Doy, C. H. (1966) *Nature (London) 211*, 736.
Doy, C. H., & Gibson, F. (1959) *Biochem. J. 72*, 586.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.
Enatsu, T., & Crawford, I. P. (1968) *J. Bacteriol. 95*, 107.
Fernley, H. N. (1974) *Eur. J. Biochem. 43*, 377.
Gaertner, F. H. (1978) *Trends Biochem. Sci. 3*, 63.
Gaertner, F. H., & Cole, K. W. (1977) *Biochem. Biophys. Res. Commun. 75*, 259.
Hankins, C. N., Largen, M., & Mills, S. E. (1975) *Anal. Biochem. 69*, 510.
Hartree, E. F. (1972) *Anal. Biochem. 48*, 422.
Hoch, S. O., & Crawford, I. P. (1973) *J. Bacteriol. 116*, 685.
Hoch, S. O., Anagnostopoulos, C., & Crawford, I. P. (1969) *Biochem. Biophys. Res. Commun. 35*, 838.
Hodo, H. G., III, Murphy, J., Hardman, J. K., & Myers, R. (1977) *Arch. Biochem. Biophys. 181*, 419.
Hunter, M. J. (1966) *J. Phys. Chem. 70*, 3285.
King, E. J. (1932) *Biochem. J. 26*, 292.
Kirschner, K., & Bisswanger, H. (1976) *Annu. Rev. Biochem. 45*, 143.
Kirschner, K., Wiskocil, R. L., Rezeau, L., & Foehn, M. (1975) *Eur. J. Biochem. 60*, 513.
Krauss, G., Römer, R., Riesner, D., & Maass, G. (1973) *FEBS Lett. 30*, 6.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Levitzki, A., & Koshland, D. E., Jr. (1976) *Curr. Top. Cell. Regul. 10*, 1.
Lingens, F., Burkhardt, H. J., & Hellmann, H. (1957) *Z. Naturforsch. B 12*, 493.
McQuade, J. F., III, & Creighton, T. E. (1970) *Eur. J. Biochem. 16*, 199.
Mosteller, R. D., Nishimoto, K. R., & Goldstein, R. V. (1977) *J. Bacteriol. 131*, 153.
Pauckert, J. L., Strauss, L. D., & Rabinowitz, J. C. (1976) *J. Biol. Chem. 251*, 5104.
Rosenthal, H. E. (1967) *Anal. Biochem. 20*, 525.
Schachman, H. K. (1957) *Methods Enzymol. 4*, 32.
Smith, O. H. (1967) *Genetics 57*, 95.
Stark, G. (1976) *Trends Biochem. Sci. 2*, 64.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237.
Sugimoto, S., & Shiio, I. (1977) *J. Biochem. (Tokyo) 81*, 823.
Van Landschoot, A., Loontiens, F. G., Clegg, R. M., Sharon, N., & De Bruyne, C. K. (1977) *Eur. J. Biochem. 79*, 275.
Welch, G. R. (1977) *Prog. Biophys. Mol. Biol. 32*, 103.
Yanofsky, C., Horn, V., Bonner, M., & Stasiowski, S. (1971) *Genetics 69*, 409.
Yphantis, D. A. (1964) *Biochemistry 3*, 297.

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

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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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they might provide a functional advantage of the covalently linked enzymes over the unassociated enzymes found in other organisms (Bisswanger et al., 1979).

In the following study the number of elementary reactions involved in binding rCdRP to the enzyme is determined by rapid-reaction techniques. The changes in protein (tryptophan) and rCdRP fluorescence upon binding are useful signals for following the reactions (Bisswanger et al., 1979). The mechanism of simultaneous binding of the ligand to two different sites is deduced by analysis of the dependence of relaxation times and amplitudes on the concentration of rCdRP and protein.

Experimental Procedures

Materials. PRA isomerase–InGP synthase and rCdRP were prepared and assayed as described in the preceding paper of this issue (Bisswanger et al., 1979). The standard buffer was 0.1 M Tris–acetate buffer, pH 7.5, at 20 °C, containing 0.07 M sodium acetate, 10 mM EDTA, and 2 mM dithioerythritol.

Methods. The temperature-jump apparatus was constructed in our department by Dr. G. Haenisch and is based on the design of Rigler et al. (1974) and Jovin (1975). A 200-W Hg–Xe arc lamp (Hanovia 901-B1) was used as the light source. A Schoeffel GM 250 A tandem grating monochromator reduced stray light to low levels. The fluorescence emitted by rCdRP was isolated by a cutoff filter (Schott-WG (420). The optical path length of the quartz temperature-jump cell (Coutts et al., 1975) equalled 7 mm. The discharge voltage was 30 kV, causing a temperature jump of 5.5 °C from an initial temperature of 14.5 °C. Rapid mixing experiments were performed with a modified Durrum stopped-flow spectrophotometer described elsewhere (Paul et al., 1980). Recordings were made at two different sweep rates, and an average of 10–20 experiments was fitted by a method developed by C. Paul. Fresh solutions were made up frequently, and enzyme assays showed that the protein activity remained unchanged after a series of temperature jumps. The amplitudes were measured relative to the total fluorescence of the equilibrium solution in the observation cell, by using the same amplification.

