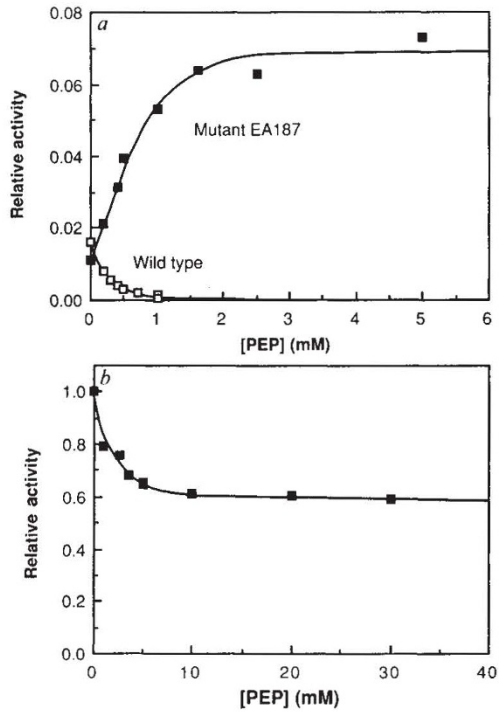

Fig. 2 *a*, Dependence on PEP concentrations of the activity of wild-type (□), and mutant Glu→Ala 187 (■), phosphofructokinases in the presence of 0.3 mM F6P and 1 mM ATP, at *p*H 8.2 and 25 °C. *b*, As *a*, but with 1 mM F6P (close to saturation). Activity of wild-type enzyme is very low above 1 mM PEP.

measurements or from competition with the binding of PEP (see below), even though the dissociation constant ($K_{R(GDP)}$) from wild-type enzyme is 40 μM. Mutation of Glu→Ala 187 has a remarkable effect on the consequences of binding PEP. At low concentrations of F6P, as found *in vivo*, where the enzyme is predominantly in the *T*-state, PEP is an activator. At 0.3 mM PEP, for example, there is a sixfold increase in activity on binding PEP (Fig. 2). Wild-type enzyme is strongly inhibited under the same conditions (Fig. 2). At high concentrations of F6P, where the mutant enzyme is close to being saturated with F6P and is predominantly in the *R*-state, there is some inhibition as a result of the binding of PEP (with a 40% reduction in rate at saturating concentrations of PEP). This is quite different from the inhibition of wild-type enzyme where the activity tends to zero.

Mutation of Glu→Ala 187 affects homotropic interactions of binding of F6P. In the absence of PEP there is a slight decrease in the Hill coefficient (n_H) from 3.7 in wild-type enzyme to 3.0 in the mutant. There is a progressive decrease in the cooperativity of F6P binding to mutant as the concentration of PEP increases, falling to only 1.1 at 10 mM PEP. The concentration of F6P required for half maximal activity ($S_{1/2}$) decreases slightly with increasing PEP concentrations. This behaviour contrasts with the effects of PEP on wild-type enzyme for which $S_{1/2}$ increases greatly as the concentration of PEP is raised.

According to the classical analysis of allostery by Monod, Wyman and Changeux[2], PEP binds to the *T*-state of wild-type enzyme[2,3]. But the effects of PEP on the mutant are consistent with PEP binding to an *R*-state. The data are not consistent, however, with the conventional two-state allosteric model[2] for the mutant because the *R*-state with PEP bound has a lower value of k_{cat} than does the *R*-state with just F6P bound. One could invoke the existence of more quaternary states, for example *T*, *R* and *R'* in a three-state model. But it is simpler to consider that the enzyme exists in two basic quaternary structures whose precise kinetic and thermodynamic properties vary slightly according to the reaction conditions and ligands present.

Table 1 Kinetic properties of wild-type and mutant enzymes

Enzyme	Specific activity* (μ mol min^{-1} mg^{-1})	Hill constant, n_H	$S_{1/2}$(F6P)† (mM)
Wild-type	190	3.7	0.33
Glu→Ala 187	87	3.0	0.75
Glu→Ala 187 +10 mM PEP		1.1	0.57

* Production of fructose-1,6-bisphosphate in the presence of 1 mM F6P, 1 mM ATP at 25 °C and *p*H 8.2 (ref. 8).

† Substrate concentration for half V_{max}.

Methods. The gene *pfkA* (ref. 9) was recloned in pEMBL8(+)[10] to give pHL1 which was constructed to give high level, inducible, expression of phosphofructokinase (data not shown). For mutagenesis, a synthetic primer which contained a single mismatch with the wild-type gene was designed to introduce an Ala codon at position 187. Site-directed mutagenesis was performed as described[11] using TG2[12] as the recipient strain in transformation. Phosphofructokinase was expressed in HE1 cells (*pro-82, ΔpfkB201, recA56, Δ(rha-pfkA)200, endA1, hsdR17, supE44/F':tra D36, proAB⁺, lacI^q, lacZΔM15*) which is a phosphofructokinase-less strain. The enzyme was purified from 24-h culture grown in 2×TY broth containing 100 μg ml^{-1} ampicillin and 70 μg ml^{-1} isopropyl thiogalactoside (IPTG) by a Blue-A column (Amicon Matrex) and by Sephacryl S-300 gel filtration chromatography. The activity of the enzyme was assayed by a coupled enzyme method[8].

Resolving the structural basis of the allosteric effects requires further high-resolution X-ray crystallographic studies. The mutagenesis studies have, however, provided definitive evidence that both inhibitor and activator bind in the same site. Mutation of Glu→Ala 187 has pinpointed one of the important residues and suggests that the crystal structure of the mutant is a candidate for further study. It may well reveal further conformational states that are important in function but not so accessible in wild-type enzyme. One of the goals of industrial protein engineering is to alter allosteric properties of regulated enzyme in order to alter yields of products[7]. This study shows that it is feasible to do this by systematically mutating a relatively small number of amino acids in effector binding sites.

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Erratum

Need for DNA topoisomerase activity as a swivel for DNA replication and for transcription of ribosomal RNA

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THIS letter was published with an incorrect title. The above title is correct.