Biochimica et Biophysica Acta, 481 (1977) 177—183

BBA 68047

IMMOBILISED MONOMERS OF HUMAN LIVER ARGINASE

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(Received September 16th, 1976)

Summary

Human liver arginase (L-arginine amidinohydrolase, EC 3.5.3.1) was immobilised by attachment to nylon with glutaraldehyde as a crosslinking agent.

Incubation of the immobilised tetrameric enzyme with EDTA followed by dialysis resulted in the dissociation of the enzyme into inactive matrix-bound and solubilised subunits. Both species recovered enzymatic activity after incubation with Mn^{2+}, and the activity of the reactivated matrix-bound subunits was nearly 25% of that shown by the enzyme initially attached to the support in the tetrameric form.

When the reactivated bound subunits were incubated with soluble subunits in the presence of Mn^{2+}, they 'picked-up' from the solution an amount of protein and enzymatic activity almost identical to that initially lost by the immobilised tetramer after the dissociating treatment with EDTA. This occurred only in the presence of Mn^{2+}.

It is suggested that the reactivation of the subunits of arginase involves the initial formation of an active monomer, which then acquires a conformation that favours a reassociation to the tetrameric state.

Introduction

The use of immobilised enzymes has proved valuable to determine whether the isolated subunits of an oligomeric enzyme are active [1—4]. For this purpose, intact oligomeric molecules are attached to a matrix via a single subunit and then those not covalently attached are removed. In this way, the catalytic activity of the immobilised subunits can be studied under conditions where reassociation is not possible [1].

This approach appears specially attractive for an enzyme such as human liver arginase (L-arginine amidinohydrolase, EC 3.5.3.1), since the catalytic activity

and the maintenance of the quaternary structure of the enzyme depend on Mn^{2+} bound to the protein [5].

In fact, withdrawal of Mn^{2+} results in dissociation of the enzyme into inactive subunits and addition of Mn^{2+} to the inactive species causes their reassociation into the tetrameric state [5]. The question we wished to answer was whether active monomers are formed prior to association or whether association is necessary for the catalytic activity of the enzyme. This paper describes the results obtained with human liver arginase attached to nylon with glutaraldehyde as a crosslinking agent [6] and evidence is presented for the existence of an active monomer of the enzyme.

Materials and Methods

Human liver arginase was purified as described by Bascur et al. [7]. According to this procedure, chromatography of a partially purified preparation on a CM-cellulose column equilibrated with 5 mM Tris · HCl (pH 7.4) resolves the enzymatic activity into two protein fractions. The experiments described here were performed with the fraction containing the highest activity, that eluting from the column at 010—0.14 M KCl. This enzyme fraction was further purified by chromatography on CM-cellulose under the conditions described for the rat liver enzyme [8], and a preparation showing a single protein band on polyacrylamide gel electrophoresis was obtained.

Immobilisation of arginase was performed as follows. A strip of nylon mesh (2 × 1 cm) was treated with glutaraldehyde diluted in 0.2 M sodium bicarbonate buffer (pH 9.0) to a final concentration of 10% (v/v). After 1 h at room temperature, with constant stirring, the support was removed and washed several times with 50 mM phosphate buffer (pH 8.0) to eliminate unreacted glutaraldehyde. The 'activated support' thus obtained was then suspended in 3 ml of a solution containing 1 mg of enzyme in 10 mM phosphate buffer (pH 8.0); the solution was stirred with a small magnetic stirrer to keep the support moving around in the tube. After 15 min at 37° C the support was taken out and washed with 10 mM phosphate buffer (pH 8.0) to remove the enzyme not coupled to it. Finally, the immobilised enzyme preparation was treated for 3 min at 0° C with 50 mM $NaBH_4$ to stabilise the linkage of the enzyme to the support and to reduce the unreacted carbonyl groups of the crosslinking agent [9]. The immobilised enzyme thus obtained is stable for at least one month when maintained at 4° C in 50 mM Tris · HCl buffer (pH 8.7).

Activity measurements on the matrix-bound enzyme were carried out by suspending the support in 2 ml of a solution containing appropriate concentrations of [*guanido*-^{14}C] arginine (specific activity 1 mCi/mol) in 50 mM glycine buffer (pH 9.5). The support was kept moving in the tube by stirring with a small magnetic stirrer. Reactions were initiated by adding the matrix-bound enzyme to the substrate buffer solution previously warmed at 37° C. After incubation for 3 min at this temperature, suitable aliquots were removed from the surrounding solution and assayed for urea as described by Righetti et al. [10]. It is important to note that under the conditions described, and with no exceptions, reactions were linear with time for at least 10 min. One enzyme

unit is defined as the amount of the enzyme that produces 1 μmol urea per min at 37° C.

In kinetic experiments designed to obtain an estimation of the K_m value for arginine, the same strip of support was used in the entire experiment. Thus, after each incubation the support was removed and washed with 50 mM glycine buffer (pH 9.5) and then incubated again with the next higher substrate concentration. When the experiment was complete, it was repeated again with the same strip of support employed in the initial experiment.