Results

Modes of Observation. It is convenient to use the fluorescence emission of rCdRP above 420 nm for following the binding kinetics. Monochromatic light of 280 nm excites the fluorescence of the bound ligand indirectly by radiationless energy transfer from tryptophan (Stryer, 1978). Under these conditions the free ligand fluoresces relatively weakly because it has an absorption minimum at 278 nm. Monochromatic light of 366 nm excites the fluorescence of the free and the bound ligand directly. The enzyme does not absorb at 366 nm, but it quenches the fluorescence of bound ligand (Bisswanger et al., 1979).

Observation of Three Relaxation Times. Solutions of enzyme and rCdRP respond to a temperature jump from 14.5 to 20 °C with three distinct relaxations. When excited at 280 nm, the ligand fluorescence decreases in the first two rapid steps ($\tau_1 \sim 1$ ms and $\tau_2 = 5$ ms; Figure 1). The third relaxation process is accompanied by an increase of fluorescence and is comparatively slow ($\tau_3 \sim 30$ ms; not shown here). Because it is not clear from these data alone how much of the third relaxation arises from the onset of cooling, the slow process was not evaluated quantitatively. Neither enzyme solutions alone nor rCdRP solutions alone give any detectable relaxation process. Since Tris has a large heat of protonation (Bernhard, 1955), the binding equilibria are perturbed by an increase of temperature and a simultaneous increase in hydrogen ion concentration.

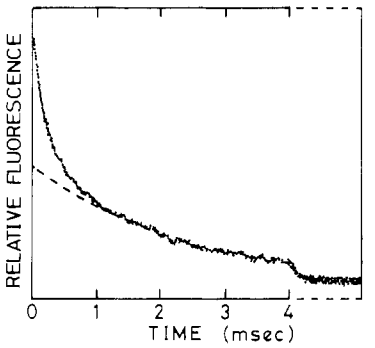

FIGURE 1: Record of progress curve from temperature-jump experiments with PRA isomerase–InGP synthase and rCdRP, showing two rapid relaxation processes. The concentration of enzyme equalled 2 μM; that of rCdRP was 22 μM. Ligand fluorescence was excited at 280 nm and observed above 420 nm. The temperature jump was from 14.5 to 20 °C, and 0.1 M Tris–acetate buffer, pH 7.5, was used. The vertical scale is fluorescence in arbitrary units. After 4 ms the sweep rate was reduced 10-fold to obtain the base line. Twenty successive experiments with four changes of the solution were averaged for display. (---) Contribution of slow relaxation.

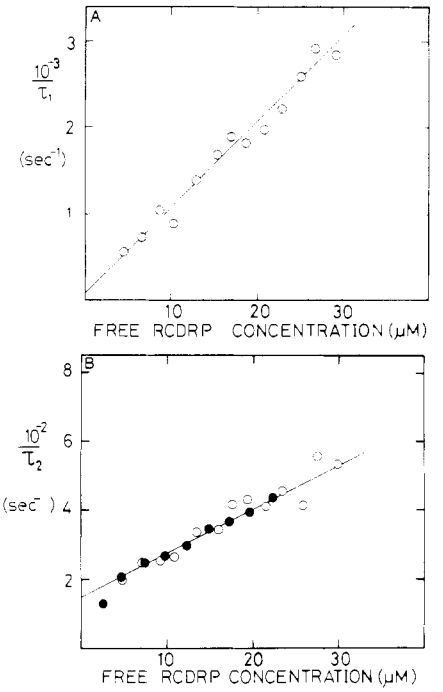

FIGURE 2: Reciprocal relaxation times $1/\tau_1$ and $1/\tau_2$ increase indefinitely with increasing excess rCdRP concentration. Conditions are as described in Figure 1. The ratio of rCdRP to enzyme concentration was kept constant at a value of 10. Each point results from an average of 15–25 progress curves. The straight lines were obtained by linear regression of $1/\tau_i$ vs. the concentration of free rCdRP estimated from the relation $[\text{rCdRP}]_f \sim [\text{rCdRP}]_0 - [\text{E}]_0$. (A) $1/\tau_1$ from temperature-jump experiments. (B) (○) $1/\tau_2$ from temperature-jump experiments; (●) $1/\tau_2$ from rapid mixing experiments.

With a 10-fold molar excess of rCdRP over enzyme concentration, $1/\tau_1$ and $1/\tau_2$ increase linearly with the free ligand concentration, which does not change appreciably during the relaxation (parts A and B in Figure 2). The slower of these processes is also observable in rapid mixing experiments, and $1/\tau_2$ has the same concentration dependence (Figure 2B).

The two rapid relaxation processes apparently arise from two independent binding steps involving rCdRP and two different sites on the enzyme. The tentative mechanism is

$$\text{LP} \underset{k_3}{\overset{k_4}{\rightleftharpoons}} \text{P} + \text{L} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{PL} \qquad (1)$$

Table I: Rate and Equilibrium Constants Determined from Concentration Dependence of the Three Relaxation Times at pH 7.5 and 20 °C

figures and eq	rate constants						equilibrium constants		
	k_1 (M^{-1} s^{-1})	k_3 (M^{-1} s^{-1})	k_2 (s^{-1})	k_4 (s^{-1})	k_5 (s^{-1})	k_6 (s^{-1})	k_2/k_1 (M)	k_4/k_3 (M)	k_6/k_5
Figure 2, eq 2-3	10^8	1.2×10^7	80	150			8×10^{-7}	1.2×10^{-5}	
Figure 3, eq 2-3	10^8	1.3×10^7	120	175			1.2×10^{-6}	1.4×10^{-5}	
Figure 5, eq 4 and 6	6×10^7		60		7	22	1×10^{-6}		3
Figures 2-6, normal mode analysis	10^8	1.4×10^7	80	140	7.5	22.5	8×10^{-7}	1×10^{-5}	3

