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A CRITICAL HISTIDINE RESIDUE IN ARGINASE FROM *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; arginase; histidine; diethyl pyrocarbonate.

Abstract—Arginase from *Phaseolus vulgaris* was inactivated by diethyl pyrocarbonate (DEPC). The bimolecular rate constant for inactivation by DEPC was 1300 M^{-1} min^{-1} and the reaction order with respect to DEPC concentration was *ca* 1. The inactivation followed a titration curve for a residue with a pK_a of 6.7 ± 0.1 at 25° and the enzymatic activity was completely restored by hydroxylamine. Results are taken as evidence for a critical, but not essential, histidine residue in the enzyme. © 1997 Elsevier Science Ltd

INTRODUCTION

Arginase (L-arginine urea amidino hydrolase, EC 3.5.3.1) is a widespread enzyme that catalyses the hydrolysis of L-arginine to L-ornithine and urea, and serves several functions in living organisms [1]. In plants cells, it is mainly involved in the production of proline and glutamate [2] and also of polyamines [3], which are important in various physiological processes in these species [4, 5].

One general property of arginase is the requirement for a bivalent metal ion, especially Mn^{2+}, for catalytic activity and structural stabilization of the enzyme [6]. On the other hand, several arginases have been cloned and their deduced amino acid sequences shows regions of high homology [7, 8], indicative of some common structural features. A critical histidine residue has been implicated by chemical modifications studies of arginase from some animal species [9–11] and site directed mutagenesis of rat liver arginase [9]. This histidine residue (His141 in the sequence of rat liver arginase), is conserved in all the arginases from bacteria, fungi and animals thus far examined [7, 8]. It is, however, absent from the sequence of the enzyme from *Arabidopsis thaliana* [12], the only plant arginase which has been cloned. Moreover, there are no reports of chemical modification studies of arginases from plant tissues. For this reason, and as a part of our studies addressed to clarify the catalytic and molecular aspects of arginase from *P. vulgaris* [5], we have now examined the effect of diethyl pyrocarbonate (DEPC) on its catalytic activity. In agreement with reports for other arginases [9, 10], we conclude that a DEPC-sensitive histidine residue is important, but not essential for activity of *P. vulgaris* arginase.

RESULTS AND DISCUSSION

Treatment of *P. vulgaris* arginase with DEPC led to inactivation which was dependent both upon the time and reagent concentration. However, even with a very large excess of DEPC, the effect reached a limiting value at about 95% inactivation. The loss of enzyme activity followed pseudo first order kinetics [Fig. 1(a)], and double-logarithmic plots of the observed pseudo-first-order rate constants against DEPC concentration [Fig. 1(b)] yielded an order of *ca* 1, indicating that modification of a single residue resulted in the loss of enzyme activity [13]. On the other hand, the results shown in [Fig. 1(c)] indicated that no reversible complex between arginase and DEPC is formed, and allowed the determination of a second order inactivation constant of 1300 M^{-1} min^{-1}.

The enzymatic activity of arginase inactivated by DEPC was totally recovered after incubation with 15 mM hydroxylamine for 12 hr at 4°. In addition to this, the pseudo-first order rate constant declined with pH in the range pH 5.5–7.0 and the calculated pK_a value for the residue whose modification inactivates the enzyme, was 6.7 ± 0.1 at 25°. These results favour the conclusion that a histidine residue is involved in inactivation of the enzyme by DEPC. Although DEPC shows strong selectivity for the modification of hystidyl residues in proteins, it also reacts with other amino acid residues. However, only modified hystidyl

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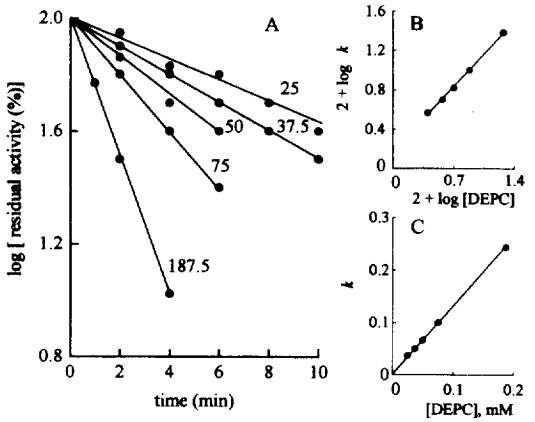

Fig 1. (A) Pseudo-first order inactivation of *P. vulgaris* arginase by diethyl pyrocarbonate (DEPC). The concentrations of DEPC, in μM units, are given by the numbers on the lines. (B) Order of the inactivation of arginase with respect to concentration of DEPC; concentration is expressed in mM units. (C) Dependence of the pseudo-first order rate constants for inactivation (k) on the concentration of DEPC. Inactivation was performed in 50 mM K-Pi buffer (pH 7.0).

and tyrosyl residues can be regenerated by treatment with hydroxylamine [14]. On the other hand, the pK_a value is within the expected range for histidine residues in proteins [15] and the pH dependence of the inactivation rate is expected if one considers that DEPC reacts with the unprotonated imidazole ring [16].

