New kinetic parameters for rat liver arginase measured at near-physiological steady-state concentrations of arginine and Mn^{2+}

Silvia MAGGINI,* Franziska B. STOECKLIN-TSCHAN, Stéphanie MÖRIKOFER-ZWEZ and Paul WALTER
Department of Biochemistry, University of Basel, Vesalianum, Vesalgasse 1, CH-4051 Basel, Switzerland

A cytosolic cell-free system from rat liver containing the last three enzymes of the urea cycle, a number of cofactors and the substrates aspartate and citrulline was shown to synthesize urea at near-physiological rates ranging between 0.40 and 1.25 $\mu\text{mol}/\text{min}$ per g of liver. This system was used to determine the kinetic parameters for arginase. With saturating amounts of Mn^{2+} (30 μM), arginine remained at a steady-state concentration of 5–35 μM depending on the aspartate and citrulline supply. V_{max} at micromolar arginine concentrations was between 1.10 and 1.25 $\mu\text{mol}/\text{min}$ per g of liver, the $K_{0.5}$ (arginine) between 6.0 and 6.5 μM and positive co-operativity was observed (Hill coefficient 2). Omission of Mn^{2+} caused a significant accumulation of arginine during the incubation, suggesting a regulatory effect of arginase. Under these conditions, V_{max} was 1.10–1.65 $\mu\text{mol}/\text{min}$ per g of liver and the K_m (arginine) increased up to 14.4–21.1 μM . The apparent K_a for Mn^{2+} in the presence of physiological concentrations of ATP, Mg^{2+} and arginine was calculated to be maximally 8 μM . Initial-velocity experiments with millimolar arginine concentrations as the direct substrate gave the following results, which are in good agreement with literature data. In the absence of Mn^{2+} , V_{max} was 71.3 $\mu\text{mol}/\text{min}$ per g of liver and the K_m (arginine) 1.58 mM. With 30 μM - Mn^{2+} , V_{max} was 69.4 $\mu\text{mol}/\text{min}$ per g of liver and the K_m (arginine) decreased to 0.94 mM. On the basis of our results, we propose the presence of high-affinity and low-affinity sites for arginine on rat liver arginase and postulate that alterations in arginase activity arising from changes in the concentration of arginine and of the cofactor Mn^{2+} may contribute to the regulation of ureagenesis *in vivo*.

INTRODUCTION

Arginase (EC 3.5.3.1) is the last enzyme of the urea cycle and catalyses the hydrolysis of arginine to urea and ornithine. The maximal activity of purified rat liver arginase has been reported to range between 580 and 1210 $\mu\text{mol}/\text{min}$ per g of liver (Schimke, 1962; McLean & Novello, 1965; Greengard *et al.*, 1970; Snodgrass *et al.*, 1978) and the K_m (arginine) value between 3 and 10 mM (Schimke, 1970; Kaysen & Strecker, 1973; Tarrab *et al.*, 1974; Soberon & Palacios, 1976; Meijer *et al.*, 1985). Since the purified arginase has by far the highest activity among the five urea-cycle enzymes, it is generally accepted that arginase never becomes rate-limiting in urea synthesis *in vivo*.

The catalytic activity and the stability of arginases from different species were found to depend on the presence of bivalent cations, particularly Mn^{2+} but also Co^{2+} , Ni^{2+} and in some instances Fe^{2+} and Cd^{2+} (Hirsch-Kolb *et al.*, 1971). The rat liver Mn^{2+} -enzyme complex is well characterized. Purified rat liver arginase is a tetramer with four Mn^{2+} -binding sites and an M_r between 115 000 and 138 000 (Hirsch-Kolb *et al.*, 1971; Tarrab *et al.*, 1974; Soberon & Palacios, 1976). The fully active enzyme contains four Mn^{2+} ions. Two ions are loosely bound and can be removed, resulting in a 50 % loss in enzyme activity. Full activity can be restored on addition of millimolar amounts of Mn^{2+} (≥ 40 mM). The other two Mn^{2+} ions are tightly bound and their removal leads to the denaturation of the enzyme with a consequent irreversible loss of enzyme activity (Hirsch-Kolb *et al.*, 1971). The concentrations of Mn^{2+} used in the experiments with the purified rat liver enzyme were in the millimolar range (Hirsch-Kolb *et al.*, 1971; Bond *et al.*, 1983, 1986), whereas the total liver concentration of this bivalent cation is between 50 and 60 μM (Failla & Kiser, 1981; Bond *et al.*, 1983) and that of isolated hepatocytes between 25 and 40 μM (Ash & Schramm, 1982; Schramm, 1986; Schramm & Brandt, 1986). In hepatocytes isolated from fed rats, the free concentration of Mn^{2+} is more

than 50-fold lower than the total concentration and amounts to only 0.6–0.8 μM (Schramm & Brandt, 1986).