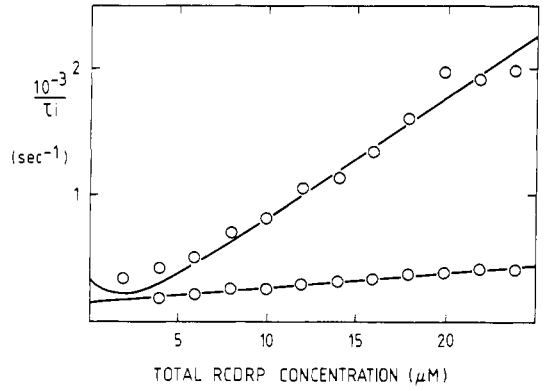

FIGURE 3: Dependence of $1/\tau_1$ and $1/\tau_2$ on the concentration of total rCdRP at constant enzyme concentration. Conditions are as described in Figure 1 with a total enzyme concentration of 2 μM. The curves are calculated by normal mode analysis with the rate constants listed in the bottom line of Table I.

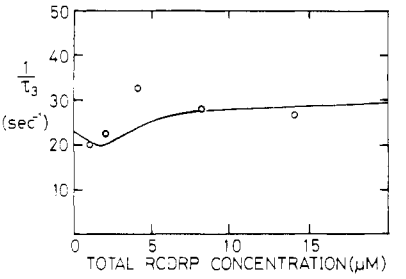

FIGURE 4: Reciprocal relaxation time of the third relaxation process ($1/\tau_3$) as obtained from rapid mixing experiments approaches a plateau at high ligand concentration. The concentration of enzyme equals 2 μM. Ligand fluorescence is excited at 366 nm. Other conditions are as described in Figure 1.

where L is rCdRP and P is the enzyme with two free binding sites. PL and LP are the two different complexes of the enzyme with rCdRP. The concentration dependences of $1/\tau_1$ and $1/\tau_2$ predicted for this mechanism are given by eq 2 and 3.

$$1/\tau_1 = k_2 + k_1[\bar{L}] \quad (2)$$

$$1/\tau_2 = k_4 + k_3[\bar{L}] \quad (3)$$

$[\bar{L}]$ is the concentration of excess free ligand.

Linear least-squares fits of the data (Figure 2) give the values of the rate and equilibrium constants listed in the first line of Table I. The values of the recombination rate constant k_1 is as large, and that of k_3 is only 10-fold smaller than the estimated lower limits for diffusion-controlled reactions [10^8 M^{-1} $s^{-1} < k < 10^9$ M^{-1} s^{-1} (Hammes & Schimmel, 1970; Pecht & Lancet, 1977)]. The comparison suggests that the two rapid processes are probably due to elementary binding reactions.

The kinetically determined equilibrium constant k_4/k_3 agrees reasonably well with the value of $K_{d,2}$ (12 μM) obtained by equilibrium studies. In contrast, k_2/k_1 is 4 times larger than the value of $K_{d,1}$ (0.2 μM) obtained by equilibrium studies (Bisswanger et al., 1979). This discrepancy suggests that the third relaxation process (τ_3) corresponds to an additional step in the binding mechanism.

Figure 3 presents the results of temperature-jump experiments conducted with constant enzyme concentration (2 μM) in which the relative concentration of total rCdRP (2–24 μM) was less than that in the experiment of Figure 2. The experiment was designed to detect additional relaxation processes that might be observable when neither the ligand nor the enzyme is present in large excess. Again, fluorescence energy transfer from the protein was used to follow the relaxation. The concentration of free ligand was calculated from the known total concentrations of rCdRP and enzyme, by using the known equilibrium values $K_{d,1}$ and $K_{d,2}$. An additional, rapid relaxation process is not observable. Linear least-squares fits of $1/\tau_1$ and $1/\tau_2$ to eq 2 and 3 as a function of the calculated free ligand concentration give the rate constants presented in the second line of Table I. The discrepancy between the values of k_2/k_1 and $K_{d,1}$ persists.

The third relaxation has a small amplitude when fluorescence of rCdRP is excited at 280 nm, but it is readily detected in temperature-jump or rapid-mixing experiments when the fluorescence is excited at 366 nm. Figure 4 presents data from rapid-mixing experiments that were performed with the same enzyme and ligand concentrations as in Figure 3. $1/\tau_3$ is practically independent of rCdRP concentration. This behavior indicates that an isomerization reaction is responsible for τ_3. The relative fluorescence amplitude increases to a sharp maximum at a ligand concentration of about 3 μM (not shown here).

Because the equilibrium dissociation constants differ 50-fold, the binding of rCdRP is limited to the high-affinity site when the concentration of enzyme is in excess (Bisswanger et al., 1979). Under such pseudo-first-order conditions, rapid mixing experiments would determine whether the slow isomerization (τ_3) is associated with rCdRP binding to the high-affinity site. The progress curves obtained from such experiments by monitoring the fluorescence of rCdRP excited at 366 nm consist of two exponential phases. The reciprocal time constant of the rapid process increases with total protein concentration, as expected for an elementary binding reaction (Figure 5A)

$$1/\tau_1 = k_2 + k_1[P]_0 \quad (4)$$

where $[P]_0$ is the total enzyme concentration. Linear least-squares fits of the data to eq 4 give the values of k_2 and k_1 listed in the third line of Table I. They agree reasonably well with those obtained from previous experiments for the more rapid of the two binding reactions.

