Mn(II) Binding Sites of Calf Liver Arginase

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Commercial calf liver arginase was further purified through gel filtration column chromatography. The enzyme is nearly homogeneous in SDS-PAGE; it contains 4 manganese atoms per molecule of enzyme. By dialysis against 1,10-o-phenanthroline at 4 °C it is possible to obtain an arginase apo-form containing 2 manganese atoms per molecule of enzyme (apo-2 form) while a treatment of the pure enzyme with o-phenanthroline at 37 °C followed by dialysis against 0.1 M NaCl is capable of producing an apo-enzyme with only 1 manganese atom per molecule (apo-1 form). The apo-2 and apo-1 arginases retain respectively about 50% and 25% of the full enzymatic activity. NMR titrations of both apo-arginases with increasing concentrations of manganese allowed us to determine the affinity constants for the binding of Mn^{2+} to the protein. It was shown that in this enzyme two manganese atoms are weakly bound, one is more strongly bound and the fourth one is bound so tightly that it is not removed under the experimental conditions used.

Introduction

Arginase is a metalloenzyme whose function is to catalyze the cleavage of L-arginine to ornithine and urea. This enzyme represents the terminal step of the urea cycle which is known to be operative only in mammalian livers. However this enzyme is present also in extrahepatic tissues in mammals and in a variety of other organisms, in such cases its physiological role being related to the ornithine catabolism [1, 2]. This enzyme has been isolated and purified from different sources, *e.g.* rat liver [3] and kidney [4] and human lung [5]. In all cases this enzyme has been shown to be an oligomer with a molecular weight of about 120000. Structural studies revealed also that rat [6, 7] and human liver [8] arginases are composed of four subunits with a molecular weight of about 30000. Biological studies showed that this enzyme is activated by manganese(II) cations and quantitative determinations of manganese content showed that, in the purified enzyme, 4 atoms of manganese are bound per enzyme molecule [9]. Furthermore it has been shown that some of the manganese atoms reversibly dissociate from the enzyme with a concomitant loss of enzymatic activity [9, 10]. In the case of rat liver enzyme, manganese(II) binding has been studied through water proton relaxation studies [10], whereas in the case of calf liver enzyme no data are available.

As this latter enzyme has been reported to differ from that of rat liver both with respect to the electrophoretic behaviour [11] and to the number of dialyzable atoms [12] we decided to study, through ^1H T_1^{-1} measurements, the interaction of this enzyme with manganese(II) aquoion.

Experimental

L-arginine (free base) and $MnCl_2 \cdot 4H_2O$ were obtained from Merck; L-arginase (calf liver) was

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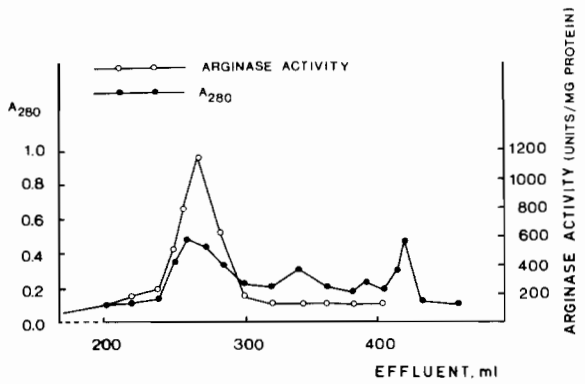

Fig. 1. Gel filtration of calf liver arginase on ACA 44.

