Steady-state kinetics of malonyl-CoA synthetase from *Bradyrhizobium japonicum* and evidence for malonyl-AMP formation in the reaction

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Malonyl-CoA synthetase catalyses the formation of malonyl-CoA directly from malonate and CoA with hydrolysis of ATP into AMP and PP_i. The catalytic mechanism of malonyl-CoA synthetase from *Bradyrhizobium japonicum* was investigated by steady-state kinetics. Initial-velocity studies and the product-inhibition studies with AMP and PP_i strongly suggested ordered Bi Uni Uni Bi Ping Pong Ter Ter system as the most probable steady-state kinetic mechanism of malonyl-CoA synthetase. Michaelis constants were 61 μ M, 260 μ M and 42 μ M for ATP, malonate and CoA respectively, and the value for $V_{\max}$ was 11.2 μ M/min. The t.l.c. analysis of the ³²P-labelled products in a reaction mixture containing [γ -³²P]ATP in the absence of CoA showed that PP_i was produced after the sequential addition of

ATP and malonate. Formation of malonyl-AMP, suggested as an intermediate in the kinetically deduced mechanism, was confirmed by the analysis of ³¹P-n.m.r. spectra of an AMP product isolated from the ¹⁸O-transfer experiment using [¹⁸O]malonate. The ³¹P-n.m.r. signal of the AMP product appeared at 0.024 p.p.m. apart from that of [¹⁶O]₄AMP, indicating that one atom of ¹⁸O transferred from [¹⁸O]malonate to AMP through the formation of malonyl-AMP. Formation of malonyl-AMP was also confirmed through the t.l.c. analysis of reaction mixture containing [α -³²P]ATP. These results strongly support the ordered Bi Uni Uni Bi Ping Pong Ter Ter mechanism deduced from initial-velocity and product-inhibition studies.

INTRODUCTION

Malonyl-CoA synthetase, which catalyses the formation of malonyl-CoA directly from malonate and CoA with the hydrolysis of ATP into AMP and PP_i, has been first purified and characterized from *Bradyrhizobium japonicum* [1]. This novel enzyme is 58 kDa monomeric and it shows a high substrate specificity for malonate, ATP and CoA. Diethyl pyrocarbonate and pyridoxal-5'-phosphate severely inhibit the enzyme, but iodoacetamide, *p*-chloromercuriphenylsulphonate, *N*-acetyl-imidazole, and phenylglyoxal do not, indicating that essential imidazole and amine groups are involved in catalysis. This enzyme has been proposed to play a role in symbiotic nitrogen metabolism between soybean (*Glycine max*) and *B. japonicum* [2]. The existence of such an enzyme, especially in malonate-grown *Pseudomonas* [3] and in rat brain mitochondria [4], has long been proposed, since malonate occurs widely in animals and plants [5,6]. Recently, malonyl-CoA synthetase from *Rhizobium trifolii* was also purified and characterized to be similar to that of *B. japonicum* in biochemical properties and in the essential groups involved in catalysis [7]. In spite of the extensive studies done with malonyl-CoA synthetase, a thorough kinetic investigation has not been reported to date. Since malonyl-CoA synthetase catalyses a reaction involving three substrates and three products (Ter Ter reaction), many different catalytic routes can be considered.

In the present study we present the results of a kinetic investigation of malonyl-CoA synthetase from *B. japonicum* and the evidence for the formation of malonyl-AMP during catalysis.

MATERIALS AND METHODS

Materials

ATP, sodium malonate, CoA, AMP, Dowex 1-X8, Inorganic

cellulose plates and Hepes were purchased from Sigma Chemical Co. H₂¹⁸O (97 atom % -enriched) was from Aldrich Chem. Co. [α -³²P]ATP (3000 Ci/mmol) and [γ -³²P]ATP (3000 Ci/mmol) were from Amersham International.

Preparation of malonyl-CoA synthetase

Malonyl-CoA synthetase from *B. japonicum* grown on glucose as a carbon source was prepared by the method reported previously [1]. Analysis of the purified enzyme by SDS/PAGE revealed that the enzyme was essentially homogeneous.