The reciprocal time constant of the slower process ($1/\tau_3$) increases with increasing excess enzyme concentration toward a plateau value of 30 s^{-1} (Figure 5B). Because $1/\tau_3$ becomes independent of protein concentration, the slow relaxation cannot be due to the association of the enzyme to dimers or higher aggregates. The increase of $1/\tau_3$ toward an asymptote is characteristic of the slow isomerization of an enzyme–ligand complex formed by a preceding rapid binding reaction (Eigen

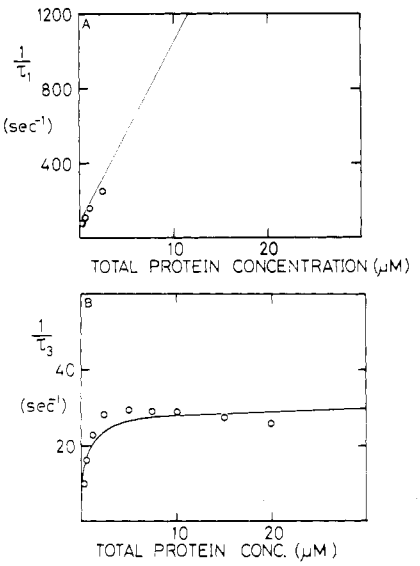

FIGURE 5: Dependence of $1/\tau_1$ and $1/\tau_3$ on the concentration of excess enzyme at constant rCdRP concentration. Rapid mixing experiments were performed with fluorescence excitation at 366 nm. Other conditions are as described in Figure 1. For the first two points $[rCdRP]_0 = 0.03$ and 0.06 μM. For the following points $[rCdRP]_0 = 0.13$ μM. (A) $1/\tau_1$ increases indefinitely with increasing enzyme concentration. The straight line was calculated with eq 4 and the rate constants listed in the bottom line of Table I. (B) $1/\tau_3$ approaches a plateau value. The curve was calculated with eq 12 and the rate constants listed in the bottom line of Table I.

& De Maeyer, 1974; Bernasconi, 1976; Lancet & Pecht, 1976).

$$P + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} PL \underset{k_5}{\overset{k_6}{\rightleftharpoons}} PL^* \qquad (5)$$

According to this mechanism (eq 5), $1/\tau_3$ should depend on the excess free protein concentration as given by

$$1/\tau_3 = k_5 + k_6[[\bar{P}]/([\bar{P}] + K_1)] \qquad (6)$$

where $[\bar{P}]$ is the concentration of free binding sites and K_1 equals k_2/k_1. $[\bar{P}]$ is approximately equal to $[P]_0$, the total protein concentration. As predicted by eq 6, $1/\tau_3$ increases hyperbolically with a half-maximal value at $[P]_0 = K_1 \sim 1$ μM (Figure 5B). From the intercept and the plateau values of $1/\tau_3$, one can estimate the following parameters: $k_5 = 7 \pm 2$ s^{-1}, $k_6 = 22 \pm 4$ s^{-1}, and $K_3 = k_6/k_5 = 3 \pm 2$ (Table I). The overall equilibrium dissociation constant $[\bar{P}][\bar{L}]/([\bar{PL}] + [PL^*]) = k_2k_5/[k_1(k_5 + k_6)]$ equals approximately 0.2 μM and agrees with $K_{d,2}$ obtained from equilibrium measurements (Bisswanger et al., 1979). We therefore assign the more rapid of the two binding reactions and the slow isomerization reaction to the active site of InGP synthase.

The relationship between the rapid binding reaction ($1/\tau_1$) and the slow isomerization reaction ($1/\tau_3$) was also studied with temperature-jump experiments at low enzyme and rCdRP concentrations and excitation at 366 nm. Only two relaxation processes are observed. The reciprocal relaxation time of the rapid process (Figure 6A) equals the values of $1/\tau_1$ and $1/\tau_2$ extrapolated to low concentrations of rCdRP (Figure 3). The reciprocal relaxation time of the slow process (Figure 6B) is of the same magnitude as $1/\tau_3$ presented in Figures 4 and 5B. The experimental error of $1/\tau_3$ is large because cooling interferes with relaxation measurements above 0.1 s. The interpretation of these results will be given below.

No isomerization appears to be associated with ligand binding to the low-affinity site. In rapid mixing experiments the binding of rCdRP to the low-affinity site is completely

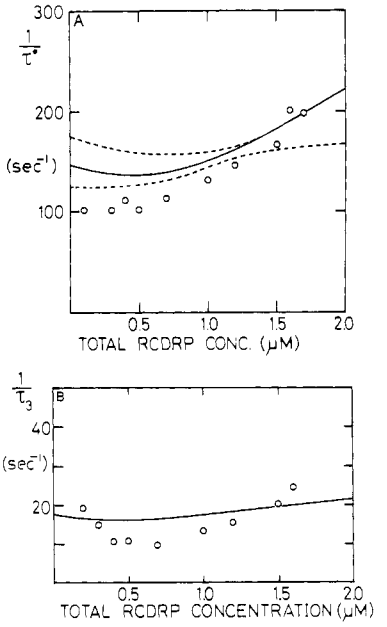

FIGURE 6: Dependence of the reciprocal mean relaxation time ($1/\tau^*$) and $1/\tau_3$ on the concentration of total rCdRP at constant, low enzyme concentration. Temperature-jump experiments were with fluorescence excitation at 366 nm. Other conditions are as described in Figure 1. The total enzyme concentration equalled 0.7 μM. (A) (—) The reciprocal mean relaxation time was calculated from eq 13 with the values of the amplitudes from Figure 9A and $1/\tau_1$ and $1/\tau_2$ obtained from normal mode analysis (cf. text); (- - -) calculated values of $1/\tau_1$ and $1/\tau_2$ obtained with the rate constants from the bottom line of Table I. (B) (—) $1/\tau_3$ calculated with eq 11 and the rate constants listed in the bottom line of Table I.

