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SUBUNIT INTERACTIONS AND IMMOBILISED DIMERS OF HUMAN LIVER ARGINASE

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Summary

Incubation of soluble human liver arginase (L-arginine amidinohydrolase, EC 3.5.3.1) with *p*-hydroxymercuribenzoate resulted in the dissociation of the enzyme into active dimers. Addition of 2-mercaptoethanol resulted in the regeneration of the tetrameric enzyme. When arginase, bound covalently to nylon, was incubated with *p*-hydroxymercuribenzoate, matrix-bound dimers were obtained. Incubation of these species with 2-mercaptoethanol resulted in stable, unmodified dimers. Based on this dissociation of arginase, a model with D_2-symmetry is suggested for this enzyme.

The specific activity, the K_m value for arginine, pH optimum and the inhibition constants for ornithine and lysine were determined for monomeric, dimeric and tetrameric forms. It is concluded that the behaviour of the active sites of the monomers is not substantially altered by the interaction of these species in the oligomeric molecule.

Introduction

We are studying the relationship between the quaternary structure of human liver arginase [1] and its biological function. It was decided to examine whether subunit interactions have any effect on the catalytic aspects of the enzyme. For this purpose, the approach of matrix-bound derivatives [2—7] was used. A previous report described the immobilisation of arginase on nylon and evidence was presented for the catalytic activity of isolated monomers of the enzyme [8]. These studies also revealed the same K_m value and specific activity for the immobilised monomers and tetramers. Thus, in terms of these kinetic parameters, the behaviour of the active sites of the monomers seems to be unaffected by the interaction of these species in the oligomeric molecule. To

test this, we examined in more detail the enzyme properties of arginase in different states of aggregation. The present report describes a procedure for the preparation of immobilised dimers and a comparison of the enzymic properties of matrix-bound monomers, dimers and tetramers. A preliminary report of this work has been presented [9].

Materials and Methods

Enzyme purification, attachment of tetrameric arginase to strips of nylon mesh (2 X 1 cm) and preparation of matrix-bound monomers were performed as described [8]. The molecular form of human liver arginase designated enzyme II by Bascur et al. [10] was used throughout.

Treatment of immobilised tetramers with *p*-hydroxymercuribenzoate was carried out by suspending the enzyme-containing support in 3 ml 1 mM *p*-hydroxymercuribenzoate, 50 mM Tris-HCl (pH 8.0). After stirring for 30 min at 37°C, the support was removed and washed twice with a total of 6 ml 50 mM Tris-HCl (pH 8.0). The solution that surrounded the matrix-bound enzyme in the incubation was added to the washings of the support and the mixture was dialyzed against 0.1 mM Tris-HCl (pH 8.0). The solution was partially lyophilised to a small volume and assayed for enzymatic activity (designated solubilised protein) and protein. Protein was determined by the method of Lowry et al. [11] with bovine serum albumin as standard.

Activity was measured by suspending the enzyme-containing support in 2 ml 50 mM glycine buffer (pH 9.5) containing appropriate concentrations of arginine. Stirring was maintained magnetically. After 3 min at 37°C, aliquots were removed from the surrounding solution and assayed for urea with α-isonitrosopropiophenone [12]. Reactions were linear for at least 10 min. One unit of arginase is defined as the amount of enzyme that produces 1 μmol urea per min at 37°C.

Since the immobilised enzymes could be reused after the assays, the same strip of support was used to measure the K_m values and inhibition constants. After each incubation, the support was removed and washed with 50 mM glycine buffer (pH 9.5) and incubated under the next conditions. The same strip was also employed for a duplicate experiment. A single strip of support was also used to determine the pH optimum of the immobilised species. This was not necessarily the same as that used in the inhibition studies.

Molecular weights were determined by gel filtration on a Sephadex G-100 column (1.7 X 50 cm) equilibrated with 50 mM Tris-HCl (pH 8.0), containing 20 mM KCl [13]. The column was calibrated with Blue Dextran 2000, tetrameric human liver arginase, bovine serum albumin, ovalbumin and horse heart cytochrome *c*.

Amino acids, Trizma Base, proteins used as molecular weight markers and *p*-hydroxymercuribenzoate were purchased from Sigma Chemical Co. Glutaraldehyde was obtained from Matheson, Coleman and Bell. Sephadex G-100 and Blue Dextran 2000 were products of Pharmacia, Uppsala. All other chemicals were analytical grade. Type 66 nylon was obtained from a local textile industry.

