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Influence of allosteric effectors on the kinetics and equilibrium binding of phosphoenolpyruvate (PEP) to phosphoenolpyruvate carboxylase (PEPC) from *Zea mays*

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Abstract

Phosphoenolpyruvate carboxylase (PEPC) the carbon dioxide processing enzyme of C_4 plants, shows the features of an allosteric enzyme. Allosteric activators such as D-glucose-6-phosphate and glycine increase the affinity of PEPC for its substrate PEP at pH 8.0 and pH 7.0. Allosteric inhibitors like L-malate and L-aspartate predominantly decrease the affinity of the carboxylase for PEP at pH 7.0. This was demonstrated by determination of the enzymatic activity and stopped flow (SF) fluorimetry. The binding reaction of PEP to PEPC from *Zea mays* was measured using the fluorescence probe 2-*p*-toluidinonaphthalene-6-sulfonate (TNS). The kinetics are described by an allosteric mechanism with a fast reversible bimolecular binding step of PEP to a high affinity (tensed) form of PEPC, which is in equilibrium with its low affinity (relaxed) form. The influence of allosteric effectors on the conformational transition step is demonstrated in support of the description of the kinetics of PEPC by applying a concerted allosteric mechanism as introduced by Monod, Wyman and Changeux. In summary, we present data for the influence of allosteric activators on the kinetics of PEP binding to PEPC and on the concentration dependence of the isomerisation reaction between two allosteric forms of PEPC.

Keywords: Phosphoenolpyruvate carboxylase (PEPC); Kinetics of phosphoenolpyruvate binding; Enzyme kinetics; Allosteric enzyme regulation; Fast reaction techniques

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1. Introduction

Phosphoenolpyruvate carboxylase (PEPC) is the key enzyme of the Hatch–Slack–Kortschak cycle in C_4-plants. The enzyme catalyses the irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of magnesium ions as cofactors yielding oxaloacetate and inorganic phosphate as products. The first step in the carboxylation of PEP is the formation of an enolate anion of pyruvate and carboxyphosphate from the adducts PEP and HCO_3^- [1]. In a second step, the enolate anion of pyruvate reacts with CO_2, which is formed by the decay of carboxyphosphate in the active site of PEPC [1]. Phosphoenolpyruvate carboxylase (PEPC) is a tetramer with a molecular weight of 400 000 consisting of four identical subunits [2–4]. The enzyme exists in different oligomeric states depending on pH, ionic strength as well as Mg^{2+} and PEP concentrations [5]. D-Glucose-6-phosphate (G-6-P), the main product of the Hatch–Slack–Kortschak cycle, is a strong activator of PEPC [6,7] while L-malate functions as an inhibitor [6,7]. D-Glucose-6-phosphate (G-6-P) lowers the inhibition of PEPC by L-malate [6,7]. The effect of D-glucose-6-phosphate (G-6-P) and L-malate is stronger at pH 7.0 than at pH 8.0 [6,7]. PEPC from *Zea mays* shows the features of an allosteric enzyme. To investigate the allosteric behaviour of PEPC, measurements of the equilibrium state and the dynamics of substrate binding at different pH-values in the absence and presence of allosteric effectors using the substrate PEP were performed. From these experiments, we expect further information concerning the concerted allosteric mechanism proposed recently [8]. The kinetic data are compatible with a concerted mechanism as introduced by Monod et al. [9] for allosteric enzymes. It was assumed that PEPC exists in two forms, I and II, with different affinities for PEP in the presence of Mg^{2+}-ions. Form I indicates a low affinity conformation, while form II binds PEP much more tightly. PEP binding to the high affinity form II can be correlated with the experimentally determined relaxation time τ_A. Both forms are in equilibrium and can be converted into each other with the rate constants $k_{B+(i)}$ in the forward and $k_{B-(i)}$ in the opposite direction. The relaxation time τ_B obtained from our stopped flow measurements can be attributed to this isomerisation process. To confirm this mechanism, the equilibrium binding of PEP to PEPC was studied by determining the enzymatic activity as a function of the substrate concentration in the absence and presence of allosteric effectors at pH 7.0 and pH 8.0. In addition, the dynamics of the allosteric transition of PEPC were measured under these conditions by the application of stopped flow (SF) fluorimetry, using the fluorescent probe 2-*p*-toluidinonaphthalene-6-sulfonate (TNS) for detection [8–10].

2. Materials and methods

2.1. Materials

Phosphoenolpyruvate (trisodium salt, hydrate) was obtained from Sigma-Aldrich (Deisenhofen, Germany) and TNS was purchased from Serva (Heidelberg, Germany).

PEPC was purified according to a modified protocol as published by Jawali [11]. In this purification scheme, the Bio-Gel A chromatography was replaced by an AcA 22 gel filtration. Prior to subsequent density gradient ultracentrifugation in an AH 627 rotor (Beckman) at 4°C, the protein solution was adjusted to 1.7 M $(NH_4)_2SO_4$ to precipitate contaminants of ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO). The precipitate was removed by centrifugation and the supernatant was brought to 65% $(NH_4)_2SO_4$ saturation. The resulting precipitate was collected by centrifugation, dissolved in 50 mM HEPES buffer at pH 7.0 (1 mM DTE and 1 mM EDTA) and dialysed against the same buffer. The protein solution was then applied to 10–30% glycerol gradients, which were fractionated after spinning at 27 000 rev./min for 24 h and the protein concentration was determined using absorption at 280 nm. The purity and enzymatic activity of the preparation were assessed by SDS-PAGE and UV-Vis spectroscopy (Fig. 1a,b). Active fractions were stored at −20°C. Prior to use, the samples were dialysed at 4°C against 100 mM

HEPES buffer of pH 7.0 or pH 8.0 containing 1 mM dithioerythritol and 1 mM EDTA for stabilisation.