Treatment with EDTA was performed essentially as described by Carvajal et al. [5]. In a typical experiment, the strip of enzyme-containing support was suspended in 2 ml of a solution of 30 mM EDTA and 50 mM Tris · HCl (pH 8.0). After incubation for 40 min at 37° C with constant stirring, the support and the surrounding solution were both transferred to the same dialysis bag and exhaustively dialyzed for 24 h at 4° C against bidistilled water. After this treatment, the support was washed with 50 mM Tris · HCl (pH 8.7), and then suspended in 2 ml of the same buffer solution. The solution in the dialysis bag was made 50 mM in Tris and the pH adjusted to 8.7 and throughout this work it will be referred to as the solubilised protein fraction.

Reactivation with Mn^{2+} was performed by incubation of the matrix-bound or solubilised protein with 5 mM $MnCl_2$ in 10 mM Tris · HCl (pH 8.7) for 20 min at 37° C. Further increase of activity was not observed after longer incubation, even with higher concentrations of the metal ion.

Protein concentrations were determined by the method of Lowry et al. [11].

[*guanido*-^{14}C] Arginine was obtained from Nuclear-Chicago Corporation. Unlabeled arginine, glycine and Trizma Base were purchased from Sigma Chemical Co., glutaraldehyde from Matheson, Coleman and Bell. All other chemicals were analytical grade or of the highest purifity available. Type 66 nylon was obtained from a local textile industry.

Results

Treatment of matrix-bound arginase with EDTA

Matrix-bound tetrameric arginase was prepared and treated with EDTA as described under Materials and Methods to remove those subunits not covalently attached to the matrix. After the final dialysis, the enzymatic activity remaining bound to the matrix and that associated to the solubilised protein were measured both in the absence of added metal ions and after activation with Mn^{2+}. The results obtained in these experiments are presented in Table I, from which it can be seen that the matrix-bound and the solubilized protein were active only in the presence of Mn^{2+}. This result is in accordance with previous information concerning the soluble enzyme [5] and indicates that after the treatment with EDTA, matrix-bound and soluble subunits were obtained from the immobilised tetramer.

Table I also shows that in the presence of Mn^{2+}, the matrix-bound subunits recover a level of enzymatic activity equal to 24.6% of that of the initial immobilised tetramer. Under the conditions used, partial inactivation of the soluble subunits, but not of the immobilised subunits, occurred, and this would explain the incomplete recovery of activity when compared with the activity

TABLE I

TREATMENT OF IMMOBILISED TETRAMER WITH EDTA

Treatment with EDTA was performed as described under Materials and Methods. Enzymatic activities were assayed both in the absence of added Mn^{2+} and after activation with 5 mM $MnCl_2$ in 10 mM Tris · HCl (pH 8.7) for 20 min at 37°C. In this table these are referred to as activities assayed without and with Mn^{2+}, respectively. The substrate concentration was 100 mM and the buffer was 50 mM glycine/NaOH (pH 9.5).

Conditions	Enzymatic activity (total units)			
	Bound protein		Solubilized protein	
	without Mn^{2+}	with Mn^{2+}	without Mn^{2+}	with Mn^{2+}
Before treatment	3.36	3.37	—	—
After treatment	0	0.83	0	2.18

of the initially bound tetrameric enzyme. In any case, this recovery was never lower than 85% and always a quarter of the initial activity was found associated with the matrix.

After the dissociating treatment, followed by reactivation with Mn^{2+}, 25% of the activity bound to the matrix would be expected on the basis of a change from a tetrameric to a monomeric state with no difference in the specific activity of these immobilised species. Then, the actual value found here (24.6%) strongly suggests that the tetramer was bound to the matrix via a single subunit and therefore that the reactivated, matrix-bound subunits represent monomers of arginase. Evidence for an equal specific activity of bound tetramer and reactivated bound subunits will be presented later in this paper.

It is important to note that the activity of the immobilised tetramer was the same when assayed in the absence of added Mn^{2+} or after an activation treatment with Mn^{2+} (Table I). Then, the amount of manganese bound to the immobilised tetramer was enough to make the enzyme fully active. This is in accordance with previous information concerning the soluble tetrameric enzyme [5].

Reassociation experiments

The reversibility of the dissociation process was also investigated. For this purpose, the Mn^{2+}-reactivated, matrix-bound species obtained after the dissociating treatment were incubated with soluble subunits in 10 mM Tris · HCl (pH 8.7) for 6 h at 4°C, first in the absence of manganese and then in the presence of 5 mM $MnCl_2$. Soluble subunits were obtained as already described [5]. Since in the presence of Mn^{2+} the soluble subunits also associate with one other, the solution containing the subunits was changed several times and this was followed by the addition of the metal ions. In this way, we intended to increase the amount of subunits associating with matrix-bound protein. After the specified 6 h of incubation, the support was washed repeatedly with 50 mM Tris · HCl (pH 8.7) and then assayed for activity. The assays with the support previously incubated with subunits in the absence of Mn^{2+} were performed after activation with 5 mM $MnCl_2$. The results obtained in these

TABLE II

REASSOCIATION EXPERIMENTS

The reactivated bound subunits were incubated with soluble subunits in the presence and absence of Mn^{2+} as described in the text. The active bound species employed were the same as described in Table I, obtained from a tetramer with an enzymatic activity corresponding to 3.36 units.

