Kinetic properties of D-fructose-1,6-bisphosphate 1-phosphohydrolase isolated from human muscle

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D-Fructose-1,6-bisphosphate 1-phosphohydrolase (EC 3.1.3.11) [Fru(1,6)Pase] was isolated from human muscle in an electrophoretically homogeneous form, free of aldolase contamination. The enzyme is inhibited by the substrate [fructose (1,6)-bisphosphate]. K_m is $0.77 \mu\text{M}$; K_{is} is $90 \mu\text{M}$. The fructose-2,6-bisphosphate [Fru(2,6)P₂], a regulator of gluconeogenesis, inhibits human muscle Fru(1,6)Pase with $K_i = 0.13 \mu\text{M}$. To determine K_m , K_{is} and K_i the integrated method was used. AMP is an allosteric inhibitor of Fru(1,6)Pase. As with other mammalian

isoenzymes, the human muscle enzyme is more strongly inhibited by AMP than is the liver isoenzyme [Dzugaj and Kochman (1980) Biochim. Biophys. Acta 614, 407–412]. Both of the inhibitors [AMP and Fru(2,6)P₂] act synergistically on human muscle Fru(1,6)Pase. K_i for Fru(2,6)P₂ determined in the presence of $0.4 \mu\text{M}$ AMP was $0.028 \mu\text{M}$. The human muscle enzyme, like other mammalian Fru(1,6)Pases, requires Mg²⁺ for its activity. The K_a for magnesium was $232 \mu\text{M}$, and h (Hill coefficient) = 2.0.

INTRODUCTION

D-Fructose-1,6-bisphosphate 1-phosphohydrolase [Fru(1,6)Pase] catalyses the hydrolysis of D-fructose (1,6)-bisphosphate [Fru(1,6)P₂] to fructose 6-phosphate (Fru6P) and P_i. Liver, muscle and brain Fru(1,6)Pase isoenzymes have been detected in mammalian tissues. The liver isoenzyme, recognized as a regulatory enzyme of gluconeogenesis [1], has also been found in kidney and intestine [1–4]. The physiological role of the muscle and brain isoenzymes is rather an enigma. Newsholme and Start postulated that the muscle isoenzyme participates in the regulation of glycolysis [5]. On the other hand, Mizunuma and Tashima [6], on the basis of the kinetic properties of the mouse muscle isoenzyme, concluded that this enzyme is inactive *in vivo* in the presence of physiological inhibitors like AMP and fructose-2,6-bisphosphate (Fru(2,6)P₂). A number of papers have been published concerning purification of liver Fru(1,6)Pase from different species, structure/function relationship, primary structure, including that of the human liver enzyme, and determination of kinetic properties [7–15]. Much less information is available on the muscle isoenzyme [12,16,17]. No purification of human muscle Fru(1,6)Pase has been reported and little information on the kinetic properties of human muscle isoenzyme, determined as a homogenate, has been reported [18].

This prompted us to focus our attention on the human muscle isoenzyme. In the present paper, a simple procedure for the isolation of human muscle Fru(1,6)Pase is presented and the enzyme's kinetic properties are described. The application of the integrated method to kinetic investigation enabled relatively easy determination of the synergistic effects of AMP and Fru(2,6)P₂ on the human muscle isoenzyme.

MATERIALS AND METHODS

Materials

Tris, Bis-Tris-Propane (BTP), NADP, NADH, AMP, EDTA, MgCl₂·6H₂O, β-mercaptoethanol, acrylamide, bisacrylamide,

NNN'-tetramethylethylenediamine (TEMED), Fru(1,6)P₂, Fru(2,6)P₂, glucose-6-phosphate dehydrogenase, triose-3-phosphate isomerase and glycerol-3-phosphate dehydrogenase were purchased from Sigma. Phosphocellulose was from Whatman, (NH₄)₂SO₄ from BDH. SDS was from Serva. Coomassie Brilliant Blue was from Fluka. Saccharose was purchased from Reachim. Malachite Green and ammonium molybdate were from POCh. All other reagents were of the highest purity commercially available.

Enzyme assays

Fru(1,6)Pase activity was measured as described by Traniello et al. [19]. The standard assay mixture (1 ml of the final volume) contained 50 mM BTP, 2 mM MgCl₂, 150 mM KCl, 1 mM EDTA, 0.2 mM NADP⁺, 5 units/ml glucose-6-phosphate dehydrogenase, 5 units/ml glucose-6-phosphate isomerase, 40 μM Fru(1,6)P₂, pH 7.5; the assay was carried out at 37 °C.