The concentration of PEPC was determined according to Warburg and Christian [12]. In addition, an extinction coefficient $\varepsilon_{280\,nm}$ of 5.46×10^5 M^{-1} cm^{-1} was used [8]. The purity of the enzyme preparations was determined by scanning SDS polyacrylamide gels (Image Documentation System CSI, Cybertech, Berlin, Germany).

2.2. Michaelis–Menten experiments

The enzymatic activity of PEPC was assayed spectrophotometrically at 340 nm by coupling the enzymatic reaction to exogenous malate dehydrogenase (MDH) at 25°C. Steady-state velocity measurements were performed by mixing 20 μl of an enzyme fraction (conc. ~ 1 mg/ml), preincubated in either 100 mM Tris–HCl buffer of pH 8.0 or 100 mM HEPES buffer of pH 7.0, both contain-

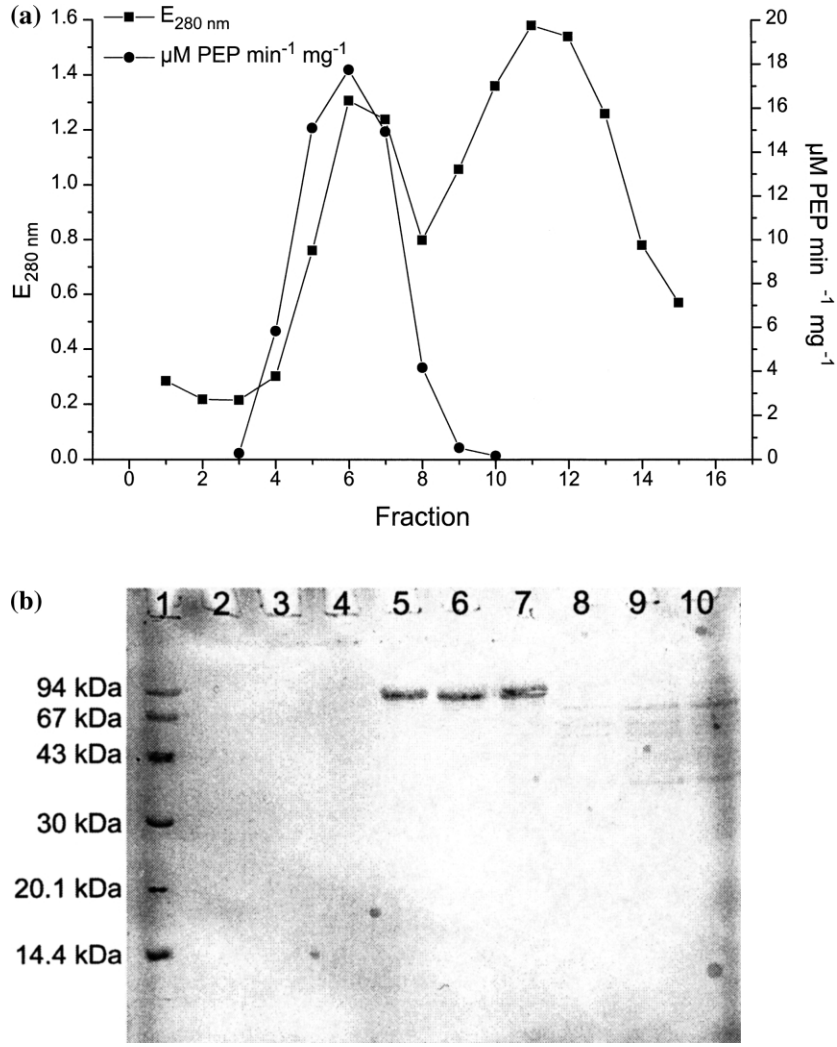

Fig. 1. (a) Protein concentration ($E_{280\,nm}$) and enzymatic activity (μM PEP min^{-1} mg^{-1}) of fraction 1–15 of a 10–30% glycerol gradient as the final purification step of PEPC. (b) Fraction 5–7 of 10–30% glycerol gradients are shown to be homogeneous by SDS-PAGE using 15% polyacrylamide.

ing 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM dithioerythritol, 1 mM EDTA, 0.2 mM NADH, 25 μg/ml MDH (1200 units/mg, Boehringer, BRD) and allosteric effectors (for concentration see results) with 50 μl solution of the substrate PEP, resulting in a total volume of 2 ml. The rate data were fitted by a non-linear regression program (ENZFITTER, Biosoft, UK) to a hyperbolic function. This program also determines the experimental errors of V_{max} and K_m.

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

where K_m is the Michaelis–Menten constant and V_{max} is the maximal rate at infinite substrate concentration $[S]$.

In the case of deviations of the analysed curves from hyperbolae, the following equation was used:

$$V = \frac{V_{max}[S]^n}{K_m + [S]^n} \quad (2)$$

In this equation, n is the Hill coefficient.