Only recently, experiments with lower Mn^{2+} concentrations were performed, and apparent K_a values for Mn^{2+} ranging between 10 and 200 μM have been determined for cytosolic mouse liver arginase (Spolarics & Bond, 1989). Also for arginase from *Saccharomyces cerevisiae* (Green *et al.*, 1990) an apparent K_a of 12 μM was measured in the presence of saturating amounts of the substrate arginine.

Experiments on Mn^{2+} activation of purified rat liver arginase have so far only been carried out with high unphysiological concentrations of both Mn^{2+} and the substrate arginine. Furthermore, ATP, a potential candidate for Mn^{2+} binding, and Mg^{2+} , known to interfere with many Mn^{2+} -stimulated enzyme reactions, were not present in the assay mixtures. In this paper, a new approach is presented that allows the investigation of the flux through the arginase reaction as well as the Mn^{2+} effect on enzyme activity under near-physiological steady-state conditions. The experimental system is a cytosolic cell-free preparation combining the complexity of whole cellular preparations with the advantage of a system *in vitro* lacking any membranes that would limit the uptake and redistribution of substrates and effectors. In contrast with the generally accepted view, the kinetic parameters determined for rat liver arginase under near-physiological conditions indicate that this enzyme may contribute to the regulation of ureagenesis *in vivo*.

EXPERIMENTAL

Materials

Urease S, glutamate dehydrogenase, malate dehydrogenase and dithiothreitol were obtained from Boehringer (Mannheim, Germany). Other enzymes, coenzymes, adenine nucleotides, GSH, 2-oxoglutarate, (amino-oxo)acetic acid and L-arginino-succinic acid were purchased from Sigma Chemical Co. (St.

* To whom correspondence should be addressed.

Louis, MO, U.S.A.). L-Arginine, L-aspartate and L-citrulline were from Serva (Heidelberg, Germany). Chelex 100 was from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and Sephadex G-25 medium from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity commercially obtainable. Solutions were prepared with twice quartz-distilled water.

In order to avoid contamination of the assay mixtures with bivalent cations, the following precautions were taken: (a) all glassware used was soaked in 2 mM-EDTA, pH 9.0, for at least 30 min and was then thoroughly washed first with deionized water and then with twice quartz-distilled water to remove all EDTA (Mörkofer-Zwez, 1983); (b) solutions were passed through Chelex 100 before use (except bivalent cations, and the doubly positive charged arginine and ornithine, which would otherwise bind to Chelex).

Male albino Wistar rats fed a chow diet *ad libitum* from Madörin (Füllinsdorf, BL, Switzerland) weighing 180–230 g were used.

Cytosol preparation

For the preparation of cytosols, 3 g wet wt. of liver from rats killed by decapitation were immediately homogenized (Potter/Elvehjem homogenizer) at 4 °C with 15 ml of a buffer containing 0.14 M-sucrose, 50 mM-potassium phosphate buffer, pH 7.2, 2 mM-GSH and 0.1 mM-EDTA in order to bind bivalent cations. The homogenate was centrifuged, the cytosol was freed from EDTA and other low- M_r compounds by gel filtration and was further treated as described previously (Stoecklin *et al.*, 1986).

Incubation procedure

The standard incubation mixture contained: 33 mM-Tris/HCl buffer, pH 7.4, 0.66 mM-GSH, 0.8 mM-dithiothreitol, 3 mM-ATP, 4 mM-MgSO₄, 80 mM-KCl and 1.67 mM-P_i, the latter originating from the added cytosol. Concentrations of other compounds are given in the Results section.

Incubations were carried out in plastic tubes at 30 °C in a final volume of 1.5 ml. After a preincubation period of 3 min, the reaction was started by addition of 0.05 ml of cytosol, containing 0.7 mg of protein. The reactions were stopped with ice-cold HClO₄ (final concentration 0.4 M). The samples were then treated as described previously (Mörkofer-Zwez *et al.*, 1981).

Analysis of metabolites

Metabolites were determined in the neutralized extracts as described previously (Stoecklin *et al.*, 1986). Urea was determined as described by Kersher & Ziegenhorn (1985). The method was modified as follows: triethanolamine/HCl buffer, pH 8.0 (final concentration in the assay mixture 150 mM), was used instead of Tris/succinate buffer, and urease was dissolved in twice-quartz-distilled water instead of phosphate buffer and glycerol, because the latter buffer gave high blank values.