Results

Effect of p-hydroxymercuribenzoate on soluble human liver arginase

It has been shown that depending on the organism used as a source, arginase is inhibited, unaffected or even stimulated by reaction with *p*-hydroxymercuribenzoate [14—17]. We observed that the activity of human liver arginase was not changed by incubation with 1 mM *p*-hydroxymercuribenzoate 50 mM Tris-HCl (pH 8.0) for 30 min at 37°C. However, this treatment resulted in the dissociation of the enzyme into dimers with a molecular weight of about 61 000 as determined by gel filtration on Sephadex G-100.

The dissociated enzyme was incubated with 1 mM 2-mercaptoethanol 50 mM Tris-HCl (pH 8.0) for 30 min at 15°C and on gel filtration, complete regeneration of the tetrameric enzyme (M_r 118 000) was observed.

Preparation of immobilised dimers of arginase

Treatment of matrix-bound arginase with p-hydroxymercuribenzoate. When matrix-bound tetrameric arginase was treated with *p*-hydroxymercuribenzoate, active protein was dissociated from the matrix and nearly a half of the starting bound activity remained as immobilised enzyme. This is shown in Table I (Expt. 1) where it can be seen also that the activities of the solubilised and bound species were not affected by 2-mercaptoethanol. Repeated washings of

TABLE I

DISSOCIATION AND REASSOCIATION OF MATRIX-BOUND ARGINASE

The *p*-hydroxymercuribenzoate-treated enzyme was also assayed after incubation with 0.1 mM 2-mercaptoethanol in 50 mM Tris-HCl (pH 8.0) for 10 min at 37°C. Treatment with EDTA was performed as described previously [8] but including 0.1 mM 2-mercaptoethanol in the incubation with the chelating agent. Activities were assayed in the absence of added Mn^{2+} and after incubation with 5 mM $MnCl_2$ for 20 min at 37°C. In reassociation experiments the bound enzyme was incubated with soluble subunits in the presence of 1 mM $MnCl_2$ in 10 mM Tris-HCl (pH 8.7) for 6 h at 4°C in the manner described previously [8]. Reassociated, matrix-bound enzyme refers to the species obtained from the bound derivative previously incubated with 2-mercaptoethanol. In these experiments, the activity of the starting bound tetramer was 3.08 total units. The substrate concentration was 100 mM and the buffer was 50 mM glycine/NaOH (pH 9.5).

	Enzymatic activity (total units)	
	Bound protein	Solubilised protein
Experiment 1		
Untreated tetramer	3.10	—
p-hydroxymercuribenzoate-treated enzyme	1.53	1.40
+ 2 mercaptoethanol	1.53	1.39
Experiment 2		
p-hydroxymercuribenzoate-treated enzyme	1.53	
After treatment with EDTA		
$-Mn^{2+}$	0	
$+ Mn^{2+}$	0.76	
Experiment 3		
p-hydroxymercuribenzoate-treated enzyme	1.51	
Reassociated matrix-bound enzyme	3.05	

the supports with 50 mM Tris-HCl (pH 8.7) did not alter the activity. Likewise, a second treatment with *p*-hydroxymercuribenzoate did not affect the level of bound enzyme.

Subunit composition of the p-hydroxymercuribenzoate-treated, matrix-bound enzyme. We showed previously that matrix-bound arginase treated with EDTA releases those subunits not covalently attached to the matrix and that in the presence of Mn^{2+}, the bound subunits recovers 25% of the activity of the starting bound tetramer [8]. The subunit composition of the *p*-hydroxymercuribenzoate-treated, immobilised enzyme was thus analyzed after EDTA treatment. The treatment with EDTA was performed in the presence of 0.1 mM 2-mercaptoethanol. As shown in Table I (Expt. 2), after the addition of Mn^{2+} the bound subunits recovered the activity expected for a change from dimers to monomers.

Reassociation experiments. The *p*-hydroxymercuribenzoate-treated derivative was incubated with soluble subunits both in the presence and in the absence of 1 mM $MnCl_2$. The matrix-bound activity increased only when the derivative was previously incubated with 2-mercaptoethanol and when Mn^{2+} was present in the incubation with soluble subunits. As shown in Table I (Expt. 3), the activity of the reassociated matrix-bound enzyme is nearly the same as that of the original immobilised tetramer. Under the same conditions, the activity of matrix-bound tetramers was not affected. These results and those obtained with the soluble enzyme indicates that after the addition of 2-mercaptoethanol, unmodified matrix-bound dimers are formed. These species were unable to interact with soluble tetramers.