2.3. Fast kinetic experiments

In stopped flow (SF) experiments, the time course of the change of fluorescence intensity during ligand binding was measured between 1 ms and 20 s under pseudo-first order conditions with an SF apparatus (SX-18MV, Applied Photophysics, UK). The fluorescence changes were detected after fast 1:1 mixing of an enzyme solution containing 20 μM TNS with a solution of substrate containing the same TNS concentration. The excitation of fluorescence was achieved by a 150-W high-pressure xenon/arc lamp at a wavelength of 360 ± 20 nm. The detection of the fluorescence was restricted to wavelengths above 400 nm by a GG 400-nm cut off filter (Schott, Germany). The enzyme concentration after mixing was 0.05 mg/ml (0.125 μM) in 100 mM HEPES buffer of pH 7.0 or 8.0 (10 mM $MgCl_2$, 1 mM dithioerythritol and 1 mM EDTA) in the absence and presence of various allosteric effectors as indicated in Section 3. The biphasic stopped flow signals were fitted via non-linear regression to two exponentials with the Origin software (MicroCal, USA), yielding rate constants and their experimental errors.

TNS was used for the detection of PEP binding to PEPC because it is only weakly fluorescent in polar solvents, but dissolved in unpolar solvents or upon binding to hydrophobic regions of proteins, the fluorescence of the dye strongly increases and the maximum of the fluorescence spectrum is shifted to shorter wavelengths. It reacts as an environmentally sensitive probe which

Table 1
Michaelis–Menten constant, K_m (PEP), V_{max} and the Hill coefficient n for the PEPC catalysed carboxylation of PEP in the presence of 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ at 25°C

pH	Allosteric effector	K_m (PEP) (mM)	V_{max} (μM PEP min^{-1} mg^{-1})	n
7.0	–	7.9 ± 0.7	16.4 ± 0.9	1.2 ± 0.09
7.0	5 mM glucose–6-phosphate	0.53 ± 0.07	15.1 ± 0.3	1.22 ± 0.1
7.0	5 mM glycine	2.4 ± 0.1	19.04 ± 0.3	1.35 ± 0.05
7.0	2 mM L-malate	37.1 ± 2.8	11.4 ± 0.4	1.58 ± 0.06
7.0	2 mM L-aspartate	59.4 ± 4.2	10.8 ± 0.9	1.45 ± 0.07
8.0	–	0.94 ± 0.05	25.1 ± 0.3	1.03 ± 0.07
8.0	5 mM glucose-6-phosphate	0.14 ± 0.01	27.05 ± 0.4	0.91 ± 0.07
8.0	5 mM glycine	0.52 ± 0.05	18.9 ± 0.4	0.76 ± 0.05
8.0	2 mM L-malate	1.06 ± 0.07	19.4 ± 0.3	0.9 ± 0.05
8.0	2 mM L-aspartate	0.89 ± 0.03	16.8 ± 0.2	1.01 ± 0.04

The Hill coefficient n was calculated according to Eq. (2). K_m and V_{max} were calculated at pH 8.0 applying Eq. (1). At pH 7.0, Eq. (2) was used.

detects polarity and viscosity changes in its surroundings [13,14]. There is no indication for the dissociation of TNS from the enzyme/TNS-complex caused by PEP binding to PEPC. Ligand binding to PEPC is not affected by TNS since the Michaelis–Menten constants of enzyme kinetic experiments are comparable with dissociation constants determined by using TNS as fluorescent probe in fast kinetic experiments (Tables 1 and 2).

3. Results

3.1. Enzyme kinetics

The specific activity of PEPC in the final purification step was 25.1 ± 1.5 units/mg (pH 8.0) which is close to the maximum of the specific activity of PEPC from *Zea mays* and agrees well with the values for the specific activity reported previously [15]. The purity of the enzyme preparations was greater than 95% as monitored by SDS polyacrylamide gels (Fig. 1a,b). For the determination of K_m (PEP) and V_{max}, the enzymatic activity of PEPC was assayed spectrophotometrically at 340 nm, as a function of the substrate concentration at pH 7.0 and 8.0 in the presence of different allosteric activators. The estimated values for K_m, V_{max} and the Hill coefficient n are given in Table 1. The graphs of the PEPC activity as a function of the PEP concentration at pH 8.0 and 7.0 in the absence and presence of 5 mM glucose-6-phosphate and 2 L-malate are shown in Fig. 2a,b.

3.2. Kinetic analysis of PEP binding by stopped flow (SF) fluorimetry

The stopped flow signals for the binding of PEP to PEPC at pH 7.0 and pH 8.0 were biphasic (Fig. 3). In both experiments, $1/\tau_A$ of the fast step depended linearly on the ligand concentration, as expected for a reversible bimolecular binding reaction under pseudo-first order conditions applying an excess of the ligand [8]. The association and dissociation rate constants k_{+A} and k_{-A} can be determined from the slopes and intercepts of the graphs of $1/\tau_A$ as a function of the ligand concentration at a constant enzyme concentration, as reported recently [8]. The reciprocal time constant of the slower reaction step