Malonyl-CoA synthetase assay

There are four different assay methods for the determination of malonyl-CoA synthetase activity [8]. Among them the malonohydroxamate assay was used for the preparation of the enzyme and ¹⁸O-transfer experiment, while the direct spectrophotometric assay, based on the measurement of the increase in absorbance at 232 nm resulting from the formation of a thioester bond of malonyl-CoA (ϵ_{232} 4.5×10^3 M⁻¹·cm⁻¹), was routinely used for kinetic experiments. The standard reaction mixture for direct spectrophotometric assay contained the following components in a final volume of 0.5 ml: 100 mM potassium phosphate buffer, pH 6.9, 2 mM sodium malonate, 5 mM MgCl₂, 0.2 mM ATP, 0.2 mM CoA and enzyme. Reactions were initiated with malonyl-CoA synthetase, and initial rates were obtained from the linear portion of the progress curve. All kinetic measurements were made in cuvettes of 1 cm path length with a Shimadzu UV-260 recording spectrophotometer equipped with a thermostatically controlled cell compartment. A constant temperature (30 °C) was maintained in the cell compartment using a circulating water system.

Abbreviation used: PEI, polyethyleneimine.

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Initial-velocity experiments

Because three substrates are involved in the malonyl-CoA synthetase reaction, initial-velocity experiments were carried out for the overall reaction in which the concentration of one substrate was varied in the presence of different levels of a second substrate, while the concentration of the third substrate was held constant at a non-saturating concentration [9]. Under all conditions the enzyme concentration ensuring a linear relationship between incubation time and rates of reaction was chosen. We observed substrate inhibition at over 40 mM for malonate, and at over 0.2 mM for ATP and CoA. However, the working concentrations of substrates were below their inhibition concentrations. The data reported here were all carried out in duplicate. The concentration of MgCl_2 was 5 mM throughout. Because the ATP concentrations utilized in the experiments never exceeded 0.1 mM, we have made the simplifying assumption that this concentration of MgCl_2 is sufficiently high that the kinetic effects of Mg^{2+} need not be considered.

Kinetic data analysis

The kinetic data were analysed graphically by constructing double-reciprocal plots of initial velocities and substrate concentrations followed by replots of slopes and intercepts against the reciprocal concentrations of changing fixed substrate in the initial-velocity studies or against the inhibitor concentration in the product-inhibition studies; all plots and their replots were linear. Kinetic data were fitted to the appropriate initial-velocity equation with the FORTRAN programs of Cleland [10] to obtain the desired kinetic parameters. Data from the initial-velocity measurements were fitted to eqns. (1) and (2).

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

$$v = VAB/(K_aB + K_bA + AB) \quad (2)$$

where V is $V_{\max}$, A and B are concentrations of substrates A and B, K_a and K_b are K_m values for A and B and K_{ia} is the dissociation constant for the reaction of A with the free enzyme. Because our measurements were made at a single constant value of the fixed substrate, the reported kinetic parameters are apparent values rather than true values.

Preparation of [^{18}O]malonic acid

[^{18}O]Malonate was synthesized by the reaction of malonyl dichloride with H_2^{18}O . H_2^{18}O (0.5 g) and malonyl dichloride (1.3 ml) were mixed in a round-bottomed flask (molar ratio 2:1). After 1 min, malonic acid remained as a solid product. It was dissolved in 20 ml of ethyl acetate and decolourized with Norit; 20 ml of benzene was added and the mixture was kept at -20°C for 12 h. [^{18}O]Malonic acid was crystallized and dried under reduced pressure. [^{18}O]Malonic acid was methylated with ethereal diazomethane and shown by g.c.-m.s. to be [^{18}O]malonic acid containing 50% [^{18}O].

^{18}O transfer experiment

Reaction mixture contained 100 mM potassium phosphate buffer, pH 6.9, 5 mM MgCl_2 , 10 mM [^{18}O]malonate, 10 mM ATP, 0.5 mM CoA, 200 mM neutralized hydroxylamine and 0.03 unit of malonyl-CoA synthetase in a total volume of 0.5 ml. Reaction was initiated with enzyme and the mixture was incubated for 6 h at 37°C . The reaction mixture was filtered by using a Centricon-30 ultrafiltration apparatus (Amicon Co.) to remove the protein, and the filtrate was loaded on to a Dowex 1-X8 column