purchased from Boheringer, Mannheim; ACA 44 from LKB; Biogel P-2 (200–400 mesh) from BioRad; *o*-phenanthroline was from Fluka. All other chemicals were of the best purity available. Arginase activity was measured by the method of Herzfeld and Raper [13] and the urea produced was assayed according to Geyer and Dabich [14]. A preincubation procedure is used for detecting the full catalytic activity of apo-arginases: approximately 0.3 μg of enzyme protein is preincubated at 37 °C for 15 min in the presence of glycine–NaOH buffer, pH 9.5 and 6 m*M* $MnCl_2$; the substrate arginine is then added and the reaction is allowed to proceed for 15 min. One enzyme unit is defined as the amount of enzyme that produces 1 μmol of urea per min at 37 °C. Specific activity is expressed in terms of enzyme units per mg of protein. Protein was measured taking as molar absorption coefficient at 278 nm the value 113,500 M^{-1} cm^{-1}, determined on a weighed sample of purified enzyme, after exhaustive dialysis against double distilled water and lyophilization. For all calculations a M.W. of 120000 was assumed [11]. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [15]. Densitometric scanning of stained gels was made at 605 nm in a Gilford 250 spectrophotometer equipped with the gel scanner accessory. Manganese content was determined by atomic absorption spectroscopy using a Perkin Elmer mod 4000 with a graphite furnace; NMR measurements were made using a CFT-20 Varian spectrometer. Samples were contained and run in a Wilmad cell with external lock. The probe temperature was 37 °C. The T_1 values were measured by the inversion recovery method.

Preparation of the enzyme

Arginase obtained from Boheringer has been purified as follows: 150 mg of calf liver enzyme were dissolved in 3 ml of 10 m*M* Tris-HCl pH 7.5, containing 25 m*M* $MnCl_2$, and applied to a column of ACA 44 (2.6 × 86 cm) equilibrated with the same buffer; 10 ml fractions were collected at a flow rate of 1.5 $ml/cm^2 \cdot h$ (Fig. 1). The fractions containing arginase activity were pooled, concentrated by ultrafiltration using an Amicon PM-10 membrane and then passed through a short column of Biogel P-2 (200–400 mesh) equilibrated with 1 m*M* Tris-HCl pH 7.5 The pooled fractions from Biogel P-2 were used for subsequent studies. Further concentration was achiev-

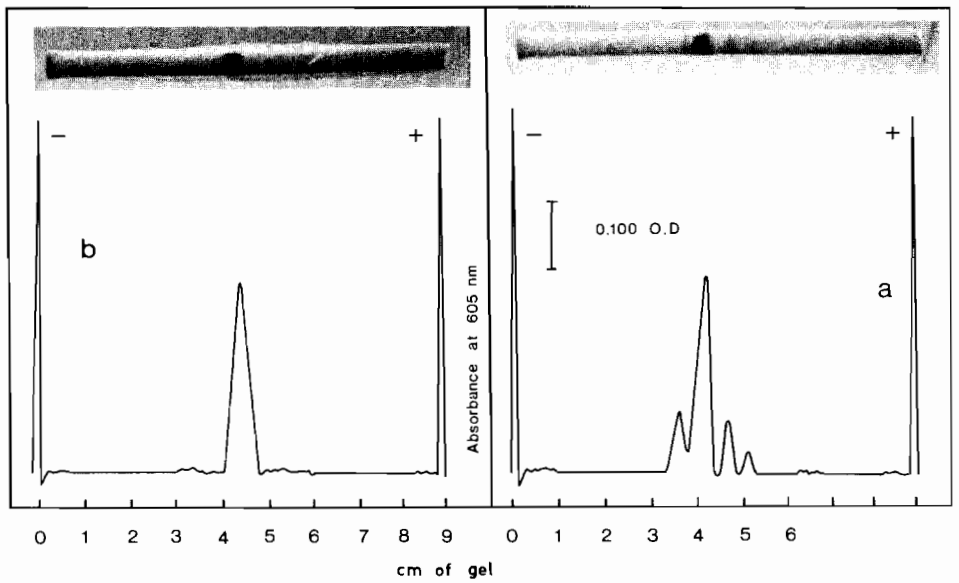

Fig. 2. SDS-PAGE of calf liver arginase before (a) and after (b) gel filtration. 20 μg protein were run in 0.5 × 10 cm gel rods at 3 mA per gel. Proteins were stained with Coomassie R 250. Densitometric scanning was made at 605 nm using a Gilford 250 recording spectrophotometer equipped with a gel scanner accessory.

TABLE I. Arginase Activity of apo-1 Enzymes Prepared by Treatment with Chelating Agents and Subsequent Dialysis. The enzyme activity was assayed with the preincubation procedure when $MnCl_2$ was included in the assay medium (100% catalytic activity) and directly when $MnCl_2$ was omitted (residual catalytic activity).