Table 2
Dissociation constants K_d (PEP) at 25°C for the binding of PEP to PEPC labelled with 20 μM TNS for fluorescence detection

pH	Allosteric effector	$k_{B-(0)}$ (s^{-1})	$k_{B+(0)}$ (s^{-1})	K_d (PEP) = K_{dII} (mM)
7.0	–	0.83 ± 0.17	0.27 ± 0.06	8.7 ± 2.4
7.0	glucose-6-phosphate	0.47 ± 0.19	0.37 ± 0.03	0.55 ± 0.34
7.0	glycine	0.995 ± 0.16	0.26 ± 0.065	1.74 ± 0.68
7.0	L-malate	0.746 ± 0.06	0.55 ± 0.04	33.1 ± 8.3
7.0	L-aspartate	0.71 ± 0.11	0.385 ± 0.07	47.4 ± 13
8.0	–	6.05 ± 0.4	1.09 ± 0.15	1.25 ± 0.2
8.0	glucose-6-phosphate	6.7 ± 0.9	1.7 ± 0.2	0.12 ± 0.03
8.0	glycine	5.99 ± 0.8	0.97 ± 0.3	0.48 ± 0.14
8.0	L-malate	4.39 ± 0.3	0.41 ± 0.06	1.01 ± 0.12
8.0	L-aspartate	7.26 ± 0.9	0.51 ± 0.2	0.96 ± 0.28

K_d (PEP) values were determined from the concentration dependence of the slow (τ_B) relaxation process observed in stopped flow experiments in the absence and presence of 5 mM glucose-6-phosphate, 5 mM glycine, 2 mM L-malate and 2 mM L-aspartate at pH 8.0 and pH 7.0 in HEPES buffer containing 10 mM $MgCl_2$, 1 mM EDTA and 1 mM DTE. Rate constant $k_{B+(0)}$ for the allosteric transition of form I into II, rate constant $k_{B-(0)}$ for the allosteric transition of form II into I in the absence and presence of 5 mM glucose-6-phosphate, 5 mM glycine, 2 mM L-malate and 2 mM L-aspartate at pH 8.0 and pH 7.0 in HEPES buffer containing 10 mM $MgCl_2$, 1 mM EDTA and 1 mM DTE. The dissociation constant K_d (PEP) is equal to K_{dII} of the allosteric mechanism for PEP binding to PEPC.

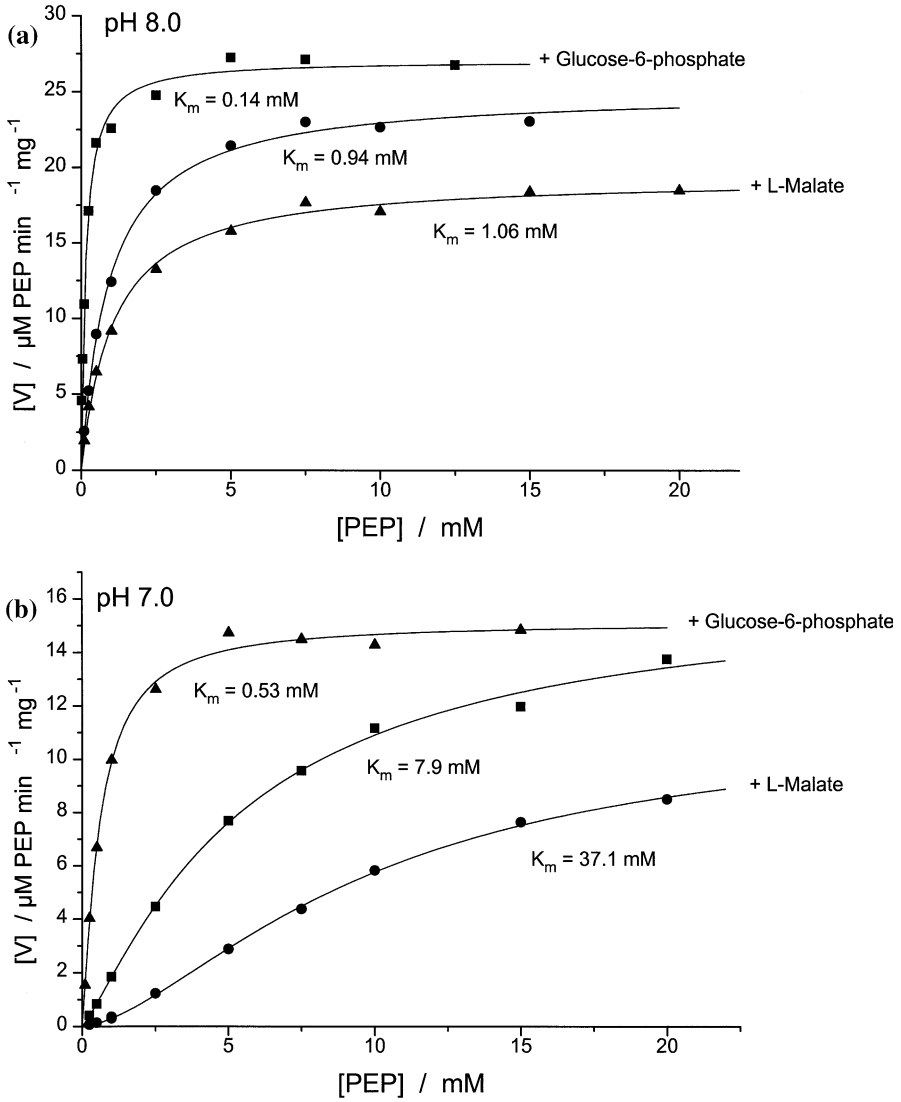

Fig. 2. Rate V (µM PEP min^{-1} mg^{-1}) of the carboxylation reaction catalysed by PEPC as a function of the PEP concentration at pH 8.0 (a) and pH 7.0 (b) in the absence and presence of 5 mM glucose-6-phosphate and 2 mM L-malate measured at 25°C by the absorption change at 340 nm.

