***N*-(5'-Phosphoribosyl)anthranilate Isomerase–Indol-3-ylglycerol Phosphate Synthetase of Tryptophan Biosynthesis**

RELATIONSHIP BETWEEN THE TWO ACTIVITIES OF THE ENZYME FROM *ESCHERICHIA COLI*

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Further evidence is presented to confirm the previous conclusion that the enzyme from *Escherichia coli* catalysing the two sequential reactions in tryptophan biosynthesis, *N*-(5'-phosphoribosyl)anthranilic acid (PRA) → 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CdRP) → indol-3-ylglycerol phosphate (InGP) + CO₂ + H₂O, consists of a single polypeptide chain. The kinetic properties of the enzyme demonstrate that intermediate CdRP formed from PRA must dissociate from the enzyme before it can be converted into InGP. It is concluded that there are two distinct and non-overlapping catalytic sites on the enzyme for the two reactions. The expected complementation between a mutationally altered form of the enzyme lacking the first reaction and a mutationally altered form lacking the second reaction has been demonstrated *in vitro* by InGP formation from PRA. This system thus exhibits intracistronic complementation with a non-oligomeric protein gene product.

The enzyme PRA isomerase–InGP synthetase† of *Escherichia coli* (Creighton & Yanofsky, 1966) and other species of Enterobacteriaceae (McQuade & Creighton, 1970) is of special significance in that it catalyses two sequential reactions (PRA isomerase and InGP synthetase) in the biosynthesis of tryptophan, although it appears to consist of a single polypeptide chain:

In certain other organisms, these two reactions are catalysed by separate proteins (Hutter & DeMoss, 1967; Enatsu & Crawford, 1968; Hoch, Anagnostopoulos & Crawford, 1969), suggesting that the occurrence of the two activities on the same protein in the Enterobacteriaceae is not dictated by the chemical basis of the enzymic catalysis. Further, mutant strains of *E. coli* lacking either of the two activities, but retaining the other activity, have been isolated (Smith, 1967).

The catalysis of two reactions by a single polypeptide chain raises the question of the relationship on the enzyme of the catalytic sites for the two reactions. There could be a single catalytic centre capable of catalysing both reactions, or there could be two distinct catalytic sites, one for each of the reactions, possibly overlapping to some extent.

Further, occurrence of the mutant strains lacking either of the two activities makes it likely that genetic complementation between mutant strains from the two categories (one lacking PRA isomerase activity, the other lacking InGP synthetase activity) would be observed. The intermediate CdRP should be produced by the protein retaining PRA isomerase activity and utilized by the protein retaining InGP synthetase activity. Since the protein is a single polypeptide chain, the two mutational events in the two strains would have occurred within copies of the same cistron, and this would thus be an example of intracistronic (interallelic, allelic, intragenic) complementation (Fincham, 1966). The occurrence of such complementation has previously been used as an indication that the mutationally altered protein normally exists in an oligomeric form.

The purpose of the present report is to confirm that the PRA isomerase–InGP synthetase of *E. coli* is a single polypeptide chain, to present

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† Abbreviations: PRA, *N*-(5'-phosphoribosyl)anthranilic acid; CdRP, 1-(*o*-carboxyphenylamino)-2-deoxyribulose 5-phosphate; InGP, indol-3-ylglycerol phosphate.

kinetic evidence that there are two distinct catalytic sites for the two reactions, and to present evidence obtained *in vitro* that the expected complementation by cross-feeding of the intermediate does take place.

MATERIALS AND METHODS

Sources of PRA isomerase-InGP synthetase. The PRA isomerase-InGP synthetase was purified to apparent homogeneity from the A2/F'A2 strain of *E. coli* as described by Creighton & Yanofsky (1966). The mutant strains *trp* C9830, *trp* C9870, *trp* C9941 and *trp* D3 were isolated in the laboratory of C. Yanofsky and obtained from B. C. Carlton. Bacterial crude extracts were prepared by growing the appropriate bacteria at 37°C in the minimal medium of Vogel & Bonner (1956) with 0.2% glucose and limiting indole (2–3 µg/ml) to the stationary phase. The harvested bacteria were disrupted by sonication in 0.1 M-potassium phosphate buffer, pH 7.0. Cellular debris was removed by centrifugation and the extract stored at –15°C.

