Table I: Reassociation of PFK by Dilution after Inactivation by Several Dissociating Agents

dissociation by	residual denaturant during reassociation (M)	second-order rate constant ($M^{-1}\cdot s^{-1}$) for regain of native fluorescence (M → D)	second-order rate constant ($M^{-1}\cdot s^{-1}$) for reactivation (M → T)	ref
7.5 M urea[a]	0.5[a]	4×10^3	10^2	this paper
2 M Gdn-HCl[a]	0.02[a]	4×10^4	10^3	*c*
0.5 M KSCN[b]	0.025[b]	2×10^5	5×10^3	*d*

[a] 0.1 M phosphate buffer, pH 7.6, containing 1 mM Mg^{2+} and 2 mM DTT. Dissociation time = 24 h. [b] 50 mM imidazole buffer, pH 7.0, containing 1 mM Mg^{2+} and 2 mM DTT. Dissociation time = 0.5 h. [c] Teschner and Garel (1989). [d] Deville-Bonne et al. (1989).

to D involves a larger area of contact between subunits than the second association step leading to N, which explains the greater stability of the R (regulatory) interface as compared to the A (active) interface (Shirakihara & Evans, 1988). It is probable that the 40-fold difference found here in the rates of subunit association is also related to the fact that these two steps do not decrease the protein surface accessible to solvent by the same account.

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Registry No. PFK, 9001-80-3; urea, 57-13-6.

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Dissection of the Effector-Binding Site and Complementation Studies of *Escherichia coli* Phosphofructokinase Using Site-Directed Mutagenesis†

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ABSTRACT: A systematic study by site-directed mutagenesis has been conducted on the effector site of phosphofructokinase from *Escherichia coli* to delineate the role of side chains in binding the allosteric activator, GDP, and inhibitor, PEP, and to search for key residues in the allosteric transtion. Target residues were identified from the crystal structure of the enzyme–nucleoside diphosphate complex. It is found that both activator and inhibitor bind to the same set of amino acid side chains. Deletion of positively charged groups (Arg21, Arg25, Arg54, Arg154, and Lys213 mutated to alanine) weakens binding of both effectors by 2–3 kcal/mol, consistent with the disruption of charged hydrogen bonds. Residue Glu187, which is known from the crystal structure to bind the coordinated Mg^{2+} ion of GDP, is found to have a unique behavior on mutation and appears to be crucial in triggering the allosteric transition. All other residues mutated simply weaken binding of both PEP and GDP in a parallel manner. However, mutation of Glu → Ala187 reverses the roles of GDP and PEP, causing GDP to become an allosteric inhibitor and PEP an activator. Mutation of Glu → Gln187 has only a small effect on the binding of PEP, and both PEP and GDP are inhibitors. Studies are described in which mutations in different subunits of a tetrameric complex complement each other. The effector site is composed of residues from two subunits. In particular, Arg21 and Lys213 in each site are from different subunits. Mutations of either one of these residues abolishes activation by GDP of the homotetramer. However, the hybrid Arg → Ala21/Lys → Ala213 is activated by GDP and its kinetic properties consistent with a heterotetramer being formed in the expected statistical distribution. Assuming this distribution, the kinetic data may be adequately described by the MWC concerted mechanism.

Phosphofructokinase catalyzes the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. The major form of phosphofructokinase in *Escherichia coli* is pfk 1, which represents about 90% of the phosphofructokinase activity in the cells. It is a tetramer of four identical subunits. The kinetic properties of pfk 1 from *E. coli* have been studied extensively by Blangy et al. (1968) and Blangy (1971). The native enzyme is allosterically regulated by its effectors, being inhibited by phosphoenolpyruvate (PEP) and activated by adenosine di-

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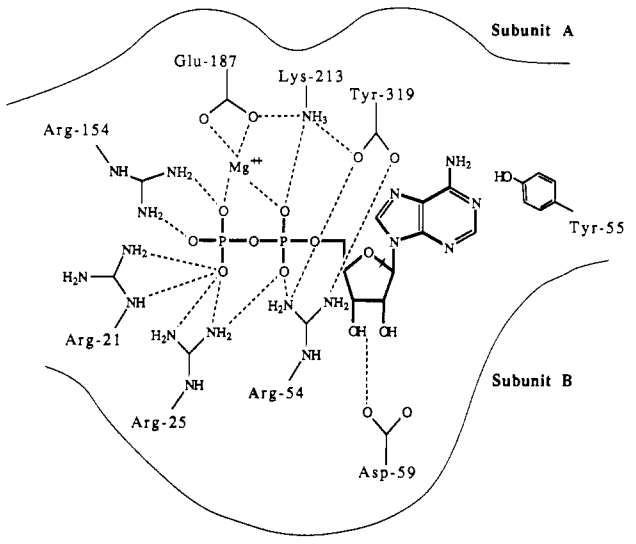


FIGURE 1: Sketch of the effector site of *E. coli* phosphofructokinase [based on Shirakihara and Evans (1988)].

phosphate (ADP) or guanosine diphosphate (GDP). The crystal structure of the *E. coli* enzyme has been determined (Shirakihara & Evans, 1988). There is evidence that effectors and inhibitors bind to the same site (Evans & Hudson, 1979). Site-directed mutagenesis studies have been initiated on the effector site of *E. coli* pfk 1 enzyme (Lau et al., 1987). Two questions have been tackled. First, do activator and inhibitor molecules bind to an overlapping set of amino acid chains? Second, if they do so, are there key "trigger" residues that are responsible for the balance between activation and inhibition?

