and a four-electron capacity, to a one-electron carrier, amicyanin.

Registry No. PQQ, 72909-34-3; methylamine dehydrogenase, 60496-14-2.

References

Ameyama, M., Matsushita, K., Ohno, Y., Shingawa, E., & Adachi, O. (1981) *FEBS Lett. 130*, 179–183.

Ameyama, M., Shinagawa, E., Matsushita, K., Takimoto, K., Nakashima, K., & Adachi, O. (1985) *Agric. Biol. Chem. 49*, 3623–3626.

Anthony, C. (1982) *The Biochemistry of Methylotrophs*, Academic, London.

Bradford, M. M. (1976) *Anal. Biochem. 73*, 248–254.

De Beer, R., Duine, J. A., Frank, J., & Large, P. J. (1980) *Biochim. Biophys. Acta 622*, 370–374.

Duine, J. A., & Frank, J. (1979) *Biochem. J. 187*, 221–226.

Duine, J. A., & Frank, J. (1981) *Trends Biochem. Sci. (Pers. Ed.) 6*, 278–280.

Duine, J. A., Frank, J., & van Zeeland, J. K. (1979) *FEBS Lett. 108*, 443–446.

Duine, J. A., Frank, J., & Verweil, P. E. J. (1981) *Eur. J. Biochem. 118*, 395–399.

Duine, J. A., Frank, J., & Jongejan, J. A. (1986) *FEMS Microbiol. Rev. 32*, 165–178.

Eckert, T. S., Bruice, T. C., Gainor, J. A., & Weinreb, S. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2533–2536.

Faraggi, M., Chandrasekar, R., McWhirter, R. B., & Klapper, M. H. (1986) *Biochem. Biophys. Res. Commun. 139*, 955–960.

Gray, K. A., Knaff, D. B., Husain, M., & Davidson, V. L. (1986) *FEBS Lett. 207*, 239–242.

Husain, M., & Davidson, V. L. (1985) *J. Biol. Chem. 260*, 14626–14629.

Husain, M., & Davidson, V. L. (1986) *J. Biol. Chem. 261*, 8577–8580.

Husain, M., & Davidson, V. L. (1987) *J. Bacteriol. 169*, 1712–1717.

Husain, M., Davidson, V. L., & Smith, A. J. (1986) *Biochemistry 25*, 2431–2436.

Kenny, W. C., & McIntire, W. (1983) *Biochemistry 22*, 3858–3868.

Lim, L. W., Mathews, F. S., Husain, M., & Davidson, V. L. (1986) *J. Mol. Biol. 189*, 257–258.

Lobenstein-Verbeek, C. L., Jongejan, J. A., Frank, J., & Duine, J. A. (1984) *FEBS Lett. 170*, 305–309.

Massey, V., & Hemmerich, P. (1978) *Biochemistry 17*, 9–17.

McIntire, W. S., & Stults, J. T. (1986) *Biochem. Biophys. Res. Commun. 141*, 562–568.

Moog, R. S., McGuirl, M. A., Cote, C. E., & Dooley, D. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8435–8439.

Sheath, P. R., Noar, J. B., Eberlein, G. A., & Bruice, T. C. (1985) *J. Am. Chem. Soc. 107*, 3328–3338.

Smith, J. M., Smith, W. H., & Knaff, D. B. (1981) *Biochim. Biophys. Acta 635*, 405–411.

van der Meer, R. A., & Duine, J. A. (1986) *Biochem. J. 239*, 789–791.

Williams, C. H., Jr., Arscott, L. D., Matthews, R. G., Thorpe, C., & Willkinson, K. D. (1979) *Methods Enzymol. 62*, 185–198.

Williamson, P. R., Moog, R. S., Dooley, D. M., & Kagan, H. M. (1986) *J. Biol. Chem. 261*, 16302–16305.

Site-Directed Mutagenesis in the Effector Site of *Escherichia coli* Phosphofructokinase

Frankie Tat-Kwong Lau,[‡] Alan R. Fersht,*[,‡] Homme W. Hellinga,[§] and Philip R. Evans[§]

Department of Chemistry, Imperial College of Science and Technology, London SW7 2AY, U.K., and Laboratory of Molecular Biology, Medical Research Council, Cambridge CB2 2QH, U.K.

Received December 29, 1986; Revised Manuscript Received March 9, 1987

ABSTRACT: A new vector for the expression of phosphofructokinase (pfk-1) was constructed with pEMBL, which allows reliable, inducible, high-expression, and facile mutagenesis of the gene. Two mutants in the effector site of the enzyme were produced by site-specific mutagenesis of residue Tyr-55 to assess the role of its side chain in binding an allosteric inhibitor, phosphoenolpyruvate (PEP), and an activator, guanosine 5′-diphosphate (GDP): Tyr-55 → Phe-55 and Tyr-55 → Gly-55. The dissociation constant of PEP from the T state is unaffected by the mutations. Mutation of Tyr-55 → Phe-55 only slightly increases the dissociation constant of GDP from the R state, indicating a minimal involvement of the hydroxyl group in binding. A 5.5-fold increase in the dissociation constant of GDP on the mutation of Tyr-55 → Gly-55 suggests a small hydrophobic interaction of the aromatic ring of the tyrosine residue with guanine of GDP.