Ultracentrifugation. Sedimentation-equilibrium measurements were made at 20°C with a Beckman-Spinco model E analytical ultracentrifuge equipped with a photoelectric scanner. The sample was placed in a 12 mm filled Epon double-sector cell, fluorocarbon FC-43 being used to form a false cell bottom. The extinction at 280 nm was used as a measure of the protein concentration. After the equilibrium measurements had been made, the extinction without the protein was determined near the meniscus after high-speed centrifugation of the protein to the bottom of the cell.

The reduced and unfolded protein used was first reduced by incubation at 37°C for 15 min in a freshly prepared mixture of 8.0 M-urea, 0.25 M-NH₄HCO₃ and 0.10 M-dithiothreitol. A one-tenth volume of freshly prepared 3.0 M-iodoacetic acid (neutralized with aq. NH₃) was added and the incubation continued for 30 min. The reaction was then terminated by the addition of 0.1 vol. of mercaptoethanol. The mixture was dialysed exhaustively at 0°C against 0.1 M-NH₄HCO₃ and freeze-dried. The dried protein was dissolved directly in 6.0 M-guanidinium chloride (Heico Ultra High Purity).

Electrophoresis. Electrophoresis on Sepharose III cellulose acetate strips (Gelman Instrument Co.) was performed at room temperature in 0.1 M-sodium borate buffer, pH 8.3, with 1 mM-mercaptoethanol and EDTA. Protein was stained with 0.5% Ponceau S in 5% (w/v) trichloroacetic acid, followed by rinsing in 7.5% (w/v) acetic acid. When urea was included in the buffer, the electrophoresis strips were fixed before staining by soaking in a mixture of 5% (w/v) trichloroacetic acid and 5% sulphosalicylic acid.

The details of the procedures used in electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate have been described elsewhere (Shapiro & Maizel, 1969; McQuade & Creighton, 1970).

Preparation of PRA and CdRP. The substrate PRA was prepared non-enzymically just before use and measured by the procedures described by Creighton (1968). This substrate has not been purified, as it is very unstable, and the preparation is assumed to be contaminated with unreacted anthranilic acid and ribose

5-phosphate and any side products. To have the PRA and CdRP substrate preparations as equivalent as possible, CdRP was prepared by a method similar to that used for PRA. The initial incubation (Creighton, 1968) of anthranilic acid and ribose 5-phosphate was prolonged for 15 h at room temperature, at which time the mixture was diluted 50-fold with water and incubated at room temperature for 1 h, to decompose any PRA present. The mixture was then diluted with an equal volume of 0.10 M-triethanolamine-HCl buffer, pH 8.6, and kept at 0°C for no more than a few hours before use. The yield of CdRP (50%) was approximately the same as that of PRA (43%), so the two substrate mixtures were probably nearly equivalent. Contamination of either of the substrate preparations by the other substrate was less than 1% in each case.

All other materials were obtained commercially and used without further purification.

Assay procedures. The enzymic activity of the PRA isomerase-InGP synthetase was determined by following InGP formation from PRA in 3.0 ml of 0.10 M-triethanolamine-HCl buffer, pH 8.6, by the increase in extinction at 280 nm (Creighton & Yanofsky, 1966; Creighton, 1968).

InGP synthetase activity was measured by the same method, by following InGP formation from CdRP. Where indicated, InGP formation was determined by the amount of 3-formylindole formed after treatment with periodate (Gibson & Yanofsky, 1960).

Tetrazolium dye reduction by CdRP was followed by the increase in extinction at 520 nm of a mixture containing 3 mg of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride, 80 µg of phenazine methosulphate, 0.36 mg of gelatin, and the appropriate source of CdRP, in 0.10 M-triethanolamine-HCl buffer, pH 8.6, and a final volume of 3.0 ml. The previously noted (Creighton, 1968) ability of the purified enzyme to convert ribose phosphate into a highly reducing form has been found to be a contaminating activity resolvable by ion-exchange chromatography on DEAE-Sephadex. The purified wild-type enzyme used here did not have significant amounts of such activity.