In order to determine Fru(1,6)Pase activity in conditions imitating the physiological state, 1 ml of the following assay mixture was used: 50 mM BTP/2 mM MgCl₂/150 mM KCl/1 mM EDTA/0.2 mM NADP⁺/15 μM AMP/1 μM Fru(2,6)P₂/5 units/ml glucose-6-phosphate dehydrogenase/5 units/ml glucose-6-phosphate isomerase/35 μM Fru(1,6)P₂/50 μg of Fru(1,6)Pase (approx. 1.4 μM catalytic sites) (pH 7.5); the assay was carried out at 37 °C.

Aldolase activity was measured in the presence of coupling enzymes, triose-3-phosphate isomerase and glycerol-3-phosphate dehydrogenase, after the decrease in absorbance of NADH. A standard mixture contained 50 mM BTP, 2 mM MgCl₂, 150 mM KCl, 1 mM EDTA, 0.2 mM NADP⁺, 5 units/ml glycerol-3-phosphate dehydrogenase and 5 units/ml triose-3-phosphate isomerase, 40 μM Fru(1,6)P₂, pH 7.5; the assay was carried out at 37 °C.

For determination of kinetic parameters 0.4 μg/ml Fru(1,6)Pase was used. One unit of enzyme activity is defined as that

Abbreviations used: Fru(1,6)Pase, D-fructose-1,6-bisphosphate 1-phosphohydrolase; Fru(1,6)P₂, D-fructose 1,6-bisphosphate; Fru(2,6)P₂, D-fructose 2,6-bisphosphate; Fru6P, fructose 6-phosphate; BTP, Bis-Tris-Propane.

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amount of the enzyme that catalyses transformation of 1 μmol of the substrate/min. Spectrophotometer determination was performed with an HP 8452A diode array spectrophotometer. The determination of $I_{0.5}$, h for AMP and K_a and h for Mg^{2+} was performed using GraFit Leatherbarrow program [20]. All other parameters were calculated with the integrated method using the Matlab program of MathWorks [21] and TURBO-Pascal of Borland Inc. Fru(1,6)Pase concentration was determined spectrophotometrically assuming that $A_{1\text{cm}}^{1\%} = 6.3$ at 280 nm. During the purification procedure, protein concentration was measured as described by Lowry et al. [22]. Magnesium concentration was determined by titration of magnesium solution with EDTA in the presence of Eriochrome Black T. AMP concentration was determined spectrophotometrically by using $15400 \text{ M} \cdot \text{cm}^{-1}$ as the molar absorption coefficient at 259 nm. To determine Fru(2,6) P_2 concentration this compound was hydrolysed with HCl at pH 2 for 10 min. Released P_i was determined as described by Tashima and Yoshimura [23]. Fru6P was determined as described by Traniello et al. [19].

Purification procedure

A 10 g portion of the human skeletal muscle (m. pectoralis), removed surgically, was homogenized in 20 ml of 1 M saccharose/1 mM EDTA (pH 7.5) for 3 min, and the homogenate was centrifuged at 13000 g for 15 min. The supernatant was filtered through glass wool and rapidly heated at 68 $^{\circ}\text{C}$ for 3 min in a water bath with stirring. Then it was rapidly cooled and centrifuged at 13000 g for 15 min. To remove aldolase, $(\text{NH}_4)_2\text{SO}_4$ was slowly added until 65 % saturation was reached. After 15 h the pellet was removed and the supernatant was dialysed overnight against 1 mM EDTA, pH 7.0. The dialysate was diluted in 2.5 vol. of 1 M saccharose/1 mM EDTA (pH 7.5) and was treated with moist phosphocellulose (1 g per 10 ml of solution). The enzyme was adsorbed at pH 8.0. After 1 h the suspension was loaded on to a Pharmacia K 16/40 column. The phosphocellulose was washed with 50 mM Tris/0.5 mM EDTA (pH 7.5) until the absorbance at 280 nm of the eluate had fallen below 0.01. Fru(1,6)Pase was eluted with the same buffer containing 3 mM Fru(1,6) P_2 and 3 mM AMP. Fractions containing Fru(1,6)Pase were pooled and dialysed against 50 mM Tris/0.5 mM EDTA (pH 7.5).