(3 cm $\times$ 2.5 cm). The adenine nucleotides were eluted with a 300 ml linear gradient of formic acid (0–5 M). AMP was eluted and its identity confirmed by t.l.c. on a PEI-cellulose plate with 0.7 M sodium phosphate buffer, pH 3.5, as a developing solvent. AMP fractions were pooled, freeze-dried, and then dissolved in 10 ml of distilled water. The pH of AMP solution was adjusted with diluted NaOH to about pH 8.5 and again freeze-dried. The freeze-dried AMP was dissolved in 1.3 ml of 20 mM Hepes buffer, pH 8.8, containing 1 mM EDTA and 20% $^2\text{H}_2\text{O}$. The solution of commercial AMP (11 mM) was prepared under the same conditions as those above and was used as a standard. The ^{31}P -n.m.r. measurements of standard and sample AMP were performed as described below.

N.m.r. measurements

The ^{31}P -n.m.r. spectra (121.47 MHz) were obtained in the Fourier-transform mode with a Varian Gemini-300 7.05 T spectrometer. N.m.r. measurements were made at $25 \pm 1^\circ\text{C}$ with a sample volume of 0.8 ml contained in a 5 mm tube. A total of 246 transients were accumulated using pulses of 45° (the 90° pulse was $16\ \mu\text{s}$) and an acquisition time of 3.0 s. A sensitivity enhancement exponential function gave a line broadening of 0.1 Hz. The chemical shifts are reported relative to 85% H_3PO_4 as an external reference.

RESULTS AND DISCUSSION

Initial-velocity studies

Evaluation of the kinetic mechanism and, in particular, the kinetic parameters of an enzyme-catalysed reaction can often be best approached through initial-velocity studies. Double-reciprocal plots of the initial-velocity data allow the differentiation of the kinetic mechanism: Ping Pong mechanisms result in one or more parallel lines in double-reciprocal plots, whereas sequential mechanisms exhibit non-parallel lines. The results of an experiment, in which the CoA concentration was varied at different fixed concentrations of malonate or ATP, are presented in double-reciprocal form in Figures 1(a) and 1(b) respectively. In both cases the lines are parallel. This behaviour is characteristic of the Ping Pong mechanism in which one or two products are released before the introduction of a final substrate. It also indicates that CoA is the final substrate introduced [11]. When the initial-velocity data of Figures 1(a) and 1(b) were plotted with malonate and ATP as the variable substrates respectively, both sets of results again yielded families of parallel lines (Figures 1c and 1d), suggesting that the malonate-CoA pair and ATP-CoA pair interaction in the malonyl-CoA synthetase-catalysed reaction is a Ping Pong mechanism. The data fitted well to eqn. (2) and the apparent values for the kinetic constants were obtained as follows: $V_{\max}$, $18.5 \pm 0.1\ \mu\text{M}/\text{min}$; K_{malonate} , $429 \pm 30\ \mu\text{M}$; K_{CoA} , $59.2 \pm 3.6\ \mu\text{M}$, at $25\ \mu\text{M}$ ATP; $V_{\max}$, $8.3 \pm 0.7\ \mu\text{M}/\text{min}$; K_{ATP} , $96.3 \pm 8.8\ \mu\text{M}$; K_{CoA} , $24.6 \pm 2.7\ \mu\text{M}$, at 0.15 mM malonate. There are three classes of Ping Pong Ter Ter mechanisms; Bi Uni Uni Bi Ping Pong Ter Ter, Bi Bi Uni Uni Ping Pong Ter Ter and Hexa Uni Ping Pong. The effects of ATP and malonate concentration changes on the initial rates (at constant non-saturating concentration of CoA) are depicted in Figures 1(e) and 1(f). The lines are intersecting, indicating the sequential addition of ATP and malonate (or malonate and ATP) prior to product release. The data fitted well to eqn. (1), and the values for $V_{\max}$, $(6.7 \pm 1.3\ \mu\text{M}/\text{min})$, K_{malonate} ($91.8 \pm 50.8\ \mu\text{M}$) and K_{ATP} ($25.5 \pm 7.3\ \mu\text{M}$) were obtained at a constant CoA concentration of 40 μM . From these results Hexa Uni Ping Pong Ter Ter