Phosphofructokinase catalyzes the phosphorylation of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate. One of the two such enzymes in *Escherichia coli*, pfk-1, is a key regulatory element in the glycolytic pathway. The kinetic properties of *E. coli* pfk-1 have been studied extensively by Blangy et al. (1968) and Blangy (1971). This enzyme exhibits strong cooperative interactions with respect to fructose 6-phosphate and shows cooperative kinetics with respect to allosteric control, being inhibited by phosphenolpyruvate (PEP) and activated by ADP or GDP. The crystal structure of the active conformation of the enzyme obtained from *Bacillus*

[‡] Imperial College of Science and Technology.
[§] Medical Research Council.

0006-2960/87/0426-4143$01.50/0

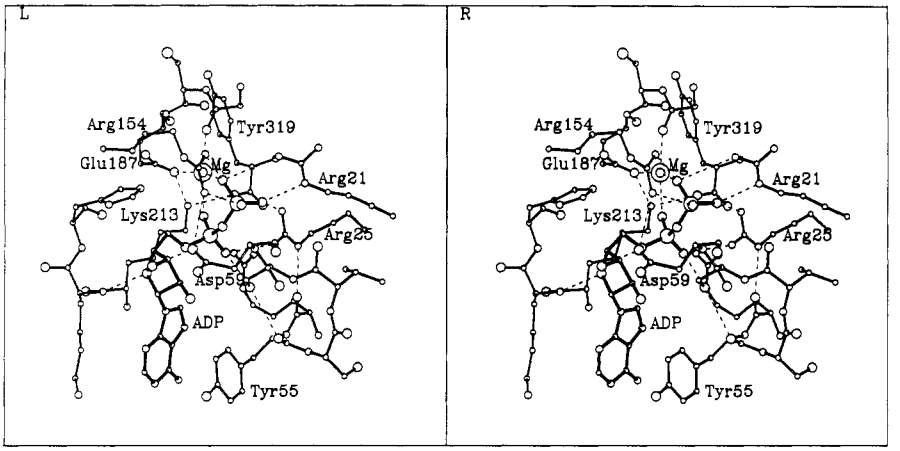

FIGURE 1: Effector binding site of phosphofructokinase from *E. coli* (P. R. Evans, unpublished results).

stearothermophilus has been solved to 2.4 Å (Evans & Hudson, 1979; Evans et al., 1981). Crystal structures of two forms of the *E. coli* enzyme have been refined to the same resolution (R state with F6P and ADP bound and T state (unligated); Y. Shirakihara, W. R. Rypniewski, and P. R. Evans, unpublished results).

The gene for pfk-1, *pfkA*, was originally identified from a set of mutants with greatly reduced phosphofructokinase activity. A recombinant plasmid pLC16-4 was constructed that carried the structural gene for this enzyme (Clarke & Carbon, 1976). The gene was sequenced and subcloned with either the single-stranded DNA phage vector M13mp8 or the high copy number plasmid pUC9 (Hellinga & Evans, 1985). The promoter contains an artifactual run of thymidines at the −35 region as a consequence of the construction of pLC16-4. The recombinants were expressed in *E. coli* DF1020 (Daldal, 1983), a strain that has had deleted the genes for pfk-1 and a second nonallosteric enzyme, pfk-2 (Babul, 1978). A number of recombinants were constructed, and a high level of expression was obtained from pHE1007 and pHE1012. However, they both have some disadvantages. pHE1012 gives exceptionally high expression, but as a consequence, there is a high tendency for selection against expression clones because of the metabolic burden. pHE1007 is more stable but is a much larger construct, carrying the genes for other proteins.

In order to have a more reliable expression system for phosphofructokinase, we decided to construct a new vector from pEMBL8(+) (Dente et al., 1983) and mHE1011 for inducible overproduction of the enzyme under the control of *lac* promoter. The pEMBL vector was chosen because of the advantages it provided for single-stranded DNA production following the superinfection with F1 phage. It greatly facilitates DNA sequencing and site-directed mutagenesis.

We have used the newly constructed vector (pHL1) to make mutations in the effector site of the enzyme. This site binds both inhibitor (PEP) and activators (GDP or ADP). This is an attractive site to study by systematic site-directed mutagenesis because the interactions with ADP have been characterized by X-ray crystallography (Figure 1). We wish to map out the energetics of these interactions to understand how one binding site is used both by activators and inhibitor and, when the structures of both R and T states are available, how these control the allosteric transition. The first target residue of the *E. coli* enzyme, Tyr-55, appears to be situated such that it may make a hydrophobic interaction with the adenine moiety of ADP or the guanine moiety of GDP in the effector-binding site. Further, the hydroxyl group of the tyrosyl residue may possibly make a hydrogen bond with adenine. In *B. stearothermophilus* phosphofructokinase, that tyrosyl residue is replaced by a glycine (Evans & Hudson, 1979; Evans et al., 1981). In order to study the importance of Tyr-55 for activator binding, mutation of Tyr-55 → Phe-55 should show whether there is any involvement of the phenolic hydroxyl group in hydrogen bonding, and Tyr-55 → Gly-55 should remove all possible hydrophobic interaction. In this study, we show that the mutation Tyr-55 → Phe-55 does not significantly change the affinity of the enzyme for GDP and the mutation Tyr-55 → Gly-55 reduces the affinity of the activator by about 5.5-fold. On the other hand, the affinity of PEP is unaffected.

