration gradient increasing from 50 to 210 mM of the same buffer. The peak of enzyme activity is eluted at 80 mM buffer. The active fractions were pooled and concentrated by adding 32.6 g of solid ammonium sulfate per 100 mL of solution and collecting the pellet after centrifugation.

*Step 4.* The precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 7.0, to a final concentration of 40–50 mg of protein per mL. The centrifuged solution was applied to the bottom of a column (5 × 90 cm) of Sephadex G-75 previously equilibrated with the same buffer. The protein was washed in by 20 mL of 20% sucrose in buffer and eluted with buffer. Active fractions were pooled and concentrated with ammonium sulfate as described above.

*Crystallization.* The precipitate was dissolved in 0.1 M potassium phosphate containing 5 mM EDTA and 2 mM DTE, pH 7.0. After determination of the concentration of ammonium sulfate conductimetrically, the solution was adjusted to a protein concentration of 30–40 mg of protein per mL and 1.2 M ammonium sulfate, pH 7.0, and clarified by brief centrifugation. Crystallization was initiated by adding seed crystals of the enzyme and slowly increasing the concentration of ammonium sulfate to 1.36 M. After 2–3 days at 4 °C, the crystals were collected by centrifugation and resuspended in the same buffer containing 1.64 M ammonium sulfate.

*Determination of Molecular Weight.* A Beckman-Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner was used for determining the sedimentation and diffusion coefficients of PRA isomerase–InGP synthase in 0.1 M Tris–acetate buffer, pH 7.5, 20 °C (Schachman, 1957). The meniscus depletion method (Yphantis, 1964) was used to determine the weight-average molecular weight of the enzyme in the same buffer. The experiment was repeated in the presence of 0.2 mM rCdRP. Electrophoresis in polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate was used to determine the molecular weight of the polypeptide chain (Laemmli, 1970; Studier, 1973). Bovine serum albumin ($M_r$ 68 000), tryptophan synthase $\beta_2$ subunit ($M_r$ 45 000), yeast glyceraldehyde-3-phosphate dehydrogenase ($M_r$ 36 000), tryptophan synthase $\alpha$ subunit ($M_r$ 29 500), and myoglobin ($M_r$ 17 000) were used as marker proteins.

*Equilibrium dialysis* was performed with the cells and membranes described previously (Kirschner et al., 1975). Six hours was allowed for equilibration at 20 °C; 12 h was allowed at 4 °C. Because rCdRP is photolyzed to anthranilate, it was important to check the radiochemical purity of the labeled ligand (Builder & Segel, 1978). The following equilibrium dialysis depletion technique was used: 0.4 mL of a solution containing 15 $\mu$M enzyme and 5 $\mu$M ligand in 0.1 M Tris–acetate buffer, pH 7.5, was dialyzed in a cell with an enlarged buffer compartment against 1.6 mL of buffer for 6 h at 20 °C. After taking samples from both chambers, we replaced the buffer and repeated the procedure twice. The total ligand contained in the enzyme chamber is depleted only by a small fraction, and the value of $K_{d,1}$ as calculated according to the mass law expression equals 0.21 ± 0.02 $\mu$M. If the ligand preparation contains weakly binding radioactive impurities, the initial value of $K_i$ is much higher but drops to a value of 0.2 $\mu$M after the second buffer exchange.

*Spectroscopic Titrations.* Fluorescence spectra were recorded with a ratio recording Schoeffel RRS 1000 fluorometer equipped with interchangeable duplex thermostated cuvette holders. An Agla syringe (Burroughs Wellcome) was used to administer small aliquots of titrant to the cuvette. Fluorescence signals were recorded for both the sample cuvette and a reference cuvette containing buffer and the titrated species. Changes in the reference signal were used to correct the sample signal for instrumental drift. Experiments were conducted in duplicate and the measurements were averaged. Absorbance spectra and difference spectra were recorded with a Cary Model 118 spectrophotometer equipped with thermostated cuvette holders.

Table I: Flow Sheet for Purification of PRA Isomerase-InGP Synthase

| fraction[a] | total protein (g) | sp act. (units mg$^{-1}$) | yield (%) |
|---|---|---|---|
| crude extract | 71.20 | 0.23 | 100 |
| DEAE-Sephacel step | 9.58 | 1.46 | 85 |
| DEAE-Sepharose step | 1.59 | 7.02 | 68 |
| Sephadex G-75 step | 0.90 | 9.13 | 50 |
| crystals | 0.83 | 8.32 | 42 |

[a] Representative purification from 600 g wet weight *E. coli* cells (for strain, cf. Experimental Procedures).