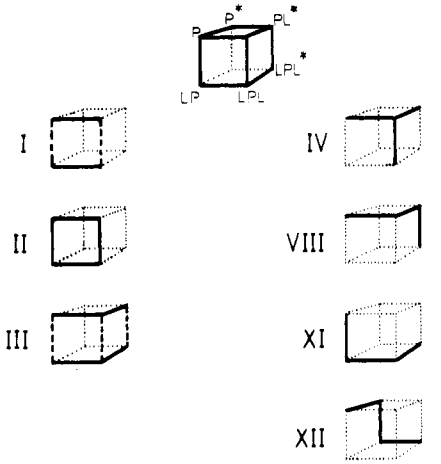

FIGURE 7: General mechanism of ligand binding to a bifunctional enzyme with isomerization of one of the binding sites. The partial schemes I–XII are special cases obtained by neglecting certain species from the general mechanism. In schemes I and III, the equilibrium and rate constants of reactions on opposite sides of the square(s) are assumed to be identical. Scheme III corresponds to the mechanism of eq 7.

equilibrated before the isomerization of the primary complex of the high-affinity site has progressed significantly (Figure 2B). This observation suggests that the two binding sites on the enzyme are independent.

The tentative mechanism inferred from the experimental results is described by a combination of eq 1 and 5:

$$LP \underset{k_3}{\overset{k_4}{\rightleftharpoons}} P + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} PL \underset{k_5}{\overset{k_6}{\rightleftharpoons}} PL^* \qquad (7)$$

The mechanism is a special case (III) of the general mechanism (Figure 7). A number of alternative mechanisms have

Table II: Relative Changes of Equilibrium Constants and Fluorescence Quantum Yields from Fitting the Amplitude and Rate Data to Mechanism III

excitation wavelength (nm)	$\frac{\Delta K_i/K_i}{\Delta K_1/K_1}$[a]			rel fluorescence quantum yield[b]				
	$i=1$	$i=2$	$i=3$	f_{LP}	f_{PL}	f_L	$f_{P_1}+f_{P_2}$	f_{PL^*}
280	1.0	0.5	1.5	1.6	1.25	0.1	1.0	1.05
366	1.0	0.5	1.5	0.9	0.44	1.0	0	0.52

[a] K_i values are equilibrium association constants. $\Delta K_1/K_1 < 0$. [b] The f_i values are the best fit apparent values of the normalized molar fluorescence changes of the species of mechanism III upon introduction into buffer. P_1 is the unoccupied site of InGP synthase and P_2 is the unoccupied site of PRA isomerase. For 280-nm excitation, the parameters are normalized by $f_{P_1}+f_{P_2}$. For 366-nm excitation, the parameters are normalized by f_L.

been studied in detail, but none was capable of explaining the observed concentration dependences of $1/\tau_1$, $1/\tau_2$, and $1/\tau_3$. Mechanism II is particularly interesting because it is capable of generating negatively cooperative binding at equilibrium. However, all three reciprocal relaxation times must increase indefinitely with increasing ligand concentration.

Because the experimentally determined value of $1/\tau_3$ does not increase with the ligand concentration (cf. Figure 4), mechanism II can also be excluded. By exclusion of alternative simple mechanisms, the mechanism of eq 7 is the simplest that is consistent with all of the data.

Data Fitting to Mechanism III. The relaxation spectrum of this mechanism as expressed by eq 7 is characterized by three time constants and three fluorescence amplitudes, all of which depend on the protein and ligand concentrations.

$$F_{rel} = F_1^0 e^{-t/\tau_1} + F_2^0 e^{-t/\tau_2} + F_3^0 e^{-t/\tau_3} \quad (8)$$

Assuming that the rate processes are always well separated from each other, the reciprocal relaxation times depend on concentrations as given by the equations

$$1/\tau_1 = k_2 + k_1([\bar{P}_1] + [\bar{L}]) \quad (9)$$

$$1/\tau_2 = k_4 + k_3\left[[\bar{L}] + [\bar{P}_2]\left(\frac{[\bar{L}] + K_1}{[\bar{L}] + [\bar{P}_1] + K_1}\right)\right] \quad (10)$$

$$1/\tau_3 = k_5 + k_6\left[\frac{[\bar{L}]([\bar{L}] + [\bar{P}_2] + K_2) + [\bar{P}_1]([\bar{L}] + K_2)}{([\bar{L}] + K_1)([\bar{L}] + [\bar{P}_2] + K_2) + [\bar{P}_1]([\bar{L}] + K_2)}\right] \quad (11)$$

where $[\bar{P}_1]$ and $[\bar{P}_2]$ are the equilibrium concentrations of the unoccupied binding sites of high and low affinity, K_1 equals k_2/k_1, and K_2 equals k_4/k_3.