SDS/PAGE of purified Fru(1,6)Pase was performed as described by Weber and Osborn [24]. A sample (1 μg) of the protein was applied on the top of the gel. Polyacrylamide-gel disc electrophoresis showed only one sharp protein band.

Determination of kinetic parameters with the integrated method

We have considered two possibilities. (a) The inhibition of Fru(1,6)Pase by substrate in the low range of substrate concentration is negligible. The reaction is described by the Michaelis-Menten equation. The integrated form of this equation is:

$$t = \frac{1}{V_{\max.}} \left\{ [S_0] - [S] + K_m \ln \left(\frac{[S_0]}{[S]} \right) \right\} \quad (1)$$

(b) The inhibition of Fru(1,6)Pase by substrate is taken into account. For the uncompetitive inhibition we can write:

$$\frac{v}{V_{\max.}} = \frac{[S]}{K_m + [S] + \frac{[S]^2}{K_{is}}} \quad (2)$$

K_m and $V_{\max.}$ correspond to those theoretical values when the enzyme would not be inhibited by substrate. K_{is} is the inhibition constant of Fru(1,6)Pase by substrate. Under rapid-equilibrium conditions K_m is equal to K_s (the dissociation constant for the substrate from catalytic site) and K_{is} is the dissociation constant for the substrate from the second class of binding site. The integrated form of eqn. (2) is as follows:

$$t = \frac{1}{V_{\max.}} \left\{ [S_0] - [S] + K_m \ln \left(\frac{[S_0]}{[S]} \right) + \frac{1}{2K_{is}} \left([S_0]^2 - [S]^2 \right) \right\} \quad (3)$$

We will obtain the same expression for non-competitive inhibition under rapid equilibrium conditions when $K_{is} \gg K_s$.

On the basis of the recorded progress of the reaction, the parameters for two considered possibilities were calculated. In the first case $V_{\max.}$, K_m and $[S_0]$, and in the second $V_{\max.}$, K_m , K_{is} and $[S_0]$, were determined. All parameters were calculated for five curves. The increase of absorbance was read at even intervals of 1 s. Calculations were performed using non-linear regression. The details of all calculations are in the appendix.

Taking into consideration the inhibition of Fru(1,6)Pase by substrate, we calculated $S_{\max.}$ and $S_{0.5}$ on the basis of eqn. (2). $S_{\max.}$ corresponds to the substrate concentration at which the maximum enzyme activity was observed. $S_{0.5}$ is equal to the substrate concentration at which Fru(1,6)Pase expresses one-half of the activity observed at $S_{\max.}$. In the presence of Fru(2,6) P_2 , a competitive inhibitor of Fru(1,6)Pase, the inhibition by substrate was also taken into account. In this case, in eqns. (2) or (3), K_m will be substituted for by $K'_{m,app.}$

$$K'_{m,app.} = K_m (1 + [I]/K_i) \quad (4)$$

$K'_{m,app.}$ was determined for each of the recorded progress of the reaction in the presence of Fru(2,6) P_2 . K_i was calculated from eqn. (4) taking K_m as the mean value calculated from eqn. (3) on the basis of experiments performed in the absence of Fru(2,6) P_2 .

RESULTS

Purification

The protocol of the purification of the human muscle enzyme is presented in Table 1. Initial total activity of aldolase in human muscle is about 100 times higher than the corresponding value of Fru(1,6)Pase (both activities expressed in units per mg wet weight of tissue). Both enzymes are competing for the same substrate. Therefore even 1 % of the initial amount of aldolase left as contamination would perceptibly affect the determination of kinetic parameters of Fru(1,6)Pase. By precipitating aldolase with $(\text{NH}_4)_2\text{SO}_4$, it was possible to obtain Fru(1,6)Pase in which contamination with aldolase did not exceed 0.1 %. In both resting and exercising muscle the steady-state concentration of Fru(1,6) P_2 is observed. Therefore in living cells aldolase does not affect the kinetic properties of Fru(1,6)Pase.

The mobility of human muscle Fru(1,6)Pase in SDS/PAGE, in the range of experimental error, was the same as the human liver enzyme. It might be deduced that the molecular mass of liver and muscle Fru(1,6)Pase is approximately the same.