## Results and Discussion

*Purification and Stability of PRA Isomerase–InGP Synthase.* The protocol described under Experimental Procedures is a substantial modification of the previous method (Creighton & Yanofsky, 1970). The chromatography of crude extract on DEAE-Sephacel replaces the precipitation of DNA by streptomycin sulfate and the subsequent precipitation with acetic acid at pH 4.9. The eluate from the first column is diluted and pumped directly onto a column of DEAE-Sepharose CL-6B, giving rise to 70% pure protein within 24 h. Results of a typical purification are presented in Table I. The overall yield of crystals and the maximum specific activity of InGP synthase (10 ± 0.5 units/mg) are greater than those obtained with the previous method.

The enzyme was pure as judged by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [$M_r$ equals 48 000 compared to values of 45 000 (Creighton, 1970) and 46 000 (McQuade & Creighton, 1970)]. A value of $M_r$ 46 000 was calculated from $D_{20,w} = 6.6 \times 10^{-7}$ cm$^2$ s$^{-1}$, $s_{20,w} = 3.2$ S (our own measurements), and $\bar{\nu} = 0.736$ cm$^3$/g (Creighton & Yanofsky, 1966). The value of $M_r$ from sedimentation equilibrium equalled 46 000 [45 000 (Creighton & Yanofsky, 1966; Creighton, 1970)]. The same value was found in the presence of 0.2 mM rCdRP, indicating that the enzyme does not dimerize when saturated with the ligand.

Mosteller et al. (1977) have shown that the InGP synthase activity is diminished more extensively than the PRA isomerase activity under certain starvation conditions during growth. Oxidation of sulfhydryl groups and exposure to heavy metal ions also lead to inactivation (Creighton & Yanofsky, 1970). Preparatory to examination of the mechanism of binding (Cohn et al., 1979), we tested the stability of the enzyme in various buffers after incubation for 16 h at 25 °C. As seen from Table II, the enzyme is most stable in potassium phosphate buffer, pH 6.5–7.5, in the presence of 1 mM dithioerythritol. The loss of activity in the cationic buffers correlates with the oxidation of dithioerythritol. The varying rates of air oxidation are due to the different content of $Cu^{2+}$ in the buffer components (unpublished work). We therefore supplemented all enzyme solutions with 5 mM EDTA.

The nature of the buffer has a pronounced effect on the dissociation constant of InGP synthase for CdRP (cf. below). As seen in Figure 1, $V_{max}$ also depends on the buffer. In 0.1

Table II: Stability of PRA Isomerase–InGP Synthase and Dithioerythritol in Various Buffers

| buffer | concn (M) | pH value | initial DTE concn[b] (mM) | synthase act. remaining after 16 h[a] (%) | DTE concn remaining after 16 h[b] |
|---|---|---|---|---|---|
| potassium phosphate | 0.1 | 6.5 | 1.0 | 99 | 1.00 |
| potassium phosphate | 0.1 | 7.5 | 1.0 | 100 | 1.00 |
| potassium phosphate | 0.1 | 7.5 | 0 | 91 | 0 |
| Tris-HCl[c] | 0.1 | 7.5 | 1.0 | 94 | 0.23 |
| Tris-HCl | 0.1 | 7.5 | 0 | 74 | 0 |
| Tricine-HCl[c] | 0.1 | 7.5 | 1.0 | 93 | 0.19 |
| triethanolamine chloride | 0.1 | 7.5 | 1.0 | 81 | 0.54 |
| Hepes chloride[c] | 0.1 | 7.5 | 1.0 | 55 | 0.01 |
| sodium pyrophosphate | 0.05 | 8.5 | 0 | 1 | 0 |

[a] 0.93 mg of enzyme per mL was incubated in the various buffers for 16 h at 25 °C and was assayed in the synthase reaction. [b] The concentration of dithioerythritol (DTE) was determined with Ellman's reagent (Ellman, 1959). [c] Buffer contained 0.1 M KCl additionally.

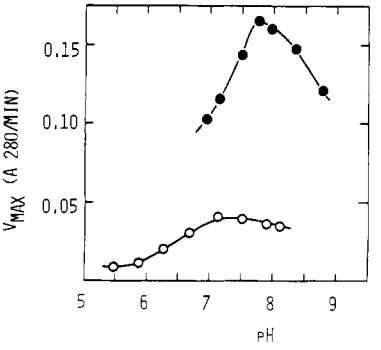



FIGURE 1: Maximal velocity of InGP synthase depends on the nature of the buffer ion. Initial rates were measured at 37 °C as described under Experimental Procedures. Enzyme concentration was 0.033 unit/mL. (○) 0.05 M potassium phosphate buffer; (●) 0.05 M Tris-HCl buffer.