Figure 1 Initial velocity patterns for the malonyl-CoA synthetase reaction

The reactions were initiated with 0.003 unit of purified malonyl-CoA synthetase. (a) Plots of $1/v$ versus $1/[CoA]$ at various levels of malonate and at a constant concentration of ATP (25 μM). Malonate concentrations were 0.04 mM (■), 0.05 mM (□), 0.067 mM (▲), 0.1 mM (*) and 0.2 mM (×). (b) Plots of $1/v$ versus $1/[CoA]$ at various levels of ATP and at a constant concentration of malonate (0.15 mM). ATP concentrations were 10 μM (■), 12.5 μM (□), 16.7 μM (▲), 25 μM (*) and 50 μM (×). (c) Double-reciprocal plots of the initial-velocity versus malonate concentration at several concentrations of CoA and at a fixed concentration of ATP (25 μM). CoA concentrations were 16 μM (■), 20 μM (□), 30 μM (▲) and 50 μM (*). (d) Double-reciprocal plots of the initial velocity versus ATP concentration at several concentrations of CoA and a fixed concentration of malonate (0.15 mM). CoA concentrations were 10 μM (■), 15 μM (□), 20 μM (▲) and 40 μM (*). (e) Double-reciprocal plots of the initial velocity versus malonate concentration at several concentrations of ATP and a fixed concentration of CoA (40 μM). ATP concentrations were 16 μM (■), 30 μM (□) and 60 μM (▲). (f) Double-reciprocal plots of the initial velocity versus ATP concentration at several concentrations of malonate and a fixed concentration of CoA (40 μM). Malonate concentrations were 0.04 mM (■), 0.05 mM (□), 0.067 mM (▲), 0.1 mM (*) and 0.2 mM (×).

mechanism could be ruled out. In the same experiment as in Figure 1(f), but with the concentration of CoA raised 4-fold (a near-saturating concentration), the intersecting pattern of double-reciprocal plots emerges (Figure 2c). The same experiments as in Figure 1(c) and Figure 1(d) were repeated with a 4-fold increase each in ATP and malonate concentration, and again parallel patterns clearly arose (Figures 2a and 2b). Thus the same patterns of the double-reciprocal plots at constant saturating and non-saturating concentration of each substrate (ATP, malonate and CoA) suggest that there is no random sequence in the malonyl-CoA synthetase-catalysed reaction.

Taken together, the initial-velocity data cited above can be fitted to the rate equation for a Bi Uni Uni Bi or a Bi Bi Uni Uni Ping Pong Ter Ter mechanism. In the absence of product, the initial-velocity equation for the two mechanisms is the same (eqn. 3):

$$v = VABC / (K_{ia}K_bC + K_cAB + K_bAC + K_aBC + ABC) \quad (3)$$

Where V is the maximum velocity, A , B and C are the concentrations of the three substrates, K_a , K_b , K_c are the Michaelis

constants for the substrates A, B and C respectively and K_{ia} is the dissociation constant for the reaction of A with free enzyme. The rate equation given in eqn. (3) may be rearranged, according to which one of the substrates is fixed, to yield eqns. (4)–(6). Varying B and C at a constant level of A:

$$v = VBC / (K_{ia}K_bC/A + K_cB + K_bC + K_aBC/A + BC) \quad (4)$$

Varying A and C at a constant level of B:

$$v = VAC / (K_{ia}K_bC/B + K_cA + K_bAC/B + K_aC + AC) \quad (5)$$

Varying A and B at a constant level of C:

$$v = VAB / (K_{ia}K_b + K_cAB/C + K_bA + K_aB + AB) \quad (6)$$

The initial-velocity data obtained above were well fitted to these equations (eqns. 4–6).

Product-inhibition studies

Although the initial-velocity data give a good deal of information about the kinetic mechanism of the malonyl-CoA synthetase reaction, our initial-velocity data should be supplemented with

Figure 2 Initial-velocity patterns at a fixed near-saturating concentration of one substrate

The reactions were initiated with 0.003 unit of purified malonyl-CoA synthetase. (a) Double-reciprocal plots of the initial velocity versus the malonate concentration at various levels of CoA and at a near-saturating constant concentration of ATP (0.1 mM). CoA concentrations were 16 μM (■), 20 μM (□), 30 μM (▲) and 50 μM (*). (b) Double-reciprocal plots of the initial velocity versus the ATP concentration at several concentrations of CoA and at a fixed near-saturating concentration of malonate (0.6 mM). CoA concentrations were 8 μM (■), 15 μM (□), 20 μM (▲) and 50 μM (*). (c) Double-reciprocal plots of the initial velocity versus the ATP concentration at various levels of malonate and at a near-saturating constant concentration of CoA (0.15 mM). Malonate concentrations were 0.08 mM (■), 0.15 mM (□) and 0.3 mM (▲).