When the concentration of ligand is in excess over that of enzyme (Figures 2 and 3), eq 9 simplifies to eq 2 and eq 10 simplifies to eq 3. When the concentration of protein is in excess over that of rCdRP, eq 9 simplifies to eq 4. For low concentrations of both enzyme and ligand (Figures 4 and 6B; $[\bar{L}]$, $[\bar{P}_1]$, and $[\bar{P}_2] < K_2$), eq 11 is simplified to

$$1/\tau_3 = k_5 + k_6\left(\frac{[\bar{L}] + [\bar{P}_1]}{[\bar{L}] + [\bar{P}_1] + K_1}\right) \quad (12)$$

As shown in Figures 4 and 6B, $1/\tau_3$ passes through a minimum under these conditions, because $[\bar{P}_1]$ decreases and $[\bar{L}]$ increases with increasing total concentration of ligand. Under conditions where the enzyme is in large excess (Figure 5B; $[\bar{L}] < K_1 < [\bar{P}_1]$, $[\bar{P}_2] < K_2$, and $[\bar{P}_1] \approx [\bar{P}_2] \approx [P]_0$), eq 11 reduces to eq 6.

In Figure 6A and, at low concentrations of rCdRP, also in Figure 3, the rapid binding processes are not well separated. Only a single rapid relaxation is observable. To see whether these observations are consistent with the mechanism, we calculated the three relaxation times and fluorescence amplitudes of eq 8 by normal mode analysis of eq 7 (cf. Appendix).

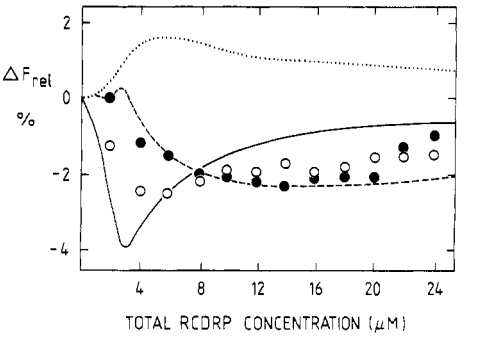

FIGURE 8: Comparison of the relaxation amplitudes F_1^0 and F_2^0 with simulated values. The experimental amplitudes correspond to the relaxation times presented in Figure 3. (○) Experimental values of F_1^0; (●) experimental values of F_2^0; (—) calculated values of F_1^0; (---) calculated values of F_2^0; (···) calculated values of F_3^0.

The last line in Table I presents the rate constants that best fit all of the rate data of Figures 3–6. Table II lists the relative changes of the equilibrium constant and of the molar fluorescence for each independent elementary reaction that give reasonable qualitative agreement between theory and the amplitude data. The data are not sufficiently precise to estimate the absolute values of the individual changes of equilibrium constants and molar fluorescence of each elementary step of the mechanism by multiple regression analysis (Jovin, 1975; Thusius, 1977). Therefore, the parameters were optimized by trial and error, starting from initial values that were derived from previous binding studies (Bisswanger et al., 1979) and the rate constants given in the last line of Table I.

The experimental values of the relaxation amplitudes belonging to Figure 3 (excitation by fluorescence energy transfer) are presented in Figure 8 and are compared to the amplitudes, calculated from mechanism III. The fit of the calculated amplitude F_1^0 to the data is poor at low rCdRP concentrations, where the two processes have similar rates (cf. Figure 3). The calculated second relaxation amplitude F_2^0 is negligible at low concentrations of rCdRP which explains why no second relaxation process was observable (Figure 3). No data are available for comparison with the calculated third relaxation amplitude F_3^0, but the opposite sign of the amplitude agrees with qualitative observations.

The comparison of calculated and observed relaxation times for the experiments at low enzyme concentration suggests that the smaller of the two observed relaxation times is an average value. The calculated concentration dependence of $1/\tau_1$ and $1/\tau_2$ presented in Figure 5A as dashed curves would not be observable as distinct processes. The calculated relaxation amplitudes corresponding to $1/\tau_1$ and $1/\tau_2$ are presented as

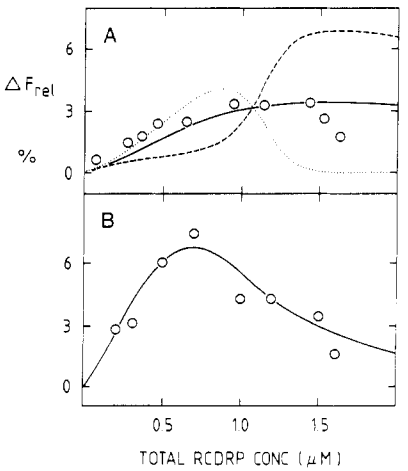

FIGURE 9: Comparison of the relaxation amplitudes F^* and F_3^0 with simulated values. The experimental values correspond to the relaxation times presented in Figure 6. (A) (O) Experimental amplitude of the rapid binding process; (—) calculated values of F^*; (- - -) calculated values of F_1^0; (···) calculated values of F_2^0. (B) (O) Experimental amplitude of the isomerization process; (—) calculated values of F_3^0.

dashed curves in Figure 9A. The reciprocal variation of F_1^0 and F_2^0 with increasing concentration of rCdRP is due to the strong coupling between the two rapid binding processes. Normal mode analysis (Bernasconi, 1976) shows that the rapid binding step reflects the reequilibration of the free ligand concentration via both binding processes (2P + 2L $\rightleftharpoons$ PL + LP). The second relaxation time is associated with a redistribution of bound ligand at constant free ligand concentration (PL + P $\rightleftharpoons$ P + LP or PL $\rightleftharpoons$ LP).