M phosphate buffer, $V_{max}$ increases with increasing pH to a plateau value above pH 7.5. In 0.1 M Tris buffer, there is a maximum at pH 7.8 with a fourfold larger value of $V_{max}$ than found in phosphate buffer.

*Synthesis and Structure of Reduced CdRP.* CdRP was synthesized from anthranilic acid and sodium ribose 5-phosphate (Creighton & Yanofsky, 1970) and subsequently reduced with sodium borohydride (Scheme II). After chromatography on DEAE-cellulose as detailed under Experimental Procedures, the material was pure as judged from thin-layer chromatography and from a quantitative comparison of its absorption spectrum (Figure 2) with that of authentic *N*-ribitylanthranilate (Lingens et al., 1957) and anthranilic acid. The compound is light sensitive, slowly decaying to products which do not bind to the enzyme.

Reduced CdRP (rCdRP) is converted to *N*-ribitylanthranilate by the action of alkaline phosphatase and is therefore probably identical with *N*-(5-phosphoribityl)-anthranilate (Scheme II). Because it differs from CdRP only by reduction of the 2′-carbonyl group, rCdRP can be regarded as a product analogue of PRA isomerase as well as a substrate analogue of InGP synthase. It is therefore potentially capable of binding to each of the two different active sites located on the bifunctional enzyme.

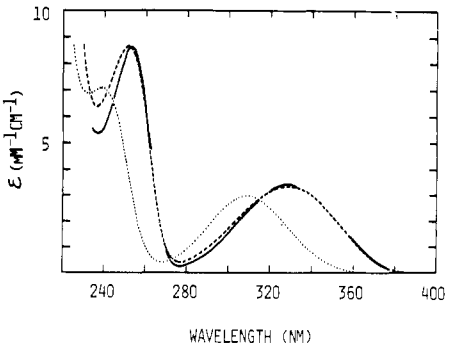


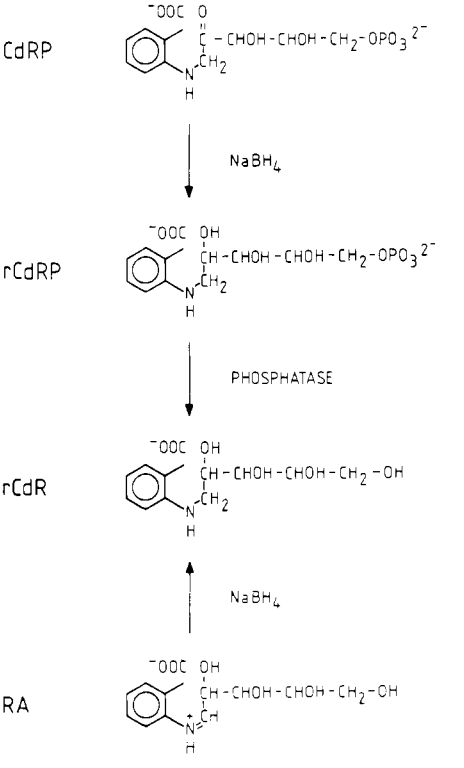

FIGURE 2: Reduced CdRP and *N*-ribitylanthranilate have the same absorption spectrum, which differs from that of anthranilate. 0.1 M potassium phosphate buffer, pH 7.0, 25 °C, was used. (—) *N*-Ribitylanthranilate; (---) rCdRP; (···) anthranilate.

*InGP Synthase Inhibited by rCdRP, InGP, and Indolepropanol Phosphate.* As a preliminary test of binding, we measured the inhibition constant of rCdRP in the standard 0.1 M Tris buffer, pH 7.5, 20 °C. The conversion of CdRP to InGP was followed by the increase of fluorescence at $\lambda >$ 320 nm [excitation at 297 nm (Hankins et al., 1975)]. The use of a sensitive fluorescence stopped-flow instrument allowed us to measure progress curves at low substrate concentrations (0.5–5 $\mu$M CdRP). The dependence of initial velocity ($v_i$) on the concentration of CdRP in the presence of a competitive inhibitor I is given by eq 1.

$$v_i/[\text{CdRP}] = (V_M - v_i)/[K_M(1 + [I]/K_i)] \quad (1)$$

As seen in Figure 3, rCdRP is a competitive inhibitor of InGP synthase with respect to CdRP. The average values from several experiments are $K_i = 0.3 \pm 0.1$ $\mu$M and $K_M = 1.2 \pm 0.1$ $\mu$M. The value of $K_M$ is lower than reported previously [$K_M = 16$ $\mu$M (Creighton & Yanofsky, 1966); $K_M = 5.8$ $\mu$M (Hankins et al., 1975)]. As shown below, this discrepancy is probably due to product inhibition by InGP. Neither ribose 5′-phosphate nor *N*-ribitylanthranilic acid inhibits InGP syn-