experiments involving product inhibitors in order to differentiate between Bi Uni Uni Bi and Bi Bi Uni Uni Ping Pong Ter Ter mechanisms and to determine the most likely pathway of substrate addition to the enzyme. The most commonly used method involves holding two substrates at non-saturating concentration while varying the third-substrate and inhibitor concentrations. The initial-velocity data are then plotted in a double-reciprocal form, and the inhibition patterns can be used to limit the possible kinetic pathways.

AMP was found to act as an uncompetitive inhibitor of CoA

Figure 3 Product inhibition patterns for the malonyl-CoA synthetase reaction

(a) Double-reciprocal plots of the initial velocity versus the CoA concentration at various concentrations of AMP. Malonate and ATP concentrations were fixed at 0.15 mM and 25 μM respectively. AMP concentrations were 40 μM (■), 20 μM (□) and 10 μM (▲); * indicates the initial velocities in the absence of inhibitor. (b) Double-reciprocal plots of the initial velocity versus the ATP concentration at various concentrations of AMP. Malonate and CoA concentrations were fixed at 0.15 mM and 40 μM respectively. AMP concentrations were 40 μM (■), 20 μM (□) and 10 μM (▲); * indicates the initial velocities in the absence of inhibitor. (c) Double-reciprocal plots of the initial velocity versus the malonate concentration at various concentrations of PP_i . ATP and CoA concentrations were 25 μM and 40 μM respectively. PP_i concentrations were 0.16 mM (■), 0.085 mM (□) and 0.05 mM (▲); * are the initial-velocity data obtained in the absence of inhibitor.

in the malonyl-CoA synthetase reaction (Figure 3a). Competitive inhibition was also observed with respect to ATP (Figure 3b). Taken along with the results from the initial-velocity experiments, these inhibition patterns indicate that AMP is the R product (final released product) and that substrate A (the first substrate to bind) is ATP and substrate B is malonate [11]. Therefore a Bi Bi Uni Uni Ping Pong Ter Ter mechanism is ruled out and a Bi Uni Uni Bi mechanism is left as the most reasonable kinetic

Figure 4 Autoradiogram of T.L.C. analysis of the partial reaction products using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

The basic reaction mixtures contained 0.1 M potassium phosphate (pH 6.9) buffer, 5 mM MgCl_2 , 0.2 pmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 0.001 unit of malonyl-CoA synthetase (6.7 pmol) in a total volume of 25 μl . Reactions were initiated with the addition of enzyme. The mixture was quickly vortex-mixed and incubated at 37 °C. Aliquots (1 μl each) were taken at the indicated time and spotted on to a PEI-cellulose plate. Sodium phosphate buffer (0.7 M, pH 3.5) was used as a developing solvent. (a) Without malonate; (b) malonate added to a final concentration of 0.2 mM. Lane 1, control (with boiled enzyme); lane 2, 1 min; lane 3, 3 min; lane 4, 5 min; lane 5, 7 min; lane 6, 9 min; lane 7, 12 min; lane 8, 15 min; lane 9, 20 min; lane 10, 10 min after the addition of 3 μl of pyrophosphatase suspension (0.4 unit) to a reaction mixture with 0.2 mM malonate incubated during 20 min. The position of ATP was identified using unlabelled ATP, while the positions of P_i and PP_i were deduced from the result of pyrophosphatase treatment.

pathway taken by the malonyl-CoA synthetase reaction. A Bi Uni Uni Bi Ping Pong Ter Ter mechanism for the malonyl-CoA synthetase reaction was further supported by product inhibition with PP_i . PP_i behaved as a mixed-type inhibitor with respect to malonate (Figure 3c). In the presence of product, new terms are present in the denominator of the velocity equation (eqns. 7–9). When C is varied in the presence of R:

$$v = VC/[K_c + C(1 + K_a/A + K_b/B + K_{ia}K_b/AB + K_{ia}K_bR/K_{ir}AB + K_aR/K_{ir}A)] \quad (7)$$