For the determination of arginine and argininosuccinic acid, an enzyme assay was developed with the following reactions:

The assay mixture contained 150 mM-triethanolamine/HCl/50 mM-potassium phosphate buffer, pH 8.0, 60 mM-2-oxoglutarate, 2 mM-ADP, 2 mM-MgCl₂, 1 mM-(amino-oxy)acetic acid and 0.25 mM-NADH. The final volume was 1 ml. Glutamate

dehydrogenase (12 units/ml) and urease (2.5 units/ml) were added, and the absorbance at 340 nm was measured after 30 min. Arginase (12 units/ml) was then added and the absorbance was again measured 45 min later. For the determination of arginino-succinic acid, argininosuccinate lyase (0.1 units/ml) was added in a further step and the absorbance was measured after 90–120 min.

Since aspartate aminotransferase and malate dehydrogenase activities were present as impurities in the arginase preparation, (amino-oxy)acetic acid was added to prevent unspecific NADH consumption due to the presence of aspartate in the neutralized probes.

Protein was determined by the biuret method against BSA standard. Protein concentration in the cytosols was 14.22 ± 1.30 mg (mean $\pm$ s.d., $n = 65$)/ml of supernatant.

Calculations

Data on metabolite formation and substrate utilization represent net values calculated from the difference between the incubated samples and the zero-time controls. Metabolite formation between two given incubation times was calculated as the difference in net metabolite concentration at the beginning and end of the time interval.

For the calculation of formation rates, 6 ml of cytosol was considered equivalent to 1 g wet wt. of liver. The dilution factor originating from Sephadex filtration of the cytosol was taken into account (for details see Mörkofer-Zwez, 1983).

Kinetic data were fitted to the Hill equation according to the method of Atkins (1973). The curves presented are those giving the best fit with experimental data.

RESULTS

Characterization of the incubation system

Formation of urea was studied in a cytosolic cell-free system prepared from livers of fed rats, containing all the enzymes required for the conversion of aspartate and citrulline into urea (Scheme 1). The concentrations of urea, arginine, malate, fumarate, argininosuccinic acid and aspartate in the gel-filtered cytosol were close to zero (less than 0.2 μ mol/g of liver).

Scheme 1. Reactions in the incubation system

Table 1. Net product formation and aspartate utilization between 10 and 20 min incubation time

Metabolite formation and utilization were determined in the standard incubation mixture described in the Experimental section. Substrates were 1 mM-aspartate and 1 mM-citrulline. Results are expressed as means $\pm$ S.E.M. from four independent experiments.

Metabolites	Metabolite formation and utilization ($\mu\text{mol}/\text{min per g of liver}$)	
	No additions	30 $\mu\text{M-Mn}^{2+}$
Aspartate used	2.74 ± 0.40	2.80 ± 0.37
Urea + arginine + argininosuccinic acid formed	2.71 ± 0.40	2.82 ± 0.44
Malate + fumarate + argininosuccinic acid formed	2.82 ± 0.41	2.74 ± 0.40

Table 1 shows the net formation and utilization of some ureagenic intermediates and substrates. The recovery was calculated in relation to the amount of aspartate used, either on the basis of the products urea + arginine + argininosuccinic acid or of the products malate + fumarate + argininosuccinic acid. The recovery was close to 100 %, indicating that no reactions other than those illustrated in Scheme 1 took place.

Time- and substrate-dependence of net metabolite formation; influence of Mn^{2+}

The time-dependent net metabolite formation with three different initial concentrations of aspartate and citrulline either in the absence or in the presence of 30 $\mu\text{M-Mn}^{2+}$ is presented in Fig. 1. From the results it can be concluded that: (a) the net

Fig. 2. Aspartate and citrulline (supplied in a 1:1 ratio) dependence of net urea formation between 10 and 20 min incubation time and of the arginine near-steady-state concentration, in the presence of 30 $\mu\text{M-Mn}^{2+}$

Net formation of urea (●) and arginine final concentration after 20 min (▲) were determined in the standard incubation mixture described in the Experimental section. Results are expressed as means $\pm$ S.E.M. ($n = 3-5$).

formation of urea is stimulated by 30 $\mu\text{M-Mn}^{2+}$; (b) the net formation of malate + fumarate is not influenced by Mn^{2+} , indicating the specific effect of the bivalent cation on the arginase step; (c) in the absence of Mn^{2+} there is an accumulation of arginine over the incubation period. The addition of 30 $\mu\text{M-Mn}^{2+}$ abolishes this effect and there is a near-steady-state concentration of arginine between 10 and 20 min.

When arginine (0.1 and 0.5 mM) was also added as a substrate together with aspartate and citrulline, the increase in arginine during the incubation was not as marked as in Fig. 1(a,c,e).