Mutations have been designed from the structure of *E. coli* enzyme in the active conformation (R state) with the activator molecule ADP bound (Figure 1) to shed light on these problems. Obvious targets are the charged residues that appear to interact with the bound ADP. These are Arg21, Arg25, Arg54, Asp59, Arg154, Glu187, and Lys213. Each has been mutated to alanine to remove the charged side chain. Residue Tyr319, which is at the end of the C-terminus, was deleted by mutating its coding sequence to a stop codon. Residue Glu187 was also mutated to Gln. Residue Asp59 was also chosen because of its putative hydrogen bonding with the ribose 3′-hydroxyl group of ADP in the crystal structure. We show that all the target residues are important for binding both effectors, PEP and GDP, and so provide evidence that the same site is used for all effector molecules. We also show that there is one key residue, Glu187, that is crucial in governing the balance between activation and inhibition.

The effector site is situated between two subunits. The seven residues mutated can be divided into two groups according to their location: Arg21, Arg25, Arg54, Asp59, and Tyr319 from subunit A; Arg154, Glu187, and Lys213 from subunit B. Experiments have been designed to see whether hybrids formed from a mutant with a mutation in the B subunit residue (Lys213) and a mutant with a mutation in the A subunit can be activated. That is, is there complementation of mutations in the effector site? We show from hybrid enzyme that complementation does occur and that its properties are still consistent with a concerted transition between R and T states as implied by the Monod–Wyman–Changeux model (Monod et al., 1965).

Materials and Methods

Materials

Deoxyoligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer. T4 DNA ligase, *E. coli* DNA polymerase I (Klenow fragment), dNTP's, and ddNTP's were obtained from Pharmacia. Uridine 5′-diphosphate was from Sigma and guanidine hydrochloride from BDH. Other biochemicals were from Boehringer. Matrex gel blue A was from Amicon. [α-^{32}P]dATP was from Amersham, and [γ-^{32}P]rATP was from New England Biolabs. HB101[F::Tn5] (Kan^R) and helper phage R408 was a gift from Dr. M. Russel, and CJ236 and CSH50 are gifts from Dr. T. A. Kunkel. Enzfitter software is a product from Elsevier-BIOSOFT.

Methods

Preparation and Purification of Mutant Phosphofructokinases. The plasmid harboring the *pfk*A gene is pHL1, which uses pEMBL8(+) as the parent vector. The preparation of single-stranded template from pHL1 and the mutagenesis for mutants RA21, RA25, EA187, and KA213 were performed as described (Lau et al., 1987). For RA54, DA59, RA154, EQ187, and ΔTyr319, single-stranded pHL1 template for site-directed mutagenesis was prepared by superinfecting rapidly dividing cells harboring pHL1 with the helper phage R408 (Russel et al., 1986). R408 is an improved helper phage over its parent phage F1-(IR1) because pHL1 template is packaged preferentially instead of the helper phage itself. Mutagenesis was performed by using uracil-substituted template DNA prepared by using CJ236 [*dut*1, *ung*1, *thi*1, *rel*A1/pCJ105(Cm^R)] (Kunkel, 1985; Kunkel et al., 1987). The preparation of uracil-containing template on a 200-mL scale was a modification of the procedure of Cutler et al. (1987). About 10 mg of uridine 5′-diphosphate was added to 200 mL of 2 × TY medium. The mutagenesis reaction was performed exactly the same as that using normal (not uracil-substituted) templates. The extended/ligated DNA mixture was used to transform CSH50 [Δ*pro-lac*, *thi*, *ara*, *str*A/F′: *tra*D36, *pro*AB$^+$, *lac*I^q, *lac*ZΔM15] or TG2 [Δ*pro-lac*, *sup*E, *thi*, *rec*A, *Srl*::Tn10(Tc^r), *hsd*,Δ5/F′:*tra*D36, *pro*AB$^+$, *lac*I^q, *lac*ZΔM15] by the $CaCl_2$ method (Maniatis et al., 1982). Colonies harboring mutant plasmids were identified by hybridization screening (Grunstein & Hogness, 1975) using ^{32}P-kinased mutagenic oligonucleotide as a probe (Zoller & Smith, 1983) or restriction site screening. Templates of mutant were prepared by using HB101[F::Tn5] cell and R408 helper phage. Mutations were verified by sequencing the entire gene with the appropriate synthetic primers (Hellinga & Evans, 1985) by dideoxy sequencing (Sanger et al., 1977). The sequences of the mutagenic primers are as follows:

Arg → Ala21	5′-	CAACCCCGG*C*AATTGCGG -3′
Arg → Ala25		GCGCAGAAG*C*AACAACCC
Glu → Ala187		CAACGAATG*CACAGCCAC
Lys → Ala213		CGTGTTTTG*C*ACCTTTCGC
Arg → Ala54		CGCTGTAAG*C*GTCTAGCTG
Arg → Ala154		AGGTGTCAG*C*CAGACGGTC
Asp → Ala59		TACCG<u>*CCG*CGG*</u>TTGATCATGG*CAGACAC
Glu → Gln187		CTTCC<u>*GGT*ACC*</u>ACAACGAATTG*ACAGCCA
ΔTyr319		TTTCC<u>*GAT*ATC*</u>ATTAT*TACAGTT

[An asterisk follows mismatched bases; for Asp → Ala59, Glu → Gln187, and ΔTyr319 mutations, new restriction sites (underlined) of *Sac*II, *Kpn*I, and *Eco*RV were introduced, respectively.]