When A is varied in the presence of R:

$$v = VA/[(K_a(1 + K_{ia}K_b/K_{ir}B + K_{ia}K_bR/K_{ir}K_{ir}B) + A(1 + K_b/B + K_c/C)] \quad (8)$$

When B is varied in the presence of P:

$$v = VB/[(K_b(1 + K_{ia}/A)(1 + K_{ib}K_cP/K_{ip}C) + B(1 + K_a/A + K_c/C + K_cP/K_{ip}C)] \quad (9)$$

The product-inhibition data obtained above were well fitted to the appropriate rate equations (eqns. 7–9). The apparent values for the inhibition constants obtained from the secondary replots were as follows: AMP has a K_i value of $69.5 \pm 6.3 \mu\text{M}$ with respect to CoA and of $51.9 \pm 1.3 \mu\text{M}$ with respect to ATP; PP_i has a K_i value of $123 \pm 14 \mu\text{M}$ with respect to malonate.

Identification of PP_i as the first product

Since the kinetic analysis shown above suggested that PP_i was the product released first, the formation of PP_i was monitored using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the partial reaction mixture containing no CoA. As shown in Figure 4, PP_i was formed after sequential addition of ATP and malonate. In order to determine the order of ATP and malonate introduced, the isolation of enzyme-ATP (or enzyme-malonate) complex from the partial reaction mixture containing only ATP (or malonate) as a substrate was attempted by gel filtration. But the attempts failed, indicating that ATP or malonate alone may not form a stable complex with the enzyme.

Figure 5 ^{31}P -n.m.r. spectrum of the AMP product of the ^{18}O transfer experiment

(a) Mixture of AMP product and standard AMP (2:1, v/v). The relative integrals of the peaks are 1.00 and 0.58. (b) AMP product. The relative integrals of the peaks are 0.93 and 1.00. (c) Standard AMP.

Evidence for the formation of malonyl-AMP

From the initial-velocity and the product-inhibition studies the formation of malonyl-AMP, an intermediate in the Bi Uni Uni Bi Ping Pong mechanism, was suggested. The formation of the intermediate was confirmed by ^{18}O transfer from $[\text{O}^{18}]\text{malonate}$ to AMP. ^{18}O -labelled malonate (50% ^{18}O) was supplied as a substrate together with ATP and CoA in the malonyl-CoA synthetase-catalysed reaction. The reaction was carried out to 55% completion by the elimination of malonyl-CoA as malonohydroxamate in the presence of hydroxylamine. AMP, one of the products, was isolated from the reaction mixture by using Dowex 1-X8 anion-exchanger. The ^{18}O content in the AMP was monitored by ^{31}P n.m.r. spectroscopy. As shown in Figure 5(b), two resonances were observed corresponding to AMP labelled with no and one atom of ^{18}O . When unlabelled AMP (used as standard) was added to the sample, the downfield resonance increased in intensity, indicating that this peak was indeed unlabelled AMP (Figure 5a). The separation between the two resonances was 0.024 p.p.m., consistent with the chemical shift per ^{18}O reported by Cohn and Hu [12]. The resonance integrals were almost identical (Figure 5b), indicating that 50% of AMP product was labelled with one atom of ^{18}O . These results clearly show that malonyl-AMP was formed during the malonyl-CoA synthetase reaction.

The formation of malonyl-AMP was also confirmed by t.l.c. analysis of a reaction mixture containing enzyme, malonate and $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, which revealed the presence of a novel nucleotide derivative that was neither AMP, ADP nor ATP. Since the malonyl-AMP-enzyme complex may be very unstable in the presence of Mg^{2+} , such as in the case of aminoacyl-tRNA synthetase [13–15], we expected that the intensity of the AMP spot would increase with time. However, as shown in Figure 6(a), the intensity in the AMP position showed only a temporary increase, concomitant with the appearance of a new spot. The intensity of the new spot, corresponding to a novel nucleotide

Figure 6 Autoradiogram of t.l.c. analysis of reaction products using $[\alpha\text{-}^{32}\text{P}]\text{ATP}$