$1/\tau_B$ decreased with increasing PEP concentrations (Fig. 4a,b and Fig. 5a,b). Such behaviour cannot be explained by the simple binding of a substrate to a binding site on the enzyme, since such a mechanism would predict a linear increase in $1/\tau$ with increasing ligand concentration (as found for the fast step). The possibility should, therefore, be considered that the slow step is due to an enzyme conformational change induced by substrate binding. In this case two possible situations exist:

(1) an induced-fit model, i.e. the binding of ligand to a single enzyme conformation induces a subsequent conformational change of the enzyme–ligand complex:

$[E + PEP \rightarrow E \cdot PEP \rightarrow (E \cdot PEP)^*]$.

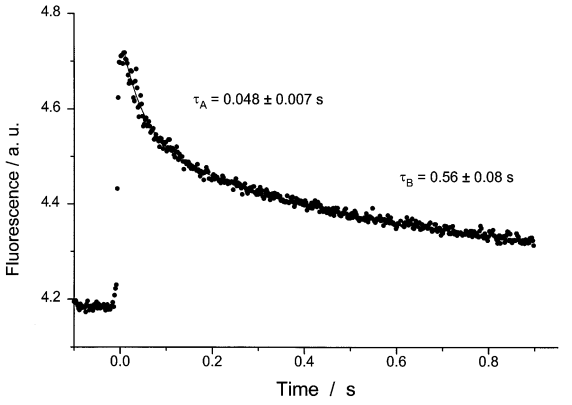

Fig. 3. Stopped flow signal for the fast binding reaction (τ_A) and the slow (τ_B) allosteric transition of PEPC in the presence of 0.5 mM PEP measured in 100 mM HEPES buffer of pH 8.0 containing 20 μM TNS, 10 mM $MgCl_2$, 1 mM dithioerythritol and 1 mM EDTA at 25°C. Enzyme concentration: 0.125 μM (0.05 mg/ml). Excitation: 360 ± 20 nm. Emission: > 400 nm.

(2) a lock-and-key model, i.e. the binding of ligand perturbs a conformational equilibrium of the enzyme by binding to one of the conformations:

$(E_I \leftrightarrow E_{II} + PEP \rightarrow E_{II} \cdot PEP)$.

For multi-subunit enzymes, the induced-fit model corresponds to the sequential (or KNF) model of allosteric enzymes proposed by Koshland et al. [16], whereas the lock-and-key model corresponds to the concerted (or MWC) model suggested by Monod et al. [9]. The induced-fit model, however, can only explain a hyperbolic (non-cooperative) or sigmoid (cooperative) increase in $1/\tau$ with increasing substrate concentration to a saturating value [17], whereas, experimentally, a hyperbolic *decrease* was observed. The induced-fit model can, therefore, be ruled out as a possible mechanism. The lock-and-key model, on the other hand, does in fact predict a hyperbolic decrease with increasing ligand concentration and would, therefore, seem to be the simplest possible mechanistic explanation for the slow phase of the kinetic traces.

Because PEPC possesses four subunits, each of which is capable of binding substrate, the observed kinetics fit within a framework for the concerted allosteric model introduced for such systems by Monod et al. [9]. In this model, it is assumed that the four subunits of PEPC are identical and bind substrates independently in each of the two states (I and II). Under these conditions, six of the nine possible relaxation processes have zero amplitude, resulting in only three observable relaxations. Two relaxation rates are characteristic for second order binding to form I or II and the third relaxation is characteristic for the isomerisation rate between form I and II; for more details see [18,19].

General reaction mechanism:

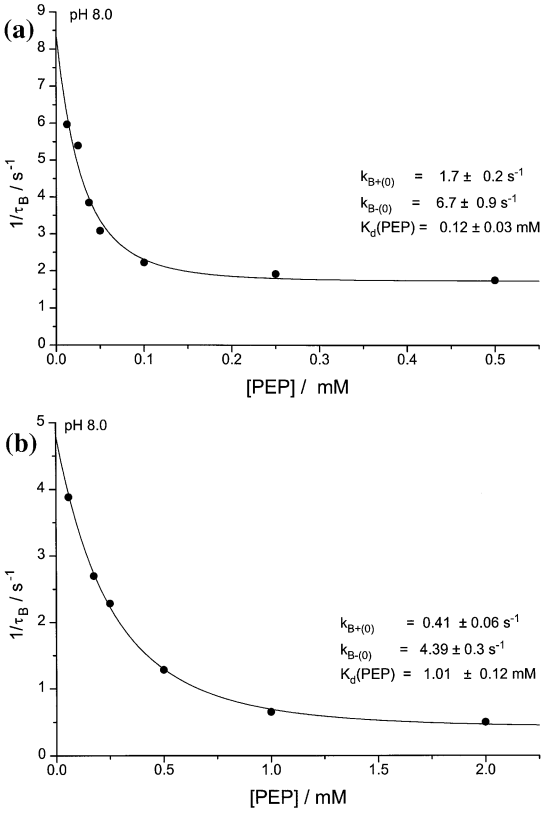

Fig. 4. Reciprocal relaxation time $1/\tau_B$ from stopped flow experiments for the slow allosteric conformational change of PEPC from *Zea mays* as a function of the PEP concentration in the presence of 5 mM glucose-6-phosphate (a) and 2 mM L-malate (b) in 100 mM HEPES buffer of pH 8.0 containing 10 mM $MgCl_2$, 5 mM dithioerythritol and 1 mM EDTA at 25°C.