Chelating agent	Specific activity	Residual catalytic activity	Manganese content
	units/mg protein	%	atoms per molecule
EDTA	196	24.8	1.1 ± 0.1
EDTA + Mn	792		
EGTA	133	17.0	1.0 ± 0.1
EGTA + Mn	780		
DTPA	159	20.4	1.1 ± 0.1
DTPA + Mn	781		
o-Phenanthroline	115	14.7	1.0 ± 0.1
o-Phenanthroline + Mn	785		
Dipyridyl	140	18.2	1.1 ± 0.1
Dipyridyl + Mn	770		
Control sample	684	85.0	3.5 ± 0.2
Control sample + Mn	805		

ed by lyophilization, when required for NMR measurements. The specific activity of the pure enzyme ranged from 900 to 1200. The preparation is nearly homogeneous as judged by SDS-PAGE (Fig. 2).

Results

Removal of the Manganese from Arginase

Following different procedures it is possible to obtain two different types of apo-arginase, one containing ≅2 residual Mn atoms, (referred hereafter as apo-2) and the other containing only one metal atom (apo-1). In order to obtain the apo-2enzyme, 30 mg of purified arginase in Tris-HCl 10 m*M*, pH 7.5, were dialyzed at 4 °C against 10 volumes of 10 m*M*, 1,10-*o*-phenanthroline for 24 h; a second dialysis step to remove *o*-phenanthroline was made against 10 m*M* Tris-HCl at pH 7.5. The sample was then concentrated by lyophilization to a concentration of about 1×10^{-4} *M*. The apo-2 enzyme so obtained had ≅2 Mn atoms as determined by atomic absorption spectroscopy and a residual catalytic activity of about 50–60%. Prolonged dialysis against Tris-HCl or water was unable to remove further manganese atoms from the arginase.

Calf liver arginase is reported to be reversibly inactivated by treatment with EDTA at 37 °C for 60 min. We checked the possible inactivating action of some other chelating agents (DTPA, EGTA, EDTA, *o*-phenanthroline and dipyridyl)* using the following procedure: 1 mg of purified arginase was dissolved, in the presence of the appropriate chelator, in 1 ml of 25 m*M* TRIS-HCl at pH 7.5, and incubated for 60 min at 37 °C. A 100 m*M* concentration was used for EDTA, DTPA or EGTA and 10 m*M* for *o*-phenanthroline or dipyridyl. A control without chelating agents was also incubated. The samples were transferred to ice and each one was then dialyzed separately at 4 °C for 5 days, with several changes, against a total volume of 3 liters of 0.1 *M* NaCl containing 1 m*M* Tris-HCl, at pH 7.5. A dialysis against water, kept at a pH of about 7.5 with ammonium acetate, was made and samples were then lyophilized. As shown in Table I, the samples treated as above contained ≅1 Mn atom per molecule of enzyme and showed a residual catalytic activity of 15–25%. The residual catalytic activity of the control sample was about 85% and the Mn content ≅3.5 atoms per enzyme molecule. It is clear that the apo-1 form is obtained only through the treatment of arginase with chelating agents at 37 °C and subsequent dialysis against large volumes of high ionic force NaCl; *o*-phenanthroline was used routinely for the preparation of apo-arginases. As will be shown further, the apo-2 and apo-1 forms of the enzyme require a different procedure for the restoration of their full catalytic activity. Whereas the apo-2 enzyme is fully reactivated by simply including $MnCl_2$ (1 to 6 m*M*) in the incubation mixture, the apo-1 requires a preincubation step of 15 min at 37 °C with Mn^{2+} before the reaction starts. To assess

*Abbreviations: NMR, nuclear magnetic resonance: PRR, proton relaxation rate; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid; DTPA, diethylenetriamine pentaacetic acid.

TABLE II. Activity of apo-2 and apo-1 Arginases in the Presence and Absence of $MnCl_2$. The assays were carried out with and without the preincubation step for both apo-enzymes.