As seen in Figure 9A, the calculated average amplitude $F^* = (F_1^0 + F_2^0)/2$ agrees reasonably well with the data. The theoretical amplitudes were used to calculate the mean reciprocal relaxation time $1/\tau^*$ according to eq 13 (Schwarz, 1968).

$$1/\tau^* = (F_1^0/\tau_1 + F_2^0/\tau_2)/(F_1^0 + F_2^0) \qquad (13)$$

As shown in Figure 6A, the experimental data agree reasonably well with $1/\tau^*$. The amplitude of the slow isomerization process also agrees with the simulated values of F_3^0 (Figure 9B).

In summary, the comparison of experimental relaxation times and amplitudes with simulated values shows that the data are consistent with mechanism III.

Discussion

The isomerization of the rCdRP–InGP synthase complex (PL $\rightleftharpoons$ PL*; eq 7) can be interpreted in two alternative ways. On one hand, the conformation of the protein in the vicinity of the active site may change in response to ligand binding. On the other hand, the isomerization may reflect the binding of rCdRP to the active site of InGP synthase in two mutually exclusive modes (Viale, 1971).

The magnitude of $1/\tau_3$ (5–30 s^{-1}) is comparable to the rate constants of ligand-induced protein isomerizations observed with other proteins, for example, alkaline phosphatase (Halford, 1972), cyclic AMP receptor protein (Wu & Wu, 1974), and immunoglobulin A (Lancet & Pecht, 1976). These reactions have been interpreted as conformation changes.

As seen from Table II, the three species of the rCdRP–enzyme complex (PL, PL*, and LP) have different efficiencies of fluorescence energy transfer from tryptophan to the bound ligand and different degrees of ligand fluorescence quenching. These differences, as well as the different absorption spectra of the two species of bound ligand (Bisswanger et al., 1976), indicate that the microenvironment of the bound fluorophore is different in the two binding sites. Moreover, the differences between the apparent relative quantum yields of the species PL and PL* suggest that the microenvironment changes during the slow isomerization step. Both the efficiency of fluorescence energy transfer to and the degree of fluorescence quenching of the bound ligand decrease as PL isomerizes to PL*.

An independent conformational change of InGP synthase can be explained simply if each active site is located in an independent domain of folding of the polypeptide chain (Kirschner & Bisswanger, 1976). Yanofsky et al. (1971) have shown that the N-terminal portion of the polypeptide chain is responsible for generating the active site of InGP synthase. The C-terminal sequence is responsible for generating the active site of PRA isomerase. The active site within the InGP synthase domain could change conformation without necessarily transmitting the change to the neighboring PRA isomerase domain.

The absence of interaction between the two binding sites excludes the possibility of overlapping active sites. The same conclusion has been drawn by Creighton (1970) on the basis of kinetic studies of the catalyzed synthesis of InGP from PRA under steady-state conditions. Nevertheless, if parallel productive and nonproductive binding (Viale, 1971) correctly explains the isomerization, nonproductive binding could still occur to some other subsite of the active site of InGP synthase.

The two alternative explanations for the isomerization reaction cannot be distinguished by kinetic experiments alone. Spectroscopic studies and determination of the protein structure are the obvious next steps in elucidating the structure–function relationships of PRA isomerase–InGP synthase.

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Appendix

The calculation of exact values of $1/\tau_1$ and F_i^0 of eq 10 is simplified because the third relaxation process is much slower than the first two. Thus, $1/\tau_1$, $1/\tau_2$, F_1^0, and F_2^0 are calculated explicitly as detailed by Bernasconi (1976). $1/\tau_3$ is calculated from eq 11, and F_3^0 is calculated from $F^0 - (F_1^0 + F_2^0)$, where F^0 is the total amplitude calculated from the equilibrium distributions of reactants and products before and after the temperature jump.

The amplitudes F_1^0 and F_2^0 are related to the individual concentration changes of the independent concentration variables X_1 = PL, X_2 = LP, and X_3 = PL* of eq 7 as

$$F_i^0 = Q_1X_1^{0i} + Q_2X_2^{0i} + Q_3X_3^{0i}$$

where $Q_1 = f_{PL} - f_{P_1} - f_L$, $Q_2 = f_{LP} - f_{P_2} - f_L$, and $Q_3 = f_{PL^*} - f_{P_1} - f_L$ with the f_i normalized according to the wavelength of excitation as given in Table II.

$X_3^{01} = X_3^{02} = 0$ because the third relaxation process does not progress significantly during the fast relaxations. $X_1^{01} = m_{22}y_1^0/\det \mathbf{M}$, $X_2^{01} = -m_{21}y_1^0/\det \mathbf{M}$, $X_1^{02} = -m_{12}y_2^0/\det \mathbf{M}$, and $X_2^{02} = m_{11}y_1^0/\det \mathbf{M}$ where the y_i^0 values are the normal concentration variables. $y_1^0 = m_{11}X_1^0 + m_{12}X_2^0$, $y_2^0 = m_{21}X_1^0 + m_{22}X_2^0$, and the X_i^0 values are the total concentration changes of PL and LP for the two rapid relaxations

$$X_1^0 = [[L][P]_0A_1/(1 + [L]A_1)][[(\Delta A_1/A_1)[1 + A_2([L] + [P_2])] - [P_2]A_2(\Delta A_2/A_2)]/[[1 + A_1([L] + [P_1])][1 + A_2([L] + [P_2])] - [P_1][P_2]A_1A_2]]$$

$$X_2^0 = [[L][P]_0A_2/(1 + [L]A_2)][[(\Delta A_2/A_2) \times [1 + A_1([L] + [P_1])] - [P_1]A_1(\Delta A_1/A_1)]/[[1 + A_1([L] + [P_1])][1 + A_2([L] + [P_2])] - [P_1][P_2]A_1A_2]]$$

where $A_1 = k_1/k_2$, $A_2 = k_3/k_4$, and $[P]_0$ is the total enzyme concentration.