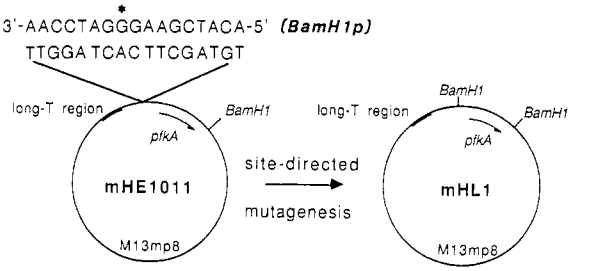

FIGURE 2: Construction of *Bam*HI site in RF form of M13 mHE1011 to generate mHL1.

Materials and Methods

Materials. Deoxyoligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer. T4 ligase, *E. coli* polymerase I (Klenow fragment), dNTP's, ddNTP's, and restriction endonuclease *Bam*HI were obtained from Pharmacia. Calf intestine alkaline phosphatase and reagents used in the coupled assay including aldolase, glycerol-3-phosphate dehydrogenase, triosephosphate isomerase, creatine kinase, creatine phosphate, ATP, NADH, fructose 6-phosphate, and phosphoenolpyruvate were obtained from Boehringer. Guanosine 6′-diphosphate and isopropyl β-D-thiogalactoside (IPTG) were purchased from Sigma. Matrex Blue-A gel was from Amicon. [α-^{32}P]dATP was purchased from Amersham. [γ-^{32}P]rATP was from New England Biolabs.

Subcloning of the pfkA Gene. The oligonucleotide primer *BamHI*p (5′-ACATCGAAG*GGATCCAA-3′) was synthesized to direct the mutation that introduced a new *Bam*HI restriction site between the artifactual long-T promoter region and the beginning of the coding region of the *pfkA* gene [where (*) denotes a mismatch]. Site-directed mutagenesis was performed on the single-stranded mHE1011 (Figure 2) essentially as described by Zoller and Smith (1983) with some

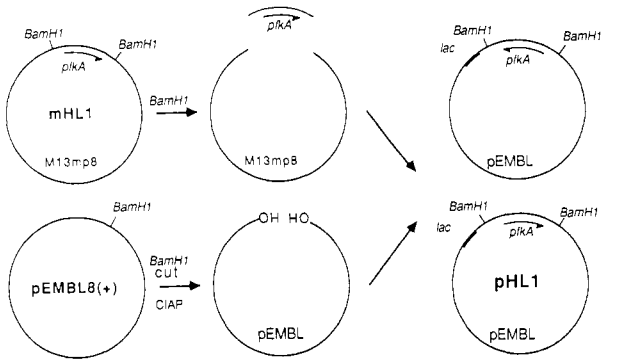

FIGURE 3: Subcloning of *pfkA* gene to generate expression/mutagenesis plasmid pHL1.

modifications. The primed template was made double stranded in vitro with DNA polymerase I (Klenow fragment) and phage T4 DNA ligase. The resulting synthetic duplex, which has the mismatched primer incorporated into it, was transfected into *BMH 71-18 mutL* (Kramer et al., 1984) to minimize mismatch repair and plated out on H-top agar with *E. coli* TG2 cells [K12, Δ(*lac-pro*),*sup*E,*thi*,*recA*$^-$,*Sr1*:Tn10^{T}c^R,-*hsd*Δ5/F′:*traD*36,*proAB*$^+$,*lac*I^q,*lacZ*Δ*M15*; Gibson, 1984]. No alkaline sucrose gradient centrifugation was performed beforehand. Mutant phage were identified by hybridization at temperatures at which only perfectly matched duplexes are stable with the synthetic *BamHI*p primer as probe. Positives were then transformed into *E. coli* TG2 and further checked for the incorporation of the correct insertion by digesting with *Bam*HI restriction enzyme. Mutant phage, mHL1, gave two fragments of 4 kb and 1.2 kb, respectively, whereas parent mHE1011 gave one fragment only.