Sample	Without preincubation	With preincubation
	Specific activity (units/mg protein)	
Apo-2	477	420
Apo-2 + Mn	868	874
Apo-1	174	160
Apo-1 + Mn	400	830

the extent of residual catalytic activity of apo-1 arginase a comparison must be made between enzymatic activity assayed without preincubation in the absence of $MnCl_2$ and with preincubation in the presence of the cation.

As shown in Table II the apo-2 enzyme needs no preincubation procedure to reach full catalytic activity. The apo-1 enzyme, on the other hand, only shows about 50% of its full activity if the assay is carried out without preincubation.

Proton Relaxation Measurements

The interaction of the apoforms with manganese-(II) aquoion was studied through proton relaxation enhancement measurements. As we are mainly interested in the determination of the number of binding sites available in each apoform and in the values of the affinity constants we found it convenient to measure the enhancements, ϵ^*, of the different solutions [16, 17], because this parameter is known to be less sensitive to systematic errors [18].

Apo-2-sample

An enzyme sample with 55% residual activity and a manganese content, determined through atomic absorption spectroscopy of 2.2 ± 0.1 atoms per enzyme molecule, was used for NMR investigations. In order to determine the enhancement factor of the removed manganese atoms a solution of $MnCl_2$ 5.2×10^{-5} was titrated with increasing amounts of arginase (2.5×10^{-5} to 5.6×10^{-5} *M*) and the values of $1/\epsilon^*$ were plotted against the inverse of the enzyme concentrations (Fig. 3). By extrapolation at infinite enzyme concentration an enhancement factor ϵ_b equal to 7.5 ± 0.5 was estimated. The number of binding sites and their affinity constants were determined by titration of the above

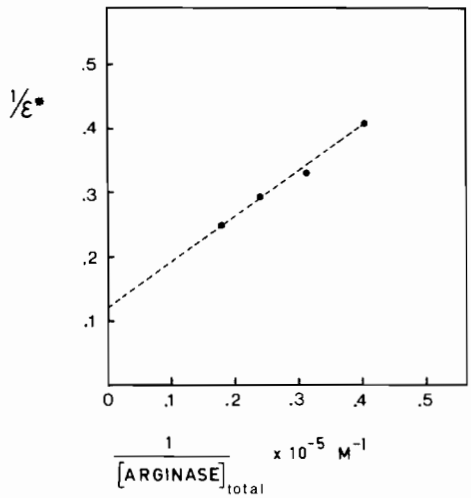

Fig. 3. Titration of Mn^{2+} (5.2×10^{-5} *M*) with apo-2 arginase preparation. Plot of reciprocal enhancement *versus* total arginase concentration.

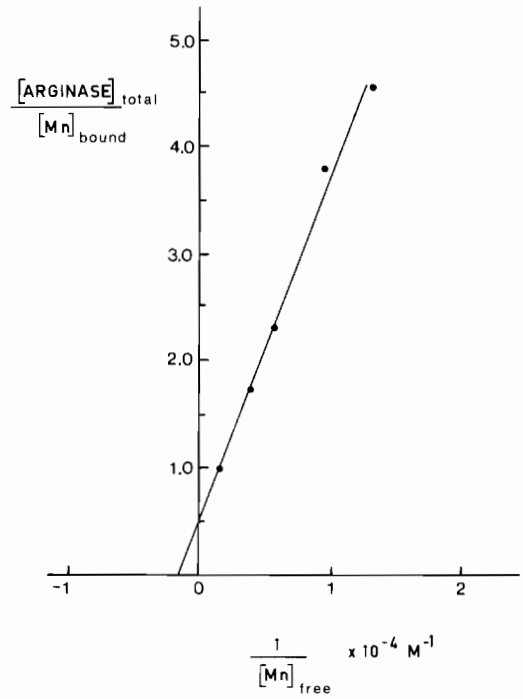

Fig. 4. Hughes and Klotz type plot of the ratio of arginase to weakly bound manganese *versus* the reciprocal of the free manganese concentration, for the apo-2 sample.

sample with increasing amounts of manganese ion. In such a titration the ϵ^* values decrease with increasing manganese concentration as it is expected to occur when equivalent binding sites are occupied. The data were then plotted according to the method of