Inhibition by substrate

Mammalian liver Fru(1,6)Pase is inhibited by the substrate, but it is often assumed that in the low range of substrate concentration this inhibition might be negligible [12,25–28]. In Figure 1 the mean values of K_m (from five curves) calculated on the basis of eqn. (1) for various initial values of substrate concentration are

Table 1 Purification of the human muscle Fru(1,6)Pase

Fraction	Volume (ml)	Protein concentration (mg/ml)	Total activity of Fru(1,6)Pase (units)	Specific activity of Fru(1,6)Pase (units/mg)	Total activity of aldolase (units)	Yield (%)
Extract	20.0	20.1	6.7	0.017	87.0	100
Heated fraction	16.5	11.5	7.4	0.039	—	110
Precipitation at 65% (NH ₄) ₂ SO ₄	17.3	5.1	7.0	0.079	3.0	105
Dialysis	20.0	4.0	9.0	0.11	2.9	134*
Phosphocellulose eluate						
Fraction 1	0.95	0.063	1.2	20.0	< 0.001	18
Fraction 2	0.80	0.23	1.2	6.5	< 0.01	18

* Dialysis removes the AMP, so an increase of activity of Fru(1,6)Pase was observed.

Figure 1 Effect of the initial concentration of substrate on the mean value of K_m

The effect of the substrate is calculated on the basis of eqn. (1), when the inhibition by substrate is regarded as negligible. K_m was calculated from five recorded curves.

presented. The plot reveals that a strong dependence of K_m on $[S_0]$ from 3.3 to 10 μM results in a decrease in K_m value from 1.6 to 0.6 μM respectively. The decrease in K_m was observed for each of the recorded curves. This phenomenon is due to the inhibition of muscle Fru(1,6)Pase by the substrate. Using eqn. (1), instead of K_m and $V_{\max}$, $K_{m,\text{app}}$ and $V_{\max,\text{app}}$ are measured, which for an uncompetitive model of inhibition are:

$$K_{m,\text{app}} = \frac{K_m}{1 + \frac{[S]}{K_{is}}}; \quad V_{\max,\text{app}} = \frac{V_{\max}}{1 + \frac{[S]}{K_{is}}}$$

So an increase in substrate concentration will be followed by a decrease in $K_{m,\text{app}}$ and $V_{\max,\text{app}}$. The difficulty in the Michaelis-Menten analysis is also caused by the large cross-correlation coefficient (> 0.96) between K_m and $V_{\max}$. Taking into account the inhibition by substrate, kinetic parameters were calculated on the basis of eqn. (3). The results are presented in Table 2. Considering the determined values of the kinetic parameters as well as confident intervals for five experimental curves, it might be concluded that the employed equation (3) is reliable and might be useful for the calculation of kinetic parameters when

Table 2 Kinetic parameters of human muscle Fru(1,6)Pase determined with the integrated method

For determination of kinetic parameters fraction no. 1 were used. Confidence intervals (shown in parentheses) were calculated as described in the Appendix.

Experimental curve	Fru(1,6)P ₂ K_m (μM)	Fru(1,6)P ₂ K_{is} (μM)	Fru(1,6)P ₂ $S_{\max}$ (μM)	Fru(1,6)P ₂ $S_{0.5}$ (μM)	Fru(2,6)P ₂ K_i (nM) [AMP] = 0 μM	Fru(2,6)P ₂ K_i (nM) [AMP] = 0.4 μM
1	0.90 (0.73–1.09)	84 (74–97)	8.7	0.64	130	42
2	0.82 (0.73–0.93)	98 (89–107)	9.0	0.61	120	49
3	0.71 (0.63–0.79)	77 (73–82)	7.4	0.52	160	38
4	0.84 (0.75–0.95)	84 (78–89)	8.4	0.60	97	28
5	0.60 (0.48–0.73)	110 (104–135)	8.1	0.46	—	—
Mean value ...	0.77	90	8.3	0.57	130	39

Figure 2 Dependence of human muscle Fru(1,6)Pase activity on substrate concentration

Experimental points of the spectrophotometrically registered progress of the reaction were used to determine the substrate concentration and the velocity of the catalysed reaction. Every sixth experimental point was used for substrate concentration calculation. For each of those points the velocity of the reaction was calculated using the least-squares method, taking additionally the five preceding and five subsequent experimental points. Those calculated values are presented as open circles. The parameters of the presented curve were calculated with the integrated method as described in the Materials and methods section.