All assay mixtures were maintained at 37°C, and all extinction measurements were made with a Gilford model 2000 recording spectrophotometer with a 10 mm path length. One unit of PRA isomerase-InGP synthetase is defined as that amount of enzyme capable of converting 1.0 µmol of PRA into InGP at the steady-state rate in 1 min under the standard assay conditions. The specific activity of the purified enzyme is approx. 4.0 units/mg of protein.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as the standard.

RESULTS

Structure of PRA isomerase-InGP synthetase. It had previously been concluded (Creighton & Yanofsky, 1966) that the enzyme consisted of a single polypeptide chain of molecular weight 4.5×10^4 because (1) there was only 1 mol of *N*-terminal residue (methionine) per 45000 g of protein; (2) digestion of the protein with trypsin plus chymotrypsin produced nearly the number of pep-

Fig. 1. Gel filtration of the *E. coli* PRA isomerase-InGP synthetase. A mixture of 2.0 ml of crude extract from strain *trp* A2/F⁺*trp* A2 (containing PRA isomerase-InGP synthetase, tryptophan synthetase β_2 subunit, and pantothenate synthetase), equine heart cytochrome *c* (Calbiochem), ox liver catalase (Worthington), *E. coli* alkaline phosphatase (Worthington), purified tryptophan synthetase α subunit (Henning, Helinski, Chao & Yanofsky, 1962), and Blue Dextran (Pharmacia) was placed on a column (2.5 cm $\times$ 72 cm) of Sephadex G-200 at 4°C equilibrated with 0.05 M-potassium phosphate buffer (pH 7.0)–1 mM-mercaptoethanol. The flow rate was 5 ml/h, and 3.1 ml fractions were collected by counting drops. Catalase and alkaline phosphatase were assayed by standard procedures using H_2O_2 and *p*-nitrophenyl phosphate as substrates, respectively. Blue Dextran and cytochrome *c* were detected by their extinction at 620 and 530 nm, respectively. The tryptophan synthetase α and β_2 subunits were assayed in the indole-tryptophan reaction in the presence of an excess of the other subunit (Smith & Yanofsky, 1962). Pantothenate synthetase was assayed by the β -alanine-dependent oxidation of NADH in a coupled system with myokinase, pyruvate kinase, and lactate dehydrogenase by the procedure of Pfeleiderer, Kreiling & Wieland (1960). The molecular weights of the standard proteins used were taken to be: catalase (x) 244 000 (Samejima, Kamata & Shibata, 1962); tryptophan synthetase β_2 subunit (O), 99 500 (Goldberg, Creighton, Baldwin & Yanofsky, 1966); alkaline phosphatase (●), 77 500 (Garen & Levinthal, 1960); pantothenate synthetase (Δ), 69 000 (Auricchio & Bruni, 1964); tryptophan synthetase α subunit (▲), 28 800 (Drapeau & Yanofsky, 1967); and cytochrome *c* (□), 13 400 (Atlas & Farber, 1956). The peak of Blue Dextran emerged in fraction 15. The arrow designates the position of the PRA isomerase-InGP synthetase.

tide spots expected on the basis of its amino acid content and molecular weight and (3) the molecular weight of the reduced protein did not decrease in 6 M-guanidinium chloride. To investigate further the molecular weight of the native protein in crude extracts, molecular-weight measurements were made by gel filtration on calibrated columns of Sephadex G-200 (Fig. 1). The PRA isomerase and InGP synthetase activities were consistently eluted

Fig. 2. Molecular-weight determination of the *E. coli* PRA isomerase-InGP synthetase in 6 M-guanidinium chloride by sedimentation equilibrium. The reduced, carboxymethylated protein was dissolved in 6.0 M-guanidinium chloride and centrifuged for 40 h to equilibrium at 20 000 rev./min and 20°C. The natural logarithm of the extinction at 280 nm is plotted against the square of the distance from the axis of rotation. The edges at the left and right of the figure indicate the solution meniscus and bottom, respectively. The solution column height was 2.4 mm.

together with a molecular weight estimated to be between 4.5×10^4 and 4.9×10^4 . These results confirm other findings that the molecular weights of the enzyme in crude extracts and of the purified protein are not significantly different (Goldberg *et al.* 1966; Ito, Cox & Yanofsky, 1969).