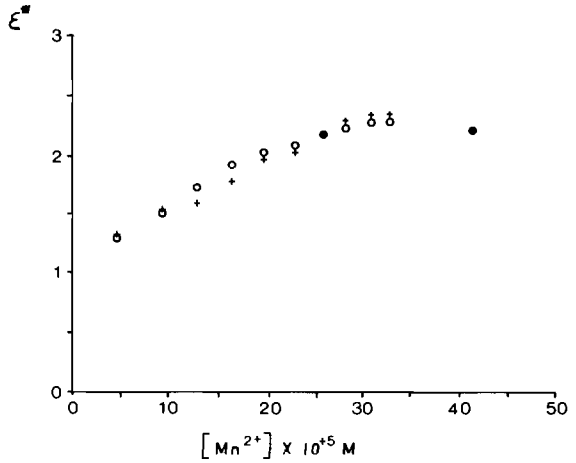

Fig. 5. Titration of apo-1 arginase (9.1×10^{-5} *M*) with increasing amount of manganese. +) Experimental; o) calculated values.

Hughes and Klotz (Fig. 4). The amount of free and bound manganese was estimated from the ϵ^* value and the known ϵ_b. In this kind of plot the ordinate intercept represents the number n of equivalent binding sites whereas the abscissa intercept represents K_A.

A number of binding sites equal to 1.9 ± 0.3 was determined with an affinity constant equal to 2 ± 0.5×10^3 M^{-1}.

Apo-1-sample

An enzyme sample with 24% of residual catalytic activity and a manganese content of 1.1 ± 0.1 g atoms of manganese per mole of enzyme was used for NMR investigations. This kind of sample showed titration curves which are, also only from a qualitative point of view, completely different from those of the apo-2-samples. Indeed, unlike what has already been described for the apo-2-sample, in this case the ϵ^* values increase when the Mn concentration increases (Fig. 5). Such a curve shows that non equivalent binding sites are present in the apo-1-arginase. Furthermore titration of a solution of $MnCl_2$ 6.37×10^{-5} *M* with increasing amounts of the apo-1-protein led to the determination of an enhancement factor at infinite enzyme concentration of 1.8 ± 0.3 (Fig. 6). Both types of titration show that this sample contains, in addition to the previously described binding sites of the apo-2-sample, a new site with different properties, *i.e.* higher affinity constant and lower enhancement factor. The higher affinity constant of this new site accounts for the more drastic conditions used for removing manganese from it. A least squares fitting analysis of the points of Fig. 5 on the basis of a model which hypothesizes the presence in the apo-1-sample of three empty sites, two of them being equivalent, was performed.

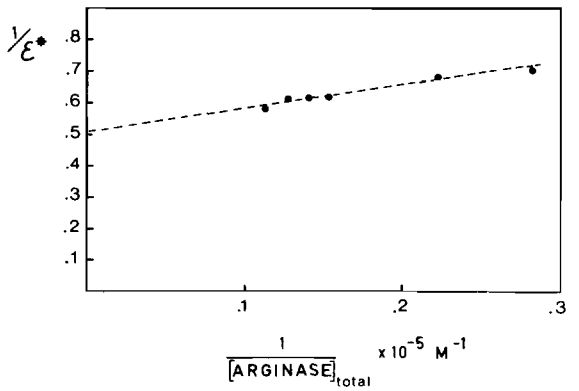

Fig. 6. Titration of Mn^{2+} (6.3×10^{-5} *M*) with an apo-1 arginase preparation.