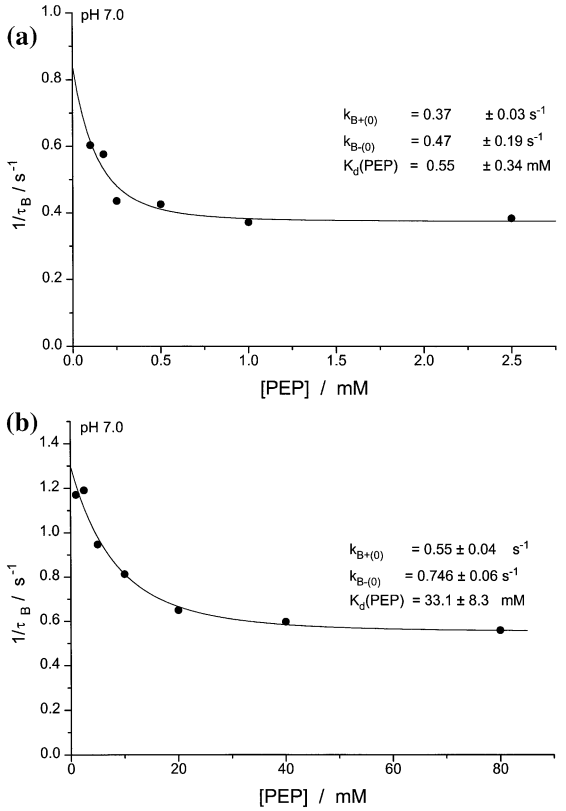

Fig. 5. Reciprocal relaxation time $1/\tau_B$ from stopped flow experiments for the slow allosteric conformational change of PEPC from *Zea mays* as a function of the PEP concentration in the presence of 5 mM glucose-6-phosphate (a) and 2 mM L-malate (b) in 100 mM HEPES buffer of pH 7.0 containing 10 mM $MgCl_2$, 5 mM dithioerythritol and 1 mM EDTA at 25°C.

$$
\begin{array}{lll}
\mathbf{4L} & + \mathbf{E_I} \overset{k_{B+(0)}}{\underset{k_{B-(0)}}{\rightleftharpoons}} \mathbf{E_{II}} & + \mathbf{4L} \\
4k_{+I} \uparrow\downarrow k_{-I} & & 4k_{+II} \uparrow\downarrow k_{-II} \\
\mathbf{3L} & + \mathbf{E_I L} \overset{k_{B+(1)}}{\underset{k_{B-(1)}}{\rightleftharpoons}} \mathbf{E_{II} L} & + \mathbf{3L} \\
\uparrow\downarrow & & \uparrow\downarrow \\
\mathbf{2L} & + \mathbf{E_I L_2} \overset{k_{B+(2)}}{\underset{k_{B-(2)}}{\rightleftharpoons}} \mathbf{E_{II} L_2} & + \mathbf{2L} \\
\uparrow\downarrow & & \uparrow\downarrow \\
\mathbf{L} & + \mathbf{E_I L_3} \overset{k_{B+(3)}}{\underset{k_{B-(3)}}{\rightleftharpoons}} \mathbf{E_{II} L_3} & + \mathbf{L} \\
\uparrow\downarrow & & \uparrow\downarrow \\
 & \mathbf{E_I L_4} \overset{k_{B+(4)}}{\underset{k_{B-(4)}}{\rightleftharpoons}} \mathbf{E_{II} L_4} &
\end{array}
$$

E_I: Form I (low affinity) of PEPC (not seen as independent relaxation in our experiments); E_{II}: Form II (high affinity) of PEPC (corresponds to τ_A); $k_{B+(i)}$: Rate constant for the isomerisation of E_{Ii} ($E_{Ii} \rightarrow E_{IIi}$) (for $i = 0$ from τ_B); $k_{B-(i)}$: Rate constant for the isomerisation of E_{IIi} ($E_{IIi} \rightarrow E_{ii}$) (for $i = 0$ from τ_B); $k_{B-(0)}/k_{B+(0)}$: allosteric constant $L = k_{B-(0)}/k_{B+(0)} = [E_{I(0)}/E_{II(0)}]$ (from τ_B); k_{+I}: Ligand association rate constant for the form I; k_{-I}: Ligand dissociation rate constant for the form I; k_{+II}: Ligand association rate constant for the form II (corresponds to k_{+A}); and k_{-II}: Ligand dissociation rate constant for the form II (corresponds to k_{-A}).