The recombinant phage mHL1 was constructed to have two convenient *Bam*HI restriction sites flanking the *pfkA* insert so that this fragment can be easily recloned. The fragment was then recloned into pEMBL8(+) vector for controlled overproduction of the mutant enzymes. The pEMBL8(+) vector was cut with *Bam*HI and treated with calf intestine alkaline phophatase to avoid self-reannealing. The RF form of mHL1 was also cut with *Bam*HI. Taking the advantage that smaller DNA fragments ligate better than larger ones, religation was performed without first isolating the 1.2-kb *pfkA* fragment, and the resulting recombinants were mainly pEMBL+*pfkA* (Figure 3). Screening for the correct orientation of the *pfkA* insert was achieved by sequencing the single-stranded pEMBL template with the insert by using a M13 universal primer. The resulting reclone was named pHL1.

Expression and Purification of pfk-1 Enzyme. The recombinant pHL1 vector was transformed by the calcium chloride procedure (Maniatis et al., 1982) into HE1 cells [*pro-82*,Δ*pfkB201*,*recA56*,Δ(*rha-pfkA*)*200*,*endA1*,-*hsdR17*,*supE44*/*F′*:*traD36*,*proAB*+,*lac*I^q,*lacZ*Δ*M15*] for expression. A single colony was picked to set up an overnight culture in 2× TY medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 1 L of medium). One hundredth of the volume was inoculated into 2× TY (about 4 L) in the presence of 100 μg/mL ampicillin and 70 μg/mL IPTG and grown for 24 h. The culture was then harvested by cooling on ice and spinning down the cells; 10 mL of low-salt buffer [50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM β-mercaptanol) was added to resuspend the cell pellets; the suspension was then sonicated and centrifuged again at 5K rpm for 20 min with a GS-3 rotor in a Sorvall RC-58 refrigerated centrifuge and then at 40K rpm for 90 min with a 45TI rotor in a Beckman L8-55 ultracentrifuge. The clear supernatant was loaded into an Amicon Matrex Blue-A column and washed with 2 column volumes of low-salt and then 4 volumes of high-salt buffer (low-salt buffer plus 1.5 M NaCl). Finally, 1.5 column volumes of an eluting solution of 2 mM ATP/10 mM Mg^{2+} in low-salt buffer was used to elute the phosphofructokinase. Phosphofructokinases were stored as a 55% ammonium sulfate precipitate. A high protein yield of about 70–100 mg of protein/L of culture resulted from the pEMBL expression system. Sephacryl S-300 gel filtration chromatography with a column volume of 22 mL and 1-cm diameter was employed to remove ammonium sulfate and ATP and gave pure enzyme for kinetic studies. The concentration of the purified protein was estimated by assuming $\epsilon_{278} = 0.6$ cm^2 mg^{-1}, and the purified protein was stored in the presence of 10 mM dithioerythritol to avoid air oxidation. Control experiments showed that the extinction coefficient was negligibly altered on mutation. The enzymes were homogeneous on polyacrylamide–sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis.

Phosphofructokinase Assays. The activity of the enzyme was assayed by a coupled enzyme method with 70 μg of aldolase, 3 μg of triosephosphate isomerase, and 30 μg of glycerol-3-phosphate dehydrogenase per assay (all from rabbit muscle), and the decrease of A_{340} with time was monitored (Kotlarz & Buc, 1982). Assays were performed in 100 mM Tris–Tris·HCl, pH 8.2 at 25 °C, in the presence of an ATP regenerating system for all experiments. The assay medium containing 10 mM $MgCl_2$, 10 mM NH_4Cl, 0.2 mM NADH, and 1 mM F6P with the ATP regenerating system of 10 μg of creatine kinase and 1 mM creatine phosphate was kept at 25 °C. Phosphofructokinase was diluted to appropriate working concentration with Tris buffer, 100 mM, pH 8.2. F6P was added subsequently when its homotropic cooperativity was studied.

A total of 0.97 mL of assay medium was pipetted into a disposable cuvette; the appropriate volume of dialyzed coupled-assay enzyme mixture, 10 μL of phosphofructokinase, and 10 μL of 100 mM ATP were added successively. Enzyme activity is expressed in units given by $(\Delta A/\text{min}) \div 12.4$. In activation studies, GDP was used instead of ADP because ADP is a competitive inhibitor with respect to substrate ATP [$K_{i(ADP)} = 2 \times 10^{-4}$ M] but GDP does not inhibit the enzyme at moderate concentrations and is also an effective activator (Blangy et al., 1968).

Production of Mutants by Oligonucleotide-Directed Mutagenesis. Single-stranded pHL1 template for site-directed mutagenesis of the *pfkA* gene was prepared by superinfecting rapidly dividing cells harboring pHL1 ($A_{660} < 0.2$) with F1-(IR1) phage and incubating at 37 °C for a further 5 h. The amount of F1(IR1) phage used to superinfect the *E. coli* TG2 cells transformed with pHL1 was first tested empirically to give good single-stranded DNA yield. Mutations of Tyr-55 → Phe-55 and Tyr-55 → Gly-55 were directed by the following oligonucleotides: 5′-GA-CAC-GCT-G*AA-ACG-GTC-T-3′ and 5′-A-CAC-GCT-G*C*C-ACG-GTC-TA-3′, respectively [where (*) denotes mismatch]. Mutagenesis using single-stranded pHL1 template was performed essentially as described in the subcloning section except that the heteroduplexes were transformed into competent *E. coli* TG2 rather than *E. coli BMH 71-18 mutL* in order to minimize the possibility of generating undesirable spurious mutations. Colonies harboring mutant plasmids were identified by hybridization screening (Grunstein & Hogness, 1975) with the mutagenic oligonucleotide as a radiolabeled probe (Zoller & Smith, 1983).