The results obtained with matrix-bound arginase are represented schematically in Fig. 1.

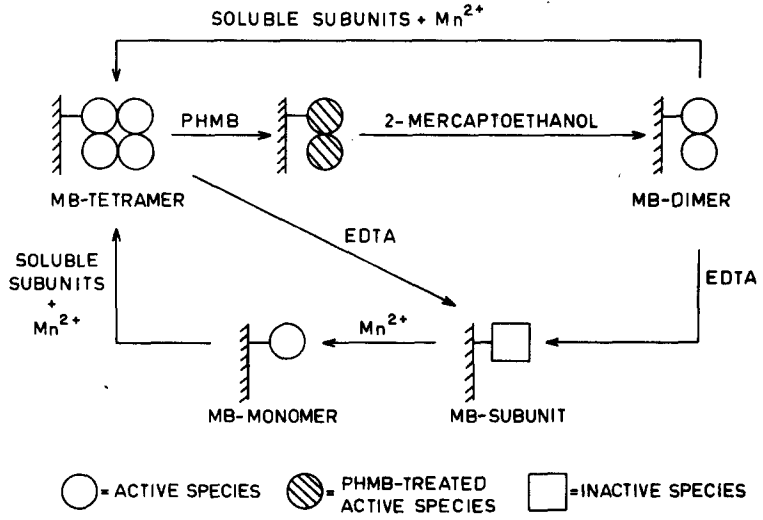

Fig. 1. Scheme representing the relationship between the matrix-bound (MB-) derivatives of arginase. PHMB = *p*-hydroxymercuribenzoate.

TABLE II

ENZYMIC PROPERTIES OF MATRIX-BOUND MONOMERS, DIMERS AND TETRAMERS

To determine the Michaelis constant, the concentration of arginine was varied from 2 to 20 mM. For K_i values, the concentration of lysine or ornithine was varied from 2 to 20 mM and the concentrations of arginine were 2, 5, 10 and 20 mM. K_i values were determined by graphical methods [18]. The buffer used in kinetic experiments was 50 mM glycine/NaOH (pH 9.5). For pH optimum, the buffer used were: 50 mM Tris-HCl (pH 7.0 to 8.7) and 50 mM glycine/NaOH (pH 8.7 to 10.0).

Matrix-bound derivative	K_m (mM)	K_i, Lysine (mM)	K_i, ornithine (mM)	pH optimum
Monomers	4.0	2.2	4.1	9.5
Dimers	4.0	2.4	4.3	9.5
Tetramers	4.0	2.3	4.2	9.5

Specific activity of the dissociated species

Because of the kind of support used, we could not measure directly the protein bound to the matrix before and after the treatment with *p*-hydroxymercuribenzoate. However, we measured the protein solubilised when the starting bound tetramer and the reassociated bound enzyme were converted to matrix-bound dimers. The values obtained were 6.2 and 6.4 μg protein, respectively. Therefore, in the reassociation experiments, the immobilised species picked up from the solution an amount of protein and enzymatic activity almost equal to that initially lost by the matrix-bound tetramer. The specific activity of the immobilised tetramers and dimers seems then to be the same.

Enzymic properties of matrix-bound species

In the following experiments the dissociated species were analyzed under conditions in which they normally exist as tetramers. Therefore, unmodified matrix-bound dimers were used.

Kinetic studies. With all three immobilised species lysine was a competitive inhibitor and ornithine a non-competitive inhibitor. As shown in Table II, no differences are seen between the matrix-bound forms with respect to inhibition constants and K_m values.

pH profiles. The same strip of enzyme-containing support was used for all pH values. The support containing the particular bound species was incubated for 15 min at 37°C in solutions at pH from 7 to 10 and then the activity was assayed at pH 9.5 to confirm that no loss of activity occurs. As shown in Table II the same pH optimum of 9.5 was obtained for the monomeric, dimeric and tetrameric forms.

The same results presented in Table II were obtained with the bound reassociated tetramers obtained after incubation of the bound monomers or dimers with soluble subunits in the presence of Mn^{2+}. The values in Table II also agree with those reported for the soluble enzyme [10].

Discussion

The experiments presented here and those reported earlier [8] indicate that the monomers of human liver arginase express the same activity and have equal

kinetic characteristics regardless of whether they exist singly or as a part of an oligomeric structure.