According to this mechanism, the enzyme could exist in the forms I and II ($E_I \Leftrightarrow E_{II}$), which are in equilibrium and bind the substrate PEP with different affinities. $1/\tau_{I,II} = 1/\tau_B$ represents the rate of allosteric transition between forms I and II of PEPC, which is a function of the PEP concentration and allows the determination of K_{dII}, equivalent with $K_{d\ (PEP)}$ [Eq. (3)] [18,19]:

$$\frac{1}{\tau_B} = \frac{1}{\tau_{I,II}} = k_{B+(0)} + k_{B-(0)} \left(\frac{1 + \dfrac{[L]}{K_{dI}}}{1 + \dfrac{[L]}{K_{dII}}} \right), \qquad \frac{[L]}{K_{dI}} = 0 \tag{3}$$

$k_{B+(0)}$: Rate constant for the isomerisation of $E_{I(0)}$, ($E_{I(0)} \rightarrow E_{II(0)}$); $k_{B-(0)}$: Rate constant for the isomerisation of $E_{II(0)}$, ($E_{II(0)} \rightarrow E_{I(0)}$); K_{dI}: Microscopic dissociation constant for the binding of PEP to form I of PEPC; and K_{dII}: Microscopic dissociation constant for the binding of PEP to form II of PEPC.

Non-linear regression analysis of $1/\tau_B$ as a function of the PEP concentration according to Eq. (3) ($1/\tau_B$ equals $1/\tau_{I,II}$) showed that in the presence of Mg^{2+}-ions, PEP mainly binds to form II of PEPC. The affinity of form I for PEP is so low that the value of L/K_{dI} approaches zero.

3.3. Influence of allosteric effectors on the concentration dependence of τ_B and the rate of the isomerisation reaction

From Eq. (3), values for $K_{dII} = K_{d\ (PEP)}$ were derived from the concentration dependence of

$1/\tau_B$ (Table 2). The isomerisation reaction between forms I and II is characterised by a relaxation time $1/\tau_{I,II} = 1/\tau_B$. According to Eq. (3), it is possible to measure the ratio k_{-A}/k_{+A} derived from the concentration dependence of relaxation process τ_A independently and calculate K_{dII} from the data in Fig. 4a,b and Fig. 5a,b as K_d (PEP). The $K_{d\ (PEP)}$ values measured in the presence of 5 mM glucose-6-phosphate, 5 mM glycine, 2 mM L-malate and 2 mM L-aspartate at pH 8.0 and pH 7.0 are in excellent agreement with the values derived for $K_{d\ (PEP)}$ by enzyme kinetic experiments (Fig. 2a,b, Fig. 4a,b, Fig. 5a,b; and Tables 1 and 2). Fitting of our kinetic data with the algorithm outlined above indicate that at [PEP] = 0, there is a ratio of forms I and II of approximately 5.5:1 ($k_{B-(0)}/k_{B+(0)} = E_{I(0)}/E_{II(0)}$) at pH 8.0 and of 3:1 at pH 7.0. In the presence of allosteric effectors no clear trend for the ratio of forms I and II ($k_{B-(0)}/k_{B+(0)} = E_{I(0)}/E_{II(0)}$) can be detected at both pH values. The rate constants for the isomerisation reaction $k_{B+(0)}$ and $k_{B-(0)}$ are significantly lower at pH 7.0 as compared to pH 8.0.

3.4. Simplified mechanism for the description of the isomerisation reaction

If one assumes that two forms of PEPC are in equilibrium and only one of the two forms can bind the substrate PEP [as suggested by fits of the experimental data to Eq. (3)], the allosteric mechanism can be approximated by the following reaction scheme:

Simplified reaction mechanism:

$$\begin{array}{lll} \mathbf{E_I} \underset{k_{B-(0)}}{\overset{k_{B+(0)}}{\rightleftharpoons}} & \mathbf{E_{II}} & + \mathbf{4L} \\ & \uparrow\downarrow K_{dII} & \\ & \mathbf{E_{II}L} & + \mathbf{3L} \\ & \uparrow\downarrow & \\ & \mathbf{E_{II}L_2} & + \mathbf{2L} \\ & \uparrow\downarrow & \\ & \mathbf{E_{II}L_3} & + \mathbf{L} \\ & \uparrow\downarrow & \\ & \mathbf{E_{II}L_4} & \end{array}$$

$k_{B+(0)}$: Rate constant for the forward isomerisation reaction; $k_{B-(0)}$: Rate constant for the backward isomerisation reaction; and K_{dII}: Microscopic dissociation constant of a binding site on E_{II} for the substrate PEP.

It can be shown that the concentration dependence of the reciprocal relaxation time of the isomerisation reaction is given by:

$$\frac{1}{\tau} = k_{B+(0)} + \frac{k_{B-(0)}}{\left(1 + \frac{[L]}{K_{dII}}\right)^4} \tag{4}$$

In the presence of saturating allosteric effector concentrations, the reaction scheme has to be modified:

Mechanism for saturating effector concentration:

$$\begin{array}{lll} \mathbf{ME_I} \underset{k_{B-(0)'}}{\overset{k_{B+(0)'}}{\rightleftharpoons}} & \mathbf{ME_{II}} & + \mathbf{4L} \\ & \uparrow\downarrow K_{dII'} & \\ & \mathbf{ME_{II}L} & + \mathbf{3L} \\ & \uparrow\downarrow & \\ & \mathbf{ME_{II}L_2} & + \mathbf{2L} \\ & \uparrow\downarrow & \\ & \mathbf{ME_{II}L_3} & + \mathbf{L} \\ & \uparrow\downarrow & \\ & \mathbf{ME_{II}L_4} & \end{array}$$

$k_{B+(0)'}$: Rate constant for the forward isomerisation reaction; $k_{B-(0)'}$: Rate constant for the backward isomerisation reaction; $K_{dII'}$: Microscopic dissociation constant of a binding site on E_{II} for the substrate PEP in the presence of an allosteric effector; and M: Allosteric effector (all binding sites for effector are occupied).