Mutations were verified by sequencing the entire gene with the appropriate primers (Hellinga & Evans, 1985) by dideoxy sequencing (Sanger et al., 1977).

RESULTS

The insertion of foreign DNA sequences in the region coding for the sequence of the α-peptide of β-galactosidase in the pEMBL vector allows "blue-white selection" in indicator plates containing IPTG and X-Gal (Dente et al., 1983). In this way, recombinant plasmids were isolated from the parent vector pEMBL8(+). Among those white colonies that gave (4 + 1.2)-kb fragments after *Bam*HI restriction enzyme treatment, roughly half of them were of the right orientation with respect to the *pfkA* insert. After the whole gene was sequenced to ensure no spurious mutations, the new construct, pHL1, was used for any further studies.

About 30% of the pEMBL colonies generated in the Tyr-55 → Phe-55 mutagenesis procedure strongly hybridized to the mutagenesis primer under stringent conditions. Single-stranded template DNA was prepared and purified from three of these mutant colonies; all of them contained the desired mutation. Other mutations were made with similar apparent efficiencies.

Phe-55 and Gly-55 mutants were purified in the same manner as wild-type enzyme. The amount of protein was estimated by the absorbance at 280 nm. The specific activities of the mutant enzymes are comparable with the wild-type protein, about 190 units/mg of protein.

Calculation of $K_{R(GDP)}$ and $K_{T(PEP)}$. Phosphofructokinase conforms to the concerted transition theory proposed by Monod et al. (1965). The equations used for the interpretation of data have been derived by Blangy et al. (1968).

As the nonexclusive binding coefficient of F6P, which is the ratio of the dissociation constant of F6P in the R state to that of the T state, is very small, the ratio between R and T conformations can be written as

$$\bar{Q} = \frac{v}{V'_{max} - v} = \frac{(1 + \alpha)^4(1 + \gamma)^4(1 + d\beta)^4}{L_0(1 + e\gamma)^4(1 + \beta)^4}$$

where $\bar{Q}$ is the ratio of the concentration of the R state to the T state; V'_{max} is the maximum velocity that can be reached in the presence of a given concentration of fructose 6-phosphate; L_0 is the allosteric constant, that is, the ratio of R/T in absence of any allosteric effector; α is the normalized concentration of F6P, that is, the ratio of the concentration of F6P to the dissociation constant of F6P in the R state; γ is the normalized concentration of GDP, that is, the ratio of the concentration of GDP to the dissociation constant of GDP in the R state; β is the normalized concentration of PEP; d is the ratio $K_{T(PEP)}/K_{R(PEP)}$; and e is the ratio $K_{R(GDP)}/K_{T(GDP)}$. Since α is constant for a fixed F6P concentration, in the presence of GDP

$$\bar{Q} = \frac{1}{L_{(\alpha)}} \frac{(1 + \gamma)^4}{(1 + e\gamma)^4} \qquad (1)$$

where

$$L_{(\alpha)} = \frac{L_0}{(1 + \alpha)^4} \qquad \gamma = \frac{[GDP]}{K_{R(GDP)}} \qquad e\gamma = \frac{[GDP]}{K_{T(GDP)}}$$

$K_{R(GDP)}$ and $K_{T(GDP)}$ are dissociation constants of ligand GDP in the R and T conformations, respectively.

The term $e\gamma$ in eq 1 can be ignored at low [GDP] since $e << 1$. Equation 1 is derived for a fixed F6P concentration in the presence of GDP and in the absence of PEP. The dissociation constant for GDP in the R state can be obtained by plotting $[v/(V'_{max} - v)]^{1/4}$ against GDP concentration to give a straight line with x intercept = $-K_{R(GDP)}$.

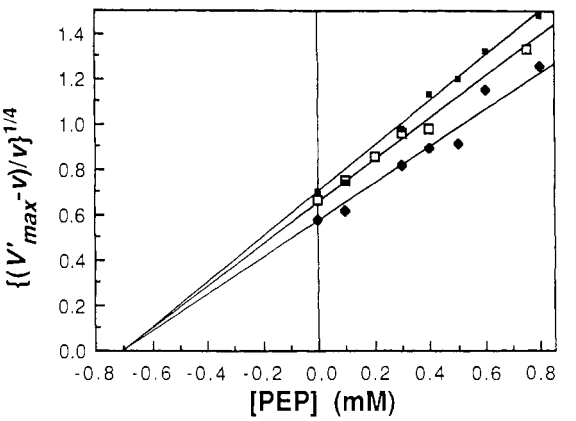

FIGURE 4: PEP inhibition of formation of fructose 1,6-biphosphate catalyzed by phosphofructokinases in the presence of 1 mM fructose 6-phosphate and 1 mM ATP at pH 8.2 and 25 °C. Wild type (□), Tyr → Phe-55 (◆), and tyr → Gly-55 (■).