Fig. 1. Time-dependence of net metabolite formation from aspartate and citrulline in the absence (a, c and e) and presence (b, d and f) of 30 $\mu\text{M-Mn}^{2+}$

(a) and (b) 0.2 mM-aspartate and 0.2 mM-citrulline; (c) and (d) 1.0 mM-aspartate and 1.0 mM-citrulline; (e) and (f) 2.5 mM-aspartate and 2.5 mM-citrulline. Net formation of urea (○, ●), malate + fumarate (□, ■) and arginine (△, ▲) were determined in the standard incubation mixture described in the Experimental section. Results are expressed as means $\pm$ S.E.M. from five independent experiments.

Fig. 3. Mn^{2+} -dependence of net metabolite formation from 1 mM-aspartate and 1 mM-citrulline between 10 and 20 min incubation time.

Net formation of urea (●), malate + fumarate (■) and arginine (▲) were determined in the standard incubation mixture described in the Experimental section. Results are expressed as means $\pm$ S.E.M. The numbers of experiments are given in parentheses (for malate-fumarate it was the same as for urea).

However, the arginine concentration after 20, 30 and 40 min incubation was about twice as high as in the presence of $30 \mu\text{M-Mn}^{2+}$ (results not shown).

The rates of urea formation ($\mu\text{mol/min per g of liver}$) calculated from the data in Fig. 1, were 0.40 (Fig. 1a), 0.52 (Fig. 1b), 0.58 (Fig. 1c), 0.90 (Fig. 1d), 0.90 (Fig. 1e) and 1.23 (Fig. 1f). These rates are in good agreement with those measured in perfused rat liver ($0.5\text{--}1.0 \mu\text{mol/min per g of liver}$) with 0.3 mM-NH_4^+ and physiological portal concentrations of glutamine (0.5 mM) as substrates (Häussinger *et al.*, 1984). Lund & Wiggins (1984, 1986) have obtained rates of urea formation ranging between 0.6 and $1.3 \mu\text{mol/min per g of liver}$ with $10 \text{ mM-NH}_4\text{Cl} + 10 \text{ mM-lactate} + 0.5 \text{ mM-oleate}$ or with 5 mM-glutamine with hepatocytes isolated from rats deprived of food for 48 h.

The formation of urea as well as the arginine concentration were measured between 10 and 20 min at intervals of 2 min in the presence of $30 \mu\text{M-Mn}^{2+}$ and were found to be linear and constant respectively (not shown).

Variations of the aspartate and citrulline load in the presence of Mn^{2+} resulted in different steady-state concentrations of arginine (Fig. 2), thus allowing the manipulation of the arginine level in our experimental system. The concentration of arginine ranged between 5 and $35 \mu\text{M}$. This is in very good agreement with the physiological values ($5\text{--}60 \mu\text{M}$; Gopalakrishna & Nagarajan, 1980).

As shown in Fig. 3, arginase activity was stimulated on addition of micromolar concentrations of Mn^{2+} . Maximal activity was obtained with $30 \mu\text{M-Mn}^{2+}$, and the increase in urea synthesis amounted to about 35%. The apparent K_a value of the arginase for its cofactor Mn^{2+} was calculated from double-reciprocal plots according to the method of Schramm (1986) and was estimated to be about $8 \mu\text{M}$.

Control experiments (results not shown) revealed that Mn^{2+} -induced stimulation of arginase activity occurred only when 0.1 mM-EDTA was present in the homogenization buffer used for cytosol preparation (see the Experimental section). When the chelating agent was omitted, Mn^{2+} had no effect on the already maximally activated arginase. No change in arginase activity was observed when additional 1.5 mM-EGTA was added to the zero Mn^{2+} probes during the incubation, indicating that arginase contained only two Mn^{2+} ions in the absence of added Mn^{2+} .

Determination of the kinetic parameters for rat liver arginase under near-physiological conditions (steady-state system)

For the determination of the V_{max} and the K_m for arginine of rat liver arginase at near-physiological substrate concentrations (steady-state conditions), arginase activity was defined as the rate of urea formation between 10 and 20 min incubation time. Different steady-state concentrations of arginine were obtained by varying the aspartate and citrulline load. The kinetic parameters were calculated from pooled data (Fig. 4). V_{max} was found to be between 1.10 and $1.25 \mu\text{mol/min per g of liver}$ and the $K_{0.5}$ for arginine between 6.0 and $6.5 \mu\text{M}$; furthermore there is good evidence that the Hill coefficient is around 2 (calculated values $2.01\text{--}2.20$). The latter values were significantly different from 1 at $P < 0.005$ and $P < 0.0002$ respectively (Student's t test).