The basic reaction mixtures contained 0.1 M potassium phosphate (pH 6.9) buffer, 5 mM MgCl_2 , 0.2 pmol $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, other components in the indicated amount and 6×10^{-4} unit of malonyl-CoA synthetase (4 pmol) in a total volume of 25 μl . All other conditions are as described in the legend of Figure 4. (a) Lane 1, control (with boiled enzyme); lanes 2–4, 1 min, 4 min and 8 min in reaction with only $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ as a substrate respectively; lanes 5–7, 1 min, 4 min and 8 min after subsequent addition of malonate to final concentration of 2 mM respectively. (b) The explanation for each lane was the same as described in (a), except for the addition of 2 mM succinate in place of malonate. (c) The basic reaction mixture was first incubated for 8 min at 37 °C. Malonate (final concentration, 2 mM) and inhibitor (in the indicated concentration) mixed previously were added and the mixtures was quickly vortex-mixed. The incubation was continued for 8 min at 37 °C. Lane 1, control (with boiled enzyme); lane 2, only malonate without inhibitor; lanes 3–6, 0.1 μM , 1 μM , 10 μM and 100 μM AMP as a competitive inhibitor respectively; lanes 7–9, 0.2 mM, 2 mM and 20 mM succinate as a non-competitive inhibitor respectively. (d) The basic reaction mixture contained $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and malonate (final concentration of 1 mM) as substrates. After 4 min, aliquots of 10 μl were taken and mixed with CoA (final concentration of 0.5 mM). The mixture containing CoA was separately incubated and aliquots of 1 μl were spotted on to a PEI-cellulose plate at the indicated time. Lane 1, control (with boiled enzyme); lanes 2–5, 1 min, 4 min, 8 min and 12 min from the reaction mixture in the absence of CoA respectively; lanes 6–7, 4 min and 8 min after the addition of CoA respectively. The positions of each nucleotide were identified using unlabelled ATP, ADP and AMP. The arrow indicates the novel nucleotide derivative, defined as malonyl-AMP.

derivative, gradually increased with the addition of malonate. However, this spot did not appear with the addition of succinate in place of malonate, indicating that the novel nucleotide derivative was formed from malonate and $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (Figure 6b). The transient formation of AMP in the presence of malonate can be explained by the formation of a relatively stable transition-state trigonal bipyramid complex of malonate and ATP. The formation of the novel nucleotide derivative was inhibited by AMP and succinate, which are known to be competitive and non-competitive inhibitors respectively [1] (Figure 6c). Also, the novel nucleotide derivative disappeared gradually with the addition of CoA, concomitant with the production of AMP (Figure 6d). Combined results of the experiments using $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ clearly indicate that the novel nucleotide derivative is malonyl-AMP.

Proposed mechanism of malonyl-CoA synthetase catalysis

The results from initial-velocity and product-inhibition studies described above support the Bi Uni Uni Bi Ping Pong Ter Ter system for the malonyl-CoA synthetase reaction. However, it is necessary to eliminate the possibility of other ter-reactant Ping Pong systems, a Theorell–Chance system or a ter-reactant Ping Pong system with a rapid equilibrium segment. First, the possibility of a Theorell–Chance-type case (Bi Uni Uni Bi Ping Pong Ter Ter; Theorell–Chance B–P) can be eliminated by looking at the result of product inhibition in which PP_i is a mixed-type inhibitor with respect to malonate at unsaturating ATP and CoA concentrations (Figure 3c). Secondly, in the Bi

Figure 7 Replot of the slopes of the lines of Figure 1(f) versus reciprocal of the malonate concentration

Uni Uni Bi Ping Pong Ter Ter system in which the first substrate binds in either direction and the respective complexes that are formed are at equilibrium, the initial-velocity equation in the absence of products and the product inhibition patterns is changed differently from the full steady-state system.

When A is varied:

$$v = VA/[K_{ia}K_b/B + A(1 + K_b/B + K_c/C)] \quad (10)$$

Compared with the equivalent velocity equation for the full steady-state system, eqn. (10) is missing the K_eAB term. Then slope $1/A$ versus $1/B$ replots go through the origin. Secondary replot using the slopes of lines in Fig. 1(f) eliminates the possibility of this system (Figure 7).

As a consequence, we propose that *B. japonicum* malonyl-CoA synthetase catalyses the reaction in the manner of an ordered Bi Uni Uni Bi Ping Pong Ter Ter mechanism as follows:

Where Mal, Mal-AMP and Mal-CoA represent malonate, malonyl-AMP and malonyl-CoA respectively.

This catalytic mechanism is further supported by the isolation of first product, PP_i, and by the identification of the key intermediate, malonyl-AMP.

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