Scheme II



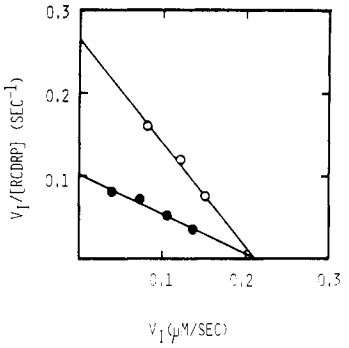



FIGURE 3: Reduced CdRP is a competitive inhibitor of InGP synthase: Eadie–Hofstee plot. Initial velocities of InGP production were evaluated with a stopped-flow apparatus as described under Experimental Procedures. 0.1 M Tris–acetate buffer, pH 7.5, 20 °C, was used. Enzyme concentration was 0.042 μM. (○) No rCdRP; (●) 0.4 μM rCdRP.

Table III: Nature of the Buffer Affects the Steady-State Parameters of InGP Synthase

| 0.1 M buffer, pH 7.5, 20 °C | substrate, CdRP $K_M$ (μM) | substrate, CdRP $k_{cat}$ ($s^{-1}$) | $K_i$ (μM) of competitive inhibitors: rCdRP | IPP | InGP |
|---|---|---|---|---|---|
| Tris-acetate | 1.2 | 7.7[a] | 0.3 | 8 | 19 |
| potassium-phosphate | 38.0 | 2.0[b] | 8.0 | 375[c] | 415[c] |

[a] Calculated from current maximum specific activity (10 units/mg of protein). [b] Calculated from Figure 1. [c] Noncompetitive inhibition.

thase. We conclude that both the phosphate and the anthranilate moieties of rCdRP are necessary for strong binding to the active site of InGP synthase.

Indolepropanol phosphate (IPP) is a chemically stable analogue of InGP (Kirschner et al., 1975). Inhibition studies similar to those depicted in Figure 3 showed that IPP is a competitive inhibitor of InGP synthase in 0.1 M Tris–acetate buffer, pH 7.5, 20 °C. The value of $K_i$ equals 8 μM. It is collected with the other steady-state constants in Table III. This finding suggests that InGP exerts competitive product inhibition. It does, and the value of $K_i$ equals 19 μM (Table III). As pointed out by Cornish-Bowden (1975, 1976), competitive product inhibition does not alter the shape of the progress curve but leads to erroneously high values of $V_{max}^{app}$ and substrate concentration dependent values of $K_M^{app}$, when the progress curves are fitted to the integrated Michaelis–Menten equation. The method of Fernley (1974) was used to analyze the progress curves, and the predictions of Cornish-Bowden (1975, 1976) were borne out by the results. As a consequence of product inhibition by InGP, initial velocities measured with concentrations of CdRP above 10 μM will tend to be underestimated, leading to erroneously high values of $K_M$ (Creighton & Yanofsky, 1966; Hankins et al., 1975).

*Inhibition of InGP Synthase by Phosphate.* In 0.1 M potassium phosphate buffer, pH 7.5, 20 °C, the values of $K_M$ for CdRP and of $K_i$ for rCdRP, IPP, and InGP are much larger than in Tris buffer (Table III). While rCdRP is a competitive inhibitor under these conditions, IPP and InGP are weak noncompetitive inhibitors. For IPP and InGP, the concentration dependence of $v_i$ is given by

$$v_i/[CdRP] = V_{max}/[K_M(1 + [I]/K_i)] - v_i/K_M \quad (2)$$

Since $k_{cat}$ is also fourfold lower in phosphate compared to Tris buffer (cf. Figure 1), the effect of phosphate cannot merely be due to competition with the phosphate moieties of substrates and inhibitors. The weak noncompetitive effects of IPP and InGP might reflect their amphiphilic nature (Hodo et al., 1977).