Previous determinations by sedimentation equilibrium of the molecular weight of the reduced protein in 6 M-guanidinium chloride had been carried out at high protein concentrations, where the hydrodynamic behaviour deviates significantly from thermodynamic ideality (Creighton & Yanofsky, 1966). Consequently, a considerable extrapolation to zero protein concentration was required to obtain the molecular weight. These studies have now been extended to low protein concentrations where non-ideality is not significant, by using u.v. absorption to measure the protein concentration gradient at equilibrium in the analytical ultracentrifuge. At an initial protein concentration of approx. 0.5 mg/ml, the reduced, carboxymethylated protein in 6.0 M-guanidinium chloride behaved as an apparently ideal solution containing a homogeneous protein species (Fig. 2). By using the value 0.736 ml/g estimated from amino acid composition of the protein (Creighton & Yanofsky, 1966) for the partial specific volume, the molecular weight was determined to be between 5.0×10^4 and 5.2×10^4 . This value is virtually the same as that determined previously by extrapolation to zero protein concentration (Creighton &

Fig. 3. Electrophoretic migration of reduced PRA isomerase-InGP synthetase in the presence of sodium dodecyl sulphate. The electrophoresis gel consisted of 5% (w/v) polyacrylamide, in the electrophoresis buffer, 0.01 M-sodium phosphate, pH 7.0, and 0.1% sodium dodecyl sulphate. The protein samples were denatured and reduced by incubation for 3 h at 37°C in the sodium phosphate buffer with 1% sodium dodecyl sulphate and 1% mercaptoethanol. Approx. 250 μ g of each protein was applied to the gel in an E-C vertical gel electrophoresis apparatus. Electrophoresis was at 280 V for 3 h. Protein was stained with Coomassie Blue. The distance migrated by each protein is plotted against its molecular weight. The standard proteins used were: bovine serum albumin (Pentex) monomer [$\times$, mol.wt. 68000 (Castellino & Barker, 1968)] and dimer [$\square$, mol.wt. 136000]; tryptophan synthetase α subunit [$\circ$, mol.wt. 28800 (Drapeau & Yanofsky, 1967)]; egg-white lysozyme (Worthington) [Δ , mol.wt. 14400 (Sophianopoulos, Rhodes, Holcomb & Van Holde, 1962)]. The distance migrated by the PRA isomerase-InGP synthetase is indicated by the vertical arrow.

Yanofsky, 1966). However, these values are somewhat higher than that determined for the molecular weight of the native protein by the same method in buffer (4.5×10^4) and by the other methods presented here; this suggests that the partial specific volume of the protein in 6.0 M-guanidinium chloride is lower than in buffer by approx. 0.01 ml/g, as has been found in other cases (e.g. Hade & Tanford, 1967; Castellino & Barker, 1968). In any case, the results are clearly incompatible with the dissociation of the protein into two or more species of significant molecular weight.

Estimates of the molecular weights of dissociated polypeptide chains may also be obtained from the relative rates of migration of the reduced proteins in polyacrylamide gels containing sodium dodecyl sulphate (Shapiro & Maizel, 1969). When compared with three standard proteins, the rate of migration of the PRA isomerase-InGP synthetase indicated a molecular weight of $4.6 (\pm 0.2) \times 10^4$ (Fig. 3). The four proteins migrated within the same gel slab, so the experimental error involved in comparing distances of migration and in keeping

the experimental conditions constant should have been minimal. Thus the reduced protein did not dissociate in the presence of the detergent.

The PRA isomerase-InGP synthetase migrated as a single band in the above experiment. If the protein dissociated into two or more non-identical polypeptide chains, two or more protein bands might have been observed. This possibility was investigated further by electrophoresis of the protein in 8 M-urea and 0.1 M-mercaptoethanol on cellulose acetate strips, conditions in which the rate of migration is less sensitive to molecular weight but more dependent upon net charge than in the previous experiment. Once again, the denatured PRA isomerase-InGP synthetase migrated as an apparent single species, as it did in the native state.

