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PIG KIDNEY DOPA DECARBOXYLASE: INACTIVATION BY IODOACETAMIDE AND SEQUENCE OF THE CARBOXYAMIDOMETHYLCYSTEINE-CONTAINING PEPTIDE

PAOLA DOMINICI*, MAURIZIO SIMMACO‡, BRUNELLA TANCINI*, DONATELLA BARRA‡ and CARLA BORRI VOLTATTORNI**

** Department of Experimental Medicine and Biochemical Sciences, University of Perugia, 06100 Perugia (Italy) and ‡ Department of Biochemical Sciences and Center of Molecular Biology, University of Roma "La Sapienza", Roma (Italy)*

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Pig kidney 3,4-dihydroxyphenylalanine (Dopa) decarboxylase is inactivated by iodoacetamide following pseudo-first order reaction kinetics. The apparent first order rate constant for inactivation is proportional to the concentration of iodoacetamide and a second order rate constant of 37 $M^{-1} min^{-1}$ is obtained at pH 6.8 and 25 °C. Cyanogen bromide fragmentation of iodo(1-^{14}C)acetamide – modified inactivated Dopa decarboxylase followed by trypsin digestion yields a single radioactive peptide. Automated Edman degradation reveals a heptapeptide sequence which contains labeled carboxyamidomethylcysteine. This finding and the results of the incorporation of the label from iodo (1-^{14}C)acetamide into the enzyme clearly indicate that the modification of 1 mol of SH per mol of enzyme dimer is responsible for the inactivation process. The labeled peptide, which was located by means of limited proteolysis on the fragment corresponding to the COOH-terminal third of the enzyme, has been aligned with a 7 amino acid stretch of Drosophila enzyme. Although this region appears highly conserved in the Dopa decarboxylase enzymes, the cysteinyl residue is not conserved. This observation together with the spectral binding properties of the iodoacetamide inactivated enzyme argue against a functional role for the modifiable cysteine in the mechanism of action of pig kidney enzyme. It is suggested that the loss of pig kidney decarboxylase activity produced by iodoacetamide modification might be attributable to steric hindrance. This could be due to the presence of the bulky acetamidic group on a cysteine residue at, or near, the active center or in a site of strategic importance to the maintenance of the active site topography.

KEY WORDS: Dopa decarboxylase, iodoacetamide, chemical modification, high-reactive cysteine, peptide sequence

INTRODUCTION

Since its discovery by Holtz *et al.* in 1938[1], Dopa decarboxylase (EC. 4.1.1.28) has been thoroughly investigated in terms of substrate specificity, inhibition and physicochemical properties[2]. Pig kidney has most frequently been used as a source of this pyridoxal-P enzyme, due to the relative abundance of the enzyme in this tissue and the good yield of the purification procedure[3]. Information has only recently been provided as to the nature of the residues at the active center of pig kidney Dopa decarboxylase. Evidence has, indeed, been presented for the location of one arginyl[4] and one histidyl residue[5] at, or near, the active center. In a continuing effort to

** Correspondence.

elucidate the chemical environment of the coenzyme-binding region, we have performed a chemical modification study with a sulfhydryl-selective reagent, iodoacetamide, in order to explore the possible involvement of cysteine residue(s) in the catalytic mechanism and/or in the binding of substrates and coenzyme. Such an occurrence would not be unexpected since previous work has indicated that Dopa decarboxylase is a sulfhydryl-containing protein and that -SH groups are required for the maintenance of decarboxylase activity. It is noteworthy that the enzyme is inhibited by a variety of sulfhydryl reagents as reported by Christenson *et al.*[6]

In this paper, we show that iodoacetamide reacts with a single sulfhydryl group which results in complete loss of enzymatic activity. The amino acid sequence of a heptapeptide containing this iodoacetamide-cysteinyl residue was determined. The comparison of the structure of this peptide with the DNA sequence of the corresponding Drosophila enzyme[7] allowed us to locate this peptide fragment in the pig kidney enzyme's primary structure. Moreover, the role of this sulfhydryl group is discussed taking into account both the effects produced by iodoacetamide modification and the observed substitution of the cysteinyl residue in pig kidney enzyme with an alanyl residue in Drosophila enzyme.