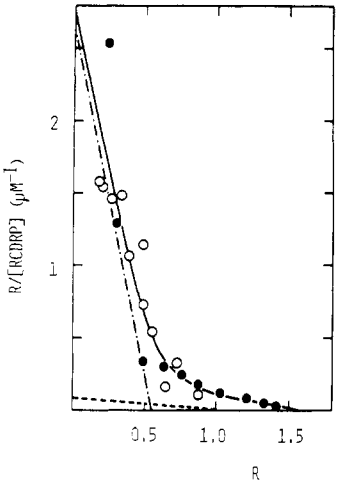



FIGURE 4: Reduced CdRP binds to two different sites on PRA isomerase–InGP synthase in 0.1 M Tris–acetate buffer, pH 7.5, 20 °C: Scatchard plot. Equilibrium dialysis was performed as described under Experimental Procedures. Total enzyme concentrations: (○) 1.5; (●) 15 μM. (—) Theoretical curve calculated for $n_1 = 0.55$, $n_2 = 1.05$, $K_{d,1} = 0.21$ μM, and $K_{d,2} = 12.5$ μM according to eq 3; (-·-) theoretical binding curve calculated for $n = 0.55$ and $K_d = 0.21$ μM; (---) theoretical binding curve calculated for $n = 1.05$ and $K_d = 12.5$ μM according to eq 4.

*Determination of the Binding Site Stoichiometry of PRA Isomerase–InGP Synthase for rCdRP by Equilibrium Dialysis.* The results of binding studies using $^3$H-labeled rCdRP and equilibrium dialysis in 0.1 M Tris–acetate buffer, pH 7.5, 20 °C, are presented in Figure 4. Two different total enzyme concentrations (1.5 and 15.0 μM) were used to improve the precision at the extremes of the saturation curve. As seen from the concave Scatchard plot, the enzyme has more than one binding site for rCdRP. Although there is considerable scatter, there is a reasonable overlap of the points obtained with the two different enzyme concentrations. The data were fitted to eq 3 by the method of Rosenthal (1967) where the binding

$$r = n_1\frac{[rCdRP]}{[rCdRP] + K_{d,1}} + n_2\frac{[rCdRP]}{[rCdRP] + K_{d,2}} \quad (3)$$

ratio $r = [rCdRP]_{bound}/[E]_0$.

The values of the thermodynamic dissociation constants of the two different enzyme–rCdRP complexes differ 60-fold ($K_{d,1} = 0.2$ μM, and $K_{d,2} = 12.5$ μM). Because $K_{d,1}$ is similar to the inhibition constant of rCdRP ($K_i = 0.3 \pm 0.1$ μM; cf. Table III), we conclude that the high-affinity binding site is the active site of InGP synthase. By exclusion, the low-affinity site must be identical with the active site of PRA isomerase. We did not attempt to measure the inhibition of the PRA isomerase reaction by rCdRP because PRA is very unstable (Creighton, 1968).

There seems to be one isomerase site ($n_2 = 1.05$) per mol of enzyme. By contrast, the number of synthase sites is only half as large ($n_1 = 0.55$). We surmise that this discrepancy could be due to the preferential instability of indoleglycerol-phosphate synthase as previously observed by Mosteller et al. (1977).

Since the enzyme is quite stable in phosphate buffer (Table II), binding studies were repeated with 0.1 M potassium phosphate, pH 7.5, 20 °C. As seen in Figure 5, the binding stoichiometry is close to $n = 2$ in this buffer. Although the data can be fitted to a linear Scatchard plot according to

$$r/[rCdRP] = (n - r)/K_d \quad (4)$$

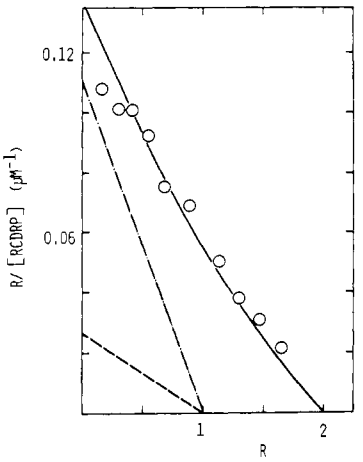



FIGURE 5: Phosphate ions increase the $K_d$ values of PRA isomerase–InGP synthetase to different extents: Scatchard plot. Equilibrium dialysis in 0.1 M potassium phosphate, pH 7.5, 20 °C, as described in Figure 4. Total enzyme concentration was 16 μM. (—) Theoretical curve calculated for $n_1 = 1.05$, $n_2 = 1.05$, $K_{d,1} = 10$ μM, and $K_{d,2} = 40$ μM according to eq 3; (–·–) theoretical binding curve for $n = 1.05$ and $K_d = 10$ μM; (- - -) theoretical binding curve for $n = 1.05$ and $K_d = 40$ μM.