Further evidence that both the PRA isomerase and InGP synthetase reactions are catalysed by the same polypeptide chain has come from the proteins of the mutant strains possessing only one of the two activities, *trp* C9830 (retaining InGP synthetase activity) and *trp* C9941 (retaining PRA isomerase activity). The enzymes from each of the strains were purified to near homogeneity by the procedure used for the normal protein. The purified proteins were indistinguishable from the normal protein in electrophoretic mobility and in trypsin-plus-chymotrypsin peptide patterns prepared by the method of Helinski & Yanofsky (1962). Thus, loss of either of the activities by mutation is not necessarily accompanied by any gross loss of protein primary structure. At least one amino acid residue would be expected to differ in each of the mutationally altered proteins, but it is not unusual that such a difference was not detected in peptide patterns of this complexity (e.g. see McQuade & Creighton, 1970).

Kinetic behaviour of the PRA isomerase-InGP synthetase. At saturating concentrations (0.2–0.3 mM) of the substrates the steady-state rate of formation of InGP with CdRP as the substrate (the InGP synthetase reaction) was found to be 1.2 times the rate with PRA as the substrate (the overall reaction). The rate of the PRA isomerase reaction was determined by adding excess of InGP synthetase activity. The mutant enzyme lacking PRA isomerase activity (but retaining InGP synthetase activity) from strain *trp* C9830 is not able to catalyse the formation of InGP from PRA, but the addition of this purified enzyme to a mixture of normal enzyme and PRA caused an increase in the rate of formation of InGP. With excess of the mutant enzyme, the rate of InGP formation from PRA increased approximately sixfold. This finding that added InGP synthetase activity stimulated the formation of InGP from PRA by the normal enzyme strongly suggests that CdRP is a free intermediate.

The kinetics of formation of InGP from PRA

Fig. 4. Kinetics of InGP formation from PRA catalysed by PRA isomerase-InGP synthetase. (a) InGP formation measured by the increase in extinction at 280nm as a function of time after addition of purified enzyme at the following concentrations (m-units/ml): ●, 5.3; ○, 4.0; ▲, 2.7; △, 1.3; ■, 0.67; □, 0.33. In each case the initial concentration of PRA was 36 μM. (b) Rate of InGP formation from the linear portions of the curves of (a) against the amount of enzyme added. (c) Abscissa intercepts of the linear curves of (a) (τ) against the reciprocal of the enzyme concentration.

support this conclusion. PRA and CdRP have virtually identical u.v. absorption spectra, which differ considerably from that of InGP (Creighton, 1968). Consequently InGP formation from either PRA or CdRP is most readily followed by the concomitant increase in extinction at 280nm, the extinction increasing by 0.43–0.45 on the formation of 0.1mM-InGP from either substrate (Creighton, 1968). With PRA as the substrate, low concentrations of the wild-type enzyme catalysed the formation of InGP at a constant rate only after a significant lag period (Fig. 4a). No such lag is detectable when CdRP is the substrate. At the very low concentrations of enzyme, the initial rate of formation of InGP from PRA was approximately zero. The

constant rate of InGP formation attained after the lag period was directly proportional to the enzyme concentration (Fig. 4b). The lag period, τ , was defined as the abscissa intercept of the extrapolated linear portion of the curve, and was found to be approximately inversely proportional to the enzyme concentration (Fig. 4c). Moreover, the addition to the reaction mixture of very small amounts of CdRP decreased the value of τ to essentially zero (Fig. 5).

The lag period is interpreted as the time during which the enzyme is utilizing PRA to attain the steady-state concentration of free CdRP for the overall reaction. The utilization of PRA for InGP formation would require its prior conversion into free

Fig. 5. Decrease by added CdRP of the lag period in InGP formation from PRA. The lag period (τ) was measured as in Fig. 4 with $36\mu\text{M}$ -PRA, 0.67m -unit of enzyme/ml, and the indicated concentrations of added synthetic CdRP.

CdRP, as the initial rate of formation of InGP was approximately zero. The inverse relationship between τ and the enzyme concentration would be expected, as decreasing quantities of enzyme would require an increasing period of time to attain the steady-state concentration of CdRP. The abolishment of the lag period by addition of CdRP to the reaction mixture is the most direct evidence in support of this interpretation. The results in Fig. 5 indicate that the steady-state concentration of CdRP under these conditions was $5\mu\text{M}$, as that was the minimal concentration of added CdRP required to decrease τ to zero.