Figure 3 Inhibition of human muscle Fru(1,6)Pase by AMP

The standard assay mixture was as described in the Materials and methods section; various concentrations of AMP were used. The inset shows the Hill plot of the data.

inhibition by the substrate is observed. The inhibition by the substrate is presented in Figure 2.

When employing eqn. (3) it is advantageous to apply $[S_d] \geq 30 \mu\text{M}$. A higher substrate concentration means stronger Fru(1,6)Pase inhibition, which enables more precise K_{is} determination.

Inhibition by AMP and Fru(2,6) P_2 and the synergetic effect of those two inhibitors

Similarly to K_m and K_{is} for Fru(1,6) P_2 , K_i for Fru(2,6) P_2 was also determined with the integrated method (see the Appendix for

Figure 4 Effect of AMP on the inhibition of human muscle Fru(1,6)Pase by Fru(2,6) P_2

Standard assay mixture was as described in the Materials and methods section. For each AMP concentration, the mean value and the maximum and minimum values of K_i for Fru(2,6) P_2 are presented. The concentrations of Fru(2,6) P_2 were 0.1 and 0.2 μM ; the concentrations of AMP were 0.06, 0.1, 0.2, 0.3, 0.4 and 1.0 μM .

Figure 5 Effect of magnesium on the activity of human muscle Fru(1,6)Pase

The standard assay mixture was as described in the Materials and methods section; various concentrations of free magnesium were used. The inset shows the Hill plot of the data.

details). K_i was calculated on the basis of four curves with 30 μM initial substrate concentration (namely two curves for 0.1 μM and two curves for 0.2 μM concentration of Fru(2,6) P_2 respectively). In the same way, K_i was determined in the presence of a different concentration of AMP. The results are presented in Table 2.

AMP is an allosteric inhibitor of human muscle Fru(1,6)Pase. $I_{0.5}$ was determined to be 0.33 μM and $h = 2.1$. The effect of AMP concentration on human muscle Fru(1,6)Pase activity is presented in Figure 3. AMP and Fru(2,6) P_2 act synergistically. The effect of AMP on the inhibition of Fru(1,6)Pase by Fru(2,6) P_2 is presented in Figure 4. For each AMP concentration, the mean value and the maximum and minimum values of four determined curves are presented.

Effect of magnesium

Human muscle Fru(1,6)Pase, like other Fru(1,6)Pases, needs bivalent cations to express its activity. The effect of free magnesium on Fru(1,6)Pase activity is presented in Figure 5. K_a was calculated to be 233 μ M, and $h = 2.0$.

Activity of Fru(1,6)Pase under close-to-physiological conditions

In vivo the Fru(1,6)Pase concentration is much higher than that used in a cuvette for the determination of kinetic properties. To determine Fru(1,6)Pase activity under close-to-physiological conditions, the experiment was performed using concentrations of Fru(1,6)Pase, substrate and inhibitors comparable with those in the muscle cell. The enzyme was inhibited by 99.9%.

DISCUSSION

The mammalian muscle Fru(1,6)Pase as compared with the liver isoenzyme is more sensitive to inhibition by AMP. $I_{0.5}$ is 10–60 times lower for the muscle than for the liver enzyme [7,12,14,16,29]. Other kinetic properties are of the same range. The reported differences are mainly due to different assay conditions (the temperature and the presence or absence of chelating agents and univalent cations) [7,12,23,29]. Human muscle Fru(1,6)Pase is synergistically inhibited by AMP and Fru(2,6)P₂. This interesting phenomenon was first observed for the mammalian liver isoenzyme [30] as well as for mouse muscle Fru(1,6)Pase [29]. Bosca et al., working on partially purified rabbit and rat muscle Fru(1,6)Pase, did not find the synergistic effect of AMP and Fru(2,6)P₂ on the two proteins [17]. Those enzymes were partially proteolysed, since the ratio of activity at pH 9.3 to activity at pH 7.5 was 0.67 for rabbit and 0.71 for rat Fru(1,6)Pase. That value for native Fru(1,6)Pase should not exceed 0.4 [31]. Proteolytic modification of Fru(1,6)Pase results in desensitization to AMP [31] and Fru(2,6)P₂ [32] inhibition and it might be the cause of the lack of synergism.