Since the arginine concentration was not constant over the incubation period in the absence of added Mn^{2+} (Fig. 1a,e,c), only a rough estimation of the kinetic parameters for rat liver arginase was possible under these conditions. With the arginine con-

Fig. 4. Kinetic data for arginase in the presence of $30 \mu\text{M-Mn}^{2+}$, fitted to the Hill equation according to the method of Atkins (1973)

●, Arginase activity = net urea formation from increasing concentrations of aspartate and citrulline (supplied in a 1:1 ratio), between 10 and 20 min incubation time, determined in the standard incubation mixture described in the Experimental section. —, Curve fitted to the Hill equation: (a) $h = 2.01$, $V_{\text{max}} = 1.22 \mu\text{mol/min per g of liver}$, $K_{0.5} = 6.43 \mu\text{M}$; (b) $h = 2.20$, $V_{\text{max}} = 1.14 \mu\text{mol/min per g of liver}$, $K_{0.5} = 6.04 \mu\text{M}$. Results are expressed as means $\pm$ S.E.M. The numbers of experiments are indicated in the Figure.

Fig. 5. Kinetic data for arginase, fitted to the Hill equation ($h = 1$) according to the method of Atkins (1973)

○, ●, Arginase activity = net urea formation from increasing initial arginine concentrations between 0 and 5 min incubation time, in the absence (○) and in the presence (●) of $30 \mu\text{M-Mn}^{2+}$. The cytosol was diluted 10-fold, MgSO_4 was 1 mM and ATP was omitted from the standard incubation mixture described in the Experimental section. —, Curve fitted to the Hill equation: ○, $V_{\text{max.}} = 71.3 \mu\text{mol/min per g of liver}$, $K_m = 1.58 \text{ mM}$; ●, $V_{\text{max.}} = 69.4 \mu\text{mol/min per g of liver}$, $K_m = 0.94 \text{ mM}$. Results are expressed as means $\pm$ S.E.M. from three independent experiments, except where indicated in parentheses.

centration after 10 min, $V_{\text{max.}}$ was $1.40 \pm 0.25 \mu\text{mol/min per g of liver}$ and K_m (arginine) was $14.4 \pm 3.6 \mu\text{M}$. The data obtained at 20 min incubation time resulted in a $V_{\text{max.}}$ of $1.35 \pm 0.25 \mu\text{mol/min per g of liver}$ and a K_m value (arginine) of $21.1 \pm 6.2 \mu\text{M}$. The Hill coefficient was 1.0 in both cases.

A comparison of the kinetic data in the absence and presence of Mn^{2+} shows that Mn^{2+} had no effect on the $V_{\text{max.}}$ of the arginase whereas it lowered the K_m value for arginine.

Determination of the kinetic parameters for rat liver arginase with initial-velocity experiments

The dependence of arginase activity on millimolar substrate concentrations is illustrated in Fig. 5. $V_{\text{max.}}$ was $71.3 \pm 1.2 \mu\text{mol/min per g of liver}$ in the absence of Mn^{2+} and $69.4 \pm 2.6 \mu\text{mol/min per g of liver}$ in the presence of $30 \mu\text{M-Mn}^{2+}$. The K_m for arginine was lowered by the presence of the bivalent cation from $1.58 \pm 0.06 \text{ mM}$ to $0.94 \pm 0.10 \text{ mM}$. The finding that Mn^{2+} had no effect on the $V_{\text{max.}}$ value, but lowered the K_m for arginine corresponded to the results obtained under near-physiological conditions. However, the kinetic parameters determined by means of the initial-velocity system were 50–200-fold higher than those measured under steady-state conditions.

The kinetic parameters at high arginine concentrations were determined at pH 7.4 and 30°C . Arginase activity is known to decrease by about 50% when the pH value is lowered from 9.7 to 7.2 (Saheki *et al.*, 1979) and it further decreases by about 50% on lowering the temperature from 37°C to 30°C . Thus it can be concluded that when millimolar concentrations of arginine are used, the kinetic parameters determined under initial-velocity conditions are in good agreement with those measured by Bond *et al.* (1983) in the rat liver cytosol ($300 \mu\text{mol/min per g of liver} \div 4 = 75 \mu\text{mol/min per g of liver}$).