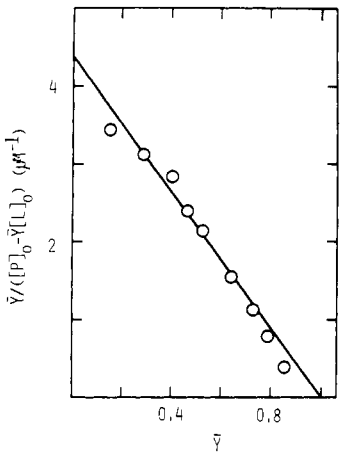



FIGURE 6: Fluorescence titration of rCdRP with increasing concentration of PRA isomerase–InGP synthase: Scatchard plot of ligand fluorescence quenching data. 0.1 M Tris–acetate buffer, pH 7.5, 20 °C, was used. Total rCdRP concentration $[L]_0 = 0.1$ μM. The total enzyme concentration $[P]_0$ was varied from 0.05 to 2.4 μM. Ligand fluorescence was excited at 324 nm, and emission was monitored at 420 nm. The degree of saturation is defined as $\bar{Y} = (F_0 - F)/(F_0 - F_\infty)$ where $F$, $F_0$, and $F_\infty$ are the values of fluorescence at a given protein concentration, in absence of protein, and at infinite protein concentration, all corrected for blank contributions from buffer and protein alone.

with $n = 1.9$ and $K_d = 15.2$ μM, the data are shown to be consistent also with the independently determined inhibition constant of the InGP synthase–rCdRP complex in phosphate buffer ($K_{d,1} = K_i = 8$ μM; cf. Table III). The best fit to eq 3 yields $n_1 = 1.0$ and $K_{d,1} = 9$ μM and $n_1 = 1.0$ and $K_{d,2} = 37$ μM. Thus, phosphate ions lead to a 50-fold increase of $K_d$ for InGP synthase and only a 3-fold increase of $K_d$ for the low-affinity site. In phosphate buffer there is only a small effect of temperature and pH value on the binding of rCdRP. The Scatchard plots appear to be linear and $n = 1.9$ and $K_d = 11.0$ for pH 6.5, 20 °C, and $n = 1.9$ and $K_d = 13.5$ for pH 7.5, 4 °C.

*Fluorometric Titration of the Enzyme with rCdRP.* The fluorescence emission of rCdRP is quenched when an excess of the PRA isomerase–InGP synthase is added. This effect has been used as a signal for measuring the kinetic progress curves in stopped-flow and temperature-jump experiments (Cohn et al., 1979). Fluorescence quenching was also employed for determining the value of $K_{d,1}$ in an independent manner by adding increasing amounts of enzyme to a constant but low concentration of ligand (van Landschoot et al., 1977; Krauss et al., 1973). Under these conditions the binding of ligand to the second site (for which $K_{d,2}$ equals 12.5 μM) is negligible because $[L] \ll K_{d,2}$ over the whole range of protein concentrations used.

The ligand fluorescence at 410 nm ($F$) decreases monotonically to an asymptotic value of $F_\infty/F_0 = 0.43$ as the total protein concentration is increased. $F_0$ and $F_\infty$ are the fluorescence intensities of rCdRP in the absence and in the presence of a saturating concentration of protein. The relative fluorescence decrease $\bar{Y} = (F_0 - F)/(F_0 - F_\infty)$ was defined as the degree of saturation and was used to calculate the concentration of free rCdRP (data not shown). The titration data are presented as a Scatchard plot in Figure 6 as given by

$$\bar{Y}/([P]_0 - \bar{Y}[L]_0) = (1 - \bar{Y})/K_d \qquad (5)$$

The points lie on a straight line extrapolating to one binding site on the ligand for the high-affinity site of the enzyme. The thermodynamic dissociation constant $K_d$ equals 0.23 μM. This simple binding proves that the enzyme binds both stereoisomers that arise from reducing the carbonyl group of CdRP with the same affinity.

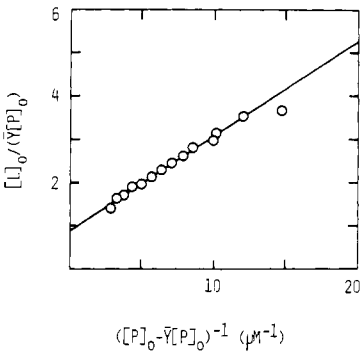



FIGURE 7: Plot of protein fluorescence quenching data according to eq 6. 0.1 M Tris–acetate buffer, pH 7.5, 20 °C, was used. The total enzyme concentration $[P]_0 = 0.4$ μM. The total rCdRP concentration was varied from 0.02 to 2.6 μM. Protein fluorescence was excited at 278 nm, and emission was monitored at 340 nm.

It is possible to measure $K_{d,1}$ also by titrating a constant quantity of enzyme with increasing amounts of ligand, since the value of $K_{d,1}$ is about 60-fold smaller than that of $K_{d,2}$ in Tris buffer (cf. Figure 4). The protein fluorescence at 340 nm (excited at 278 nm) is quenched by added rCdRP. The ligand has an absorption minimum at 278 nm (cf. Figure 2) and does not fluoresce at 340 nm.