The elements m_{ij} of the transformation matrix **M** are as follows (Bernasconi, 1976): $m_{11} = 1$, $m_{12} = -a_{12}/(a_{22} - 1/\tau_1)$, $m_{21} = -a_{21}/(a_{11} - 1/\tau_2)$, and $m_{22} = 1$; det **M** $= m_{11}m_{22} - m_{12}m_{21}$. The a_{ij} values are the elements of the coefficient matrix **A**: $a_{11} = k_2 + k_1([L] + [P_1])$, $a_{12} = k_1[P_1]$, $a_{21} = k_2[P_2]$, and $a_{22} = k_4 + k_3([L] + [P_2])$.

The reciprocal relaxation times are given as the roots of the secular equation

$$1/\tau_{1,2} = (a_{11} + a_{22})/2 \pm [(a_{11} + a_{22})^2/4 - a_{11}a_{22} + a_{12}a_{21}]^{1/2}$$

The equilibrium concentrations of the reactants and products were calculated from the mass law relations governing mechanism III (eq 7):

$$[P_1] = [P]_0/[1 + [L]A_1(1 + A_3)]$$

$$[PL] = [L][P]_0A_1/[1 + [L]A_1(1 + A_3)]$$

$$[PL^*] = [L][P]_0A_1A_3/[1 + [L]A_1(1 + A_3)]$$

$$[P_2] = [P]_0/(1 + [L]A_2)$$

$$[LP] = [L][P]_0A_2/(1 + [L]A_2)$$

where $A_3 = k_6/k_5$. The relative fluorescence changes were obtained by dividing F_1^0, F_2^0, and F_3^0 by the calculated total fluorescence of the equilibrium solution before the temperature jump:

$$F_{eq} = Q_1[PL] + Q_2[LP] + Q_3[PL^*] + (f_{P_1} + f_{P_2})[P]_0 + f_L[L]_0$$

with the f_i normalized according to the wavelength of excitation as given in Table II.

A PDP 11-40 computer was programmed to calculate F_i^0 and $1/\tau_i$ as a function of total rCdRP concentration. An iterative Newton–Raphson program was used to calculate [L] from $[L]_0$, $[P]_0$, and the constants given above.

References

Bernasconi, C. (1976) in *Relaxation Kinetics*, Academic Press, New York.

Bernhard, S. A. (1955) *J. Biol. Chem. 218*, 961–969.

Bisswanger, H., Kirschner, K., Cohn, W., Hager, V., & Hansson, E. (1979) *Biochemistry* (preceding paper in this issue).

Coutts, S. M., Riesner, D., Römer, R., Rabl, C. R., & Maass, G. (1975) *Biophys. Chem. 3*, 275–289.

Creighton, T. E. (1970) *Biochem. J. 120*, 699–707.

Creighton, T. E., & Yanofsky, C. (1970) *J. Biol. Chem. 241*, 4616–4624.

Eigen, M., & De Maeyer, L. (1974) *Tech. Chem. (N.Y.) 6 (Part II)*, 63–146.

Gaertner, F. H. (1978) *Trends Biochem. Sci. 3*, 63–65.

Halford, S. E. (1972) *Biochem. J. 126*, 727–738.

Hammes, G. G., & Schimmel, P. R. (1970) *Enzymes, 3rd Ed. 2*, 67–114.

Jovin, T. M. (1975) in *Biochemical Fluorescence: Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol I, pp 305–374, Marcel Dekker, New York.

Kirschner, K., & Bisswanger, H. (1976) *Annu. Rev. Biochem. 45*, 143–166.

Lancet, D., & Pecht, I. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3549–3553.

Pauckert, J. L., Strauss, L. D., & Rabinowitz, J. C. (1976) *J. Biol. Chem. 251*, 5104–5111.

Paul, C., Kirschner, K., & Haenisch, G. (1980) *Anal. Biochem.* (in press).

Pecht, I., & Lancet, D. (1977) *Mol. Biol., Biochem. Biophys. 24*, 306–338.

Rigler, R., Rabl, C. R., & Jovin, T. M. (1974) *Rev. Sci. Instrum. 45*, 580–588.

Schwarz, G. (1968) *Biopolymers 6*, 873–880.

Stark, G. R. (1976) *Trends Biochem. Sci. 2*, 64–66.

Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.

Thusius, D. (1977) *Biophys. Chem. 7*, 87–93.

Viale, R. O. (1971) *J. Theor. Biol. 31*, 501–507.

Wu, C.-W., & Wu, F. Y.-H. (1974) *Biochemistry 13*, 2573–2578.

Yanofsky, C., Horn, V., Bonner, M., & Stasiowsky, S. (1971) *Genetics 69*, 409–433.