Conditions	Enzymatic activity (total units)
Control (before reassociation)	0.83
After incubation with soluble subunits	
in the absence of Mn^{2+}	0.82
in the presence of Mn^{2+}	3.34

experiments are presented in Table II, where it can be seen that: (a) the activity bound to the matrix increased only when the incubation with the subunits was performed in the presence of Mn^{2+}, and (b) the activity of the reassociated matrix-bound enzyme is nearly the same as that of the original immobilised tetramer.

As expected, when the reassociated matrix-bound enzyme was again treated with EDTA, the activity measured in the presence of manganese corresponded to a 25% of the starting bound activity.

Amount of protein solubilised after treatment with EDTA

Due to the type of support we used, the entire sequence of experiments described above was always performed with the same strip of nylon mesh, so we could not measure directly the amount of protein bound to the matrix

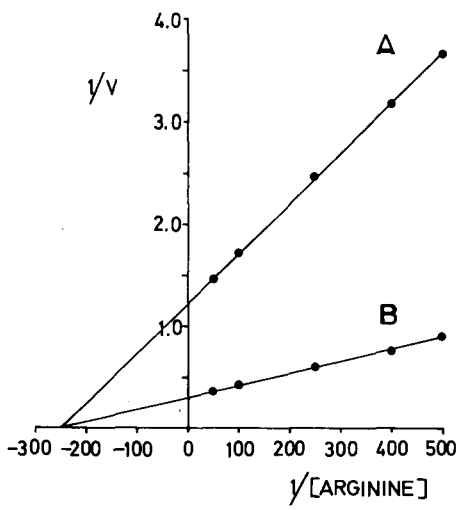

Fig. 1. Dependence of arginase activity on arginine concentration. A, reactivated bound subunits; B, immobilised tetramer. Velocity is expressed as μmol urea produced per min and arginine concentrations in molar units. With reassociated matrix-bound enzyme, the results were almost exactly the same as those shown for immobilised tetramer.

before and after the treatment with EDTA. However, we measured the total amount of protein solubilised after the dissociating treatment. This was done both with the starting matrix-bound tetramer and with the 'reassociated matrix-bound enzyme', and the values obtained were 10.8 and 10.4 μg of protein respectively. Clearly, in the reassociation experiments, the bound species picked up an amount of protein and enzymatic activity almost equal to that initially lost by the immobilised tetramer.

The suggestion made here that the specific activity of the bound tetramer and reactivated bound subunits are the same seems then strongly favoured.

Michaelis constant of immobilised species for arginine

To obtain some information on the enzyme properties of the immobilised species described here, their K_m values for arginine were determined. From the data presented in Fig. 1 the same value (4 mM) was calculated for the bound tetramer, reactivated bound subunits and the reassociated bound enzyme. The same value was obtained for the soluble enzyme and this is in accordance with the value reported by Bascur et al. [7].

Discussion

The experiments reported here corroborate the conclusion which we presented previously [5], namely that manganese plays a role in the maintenance of the quaternary structure of human liver arginase. The present study further shows that activating and structural effects of manganese on arginase can be analysed as separate phenomena.

Reactivation of the subunits of arginase apparently involves the initial formation of an active monomer of the enzyme, with these active low molecular weight species then acquiring a conformation that favours their association to the tetrameric state. This suggestion is based on the following findings reported here: (a) matrix-bound subunits, as well as those in solution, are inactive in the absence of Mn^{2+}; (b) immobilised subunits, which are prevented from reassociation, become catalytically active when they add Mn^{2+}; and (c) association of bound monomers with soluble subunits, to regenerate a matrix-bound tetramer, only occurs when manganese is included in the incubation mixture.

Low molecular weight species of arginase have repeatedly been detected by others [12—16]. However, in most cases, the question whether these species were active or whether association to an active molecule must occur, remained unsolved. The most clear exceptions are the experiments reported by Vielle-Breitburd and Orth [15], suggesting an active monomer of rabbit liver arginase and the isolation of a low molecular weight arginase from the earthworm gut [16]. The approach introduced by Chan [1] and applied here might be used to separate activating and structural effects of manganese on other arginases not yet studied in this respect. The principal advantage of this method is that the oligomeric and monomeric forms can be compared under normal assay conditions, and would permit, in the future, analysis in more detail of the specific role played by the metal ion in the catalytic action of arginase. Experiments of this kind are now in progress in our laboratory.

Acknowledgements

This work was supported by Public Health Service Grant 5R 22 3741-12 from the National Institutes of Health. The authors wish to thank Dr. C.C. Allende for helpful discussions in the preparation of the mansucript. The technical assistance of Mr. R. Campos is also appreciated.

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