If CdRP is an obligatory free intermediate, the lag period would be expected to be:

$$\tau = \frac{\bar{b}}{v_A} \quad (1)$$

where $\bar{b}$ is the steady-state concentration of CdRP and v_A is the initial rate of formation of CdRP from PRA. The results in Fig. 5 indicate that under the conditions used $\bar{b}$ is $5\mu\text{M}$, and v_A is approximately $6\mu\text{mol/min}$ per unit of enzyme. τ would then be expected to be approx. $50\text{s}\cdot\text{unit}\cdot\text{l}^{-1}$. The slope of the curve in Fig. 4(c) of τ as a function of the reciprocal of enzyme concentration yields a value of $48\text{s}\cdot\text{unit}\cdot\text{l}^{-1}$. The magnitude of the lag period is thus in excellent agreement with that expected.

The concentration of CdRP in a reaction mixture can also be determined chemically, as it is substantially more reactive in reducing tetrazolium dyes than PRA or InGP (Creighton, 1968). Tetrazolium reduction by synthetic CdRP was found to proceed without a significant lag period, and the rate of reduction was very nearly directly proportional to the concentration of CdRP (Fig. 6).

Fig. 6. Rate of tetrazolium reduction by synthetic CdRP. The rate of increase in extinction at 520nm of the tetrazolium mixture (see the Materials and Methods section) is plotted against the concentration of added CdRP.

This curve was then used as a standard to determine the CdRP concentration from the rate of tetrazolium reduction in further experiments.

When the tetrazolium dye mixture was incorporated into the standard reaction mixture containing PRA and the enzyme, the dye was reduced to a significant extent (Fig. 7a), indicating the presence of CdRP. The rate of tetrazolium reduction was linear after an initial lag period, the extent of which was approximately inversely proportional to the amount of enzyme added and comparable with that observed in InGP formation (Fig. 4). However, at high concentrations of enzyme, the rate of reduction reached a maximum and then decreased rapidly, presumably owing to the disappearance of PRA. The linear portion of the curve was taken as a measure of the apparent steady-state concentration of CdRP (Fig. 7b). Under normal conditions, this would be expected to be independent of the enzyme concentration. However, the presence of the tetrazolium dye will alter the steady-state concentration of CdRP, as it will react with some of the CdRP:

The true steady-state concentration of CdRP in the

Fig. 7. Determination of the steady-state concentration of CdRP by the rate of tetrazolium reduction upon addition of PRA isomerase-InGP synthetase to PRA. (a) Tetrazolium reduction as a function of time after addition of enzyme at 4.0 (○), 2.7 (●), 1.3 (▲) and 0.67 (△) m-units/ml to a mixture of PRA ($36\mu\text{M}$) and the tetrazolium reagents (see the Materials and Methods section). The extinction at 520nm is compared with that of the same mixture without enzyme. (b) Steady-state concentration of CdRP determined from the rate of tetrazolium reduction of the linear portion of each curve in (a) as a function of the concentration of enzyme. The data for the two highest concentrations of enzyme are not shown in (a).

absence of tetrazolium dye will be approximated when v_3 is insignificant relative to v_2 (which is proportional to the amount of enzyme added). This may explain the increasing rate of tetrazolium reduction with increasing enzyme concentration at low concentrations (Fig. 7b). Further calculations support this interpretation. It is necessary that v_2 be just sufficiently large to make v_3 insignificant at an enzyme concentration of 3 m-units/ml, where the plateau is first reached in the steady-state concentration of CdRP with increasing concentrations of enzyme (Fig. 7b). At this concentration of enzyme the steady-state rate is $3\mu\text{M}/\text{min}$, which is the value of $v_2 + v_3$. The reduced form of the tetrazolium dye has a molar extinction coefficient at 520nm of 11800 and three equivalents of reducing compound are required per equivalent of dye (Margolies & Goldberger, 1966). The rate of tetrazolium reduction observed thus indicates that the concentration of CdRP is being decreased by reaction

Fig. 8. Dependence of the steady-state concentration of CdRP on the PRA concentration. The steady-state concentration of CdRP was measured as in Fig. 7(a) with 2.7 m-units of enzyme/ml. The PRA concentration given is the initial concentration; it was not corrected for the decrease due to conversion into CdRP and InGP.

with the dye at a rate (v_3) of $0.4\mu\text{mol}/\text{min}$. Consequently, v_2 is 6.5 times v_3 at this concentration of enzyme.