MATERIALS AND METHODS

Materials

L-5-hydroxytryptophan (L-5HTP), L-Dopa, D-Dopa, pyridoxal-P and 2,4,6-trinitrobenzene-1-sulfonic acid were obtained from Sigma Chemical Company; iodoacetamide and iodoacetic acid, purchased from Fluka, were recrystallized from diethyl ether. Iodo(1-^{14}C)acetamide (24.4 mCi/mmol) was obtained from New England Nuclear. Other chemicals were of the highest grade available from commercial sources.

Dopa decarboxylase was purified from pig kidney by the method of Borri Voltattorni *et al.*[8] and its concentration was expressed as bound coenzyme determined by releasing the bound pyridoxal-P into 0.1 N NaOH using 6600 $M^{-1}\,cm^{-1}$ as the molar extinction coefficient for pyridoxal-P concentration. The enzyme was depleted of excess dithiothreitol by exhaustive dialysis against 100 mM potassium phosphate buffer, pH 6.8, immediately before use.

Spectral data were obtained in a Cary 219 spectrophotometer equipped with a thermostatted cell compartment.

Enzyme assay and inactivation

Enzymatic activity was measured by the assay method of Sherald *et al.*[9], according to the modifications introduced by Charteris and John[10]. The standard reaction contained in a final volume of 250 μl: 100 μl of 0.1 M potassium phosphate buffer, pH 6.8, 25 μl of 50 mM L-Dopa, and enzyme (5–7 μg). After incubation at 25 °C for 5 min, the reaction was stopped by heating at 100 °C for 1 min. Benzene (1.5 ml) and 2.4.6-trinitrobenzene-1-sulfonic acid (1 ml of a 43 mM solution in 0.1 M potassium phosphate buffer, pH 7.5) were added and the trinitrophenylamine derivative was determined in the benzene layer with a Beckman DB-GT spectrophotometer using 12.400 as the molar extinction coefficient for trinitrophenyldopamine[11]. The inactivation of Dopa decarboxylase was carried out by incubating the enzyme with an

appropriate amount of iodoacetamide in 100 mM potassium phosphate buffer, pH 6.8, at 25 °C. The extent of inactivation was monitored by measuring residual enzyme activity, as described previously, in 5 μl aliquots removed at appropriate times from the reaction mixture. In the enzyme protection experiments, substrates or substrate analogs were incubated during the preincubation period prior to the addition of the chemical modifier.

Incorporation of iodo(1-^{14}C)acetamide into Dopa decarboxylase

Dopa decarboxylase (10–12 nmol) was incubated with 0.5 mM iodo(1-^{14}C)acetamide (1.15×10^6 cpm/μmol) in 0.7 ml of 100 mM potassium phosphate buffer, pH 6.8, at 25 °C. At intervals, aliquots (0.15 ml) were withdrawn, cooled to 0 °C and 0.15 mg of bovine serum albumin was added. Proteins were precipitated with trichloroacetic acid 20%(w/v), containing 0.1 M-mercaptoethanol, filtered through Millipore filters (type HA-0.45 μm) and washed with a 5% trichloroacetic acid solution until no radioactivity could be detected in the wash. Radioactivity was determined in 10 ml Packard emulsifier scintillator using an Intertechnique liquid scintillation counter. In some experiments unreacted thiol reagent was removed by gel filtration on Sephadex G-25 column (20×0.8 cm) using 100 mM potassium phosphate buffer, pH 6.8 for elution. Radioactivity was determined as described above.

Separation of fragments obtained by limited tryptic proteolysis of iodo(1-^{14}C)acetamide-treated Dopa decarboxylase.

Dopa decarboxylase (30 nmol) was incubated with 0.5 mM iodo(1-^{14}C)acetamide in 100 mM potassium phosphate buffer, pH 6.8, for 2 h at 25 °C. Excess iodoacetamide was eliminated by exhaustive dialysis against 10 mM sodium phosphate buffer, pH 7.0. The alkylated sample, which had incorporated approximately 1 mol of label per mol of protein, was treated with trypsin at a ratio of protease to Dopa decarboxylase of 1/50 (w/w). After 10 min at 25 °C, the reaction was stopped by the addition of soybean trypsin inhibitor, in a two-fold molar excess over trypsin, and the sample lyophilized. The residue was dissolved in 200 μl of 0.1% trifluoroacetic acid and chromatographed on a reverse-phase 10 μm macroporous Viosfer C_8 column (4×150 mm) and eluted with a linear gradient of water/acetonitrile containing 0.1% trifluoroacetic acid. The eluant was monitored at 280 nm and the radioactivity determined in a 50-μl of each fraction.