Expression and purification of wild-type and mutant phosphofructokinases were performed as before (Lau et al., 1987). The concentration of the purified phosphofructokinase

was estimated by assuming $\epsilon_{278} = 0.6$ cm^2 mg^{-1}.

Preparation of Hybrid Mutants. Denaturation and renaturation studies were performed according to the method of Martel and Garel (1984). Equimolar concentrations of PFK(Arg → Ala21) and PFK(Lys → Ala213) mutants, in starting buffer at 50 mM Tris·HCl, pH 7.5, and 1 mM EDTA, were mixed and denatured at 27 °C in approximately 6 M Gd·HCl and 10 mM $MgCl_2$, the final concentration of mutant proteins being about 2.4 μM (monomer concentration) for each mutant (6 M Gdn·HCl solution can be made conveniently by dissolving 1 g of crystalline Gdn·HCl into 1 mL of starting solution). Renaturation of the hybrid enzymes was achieved by diluting the denatured protein solution 100-fold or more at 27 °C in 100 mM Tris, pH 8.2, 1 mM ATP, 10 mM $MgCl_2$, 10 mM dithiothreitol (a residual Gdn·HCl concentration of 60 mM or less was shown not to interfere with the enzyme assay).

Assay of Phosphofructokinase. The activity of the enzyme was assayed by a coupled enzyme method that monitors the decrease of NADH in the reaction mixture using aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase. Creatine phosphate and creatine kinase were used as an ATP regenerating system (Kotlarz & Buc, 1982). In activation studies, GDP was used instead of ADP because ADP is, in addition to being an effector, a competitive inhibitor with respect to substrate ATP ($K_{i(ADP)} = 2 \times 10^{-4}$ M).

Calculation of Kinetic Results. Nonlinear regression analysis of experimental data was performed by using the program Enzfitter with an IBM-PC XT computer (Leatherbarrow, 1987), Michaelis–Menten kinetics for variation of [ATP], sigmoidal saturation kinetics for variation of [F6P] using the Hill plot, and fourth-root plots to obtain $K_{R(GDP)}$ and $K_{T(PEP)}$, which are the dissociation constants of GDP from the R state and of PEP from the T state, respectively (Lau et al., 1987; Blangy et al., 1968). Since $K_{T(GDP)} \gg K_{R(GDP)}$, plotting $[v/(V_{max}'v)]^{1/4}$ against GDP concentration (where v is the initial velocity and V_{max}' is the maximum velocity that can be reached if all the protein is in the R conformation) at a fixed concentration of F6P gives a straight line with x intercept of $-K_{R(GDP)}$. Similarly, as $K_{R(PEP)} \gg K_{T(PEP)}$, a plot of $[(V_{max}' - v)/v]^{1/4}$ against [PEP] concentration gives a straight line with x intercept of $-K_{T(PEP)}$.

Results

Production of Mutants. The average frequency of obtaining mutants (RA21, RA25, KA213, and EA187) by using normal (that is, not uracil-substituted) template DNA was less than 20%. Sometimes, no mutants were detected on screening 200 colonies. This may have resulted from using cells with *mut*L$^+$ genotype, which allows mismatch repair that removes the induced mutations. Mismatch-repair deficient strains like BMH71-18 *mut*L (Kramer et al., 1984) were not used because undesired spurious mutations accumulate in pHL1 transformed cells that are grown up as colonies. RA54, RA154, DA59, EQ187, and ΔTyr319 mutations were induced by using the uracil-substituted template method (Kunkel et al., 1987) and gave an average mutation frequency of 30%. This frequency compares well with the estimated frequency when *E. coli* DNA polymerase (Klenow fragment) is used for the extension reaction (Bio-Rad, 1987). For DA59, EQ187, and ΔTyr319, longer mutagenic primers were used to cover the desired point mutation for a single residue change and a silent mutation that introduced a new and unique restriction site to the whole plasmid (pHL1). This allows a rapid screening for mutants by simple restriction endonuclease catalyzed cleavage of double-stranded DNA.

Table I: Kinetic Properties of Wild-Type and Mutant Enzymes

enzyme	$K_{R(GDP)}$ (mM)	$K_{T(PEP)}$ (mM)
wild type	0.041	0.7
Arg → Ala21	*a*	90
Arg → Ala25	1.2	117
Arg → Ala54	5.6	25
Asp → Ala59	1.3	32
Arg → Ala154	4.4	32.5
Lys → Ala213	*a*	32
Glu → Ala187	*b*	*c*
Glu → Gln187	*b*	1.1
ΔTyr319	1.7	27

[a] No activation is seen up to 5 mM GDP. [b] GDP causes allosteric inhibition; $K_{T(GDP)}$ is 11.5 and 8.3 mM for Glu → Ala187 and Glu → Gln187 mutants, respectively. [c] PEP causes activation at low F6P concentration; apparent $K_{R(PEP)}$ is 1.0 mM. At high F6P concentration, PEP inhibits the enzyme, so that V_{max} decreases by 40% at saturating PEP concentration.