The inhibition of mammalian liver Fru(1,6)Pase by excess substrate was reported [12,25–28]. A similar phenomenon was observed in the case of the human muscle enzyme. The inhibition of Fru(1,6)Pase by a substrate raises a question concerning determination of K_m . Usually this problem has been neglected and K_m has been calculated without taking into account inhibition by substrate. Assuming the allosteric character of substrate binding to the catalytic site as well as to inhibitory sites, Vargas et al. [28] calculated the corresponding binding constant. They found that the binding constant of substrate to the rat kidney Fru(1,6)Pase catalytic site is approx. 100 times higher than the binding constant of substrate to inhibitory site [28]. Investigating the effect of temperature on kinetic properties of human and rabbit liver Fru(1,6)Pase, we have found slight co-operativity in the binding of substrate to Fru(1,6)Pase at 25 °C, which disappeared at 37 °C [7]. All determination of the human muscle enzyme kinetic properties were performed at the physiological temperature. Therefore we considered the non-allosteric type of inhibition. There are two possibilities: uncompetitive or non-competitive type of inhibition. As we have shown in the Materials and methods section, those two models under rapid equilibrium conditions when $K_{is} \gg K_s$ are not kinetically distinguishable. Liu and Fromm [33] suggested that the site of binding of Fru(2,6)P₂ to Fru(1,6)Pase partially overlaps the catalytic site. It might be the same site to which the second substrate molecule binds; in this case the inhibition by substrate would be uncompetitive. The catalytic site of Fru(1,6)Pase has been determined [34] but the Fru(2,6)P₂ binding site has not yet been determined. Because

the binding constant of the substrate to the active site is much higher than that for the inhibitory site, at low concentrations of the substrate the inhibition of Fru(1,6)Pase is not perceptible. On the other hand it is necessary to take it into account when the integrated method of kinetic parameters determination is employed.

The basic question concerning muscle Fru(1,6)Pase is its physiological role. It seems that muscle Fru(1,6)Pase in the living cell is inactive. On the basis of the determined kinetic parameters of Fru(1,6)Pase and effector concentrations in human rest muscle [Fru(1,6)P₂ = 35 nmol/g, Fru(2,6)P₂ = 2.4 nmol/g, AMP = 15 nmol/g] and in human exercising muscle [Fru(1,6)P₂ = 95 nmol/g, AMP = 22 nmol/g] [35,36] [Fru(2,6)P₂ concentration in human exercising muscle is unknown] one may conclude that in both resting and exercising muscle, Fru(1,6)Pase is almost completely inhibited, expressing no more than 0.1 % of its activity. A determination of kinetic properties was performed at low Fru(1,6)Pase concentrations not exceeding 10 nM. In the living cell, Fru(1,6)Pase concentration is approx. 100 times higher, comparable with Fru(2,6)P₂ concentration. But even employing 0.35 μ M Fru(1,6)Pase in the presence of 35 μ M substrate/1 μ M Fru(2,6)P₂/15 μ M AMP the enzyme was 99.9 % inhibited. On the other hand, in mammalian muscle synthesis of glycogen from lactate was observed [37–39]. Thus *in vivo* Fru(1,6)Pase must be active.

It was reported that several glycolytic enzymes are immobilized on the cytoskeleton [40,41]. It is feasible that Fru(1,6)Pase is also immobilized. The binding of enzyme to the cytoskeleton changes its kinetic properties. 6-Phosphofructo-1-kinase immobilized on actin is not activated by AMP and Fru(2,6)P₂. Similarly, immobilization of Fru(1,6)Pase might result in desensitization to AMP and Fru(2,6)P₂ inhibition. Supposedly, the physiological role of Fru(1,6)Pase in muscle is not limited to participation in glycogen synthesis but, as Newsholme and Start [5] postulated, the enzyme also takes part in the regulation of glycolysis.

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APPENDIX

The elaborated method enables us to determine the kinetic parameters on the basis of one record of the progress of the reaction. The integrated form of the Michaelis–Menten equation is written as eqn. (1) of the main paper. If the reaction is inhibited by substrate according to eqn. (3) of the main paper, K_{is} might also be calculated from one record of the reaction progress.