In the presence of PEP

$$\bar{Q} = \frac{v}{V'_{max} - v} = \frac{(1 + \alpha)^4(1 + d\beta)^4}{L_0(1 + \beta)^4} \qquad (2)$$

where

$$\beta = \frac{[PEP]}{K_T(PEP)} \qquad d\beta = \frac{[PEP]}{K_{R(PEP)}} \qquad d = K_{T(PEP)}/K_{R(PEP)}$$

β is the normalized concentration of PEP. $K_{R(PEP)}$ and $K_{T(PEP)}$ are dissociation constants of ligand in the R and T conformations, respectively.

Equation 2 is derived for the presence of PEP but for the absence of GDP. For the calculation of $K_{T(PEP)}$, a plot of $[(V'_{max} - v)/v]^{1/4}$ against PEP concentration may be used at low [PEP] since the term $d\beta$ in eq 2 may be ignored at $d << 1$. The values of $K_{R(GDP)}$ and $K_{T(PEP)}$ are assumed to be independent of the fructose 6-phosphate concentration in the classical Monod–Wyman–Changeux model, and this is found for wild-type enzyme (Blangy et al., 1968).

$K_{T(PEP)}$, the dissociation constant of PEP in the less active T conformation, of phosphofructokinase was determined from inhibition studies to be 700 μM. The binding is not affected by the mutations in residue Tyr-55 (Figure 4). The value is consistent with that for wild type found by Blangy et al. (1968). It can be concluded that Tyr-55 is not involved in inhibitor binding.

Activation studies using GDP at different F6P concentrations showed that mutation of Tyr-55 → Phe-55 results in only a slightly higher value for $K_{R(GDP)}$ (58 μM for Phe-55 vs. 41 μM for wild type), which implies a minimal involvement of the hydroxyl group in the binding of the activator (Figures 5 and 6). The value of $K_{R(GDP)}$ is also consistent with the value of Blangy et al. (1968). However, the value of $K_{R(GDP)}$ for the Gly-55 mutant is increased to 233 μM (Figure 7), which suggests a small hydrophobic interaction of the aromatic ring of the tyrosine residue with the adenine group of GDP (or ADP).

The energy involved in the hydrophobic interaction is estimated from

$$\Delta G = -RT \ln \frac{(1/K_{R(GDP)})_{Gly^{55}}}{(1/K_{R(GDP)})_{wild\text{-}type}}$$

to be 1.0 kcal/mol.

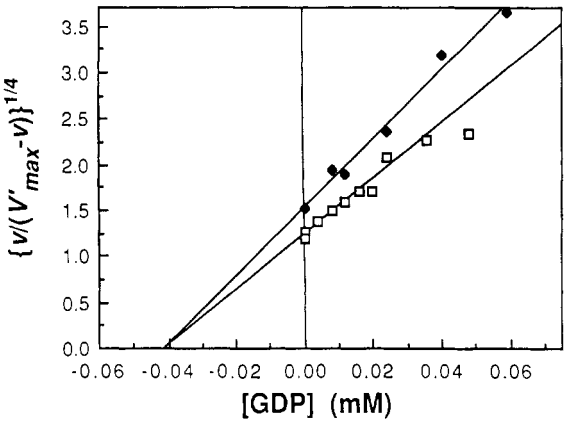

FIGURE 5: GDP activation of formation of fructose 1,6-bisphosphate catalyzed by wild-type phosphofructokinase in the presence of 0.5 (□) and 0.7 mM (◆) fructose 6-phosphate and 1 mM ATP in pH 8.2 and 25 °C.

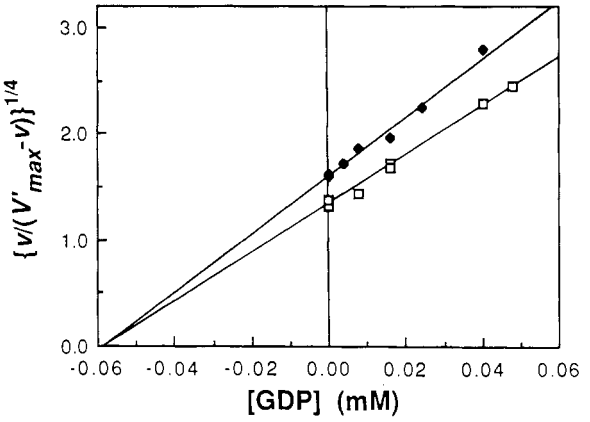

FIGURE 6: GDP activation of formation of fructose 1,6-bisphosphate catalyzed by phosphofructokinase Tyr → Phe-55 in the presence of 0.5 (□) and 0.7 mM (◆) fructose 6-phosphate and 1 mM ATP at pH 8.2 and 25 °C.