The CdRP steady-state concentration estimated from the rate of tetrazolium reduction at the intermediate concentrations of enzyme is approximately $5\mu\text{M}$, the same value as that estimated from the lag period in InGP formation at the same concentration of PRA (Fig. 5). The dependence of this parameter on the substrate concentration is shown in Fig. 8. If the two reactions are independent and CdRP is an obligatory free intermediate, its steady-state concentration would be expected to be given by:

$$\bar{b} = \frac{K_B}{\left(\frac{V_B}{\bar{v}} - 1\right)} \quad (2)$$

where $\bar{v}$ is the steady-state rate, V_B is the maximum rate of reaction with CdRP as substrate, and K_B is the Michaelis constant for CdRP, which has previously been shown to be approximately $5\mu\text{M}$ (Creighton & Yanofsky, 1966). The ratio $V_B/\bar{v}$ at saturating concentrations of PRA was found above to be 1.2. The maximum value for $\bar{b}$ would then be expected to be $5 \times K_B$, or $25\mu\text{M}$. The plateau value in Fig. 8 is 12–13 μM , which is within the range expected from the experimental error in the determination of the above parameters, particularly the Michaelis constant for CdRP.

The decrease in $\bar{b}$ at low substrate concentrations would be expected to be due primarily to a decrease in $\bar{v}$. The apparent Michaelis constant for PRA measured in the steady-state formation of InGP was found to be in the range 5–10 μM . However, the nature of the expression for $\bar{b}$ (eqn. 2) greatly exaggerates the dependence on substrate concentration in this case, as $\bar{b}$ should be one-half the maximum at a PRA concentration six times its apparent Michaelis constant. The dependence of

Table 1. *InGP* formation from PRA and CdRP by crude extracts of mutant strains of *E. coli*

InGP formation was measured by the periodate method (Gibson & Yanofsky, 1960). The reaction mixtures contained crude extract, either PRA (0.11 μ mol) or CdRP (0.16 μ mol), and either 0.10 M-triethanolamine-HCl buffer, pH 8.6, or 0.10 M-tris-HCl buffer, pH 7.8, respectively, in a total volume of 0.50 ml. With the pairwise combinations of the extracts, very nearly equal amounts of protein from the crude extracts were added individually to the reaction mixtures. Zero *InGP* formed in each case means less than 0.2 nmol/20 min per mg of protein, the lower limit of detection under the conditions used.

Strain	InGP formed (nmol/20 min per mg of protein) from	
	PRA	CdRP
<i>trp</i> D3	140	400
<i>trp</i> C9830	0	497
<i>trp</i> C9941	0	0
<i>trp</i> C9870	0	0
<i>trp</i> C9830 + <i>trp</i> C9941	149	298
<i>trp</i> C9830 + <i>trp</i> C9870	0	304
<i>trp</i> C9941 + <i>trp</i> C9870	1	0

the steady-state concentration of CdRP on the PRA concentration shown in Fig. 8 is thus consistent with the expectations of eqn. (2).

Complementation of mutationally altered proteins in vitro. Extracts of the mutant strains of *E. coli* lacking PRA isomerase (*trp* C9830), *InGP* synthetase (*trp* C9941), and both activities [*trp* C9870, which carries an 'amber' nonsense mutation in the *trp* C gene (Yanofsky & Ito, 1966)] were investigated for their abilities to catalyse the formation of *InGP* from either PRA to CdRP individually and in pair-wise combinations (Table 1). For comparison, an extract from a mutant strain (*trp* D3) unable to synthesize PRA from anthranilic acid is included. As expected, none of the strains lacking either PRA isomerase or *InGP* synthetase could produce *InGP* from PRA whereas *trp* C9830, lacking only the PRA isomerase activity, could utilize CdRP. Complementation between *trp* C9830 and *trp* C9941 was observed, as this mixture could catalyse the overall conversion of PRA into *InGP*. Such complementation may also be readily demonstrated with the purified enzymes.

DISCUSSION

The results presented here completely support the previous conclusion (Creighton & Yanofsky, 1966) that the PRA isomerase-*InGP* synthetase of *E. coli* consists of a single polypeptide chain of molecular weight approx. 4.5×10^4 . The inability of the various denaturing reagents to dissociate the protein indicates that if the enzyme consists of

multiple polypeptide chains, they must be held together by covalent forces other than disulphide bonds. Precedents for such a structure are virtually non-existent.