Protection against inactivation by substrates and competitive inhibitors can give information of the location and possible roles of specific amino acid residues on the enzyme. Arginase from *P. vulgaris* is characterized by high values of K_m for arginine and of K_i for the competitive inhibitor lysine [5]. As an example, the K_m for arginine is about 93 mM at pH 7.5 [5]. Therefore, very high concentrations would be required to observe any significant effect of arginine or lysine on the rate of inactivation of the enzyme by DEPC. However, any attempt to investigate this aspect was impractical because the required high concentrations of the amino acids reacted with DEPC [14], producing apparent protection by lowering the effective concentration of DEPC. This was an impediment to observe any effect of arginine or lysine on the rate of inactivation produced by chemical modification of the DEPC-sensitive histidine residue.

DEPC also inactivates other arginases [9–11] and site directed mutagenesis has identified His141 as the DEPC-sensitive residue that is critical in the catalytic mechanism of rat liver arginase [9, 10]. Since this histidine residue is conserved among most of the arginases examined to date [7, 8], we may assume that one equivalent residue corresponds to the DEPC-sensitive histidine in *P. vulgaris* arginase. Moreover, our present findings that *P. vulgaris* arginase is only partially inactivated by DEPC, is in agreement with a previous conclusion that His141 contributes to, but is not essential for, the catalytic activity of the rat liver enzyme [9, 10]. The non-essentiality of this residue is also in agreement with its absence from the sequence of arginase from *A. thaliana* [12]. According to a recently postulated mechanism, the DEPC-sensitive histidine may serve as a catalytic proton shuttle from bulk solvent to the ε-amino group of ornithine, before product dissociation [17]. Direct proton transfer with bulk solvent would explain the significant residual activity of rat liver arginase in which His141 has been chemically modified [9, 10] or changed by mutation [9]. The residual activity of DEPC-inactivated arginase from *P. vulgaris* would be also explained in the same manner.

EXPERIMENTAL

Materials. Diethyl pyrocarbonate (DEPC) and all biochemicals were obtained from Sigma. All other chemicals were of the highest purity obtained commercially and were used without further purification. Seeds of *P. vulgaris* were obtained from local farmers and arginase was purified as described in ref. [5]. The enzyme used in these studies was previously activated by incubation with 2 mM $MnCl_2$ for 10 min at 37° in 50 mM Tris–HCl (pH 8.7). The enzyme activated in this manner, expressed maximal activity.

Inactivation by DEPC. Reactions with DEPC, were started by the addition of the chemical modifier to the enzyme in the appropriate buffer soln at 25°. Several times, aliquots were removed, quenched by addition of 5 mM imidazole and assayed for enzymatic activity, following the formation of urea from arginine [5]. Due to the reversal of the DEPC modification of histidine at the pH optimum of 9.6, arginase activity was determined in 50 mM K-Pi buffer (pH 7.5). $MnCl_2$ (final concn, 2 mM) was always added to the substrate buffer soln.

The pH dependence of the inactivation reaction was determined in 50 mM K-Pi buffer (pH 5.5–7.5); routinely, reactions with DEPC were carried out at pH 7. For the reactivation experiments, the enzyme was inactivated with 0.3 mM DEPC until a *ca* 10% residual activity. Then, 15 mM neutral NH_2OH was added and activity recovery at 4° was monitored at specific time intervals.

The concns of solns of DEPC, prepd in ice-cold EtOH just before use, were evaluated by reacting aliquots with 10 mM imidazole at pH 7 ($\varepsilon_{242\ nm}$ = 3200 M^{-1} cm^{-1}) [14]. Under the conditions used in this study, the activity of arginase was not altered by EtOH in the absence of DEPC.

Data analysis. All curves were computer fitted to the appropriate equations [13]. The pseudo-first-order rate constants (k) of inactivation were calcd from the slope of the plots of logarithm of the residual activity against the time of reaction. The order of the reaction was estimated from the slopes of the double logarithmic pots of k *vs* DEPC concn. The second-order rate constant was calcd from the slopes of the linear

plots of k against DEPC concn. The pH dependence of the inactivation reaction was analysed using the program ENZFITTER (Biosoft, Elsevier, Cambridge).

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