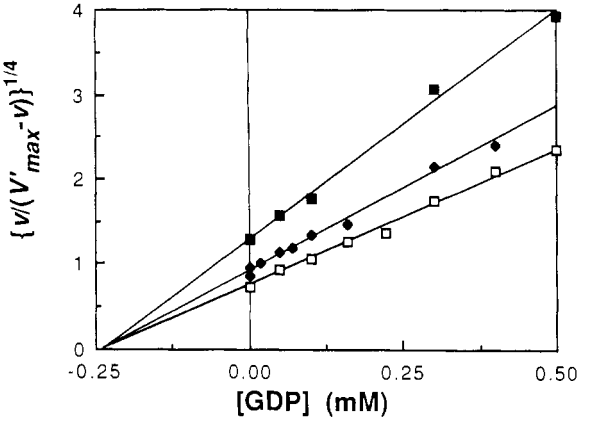

FIGURE 7: GDP activation of formation of fructose 1,6-bisphosphate catalyzed by phosphofructokinase Tyr → Gly-55 in the presence of 0.5 (□), 0.7 (◆), and 1.0 mM (■) fructose 6-phosphate and 1 mM ATP at pH 8.2 and 25 °C.

The result is also consistent with the idea of Evans and Hudson (1979) that the key interaction is with the β-phosphate of the nucleoside diphosphate rather than with the base.

Discussion

The new construct, pHL1, is highly suitable for both mutagenesis and expression. Single-stranded DNA can be obtained readily by superinfecting with F1 phages for mutagenesis and subsequent sequencing. There is, therefore, no need for recloning into M13 derivatives. Theoretically, plasmid and single-stranded DNA production and expression of phosphofructokinase can be performed by using just *E. coli* HE1 without resort to other strains. However, the *pfk*-minus strain, HE1, has a tendency to lose the F pili, which are essential for the infection by F1 phage, a necessary feature for packaging of single-stranded DNA (Dente et al., 1983). In practice, genetic manipulations were performed with *E. coli* TG2 cells, and the expression of phosphofructokinases was performed with *E. coli* HE1.

The expression of phosphofructokinase is very reliable from pHL1. The level of expression is nearly as high as pHE1007 (Hellinga & Evans, 1985) after the induction of IPTG and gives about 70–100 mg/L of culture. A further advantage of pHL1 is that it produces only phosphofructokinase-1 protein. If mutants of phosphofructokinase that are deleterious to growth are made, the delayed IPTG-induced expression will maximize expression. However, the pHL1 construct is still somewhat leaky, producing some protein without induction. If highly lethal mutants are involved, a better repressed vector should be employed. The *pfkA* insert, which is flanked by *Bam*H1 sites in pHL1, allows any further recloning to be performed easily.

In the study of Blangy et al. (1968) of the activation of *E. coli* phosphofructokinase, different nucleoside diphosphates were found to activate the enzyme to various extents. Purine compounds are the more potent. This may perhaps be due to a greater hydrophobic interaction of the purine groups with the effector-binding site compared with the pyrimidine groups. There are not many residues near the effector site that could enhance nucleoside binding. One of them is Lys-211. This residue is conserved in different species. The C-terminal half of the rabbit muscle enzyme has a lysine at the equivalent position, but this is replaced by threonine in the amino-terminal half of the enzyme that contains gene duplication (Poorman et al., 1984). There is conservative replacement by an arginine in phosphofructokinase from *B. stearothermophilus* (Kolb et al., 1980). The other likely residue is Tyr-55, which appears to be located such that it could provide significant binding energy.

Our study shows that the side chain of Tyr-55 contributes only a small binding energy for binding of base. The calculated value of 1.0 kcal/mol for the apparent binding energy of the aromatic ring of Tyr-55 with GDP is considerably less than expected for an effective hydrophobic interaction and is at the level expected for just a single methyl group (Fersht, 1985). This suggests that there are important interactions elsewhere for binding the nucleoside diphosphates. It was suggested from an X-ray crystallographic study of the structure of phosphofructokinase from *B. stearothermophilus* that the key interaction with ADP in the effector site appears to be with the β-phosphate group (Evans & Hudson, 1979). The diphosphate of ADP points into a cleft, which can explain the specificity of the site for ADP rather than AMP and ATP and its lack of specificity for the base.

More point mutations are being constructed around the α- and β-phosphate binding site in order to understand the interaction energies in the diphosphate group binding cleft. Any effects specific to GDP will be compared with those of PEP to delineate the fine balance between activation and inhibition in the same effector-binding site.

References

Babul, J. (1978) *J. Biol. Chem. 253*, 4350–4355.
Blangy, D. (1971) *Biochimie 53*, 135–149.
Blangy, D., Buc, H., & Monod, J. (1968) *J. Mol. Biol. 31*, 13–35.