Preparation of Single-Stranded pEMBL. Although the system using TG2 and f1(IR1) helper phage in preparing pEMBL templates did work, it was not entirely reliable. It happens occasionally that, perhaps due to the loss of the F episome during the cell growth in rich media like 2TY, it renders the entrace of any helper phage impossible (Caro & Schnos, 1966). As a result, no packaged single-stranded DNA secretes from the cells because the presence of helper phage in the cells is essential for the packaging of pEMBL DNA. Russel et al. (1986) used HB101[F::Tn5] cells for template DNA preparations. It allows the selection of the F sex factor in the presence of kanamycin, and it makes the procedure of template preparation more reliable. At the same time, ampicillin is used to select for the pEMBL derivative, pHL1. In fact, this strategy can be employed whenever the entrance of phage into the cell is essential for phage propagation (like M13) or template DNA preparation (like pEMBL using helper phage). The improved helper phage R408 also gives a better yield of pEMBL template DNA.

Kinetic Results. All the mutant phosphofructokinases purified are active. They all follow Michaelis–Menten kinetics with respect to ATP concentration and sigmoidal kinetics with respect to F6P concentration as found for wild-type enzyme (Blangy et al., 1968). That is, the binding of both substrates is not obviously affected. However, the effects of effector molecules, PEP and GDP, on the enzyme activities are significantly altered by the mutations.

Table I summarizes the changes in the dissociation constants of both effectors to the wild-type and mutant enzymes. It is obvious that all the mutations affect the binding of GDP and PEP. The apparent binding energies with the allosteric effect or (Fersht, 1988) are calculated by using

$$\Delta G = RT \ln (K_{R(GDP)mutant}/K_{R(GDP)wild\text{-}type})$$

or

$$\Delta G = RT \ln (K_{T(PEP)mutant}/K_{T(PEP)wild\text{-}type})$$

and are shown in Table II. For mutants Arg → Ala21 and Lys → Ala213, no obvious binding (or activation) is observed up to 5 mM GDP; we estimate the apparent binding energies of the Arg → Ala21 and Lys → Ala213 mutants by assuming the values of $K_{R(GDP)}$ have increased to 10 mM or more.

GDP Binding. Residues mutated in this study include the following: Arg21, Arg25, Arg54, Arg154, and Lys213, which form the α- and β-phosphate binding site; Glu187, which binds the Mg ion coordinated to the diphosphate of ADP; Asp59, which is hydrogen-bonded to the O3′ hydroxyl group of the ribose ring; and the C-terminus (Tyr319), which interacts with

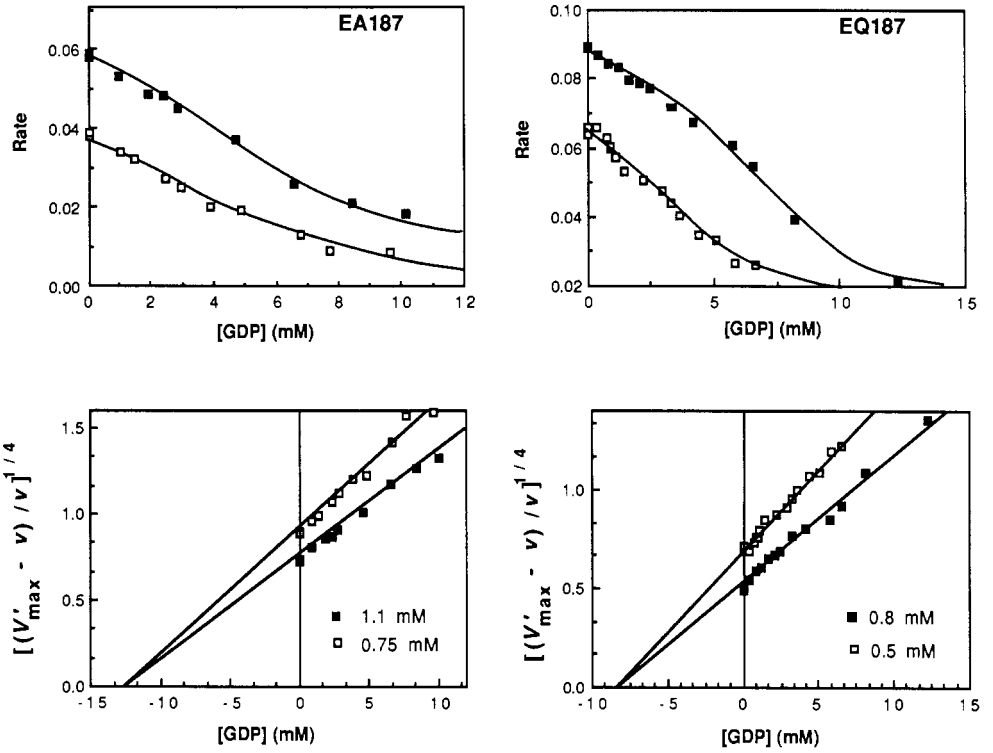


FIGURE 2: Inhibition of EA187 and EQ187 mutant enzymes by GDP. Rate of reaction is the rate of decrease of absorbance at 340 nm (ΔA_{340}/min). Fourth-root plots using the standard inhibition equation are obtained by transforming the original data as $[(V_{max}' - v)/v]^{1/4}$. F6P concentrations used in the assays are (□) 0.5 and (■) 0.8 mM for EQ187 and (□) 0.75 and (■) 1.1 mM for EA187 mutant.