The aim of the experiments reported in Fig. 6 was to measure within the same experiment the switch from the high-affinity to the low-affinity sites. For this purpose, increasing concentrations

Fig. 6. Effect of arginine addition 10 min after the start of the reaction on urea formation from 1 mM-aspartate and 1 mM-citrulline, in the presence of $30 \mu\text{M-Mn}^{2+}$

Urea formation between 12 and 15 min incubation time (●) was determined in the standard incubation mixture described in the Experimental section. Arginine (10, 25, 50, 75, 100, 200, 300 and $500 \mu\text{M}$) was added 10 min after the start of the reaction. Results are expressed as means $\pm$ S.E.M. from five independent experiments.

of arginine ranging between 0.01 mM and 0.5 mM were added to the steady-state system 10 min after the start of the reaction, i.e. when the arginine concentration reached the steady-state value (Fig. 1). Urea formation as well as arginine concentration were measured 2 min and 5 min after arginine addition (12 min or 15 min after the start of the reaction by cytosol addition). The switch from the high- to the low-affinity sites of rat liver arginase occurred with arginine concentrations of about $50 \mu\text{M}$. For arginine concentrations below $50 \mu\text{M}$, the curve in Fig. 6 resembles that of Fig. 4; $V_{\text{max.}}$ is less than $2 \mu\text{mol/min per g of liver}$ and K_m (arginine) can be estimated to be below $10 \mu\text{M}$. After the switch to the low-affinity sites, the curve looks like the one in Fig. 5 determined in the initial-velocity system ($V_{\text{max.}}$ is about $80 \mu\text{mol/min per g of liver}$ and the K_m (arginine) approx. 1.5 mM).

DISCUSSION

Regulation of rat liver arginase by Mn^{2+}

The usual approach for evaluating metal ion activation of an enzyme-catalysed reaction involves measurement of initial rates at saturating substrate concentrations. However, this approach is insufficient for evaluating Mn^{2+} effects *in vivo*, as pointed out by Schramm (1986). According to this author, experiments should be carried out at different non-saturating substrate concentrations, since saturation with substrate often influences the kinetic parameters for metal ions. Our results in Fig. 1 indeed show that Mn^{2+} -induced activation of the arginase reaction occurs at different non-saturating arginine concentrations ranging between 5 and $30 \mu\text{M}$.

As further proposed by Schramm (1986), experiments should be carried out in the presence of physiological concentrations of ATP (a candidate for Mn^{2+} binding) and Mg^{2+} . In fact, enzymes that are influenced by cellular concentrations of Mn^{2+} must be able to discriminate between millimolar concentrations of Mg^{2+} (physiological range of the free cation is around 1 mM) and micromolar concentrations of Mn^{2+} . If the kinetic constant for Mn^{2+} measured under these conditions is below $10 \mu\text{M}$, then the enzyme is a candidate for regulation by Mn^{2+} *in vivo* (Schramm, 1986). In contrast with the experiments described so far in the

literature (Hirsch-Kolb *et al.*, 1971; Bond *et al.*, 1983, 1986; Spolarics & Bond, 1988, 1989; Green *et al.*, 1990), both ATP (3 mM) and free Mg^{2+} (around 1 mM) were present at physiological concentrations in our studies. Furthermore, the steady-state concentrations of arginine ranging between 5 and 35 μM corresponded to its physiological concentration in liver (Gopalakrishna & Nagarajan, 1980), whereas in the experiments described so far in the literature millimolar concentrations were used. The apparent K_a value for Mn^{2+} of 8 μM (Fig. 3) is below the value of 10 μM set by Schramm (1986) as the limit under which an enzyme can be considered as a possible target for Mn^{2+} activation *in vivo*. The real K_a value, expressed in terms of free- Mn^{2+} , will be much lower than 8 μM . In fact, the latter value represents the total concentration of the cation, i.e. free and bound Mn^{2+} . In our experiments, the concentration of free Mn^{2+} could not be measured, but we estimate on the basis of computer calculations that it was less than 10% of the total concentration, because of binding to adenine nucleotides and cytosolic proteins.

It is well documented (Failla, 1986) that changes in the plasma levels of pancreatic and adrenal hormones, and perhaps hormones of the pituitary-gonadal axis, mediate tissue-specific modifications in Mn^{2+} distribution. Such hormone-induced alterations in the cellular distribution of Mn^{2+} are essential for adaptation to different environmental and physiological conditions. Accumulation of Mn^{2+} in the liver, but not in the kidney and duodenum, is known to be stimulated by depression of the plasma insulin/glucagon ratio, as it occurs in diabetes mellitus. A 1.5-fold increase in total Mn^{2+} concentration (from 60 μM to 100 μM) in livers of diabetic rats was reported by Failla (1986) and Bond *et al.* (1983, 1986), who suggested that the elevated concentration of Mn^{2+} could explain in part the increased arginase activity.