Above 387 nm, there is an increase of fluorescence above the extent observed for the ligand alone (data not shown). This indicates that radiation-less fluorescence energy transfer occurs from a tyrosine or tryptophan of the enzyme to the anthranilic acid moiety of the ligand (Stryer, 1978). Up to a total ligand concentration of 0.8 μM, there is a well-defined isoemissive point at 387 nm. This finding indicates that only one class of binding sites is involved in the distribution of the ligand between free and bound forms (Daniel & Weber, 1966). The protein fluorescence at 340 nm does not decrease to a plateau with increasing ligand concentration (data not shown). The slanted asymptote probably reflects the binding of ligand to the weak binding site ($K_{d,2} = 12.5$ μM) which may be approximated by a linear contribution between 0 and 2 μM total ligand concentration. The relative fluorescence change $\bar{Y} = (F_0 - F)/(F_0 - F_\infty)$ (corrected for the slanted asymptote) was

Table IV: Same $K_d$ Value Is Obtained for the Synthase-rCdRP Complex by Different Methods

| method[a] | InGP synthase $n_1$ | InGP synthase $K_{d,1}$ ($\mu$M) | PRA isomerase $n_2$ | PRA isomerase $K_{d,2}$ ($\mu$M) | ref |
|---|---|---|---|---|---|
| equilibrium dialysis | 0.6 | 0.21 | 1.1 | 12.5 | Figure 4 |
| fluorescence titration | | | | | |
| ligand fluorescence | 1.0 | 0.23 | | | Figure 6 |
| protein fluorescence | 0.9 | 0.22 | | | Figure 7 |

[a] 0.1 M Tris-acetate, pH 7.5, 20 °C.

defined as the degree of saturation, and the binding data are presented in Figure 7 according to

$$[L]_0/(\bar{Y}[P]_0) = n + K_d/([P]_0 - \bar{Y}[P]_0) \quad (6)$$

The saturation curve appears to be hyperbolic. $K_d$ equals 0.22 $\mu$M and the value of $n$ = 0.88. The values of $n_1$ and $K_{d,1}$ describing the binding of rCdRP to the InGP synthase site as obtained by different methods are collected in Table IV. The more rapid titration methods give values of $n_1$ close to unity, and $K_{d,1}$ agrees well with the value from equilibrium dialysis.

*Difference Absorption Spectra of Enzyme-Bound rCdRP.* Upon binding to the bifunctional enzyme, the absorption spectrum of the ligand is shifted to longer wavelengths (Figure 8A). Calculations indicate that the difference spectrum corresponds to 99% saturation of the high-affinity site 1 and 78% saturation of the low-affinity site 2. The difference spectrum of ligand bound exclusively to site 1 was measured with an excess of total protein ($[P]_0$ = 100 $\mu$M) over total ligand ($[L]_0$ = 40 $\mu$M) and is shown in Figure 8B. The difference spectrum of ligand bound to site 2 was calculated from the composite difference spectrum (Figure 8A) and the intrinsic difference spectrum of site 1 and is also shown in Figure 8B. Both difference spectra reflect a similar kind of perturbation of the bound ligand but differ quantitatively, reflecting differences in the microenvironment of each site. As a consequence, when the enzyme is titrated with increasing ligand concentrations, the difference spectra possess no isosbestic point (data not shown).

## Conclusion

rCdRP is well suited as a probe for the two different active sites of PRA isomerase–InGP synthase. The two sites are characterized by different affinities for the substrate analogue. This can be rationalized by the fact that PRA possesses a furanose ring whereas the deoxyribulose side chain of CdRP is extended (Doy, 1966; Creighton, 1968). Thus, rCdRP is sterically more similar to CdRP than to PRA and should bind a priori more strongly to InGP synthase than to PRA isomerase. The reduction of the affinities of rCdRP for the two active sites by phosphate deserves further study. It is likely that phosphate ions act indirectly, e.g., via a structural change induced in the enzyme by bound phosphate.

Both the absorption and the fluorescence emission spectra of bound rCdRP differ from those of the free ligand. These spectral changes have been very useful in determining whether the different active sites interact. The concave Scatchard plot (Figure 4) could be the result of negative cooperativity (Levitzki & Koshland, 1976) between two intrinsically identical binding sites. This possibility is not ruled out by finding the same value for $K_{d,1}$ in the fluorometric titration where excess enzyme is added to rCdRP (Figure 6). It is clear, however, that the possible negative interaction between the binding sites does not involve a ligand-induced protein association equilibrium. Direct evidence on this question was obtained through studies of the mechanism of binding of rCdRP by rapid reaction techniques [cf. Cohn et al. (1979)].