Table II: Changes in Free Energy of Binding of Effectors on Mutation

mutation	binds to[a]	ΔG (kcal/mol) GDP	PEP
Arg → Ala21	β	>3.2[b]	2.9
Arg → Ala25	α, β	1.95	3.0
Arg → Ala54	α	2.84	2.1
Asp → Ala59	ribose	2.0	2.3
Arg → Ala154	β	2.70	2.2
Glu → Gln187	Mg^{2+}	c	0.27
Lys → Ala213	α	>3.2[b]	2.2
ΔTyr319	(Arg54, Lys213)	2.2	2.2

[a] Describes whether the target side chain binds to the α- or β-phosphate or ribose group of the nucleoside 5'-diphosphate. [b] $K_{R(GDP)}$ is probably higher than 10 mM for the two mutants. [c] GDP inhibits Glu → Gln187 allosterically instead of activating.

residues Arg54 and Lys213. The interactions are inferred from the 2.4-Å resolution structure of the R-state *E. coli* phosphofructokinase (Shirakihara & Evans, 1988). A summary of the apparent binding energies, involving the described mutations, on GDP binding is shown in Table II. The changes in apparent binding energies are typically between 2 and 3 kcal/mol except for Arg → Ala21 and Lys → Ala213 mutants, which involve more than 3 kcal/mol.

PEP Binding. Apparent binding energies of 2.9, 3.0, and 2.3 kcal/mol are obtained for Arg → Ala21, Arg → Ala25, and Arg → Ala154 mutants, respectively. Truncation of Arg54 and Lys213, which involve only the α-phosphate binding, give lower values of 2.1 and 2.3 kcal/mol, respectively. The ΔTyr319 mutant affects PEP binding by the same amount of energy, 2.2 kcal/mol, as for GDP binding. Mutation of Asp59, which is negatively charged under the experimental conditions, also causes a change of 2.3 kcal/mol.

Glu187 Mutations. Mutation of residue Glu187 merits special attention. As reported previously, the Glu → Ala187 (EA187) mutant is activated by PEP in the presence of low

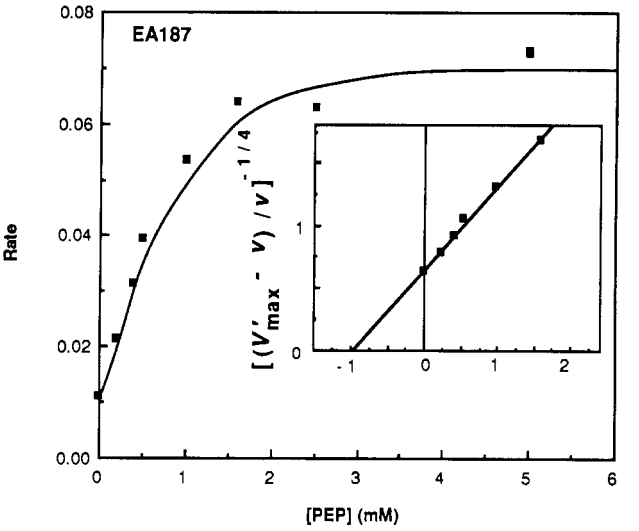


FIGURE 3: Activation of the EA187 mutant by PEP at [F6P] = 0.3 mM.

F6P concentration and inhibited by the same effector in the presence of high or near-saturating F6P concentration (Lau & Fersht, 1987). In contrast to the absence of response to GDP at low concentrations of F6P (1 mM) the EA187 mutant, instead of being activated, is inhibited by GDP (Figure 2). The GDP inhibition, if fitted by using the standard inhibition equations, gives $K_{T(GDP)}$ = 11.7 mM. Conversely, when the activation by PEP at 0.3 mM F6P is plotted by using the standard activation equation, an apparent value $K_{R(PEP)}$ of 1.0 mM is obtained (Figure 3). At F6P concentrations near its saturation (for example, 1 mM), saturating concentrations of PEP inhibit the mutant by about 40% only, unlike the wild-type enzyme that is inhibited to virtual completion. The conversion between activation and inhibition due to PEP at low and high F6P concentrations is apparent in Figure 4, which

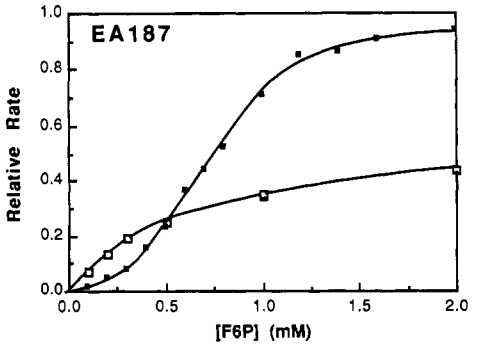


FIGURE 4: Rate dependence of the EA187 mutant on F6P concentration in the absence (■) and presence (□) of 10 mM PEP.