The results presented here show that the flux through rat liver arginase under near-physiological conditions is sensitive to changes in Mn^{2+} concentration within the physiological micromolar range. Thus we postulate that changes in the hepatic Mn^{2+} content, e.g. hormone-induced changes (Failla, 1986) or changes related to dietary conditions (Schramm, 1986), can modulate arginase activity *in vivo*.

Possible experimental causes for the discrepancy in the kinetic parameters for rat liver arginase determined under near-steady-state conditions and by initial-velocity measurements

The kinetic parameters for rat liver arginase determined under near-steady-state conditions were 50–200-fold lower than those determined by initial-velocity experiments. A possible explanation for this discrepancy could be the different compositions of the assay mixtures used for the two systems. However, it could be demonstrated (Table 2) that the arginase activity determined with the high arginine concentrations did not change on addition

of ATP, aspartate, citrulline, malate and/or fumarate to the incubation mixture.

Another important difference between the steady-state and the initial-velocity system is the concentration of metabolites other than arginine. The low V_{max} value determined in the steady-state system could be caused by the fact that the ratio arginine/non-arginine metabolites is much lower than in the initial-velocity system. The control experiments (Table 2) show that when similar concentrations of aspartate, citrulline, ATP, malate and/or fumarate were added, the discrepancy between the measured kinetic parameters was still observed.

As discussed by Bosca *et al.* (1985), addition of poly(ethylene glycol) increases local protein concentrations thus allowing study of enzymes in the physiological state of aggregation. The presence of the crowding agent did not affect the kinetic parameters for arginase in the initial-velocity system (Table 2), indicating that 10-fold dilution of the cytosol either did not cause dissociation of the arginase tetramer in the subunits or dissociation had no effect on arginase activity.

The direct proof that the discrepancy between the kinetic parameters determined at low and high concentrations of arginine is not due to an experimental artifact is given by the results of Fig. 6. It was possible to detect the switch from low activity but high affinity (steady-state system, Fig. 4) to high activity but low affinity (initial-velocity system, Fig. 5) within the same experiment.

A model for the regulation of rat liver arginase

The amino acid sequence for the subunits of rat liver arginase has been deduced from the nucleotide sequence of cDNA clones (Kawamoto *et al.*, 1987). The enzyme consists of four subunits with identical amino acid sequence, suggesting that the catalytic sites of the enzyme may also be identical. However, rat liver arginase has an absolute requirement for Mn^{2+} and the enzyme always contains at least two Mn^{2+} ions. It is thus likely that the binding of the cofactor to a pair of the subunits causes a conformational change in the three-dimensional structure of the enzyme, leading to catalytic sites with different affinities for arginine. It is impossible to prove this hypothesis by means of kinetic experiments since Mn^{2+} -free arginase is not active and is known to denature (Hirsch-Kolb *et al.*, 1971). Unfortunately, X-ray and n.m.r. analyses of arginase structure are not yet available.

It has been postulated (Hirsch-Kolb *et al.*, 1971) that in the fully active enzyme containing four Mn^{2+} cations there may be equivalence between all four subunits. However, the results of Fig. 4 indicate positive co-operativity in the presence of saturating Mn^{2+} concentrations. This means that, in the fully active enzyme also, the catalytic sites may be different.

In conclusion, although the amino acid sequence of the arginase subunits is the same, there are some indications that the catalytic sites may not be identical, due to the binding of the

Table 2. Influence of various factors on arginase activity measured in the initial-velocity system in the presence of 30 μM - Mn^{2+}

Incubation time was 5 min. Results are expressed as means $\pm$ S.E.M. from three independent experiments.

Arginine concentration (mM)	Additions ...	Arginase activity (μ mol/min per g of liver)				
		None	3 mM-ATP + 4 mM- $MgSO_4$	1 mM-Aspartate + 1 mM-citrulline	10% (w/v) Poly(ethylene glycol)	0.5 mM-Malate + 0.5 mM-fumarate
0.05		2.48 $\pm$ 0.50	2.71 $\pm$ 0.69	2.56 $\pm$ 0.41	2.28 $\pm$ 0.17	—
0.50		12.45 $\pm$ 2.71	14.07 $\pm$ 3.79	10.97 $\pm$ 2.13	12.38 $\pm$ 2.27	—
1.00		20.47 $\pm$ 1.31	—	—	—	18.51 $\pm$ 1.69

activator Mn^{2+} . This excludes negative co-operativity as a possible interpretation for the kinetic parameters determined for rat liver arginase at low and high substrate concentrations. In fact, it is well established that non-equivalence of the catalytic sites can give the appearance of negative co-operativity (for a review see Ricard & Cornish-Bowden, 1987). Furthermore, the curve of Fig. 6 is not characteristic for negative co-operativity but rather for an enzyme containing low-affinity and high-affinity catalytic sites (Gache *et al.*, 1976; Pershadsingh & McDonald, 1980).