Nevertheless, the enzyme normally catalyses *in vivo* two sequential reactions in tryptophan biosynthesis. The kinetic data *in vitro* presented here clearly demonstrate that the intermediate, CdRP, does not remain bound to the enzyme. The lag period before the overall enzyme reaction approaches the steady-state condition and the negligibly small initial rate of formation of *InGP* from PRA indicate the obligatory nature of the dissociation of CdRP from the enzyme. In general, such non-linear kinetic behaviour may have any one of numerous causes. However, in this case the abolition of the lag period by the addition of very small quantities of CdRP and the general consistency of the kinetic behaviour strongly support the interpretation that the CdRP formed from PRA must dissociate from the enzyme before being enzymically converted into *InGP*. Therefore, the binding of CdRP must be different for the two reactions, and distinct catalytic sites on the enzyme for the two reactions are defined. If the two sites overlapped, PRA and CdRP should compete for the enzyme, and the steady-state concentration of CdRP should have been directly proportional to the PRA concentration. However, the steady-state concentration of CdRP was found to be independent of the PRA concentration at saturating concentrations of the latter (Fig. 8), strongly suggesting that the two active sites do not overlap.

It is also pertinent to examine the effect of ligand binding at one active site on the catalytic properties of the other. The kinetic behaviour observed here was consistent with the two active sites being independent, although the parameters measured would not be expected to be particularly sensitive to any such effects. A possible exception was the unexplained observation that the velocity of the PRA isomerase reaction in the steady state was only one-sixth that in the absence of CdRP. However, interactions between the active sites are not the only possible explanations for the phenomenon. CdRP could be a potent product inhibitor of the PRA isomerase reaction, or the reaction could be significantly reversible. Analogous isomerase reactions (e.g. ribose 5-phosphate $\rightleftharpoons$ ribulose 5-phosphate) have equilibrium constants of an order of magnitude of unity (Axelrod & Jang, 1954), but the properties of the PRA isomerase-*InGP* synthetase have not made it possible to test for reversibility in this case. However, the results in Fig. 8 are not compatible with either product inhibition or reaction reversibility, as in either case the steady-state concentration of CdRP should have been proportional to the concentration of PRA at saturating concentrations

of the latter. Consequently no definitive conclusions on this subject may be drawn from this study.

The observations of this investigation also leave unanswered the question as to why the PRA isomerase and InGP synthetase reaction are catalysed by a single protein in the Enterobacteriaceae. With the exception of the phenomenon just discussed, this multifunctional enzyme was kinetically indistinguishable *in vitro* from two independent enzymes. However, uncertainty about the state of the enzyme *in vivo* (see below) leaves open the possibility that the enzyme may behave differently *in vivo*.

The complementation *in vitro* observed here between mutationally altered forms of the enzyme is simply the result of the coupling of two reactions, and is pertinent to genetics only because the two sequential reactions are catalysed by a single polypeptide chain. The possibility of this type of intracistronic complementation had previously been recognized (Fincham, 1966), but until now no example has been known. This type of intracistronic complementation may be readily distinguished from that resulting from the formation of hybrid oligomeric enzymes (Schlessinger & Levinthal, 1965) by its demonstration *in vitro* on simply mixing the altered proteins and by the existence of the two half-reactions and a free intermediate in the overall reaction in the complemented system. On the other hand, the type of complementation observed here may not be readily distinguishable from interacistronic complementation in a system in which physical aggregation of wild-type gene products is required for biological activity. Purification and characterization of the gene product(s) are required. The major significance of the complementation described here is to illustrate that the observation of intracistronic complementation should not automatically be taken as evidence for the oligomeric nature of the protein product.

The complementation between mutant strains each lacking one of the two activities illustrated here *in vitro* has been observed *in vivo* in *E. coli* (Liu & Smith, 1968). However, additional instances of positive complementation have also been observed that are not explained by the findings of this investigation. These may be the result of aggregation of the protein *in vivo* under conditions that have not been duplicated *in vitro*. Nevertheless, the complementation *in vitro* described here is explained by simple coupling of two sequential enzyme reactions and serves to illustrate the possibility of intracistronic complementation with a non-oligomeric protein.

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