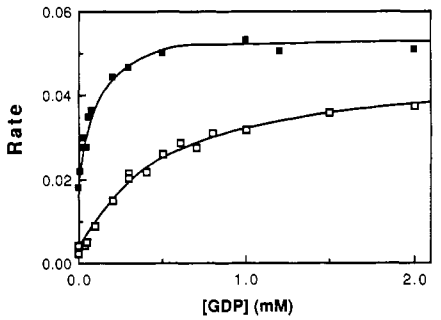
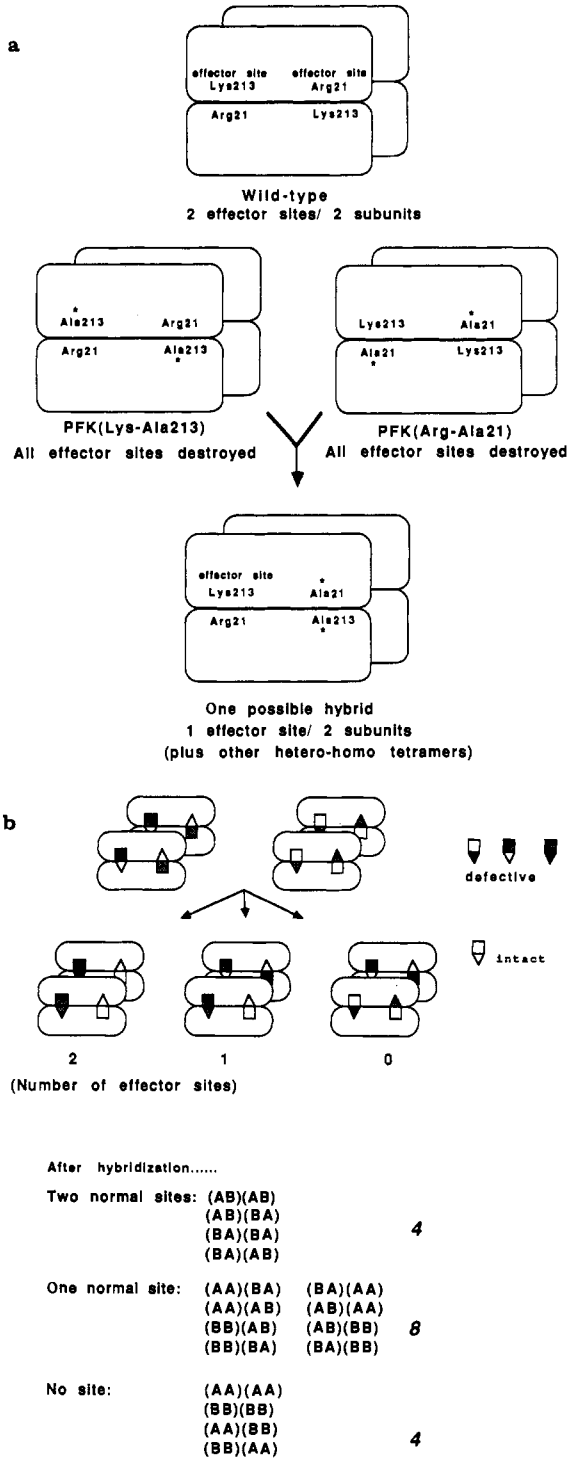


FIGURE 5: Activation of the hybrid enzyme mixture by GDP at two F6P concentrations [(□) at 0.1 mM and (■) at 0.3 mM F6P concentration]. The curves are fitted to eq 1 for both experiments by using a nonlinear regression alogrithm. Note also that RA21 and KA213 enzymes, when denatured and renatured separately, are not activated at all under similar reaction conditions. Wild-type enzyme is activated more rapidly because of the presence of all four effector sites per tetramer.

shows the rate dependence of the mutant on F6P concentration in the absence and presence of 10 mM PEP. A second more conservative mutation Glu → Gln187 (EQ187), which is isosteric and retains the ability to form hydrogen bond, was constructed. The EQ187 mutant is inhibited by PEP, giving $K_{T(PEP)}$ = 1.1 mM; the allosteric inhibition is complete, and the value of $K_{T(PEP)}$ is comparable to the wild-type value of 0.7 mM. Yet this mutant was shown to be inhibited by GDP similarly to the EA187 mutant and gives $K_{T(GDP)}$ = 8.1 mM. This value is close to 11.7 mM from EA187 mutant.