The observed enhancement was fitted to the following equation

$$\epsilon^* = \Sigma_i \epsilon_i [X_i]$$

where ϵ_i represents the enhancement factor of each X_i species. From this relation it is possible to evaluate the ϵ_i and the affinity constants K_i for which the difference between the observed and the calculated ϵ^* values is minimum. The following equilibria were assumed

$$Mn + Arg \xrightarrow{K_1} Mn(1)Arg$$

$$Mn^{2+} + Mn(1)Arg \xrightarrow{K_2} Mn(1), Mn(2,3)Arg$$

$$Mn^{2+} + Mn(1)\,Mn(2,3)\,Arg \xrightarrow{K_3}$$

$$Mn(1)Mn(2)Mn(3)Arg$$

Where Arg stands for the apo-1 form and the numbers identify the sites. The sites 2 and 3 are assumed to be equivalent and independent (this gives $K_2 = 2K_3$). The enhancement factors of each site are indicated as ϵ_1' and $\epsilon_2' = \epsilon_3'$. The best fitting values of these parameters are the following:

$$K_1 \geqslant 10^5\ M^{-1} \qquad \epsilon_1' = 1.3 \pm 0.6 \qquad \epsilon_2' = 6.5 \pm 1.5$$

$$K_3 = 4.2 \pm 1.7 \times 10^3\ M^{-1}$$

The values for sites 2 and 3 obtained by calculation using this rather simplified model of the enzyme are in good agreement with the results obtained experimentally from the apo-2-sample on sites 2 and 3 and support the approximation used.

Discussion

Arginase is a metalloenzyme which contains both dissociable and non dissociable manganese. In this

respect it resembles pyruvate carboxylase [19]. Depending on the type of procedure used (see Results) it is possible to obtain a calf liver arginase sample with a residual manganese content in the range of 2.1–2.5 or 1.1–1.5 atoms per arginase molecule. ^1H T_1^{-1} measurements in the presence of increasing amounts of added manganese(II), indicate the presence, in the samples referred to as apo-2 arginase, of two sites which bind Mn(II) with the same affinity constant whereas in the apo-1 samples a further binding site to which Mn(II) appears more firmly bound, is detected. It is apparent therefore that it is possible to remove selectively two or three manganese atoms from calf liver arginase. The fitting or the relaxation enhancement curves allowed us to estimate the affinity constants of the binding sites. Values differing by about two orders of magnitude were found for the two sets and this explains why it is possible to remove initially two manganese atoms without affecting the occupancy of the other binding sites. The four manganese atoms which are presented in calf liver enzyme can therefore be assigned to three classes of sites, which differ both in their affinity constants and in their enhancement factors. Two manganese atoms can be considered equivalent and are weakly bound with an affinity constant of the order $\cong 10^3$ M^{-1}. One manganese atom is more tightly bound with a constant $\geqslant 10^5$ M^{-1} and can be removed by treatment with complexing agents at 37 °C followed by prolonged dialysis. The fourth manganese atom is so strongly bound that it is not removed under the experimental conditions used. The calf liver enzyme appears therefore to differ from the rat liver enzyme both with respect to the number of dialyzable manganese atoms and the values of the affinity constants. The rat liver arginase has been reported to lose only two manganese atoms which show an affinity constant of 2×10^4 M^{-1}, whereas for the two other binding sites an affinity constant $\cong 10^7$ M^{-1} was estimated [10]. In the calf liver enzyme the different classes of sites also show very different enhancement factors, the looser binding sites showing an enhancement factor more than two times greater than that of the tight one. In some cases it has been reported that removal of manganese from the enzyme is accompanied by dissociation of the enzyme into subunits [6, 8]. This in principle would lead to a decrease in the T_1^{-1} enhancement factors for the samples with lower manganese content, owing to a decrease in the rotational correlation times of the subunits with respect to the tetramer [18]. However it has been demonstrated that in the case of calf liver enzyme this dissociation into subunits does not occur, the tetrameric structure being conserved also in the absence of manganese [11]. A further point which deserves some consideration is that apo-1 and apo-2 samples differ also with respect to the procedure which is necessary to restore their full catalytic activity. Indeed, whereas apo-2 samples attain full activity by simply including $MnCl_2$ in the assay mixture, for the apo-1 samples it is necessary to perform a preincubation procedure of 15 min in the presence of Mn^{2+}, in order to reach a full reactivation. The removal of the third manganese atom is probably accompanied by conformational changes in the structure of the enzyme which possibly alter the binding capability of the substrate. This has been demonstrated to occur in the case of rat liver enzyme where it has been shown that manganese induces optical activity in the 280 nm band and furthermore that the spectral changes correlate with the reactivation of the enzyme [20].

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