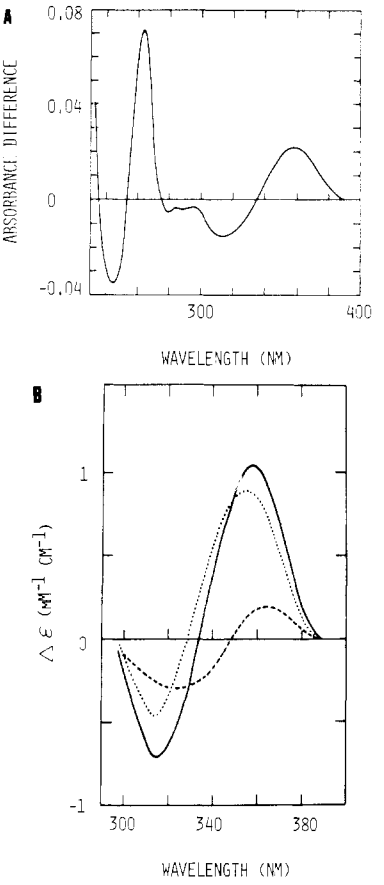



FIGURE 8: Absorbance difference spectrum of enzyme-bound rCdRP vs. free rCdRP in 0.1 M Tris–acetate buffer, pH 7.5, 20 °C ($d$ = 0.44 cm). (A) Difference spectrum of partially saturated enzyme. The concentrations of total enzyme and ligand were 57.8 and 150 $\mu$M, respectively. The free ligand concentration was calculated to be equal to 48 $\mu$M, assuming $n_1 = n_2 = 1$, $K_{d,1}$ = 0.21 $\mu$M, and $K_{d,2}$ = 12.5 $\mu$M (cf. Table IV). (B) Normalized difference spectra between rCdRP (---) bound to the active site of InGP synthase and rCdRP (···) bound to the low-affinity site. (—) Linear superposition of partial difference spectra.

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# *N*-(5-Phosphoribosyl)anthranilate Isomerase–Indoleglycerol-phosphate Synthase. 2. Fast-Reaction Studies Show That a Fluorescent Substrate Analogue Binds Independently to Two Different Sites†

Willy Cohn,‡ Kasper Kirschner,* and Carlton Paul

ABSTRACT: The mechanism of binding of reduced 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate (rCdRP) to two different binding sites on the bifunctional enzyme is determined by kinetic studies, using temperature-jump and stopped-flow equipment with fluorescence detection. Two rapid binding processes and a comparatively slow isomerization process are observed over a wide range of enzyme and rCdRP concentrations. Kinetic measurements with low concentrations of rCdRP show that the isomerization is coupled only to the more rapid of the two binding reactions that involves the active site of indoleglycerol-phosphate synthase. The slower of the two binding reactions represents rCdRP binding in one step to the active site of (phosphoribosyl)anthranilate isomerase. The simplest mechanism explaining quantitatively the dependence of the relaxation times on concentration consists of rCdRP binding to two sites on the enzyme that are intrinsically different and independent, even to the extent that a ligand-induced isomerization of one site is not transmitted to the other site. Simulation studies show that the concentration dependences of the amplitudes of the three relaxation processes are also consistent with the mechanism. The results are discussed in terms of two autonomous domains of folding of the polypeptide chain.

Phosphoribosylanthranilate (PRA)[1] isomerase–InGP synthase from *Escherichia coli* is a monomeric protein that catalyzes two sequential metabolic steps in the biosynthesis of tryptophan (Creighton & Yanofsky, 1970; Creighton, 1970). This protein is a simple example of multifunctional enzymes (Kirschner & Bisswanger, 1976; Stark, 1976; Pauckert et al., 1976; Gaertner, 1978). We have used ligand binding at equilibrium as a method to determine the dissociation constants of two different binding sites. Reduced CdRP (rCdRP) binds strongly to the active site of InGP synthase and 50-fold more weakly to a second site that is presumably identical with the active site of PRA isomerase (Bisswanger et al., 1979).

Equilibrium data alone, however, cannot reveal negative or positive interactions between the different binding sites. It is important to know whether such interactions occur because

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[1] Abbreviations used: PRA isomerase–InGP synthase, (phosphoribosyl)anthranilate isomerase–indoleglycerol-phosphate synthase (EC 4.1.1.48); CdRP, 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate; rCdRP, reduced CdRP; PRA, *N*-(5-phosphoribosyl)anthranilate; InGP, indoleglycerol phosphate.

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