Hybrid Mutants. The mutants Arg → Ala21 (on subunit A) and Lys → Ala213 (on subunit B) are not activated by GDP. Hybrid mutants were prepared from the random reshuffling of subunits from these two mutants by denaturation and renaturation. The kinetics of the "hybrid enzyme" mixture is cooperative with respect to substrate F6P as is the wild type; the Hill constant and half-saturation concentration are 2.8 and 0.45 mM, respectively (values for the wild-type enzyme are 3.7 and 0.33 mM, respectively). The hybrid enzymes were found to be activated significantly by low concentrations of GDP (Figure 5). Control experiments of denaturing and renaturing the parental mutant enzymes separately showed that the reconstituted homotetramers cannot be activated to any observable extent by up to 5 mM GDP, as before denaturation. A schematic presentation of the positive complementation to give intact effector sites from the defective parental enzymes is shown in Figure 6a. Provided the recombination is purely random, a distribution of 1:2:1 should be obtained for hybrids with zero, one, and two intact sites when started from an equimolar mixture (Figure 6b). To describe the activation kinetics, the concerted model (Monod et al., 1965), which was found to be applicable for wild-type enzyme (Blangy et al., 1968), was adopted in deriving equations for



After hybridization.....

Two normal sites:	(AB)(AB)		
	(AB)(BA)		
	(BA)(BA)		4
	(BA)(AB)		
One normal site:	(AA)(BA)	(BA)(AA)	
	(AA)(AB)	(BA)(AA)	
	(BB)(AB)	(AB)(BB)	8
	(BB)(BA)	(BA)(BB)	
No site:	(AA)(AA)		
	(BB)(BB)		
	(AA)(BB)		
	(BB)(AA)		4

FIGURE 6: (a) Schematic representation of hybrid formation. Wild-type enzymes have four effector sites per tetramer. Parent mutant enzymes Arg → Ala21 and Lys → Ala213 have no intact effector site at all. Recombination of two mutant monomers to give one effector site per two subunits is shown. (b) Combination of monomers to form no, one, or two sites per tetramer. PFK(Arg → Ala21) mutant is represented by (AA)(AA) and PFK(Lys → Ala213) by (BB)(BB). Note that ( )( ) represents two pairs of effector sites, and only (AB) or (BA) will give one complemented site. The resultant ratio follows the binomial distribution.

enzymes with fewer numbers of effector sites.

In the Monod–Wyman–Changeux model (Monod et al., 1965), the effect of an allosteric effector on enzyme activity is due exclusively to a shift in the equilibrium R ↔ T. In other words, the only quantity that changes is the apparent allosteric constant L. Assuming an exclusive binding of GDP to the R conformation, the kinetic equations can be written

for hybrids with no complemented effector site

$$\bar{Q} = \frac{\bar{R}}{\bar{T}} = \frac{v}{V_{max}' - v} = \frac{1}{L_\alpha}$$

with one effector site per tetramer only

$$\frac{v}{V_{max}' - v} = \frac{1}{L_\alpha}(1 + \gamma)$$

with two effector sites per tetramer

$$\frac{v}{V_{max}' - v} = \frac{1}{L_\alpha}(1 + \gamma)^2$$

where $L_\alpha = T/R$, the allosteric constant, at a certain F6P concentration, V_{max}' is the maximal activity attainable if all the enzyme is in the R state, and $\gamma = [GDP]/K_{R(GDP)}$, the reduced activator concentration.

By rearrangement of the above equations, and considering the population distribution of the three types of species, we have the rate equation

$$v = \frac{V_{max}'}{4}\left(\frac{1}{1 + L_\alpha} + \frac{2}{1 + L_\alpha/(1 + \gamma)} + \frac{1}{1 + L_\alpha/(1 + \gamma)^2}\right) \quad (1)$$

The overall observed rate is the summation of activities contributed by the hybrids.

The data from experiments were analyzed by using eq 1. The dissociation constants of GDP to the R state of the hybrid enzymes are found to be 40 and 43 μM at F6P concentrations of 0.1 and 0.3 mM, respectively.

Discussion

A systematic study of mutating amino acid side chains that bind to the allosteric effector of phosphofructokinase has now been made. It was shown previously that, on mutating residue Tyr55, $K_{T(PEP)}$ is totally unaffected and there is only a small hydrophobic interaction of the aromatic ring of the tyrosine residue with guanine of GDP (Lau et al., 1987). The present study has provided insights on the binding of PEP and GDP with charged side chains. It is found that the same set of residues is used in the binding of both activator, GDP, and inhibitor, PEP. On unique residue, Glu187, is found to be a trigger residue for the allosteric transition and is discussed separately.

GDP Binding. Site-directed mutagenesis does not in general provide an accurate measurement of the intrinsic binding energy but gives an apparent binding energy (Fersht, 1988). The apparent binding energies of the positively charged side chains with the diphosphate moiety of GDP are evenly distributed between the α- and β-phosphate binding pockets. The individual values of 2–3 kcal/mol are in the range now expected for the deletion of a hydrogen-bond donor/acceptor to a charged group (Fersht, 1988). On deletion of a charged side chain, water enters the effector site and acts as the hydrogen-bond donor/acceptor. It is likely that the specificity for binding GDP or ADP stems mainly from interactions with the diphosphate and the ribose ring. The present data show the importance of the interactions with pyrophosphate. The earlier study on mutation of Tyr55 showed that binding to the base in GDP may be unimportant (Lau et al., 1987), and Blangy et al. (1968) showed that CDP and UDP activate wild-type enzyme. Further, AMP is unable to activate the enzyme (Blangy et al., 1968), and this is probably due to the loss of