Clarke, L., & Carbon, J. (1976) *Cell (Cambridge, Mass.) 9*, 91-99.
Daldal, J. (1983) *J. Mol. Biol. 168*, 285-305.
Dente, L., Cesareni, G., & Cortese, R. (1983) *Nucleic Acids Res. 11*, 1645-1655.
Evans, P. R., & Hudson, P. J. (1979) *Nature (London) 279*, 500-504.
Evans, P. R., Farrants, G. W., & Hudson, P. J. (1981) *Philos. Trans. R. Soc. London, B 293*, 53-62.
Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, 2nd ed., Freeman, New York.
Gibson, T. J. (1984) Ph.D. Thesis, University of Cambridge.
Grunstein, M., & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3961-3965.
Hellinga, H. W., & Evans, P. R. (1985) *Eur. J. Biochem. 149*, 363-373.
Kolb, P., Hudson, P. J., & Harris, J. I. (1980) *Eur. J. Biochem. 108*, 587-597.
Kotlarz, D., & Buc, H. (1982) *Methods Enzymol. 90*, 60-70.
Kramer, W., Drutsa, V., Jansen, H. W., Kramer, B., Pflugfelder, M., & Fritz, H. J. (1984) *Nucleic Acids Res. 12*, 9441-9456.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, 1st ed., pp 249-251, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88-118.
Poorman, R. A., Randolph, A., Kemp, R. G., & Heinrikson, R. L. (1984) *Nature (London) 309*, 467-469.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468-500.

GTP Hydrolysis during Microtubule Assembly†

E. Timothy O'Brien,* William A. Voter, and Harold P. Erickson
Duke University Medical Center, Durham, North Carolina 27710
Received November 18, 1986; Revised Manuscript Received March 10, 1987

ABSTRACT: The GTP cap model of dynamic instability [Mitchison, T., & Kirschner, M. W. (1984) *Nature (London) 312*, 237] postulates that a GTP cap at the end of most microtubules stabilizes the polymer and allows continuing assembly of GTP-tubulin subunits while microtubules without a cap rapidly disassemble. This attractive explanation for observed microtubule behavior is based on the suggestion that hydrolysis of GTP is not coupled to assembly but rather takes place as a first-order reaction after a subunit is assembled onto a polymer end. Carlier and Pantaloni [Carlier, M., & Pantaloni, D. (1981) *Biochemistry 20*, 1918] reported a lag of hydrolysis behind microtubule assembly and a first-order rate constant for hydrolysis (k_h) of 0.25/min. A lag has not been demonstrated by other investigators, and a k_h value that specifies such a slow rate of hydrolysis is difficult to reconcile with reported steady-state microtubule growth rates and frequencies of disassembly. We have looked for a lag using tubulin free of microtubule-associated protein at concentrations of 18.5-74 μM, assembly with and without glycerol, and two independent assays of GTP hydrolysis. No lag was observed under any of the conditions employed, with initial rates of hydrolysis increasing in proportion to rates of assembly. If hydrolysis is uncoupled from assembly, we estimate that k_h must be at least 2.5/min and could be much greater, a result that we argue may be advantageous to the GTP cap model. We also describe a preliminary model of assembly coupled to hydrolysis that specifies formation and loss of a GTP cap, thus allowing dynamic instability.

Under defined conditions, microtubules contain approximately one GTP and one GDP molecule per dimeric tubulin subunit, while each free tubulin subunit in solution binds two molecules of GTP with high affinity (Weisenberg et al., 1968, 1976; Gaskin et al., 1974; Kobayashi, 1974; Hamel et al., 1986). Although it has been established that GTP is hydrolyzed during or soon after a tubulin subunit is incorporated into the microtubule polymer (Kobayashi, 1975; David-Pfeuty et al., 1977; McNeal & Purich, 1978; Carlier & Pantaloni, 1981; Hamel et al., 1982; Caplow et al., 1985), hydrolysis is not necessary for assembly. Assembly can take place in the presence of nonhydrolyzable GTP analogues (Weisenberg et al., 1976; Arai & Kaziro, 1976; Purich & MacNeal, 1978) but cannot be initiated from soluble subunits if only GDP is present (Gaskin et al., 1974; Olmstead & Borisy, 1975; Carlier & Pantaloni, 1978). Microtubules formed with nonhydrolyzable GTP analogues are generally much more stable than those formed in the presence of GTP, and this observation led to the suggestion that hydrolysis is not necessary for successful binding of a tubulin subunit to the end of a polymer but is necessary to allow normal disassembly (Weisenberg et al., 1976; Weisenberg & Deery, 1976; Arai & Kaziro, 1976).

To understand the role that hydrolysis of GTP might play in the dynamics of microtubule assembly and disassembly, it is important to know when GTP hydrolysis takes place during the assembly reaction. The idea that hydrolysis of GTP might be concomitant with tubulin assembly has been tested by a number of investigators. Before 1981, most results were consistent with the assumption that hydrolysis was tightly

† This work was supported by National Institutes of Health Grant GM-28553.
* Address correspondence to this author at the Department of Anatomy, Duke University Medical Center.

0006-2960/87/0426-4148$01.50/0