Because the measured value is absorbance (A) instead of $[S]$, the concentration eqn. (1) of the main paper might be written as

$$t = \frac{A - A_0 + K'_m \ln \left(\frac{A_{\max} - A_0}{A_{\max} - A} \right)}{V'_{\max}} \quad (\text{A1})$$

and eqn. (3) of the main paper as

$$t = \frac{A - A_0 + K'_m \ln \left(\frac{A_{\max} - A_0}{A_{\max} - A} \right) + 0.5k'(A - A_0)(2A_{\max} - A - A_0)}{V'_{\max}} \quad (\text{A2})$$

where A_0 is the initial absorbance, $A_{\max}$ is the absorbance at time t , $V'_{\max} = 0.00622 V_{\max}$, $K'_m = 0.00622 K_m$ and $k' = 1/0.00622 K_{is}$.

This form of the equation is advantageous, since one can avoid systematic errors when calculating the measured value of absorbance on the corresponding value of concentration. For the calculation knowledge of the $A_{\max}$ value is necessary. When k'

= 0 eqn. (A2) is transformed to eqn. (A1); therefore only eqn. (A2) will be considered. To describe the experimental curve with eqn. (A2) it is necessary to determine A_0 , $A_{\max}$, $V'_{\max}$, K'_m and k' . The initial absorbance of A_0 was calculated before the other parameters were determined. The reaction that was used to calculate kinetic parameters started at the initial concentration of approx. 40 μM Fru(1,6) P_2 . At the beginning some fluctuation of absorbance was observed. Therefore, for the calculation, this part of the curve was used when the concentration of substrate did not exceed 30 μM . The A_0 value was approximated on the basis of approx. 30 measured points localized symmetrically about the first point of that part of curve used for the calculation. All measured points used for A_0 in the calculation corresponded to this concentration of substrate, which was much higher than K_m , and the linear least-squares method could be used. To find the optimal values of $V'_{\max}$, K'_m , k' and $A_{\max}$ the non-linear least-squares method was applied. The sum of the squares of the residuals was minimized using the Nelder–Mead simplex algorithm [21,42]. For optimization it was necessary to disentangle eqn. (A2) from the A value. Since analytically this is not possible, the numerical procedure to find the zero of a function was applied. For the successive values $V'_{\max}$, K'_m , k' and $A_{\max}$, established by the optimization procedure, the theoretical value of absorbance ($A_{i,\text{theor.}}$) at each time t_i was calculated:

$$A_{i,\text{theor.}} = f(t_i) \quad i = 1, 2, \dots, n$$

where n = number of measuring points.

These values were used to calculate the mean squared error of

Table A1 Kinetic parameters calculated on the basis of selected curve of the reaction

(a) Calculations were performed assuming inhibition by substrate. (b) Calculations were performed assuming lack of inhibition by substrate. In Grid Search Method following partition of parameters axes the values were: $V_{\max} = 6.8 \times 10^{-4}$ mM/s, $K_m = 1.1 \times 10^{-2}$ mM, $A_{\max} = 1.4 \times 10^{-5}$, $K_{is} = 1.7 \times 10^{-2}$ mM.

Parameters	(a) Value	Confidence interval [42]	Confidence interval [43]	(b) Value	Confidence interval [42]
A_0	0.11921	—	—	0.22850	—
$A_{\max}$	0.29908	0.29890–0.29926	0.29889–0.29927	0.29902	0.29837–0.29968
$V'_{\max}$	0.21350	0.2096–0.2176	0.2103–0.2171	0.19530	0.1924–0.2056
K'_m	0.82000	0.73–0.93	0.74–0.91	0.63000	0.55–0.97
K_{is}	98.10000	89.3–107.2	90.3–105.8	—	—
Least-squares norm	8.1×10^{-8}	—	—	8.7×10^{-8}	—

approximation. In Table A1 the results of the calculation for one of the recorded curves are presented. Values of $V'_{\max}$, K'_m and k' were used to calculate $V_{\max}$, K_m and K_{is} . The confidence intervals of determined parameters were calculated as described by Johnson and Faunt [42]. For comparison, confidence intervals were calculated with the Grid Search Method [43]. The results of

these calculations are presented in Table A1. A good agreement between the two methods was obtained. The Grid Search Method is time consuming, so the Johnson and Faunt method was preferred. Calculations were performed on a PC 386 employing the Matlab program of MathWorks [21]. Our program was also written with TURBO-Pascal of Borland.