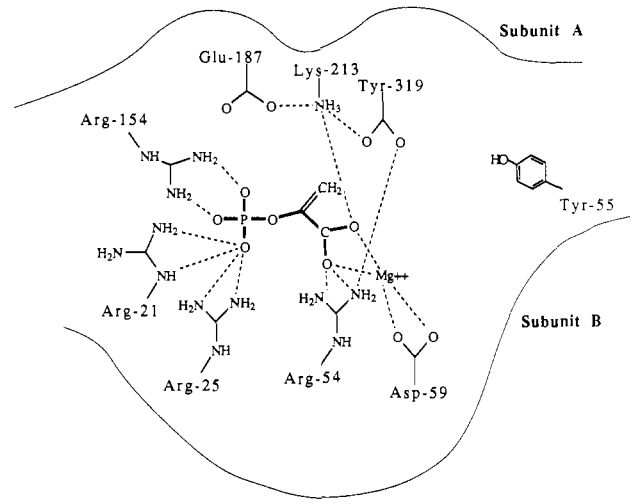


FIGURE 7: Proposed structure of a PEP-bound effector site of *E. coli* phosphofructokinase.

energy equivalent to the summation of the apparent binding energies to the β-phosphate position. The truncation of residues Arg21, Arg25, Arg54, Arg154, and Lys213 involves the removal of a charged interaction.

The truncation of the negatively charged Asp59 loses 2.0 kcal/mol of binding energy. Asp59 makes a hydrogen bond with the 3′-OH of the ribose ring. The apparent energy is at the upper limit for deleting a donor to an uncharged acceptor (Fersht, 1988).

Truncation of the C-terminal Tyr319 to the ΔTyr319 mutant also loses 2.2 kcal/mol of binding energy. In this case, there are no direct interactions between the mutated group and the bound ADP (Shirakihara & Evans, 1988). However, the carboxylate of Tyr319 does make interactions with the positively charged side chains of Arg54 and Lys213 that do directly interact (Figure 1). Mutation of Tyr319 possibly disrupts the alignments of the active site. The interpretation of the binding of Tyr319 emphasizes the importance of direct crystallographic information. In the absence of direct information on the structure of the complex, it would be only too easy to assume from mutagenesis that Tyr319 binds directly with the effector.

PEP Binding. Inorganic phosphate is found to bind in the position of the effector site occupied by the β-phosphate of GDP (Evans & Hudson, 1979). It is thus likely that the phosphate group of PEP binds to the β-phosphate pocket as predicted by Evans et al. (1981). Although the detailed structures of the effector site in the R and T states could be quite different, it is likely that residues Arg21, Arg25, and Arg154 form the PEP phosphate binding pocket, while Arg54 and Lys213 form part of the PEP carboxylate binding pocket (Figure 7). Since the Glu → Gln187 mutant is still inhibited by PEP in a manner similar to the wild-type enzyme, it is unlikely that a Mg^{2+} ion is coordinated to the phosphate. The role of Asp59 in the binding of PEP is unknown, but the possibility that a metal ion (perhaps Mg^{2+} ion) is coordinated to the carboxylate group of PEP rather than the phosphate group must be considered.

Glu187 Mutations. The residue Glu187 is found to be important in triggering the allosteric transition (Lau & Fersht, 1987). It was found previously that mutation of Glu187 to alanine caused PEP to become an activator at low concentrations of F6P. We now find that the mutant EA187 is allosterically inhibited by GDP (Figure 2). The importance of the nature of residue 187 is highlighted by a second mu-

tation in which Glu is converted to its isosteric analogue Gln. The mutant EQ187 is inhibited by both GDP and PEP. The PEP inhibition is similar to that of wild type, and the GDP inhibition is similar to that of EA187 mutant. Both GDP and PEP must bind to the T state of the mutant EQ187. As Glu187 is involved in binding the coordinated Mg^{2+} ion, the metal ion must be crucial in the allosteric transition. In mammalian hemoglobin, all effectors function as inhibitors by strengthening the existing interactions or generating new linkages in the T state (Perutz, 1978). Perhaps in phosphofructokinase the interaction between the coordinated Mg^{2+} of GDP with Glu187 provides the crucial stabilization of the R state. However, it is not clear how PEP activates the mutant EA187.

Hybrid Mutants. There is an interesting possibility arising from the effector site being composed of residues from two subunits. That is, can mutations in one subunit be complemented by hybridization with another mutant with a mutation in the other subunit? This is found to be so. Hybrid mutants formed from Arg → Ala21 and Lys → Ala213, unlike their parental enzymes, can be activated by GDP (Figure 5). Although a detailed kinetic analysis is not essential in the interpretation of results in this study, the activity of the hybrids is still consistent with the simple Monod–Wyman–Changeux scheme. Assuming the hybrids are formed in the ratio of 1:2:1, we can derive an equation (eq 1) based on the concerted MWC model (Monod et al., 1965) which fits the experimental results well. In addition, the values of $K_{R(GDP)}$ obtained for the hybrid mutants at two different substrate concentrations (40 and 43 μM) are the same as that of the wild type (41 μM) within experimental errors. This shows that the complemented effector sites appear to behave the same as a native wild-type effector site.

The protein engineering experiments have thus extended knowledge obtained from protein crystallography. The novel findings on the crucial role of Glu187 in triggering the conformational transition on binding of effectors suggests further X-ray studies to be performed on the mutants.

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