The possibility that there are two different arginases in the cytosol is also very unlikely. In fact, the existence of arginase isoenzymes in the rat liver has not been proved unambiguously. Tarrab *et al.* (1974) claimed that there were at least two molecular forms of arginase in rat liver. However, they reported only minor differences in the energy of activation and in the molecular mass, whereas the kinetic parameters for the two molecular forms were identical.

Therefore the most plausible explanation for the discussed discrepancy in the kinetic parameters is the presence of high- and low-affinity sites for arginine on rat liver arginase. At high (millimolar) arginine concentrations, urea formation is a classical titration curve of the Michaelis-Menten type with a Hill coefficient of 1. At low (micromolar) arginine concentrations Michaelis-Menten behaviour is no longer followed, positive co-operativity is observed instead and the Hill coefficient is around 2. The ratio of apparent affinities of the high-affinity and low-affinity sites at pH 7.4 is about 170.

There are other examples of enzymes with high- and low-affinity sites reported in the literature. Beside $(Na^+ + K^+)$ -ATPase (Gache *et al.*, 1976; Serspersu *et al.*, 1990) and $(Ca^{2+} + Mg^{2+})$ -ATPase (Pershadsingh & McDonald, 1980), the most important examples are the $\alpha\beta_2$ complex of tryptophan synthetase from *Escherichia coli* (Weisheit & Kirschner, 1976; Napier & Hardman, 1984) and phosphofructokinase (Kessler *et al.*, 1986).

Arginine is known to be involved in several metabolic processes other than the urea cycle. These include protein synthesis, polyamine synthesis and the synthesis of creatine (Visek, 1986). It is well documented that the hepatic arginine content undergoes marked fluctuations in response to dietary arginine, although there was some controversy in the literature regarding the concentration range (Stewart & Walser, 1980; Morimoto *et al.*, 1990). According to Stewart & Walser (1980), hepatic arginine concentration increases to 0.2–1.0 mM in rats after injection of a mixture of amino acids including arginine. Morimoto *et al.* (1990) reported that the portal arginine concentration remains in the micromolar range and increases 4-fold (maximal value 180 μ M) in rats changing from protein-free to a 60% protein diet. In any case, fluctuations of the arginine load in response to different dietary conditions are documented and it is important that regulation of arginase activity, the major arginine-metabolizing enzyme in the liver, can occur over a wide range of arginine concentrations.

Does rat liver arginase play a role in the regulation of ureagenesis?

Previous studies have shown that purified rat liver arginase has by far the highest activity among the enzymes of the urea cycle (for a review see Meijer & Hensgens, 1982). The present work demonstrates, however, that under near-physiological conditions, the activity of rat liver arginase is of the same order of magnitude as the activities of argininosuccinate synthetase and argininosuccinate lyase (Table 1, Fig. 4). Therefore the function of arginase *in vivo* should be re-evaluated. Its possible role in the regulation of urea synthesis must especially be considered in relation to mitochondrial carbamoyl phosphate synthetase I, the

first enzyme in ureagenesis, known to play an important role in the regulation of this pathway (Meijer & Hensgens, 1982).

N-Acetylglutamate is an essential activator of carbamoyl phosphate synthetase I and its synthesis is stimulated by arginine (Shigesada & Tatibana, 1971). Arginine concentrations between 30 and 50 μ M, as used in the present study, have been reported to give half-maximal activation of *N*-acetylglutamate synthesis (Kawamoto *et al.*, 1985; Morimoto *et al.*, 1990). Regulation of arginase activity and the resulting variations in arginine concentration can therefore influence the synthesis of *N*-acetylglutamate and thus indirectly play a role in the regulation of carbamoyl phosphate synthetase I.

In the cytosolic system used throughout this work, the dilution of the proteins was too high to allow enzyme-enzyme interactions and thus channelling of metabolites. *In vivo*, channelling through the urea cycle has been proposed to occur (Powers-Lee *et al.*, 1987; Cohen *et al.*, 1987; Cheung *et al.*, 1989; Watford, 1989), but its function remains to be established.

In conclusion, the kinetic parameters presented here indicate that the physiological role of arginase should be reconsidered. Alterations in enzyme activity arising from changes in the concentrations of its substrate arginine, of Mn^{2+} or of the known arginase inhibitors ornithine (Meijer *et al.*, 1985) and branched-chain amino acids (Bond *et al.*, 1986) may contribute to the regulation of ureagenesis *in vivo*.

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