Subunit interactions seems then to have little or no effect on the catalytic aspects of human liver arginase. Therefore, as with other oligomeric enzymes which show no cooperativity, the significance of the tetrameric structure of arginase remains to be determined. An interesting possibility is that the quaternary structure of arginase is required to interact with other functionally associated enzymes. An interaction of this kind occurs in *Saccharomyces cerevisiae* [19—21] and provides an 'epi-enzymatic' regulatory mechanism, which is mediated by the stoichiometric binding of ornithine carbamoyltransferase with arginase. A similar regulatory mechanism has been shown in *Bacillus subtilis* [22]. In the liver cells, the intracellular compartmentation of the urea cycle enzymes prevent the physical integration of these two enzymes. However an interaction of arginase with the cytosol enzymes involved in the formation of arginine from citrulline is possible. Soberón et al. [23—24] have postulated that, in order to function efficiently, the urea cycle enzymes should be physically linked. These researchers reported that in rat liver homogenates arginase seems to be more readily available for arginine generated from citrulline than from a different source [25]. It would be of interest, therefore, to study whether a binding capacity for some other functionally associated enzymes also exist in mammalian arginase, as suggested by Soberón et al. [23—25]. It would then be possible to understand the physiological significance of the quaternary structure of the enzymes in these systems.

In this work, we have used the dissociating effect of *p*-hydroxymercuribenzoate on human liver arginase to prepare matrix-bound dimers of the enzyme and attention was not focused on more specific aspects of the dissociation process. In any case, it is clear that at least two kinds of binding sets can be distinguished in this dissociation. On this basis, a model with D_2 symmetry is highly favoured for human liver arginase. Future work will be directed to analyze the mechanism of the dissociation process and to study in more detail the molecular symmetry predicted from the studies reported here.

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References

1 Carvajal, N., Venegas, A., Oestreicher, G. and Plaza, M. (1971) Biochim. Biophys. Acta 250, 437—442
2 Chan, W.W.C. (1970) Biochem. Biophys. Res. Commun. 41, 1198—1204
3 Chan, W.W.C. and Mawer, H.M. (1972) Arch. Biochem. Biophys. 149, 136—145
4 Chan, W.W.C., Schutt, H. and Brands, K. (1973) Eur. J. Biochem. 40, 533—541
5 Chan, W.W.C. (1976) Can. J. Biochem. 54, 521—528
6 Fell, D.A. and Brandon, C.J. (1975) Biochem. Biophys. Res. Commun. 67, 1013—1018
7 Nagradova, N.K., Golovina, T.O. and Mevkh, A.T. (1974) FEBS Lett. 49, 141—145
8 Carvajal, N., Martínez, J. and Fernández, M. (1977) Biochim. Biophys. Acta 481, 177—183
9 Martínez, J., Carvajal, N., Fernández, M., Montes de Oca, F. and Rodríguez, J. (1977) Abstract from the second Regional Meeting of P.A.A.B.S., Córdoba, Argentina

10 Bascur, L., Cabello, J., Véliz, M. and González, A. (1966) Biochim. Biophys. Acta 128, 149—154
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Archibald, R.M. (1945) J. Biol. Chem. 157, 507—518
13 Andrews, P. (1964) Biochem. J. 91, 223—233
14 Mora, J., Tarrab, R. and Bojalil, L.F. (1966) Biochim. Biophys. Acta 118, 206—209
15 Hartenstein, R. (1971) Comp. Biochem. Physiol. 40B, 781—795
16 Reddy, S.R.R. and Campbell, J.W. (1969) Comp. Biochem. Physiol. 28, 515—534
17 O'Malley, K.L. and Terwilliger, R.C. (1974) Biochem. J. 143, 591—597
18 Dixon, M., and Webb, E.C. (1964) Enzymes, 2nd edn. pp. 315—331, Academic Press, New York
19 Wiame, J.M. (1971) Curr. Top. Cell. Regul. 4, 1—38
20 Messenguy, F., Penninckx, M. and Wiame, J.M. (1971) Eur. J. Biochem. 22, 277—286
21 Penninckx, M. (1975) Eur. J. Biochem. 58, 533—538
22 Issaly, J.M. and Issaly, A.S. (1974) Eur. J. Biochem. 49, 485—495
23 Soberón, G., Ortíz Pineda, J. and Tarrab, R. (1967) Monogr. Nat. Cancer Inst. No. 27, p. 283
24 Soberón, G., Tarrab, R. and Palacios, R. (1969) Bol. Estud. Med. Biol. Mex. 26, 15
25 Palacios, R., Huitrón, C. and Soberón, G. (1970) Biochem. Biophys. Res. Commun. 38, 438—443