It can be shown that the concentration dependence of the reciprocal relaxation time of the isomerisation reaction in the presence of allosteric effectors is given by:

$$\frac{1}{\tau} = k_{B+(0)'} + \frac{k_{B-(0)'}}{\left(1 + \frac{[L]}{K_{dII'}}\right)^4} \tag{5}$$

For both reaction schemes, the same concentration dependence of τ_B is observed as for the allosteric mechanism.

4. Discussion

The experimental approach followed in this investigation was to combine thermodynamic and fast kinetic measurements (SF) to study the influence of allosteric effectors on the binding of PEP to PEPC. To monitor the binding events at the active site of PEPC from *Zea mays*, we used the fluorescent dye TNS as sensitive reporter fluorophore. TNS was introduced by Edelman and McClure as a probe for the detection of conformational changes of proteins [10,20–23] and was further characterised by Frank et al. [23]. Binding of PEP to PEPC is a fast reaction which occurs in the millisecond time range. Therefore, the kinetic analysis needed the application of fast reaction methodology such as stopped flow (SF) fluorimetry. From the stopped flow signals, it was apparent that the binding of PEP to PEPC is biphasic at pH 8.0. Similar results were obtained at pH 7.0. The fast step with a relaxation time τ_A could be attributed to a reversible bimolecular binding reaction of PEP to PEPC. $1/\tau_A$ for this process was a linear function of the PEP concentration under pseudo first-order conditions. As demonstrated previously, the dissociation constants K_d (PEP) for the PEPC/PEP complex derived from the ratio of the kinetic constants $[k_{-A}/k_{+A} = K_d(\text{PEP})]$ agree well with the value determined for K_m (PEP) from Michaelis–Menten enzyme kinetic experiments [8]. These former results demonstrated that the fluorescent probe TNS can efficiently be used to study ligand binding to PEPC and does not disturb the ligand binding in the concentration range used here (see also Tables 1 and 2). The reciprocal time constant $1/\tau_B$ of the second slower process which decreases with increasing PEP concentration is typical for an allosteric transition between the two conformational states of PEPC I and II. As outlined in Section 3, this behaviour cannot be explained by a sequential model involving a conformational change of an enzyme–ligand complex. The values of K_d (PEP) $= K_{d\text{II}}$ [see Eq. (3)] derived from the concentration dependence of $1/\tau_B = 1/\tau_{\text{I,II}}$ in the presence of allosteric effectors are in excellent agreement with K_m of the enzyme kinetic experiments (Tables 1 and 2). At pH 8.0, D-glucose-6-phosphate is a strong activator decreasing K_d (PEP) to 0.12 ± 0.03 mM. L-Aspartate and L-malate neither activate nor inhibit PEPC at pH 8.0. In contrast to pH 8.0, L-aspartate and L-malate are strong inhibitors of PEPC at pH 7.0, whereas D-Glucose-6-phosphate is still a strong activator at pH 7.0. Our present results with effectors support the description of the kinetics of PEP binding to PEPC applying an allosteric mechanism. Previous studies of other authors [6,7,11,15,24–28] showed that many phosphoenolpyruvate carboxylases from various sources are allosteric in nature. Their activities are regulated by a variety of effectors [24]. The enzyme from *Escherichia coli*, for example, is inhibited by L-aspartate and L-malate and is activated by acetyl-coenzyme A, D-fructose 1,6-bisphosphate, guanosine-5′-triphosphate (GTP) and some long-chain fatty acids [25,26]. Recently, the structure of PEPC from *Escherichia coli* co-crystallised with the allosteric inhibitor L-aspartate bound was determined [29]. The bacterial enzyme is very similar to the plant enzyme in its primary structure except for the extra residues at the N-terminus in plant PEPC [29]. Therefore, the three-dimensional structure of PEPC from *Escherichia coli* can be directly transferred to plant PEPC [29]. The binding site of L-aspartate is located approximately 20 Å away from the catalytic site. One of the four residues involved in effector binding was identified as Arg-587, which is part of a glycine-rich loop essential for catalytic activity. L-Aspartate causes inhibition of PEPC activity by shifting this glycine-rich loop and another mobile loop (Lys-702 to Gly-708) away from the active site [29]. For PEPC from *Zea mays*, a time-dependent reversible inactivation and desensitisation against the activator D-glucose-6-phosphate by pyridoxal 5′-phosphate was reported [30]. Inactivation and desensitisation are not connected, since saturating PEPC with high PEP concentrations prevent inactivation, but desensitisation is still observed. D-Glucose-6-phosphate it-

self offers significant protection against desensitisation. These experiments indicate different allosteric binding sites to the catalytic sites of PEPC from *Zea mays* for at least D-glucose-6-phosphate. PEPC is regulated by the activator glucose-6-phosphate and the inhibitor L-malate [6,7]. Hitherto, only a few kinetic studies on the allosteric regulation of PEPC have been performed. In summary, in this investigation we present for the first time kinetic data showing the influence of allosteric effectors on the dynamics of PEP binding to PEPC and on the rate of the isomerisation reaction between the two allosteric forms of PEPC. The agreement with Michaelis–Menten kinetics shows that we have observed all the essential rate constants of the enzyme reaction mechanism.

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