Isolation and sequence determination of the peptide containing the iodo(1-^{14}C)acetamide-modified amino acid residue.

A solution of 40 mg of Dopa decarboxylase, which has incorporated 1.07 mol iodo(1-^{14}C)acetamide/mol enzyme, was made 6 M in guanidine hydrochloride and adjusted to pH 8.0. After 50 min incubation at 30 °C, unlabeled iodoacetic acid was added to a final concentration of 50 mM. This solution was incubated for 90 min, dialysed against several changes of 5% acetic acid and lyophilized. The lyophilized protein was dissolved in 70% formic acid to a final concentration of 10 mg/ml, treated with excess cyanogen bromide for 18 h at room temperature and again lyophilized. The digest was dissolved in 6 ml of 10% acetic acid and fractionated on a Sephadex G-50 Superfine column (2.5×140 cm) in 10% acetic acid, collecting 4 ml fractions. The radioactive

fractions were pooled, lyophilized and subdigested in 0.1 M ammonium bicarbonate at 37 °C with trypsin (Worthington, TPCK-treated) (enzyme/substrate ratio: 1/30). After 12 h the peptide was purified using a Beckman model HPLC on a reverse phase column (Aquapore RP 300, 4.6 × 250 mm, Brownlee Labs) and eluted with a gradient of the following solvents: solvent A: 0.2% trifluoroacetic acid; solvent B: 0.1% trifluoroacetic acid in acetonitrile: isopropanol (4/1 v/v) at a flow rate of 1.2 ml/min. Elution of peptides was monitored at 214 nm on a Beckman 160 spectrophotometer and radioactivity determined as described above. The labeled peptide (1 nmol) was sequenced automatically by using an Applied Biosystems model 470 A gas-phase sequencer, equipped with an Applied Biosystems model 120 APTH analyzer. An aliquot corresponding to 40% of the material from each degradation cycle was directly injected onto the HPLC column for on-line detection of the PTH-amino acids. The remaining 60% of the sample was automatically delivered to the fraction collector and counted for radioactivity.

RESULTS

Inactivation and modification of Dopa decarboxylase by iodoacetamide

The time course for the inactivation of holoDopa decarboxylase by iodoacetamide at 25°C in 100 mM potassium phosphate buffer, pH 6.8, is shown in Figure 1. As can be seen the inactivation process follows a pseudo-first order kinetics. When the pseudo-first order rate constants, k_{app}, were plotted against iodoacetamide concentration, a linear relationship was obtained (inset of Figure 1). These data indicate that there is no reversible complex formed prior to the inactivation process. The second-order rate constant, estimated from the slope of the graph in the inset, was $37\ M^{-1}\,min^{-1}$. The number of moles of inactivator reacting per mole of enzyme was calculated by plotting the log of the apparent first order rate constants for inactivation versus the log of iodoacetamide concentration, and determining the slope of the resulting line (data not shown). A slope of 1.15 was obtained; this suggests that the reaction of 1 mol iodoacetamide per mol of enzyme dimer is responsible for inactivation. Dopa decarboxylase is also inactivated by iodoacetate. However, under the same experimental conditions used for iodoacetamide, iodoacetate is about 10 times less active than iodoacetamide. It is possible that a negative charge on the iodoacetate molecule results in more difficult access to the residue(s) being attacked.

Figure 2 emphasizes the correlation between loss of enzyme activity and extent of iodo(1-^{14}C)acetamide incorporation. Approximately 1 mol of label is incorporated per mol of holoenzyme dimer resulting in a complete loss of activity. L-5HTP completely protects against iodoacetamide incorporation and inactivation at all time intervals in which the enzyme was present as enzyme-substrate intermediate complex. In contrast, D-Dopa only affords a partial protection even at concentration up to 20-fold the K_D value determined spectrophotometrically[12]; so, at 0.44 mM iodoacetamide, 7.5 mM Dopa results in an approximately 45-fold reduction in the apparent first-order rate inactivation constant (data not shown).

Properties of modified enzyme

Iodoacetamide-inactivated holoDopa decarboxylase ($\ll 1\%$ residual activity) shows characteristic spectral features between 300 and 500 nm and a coenzyme content

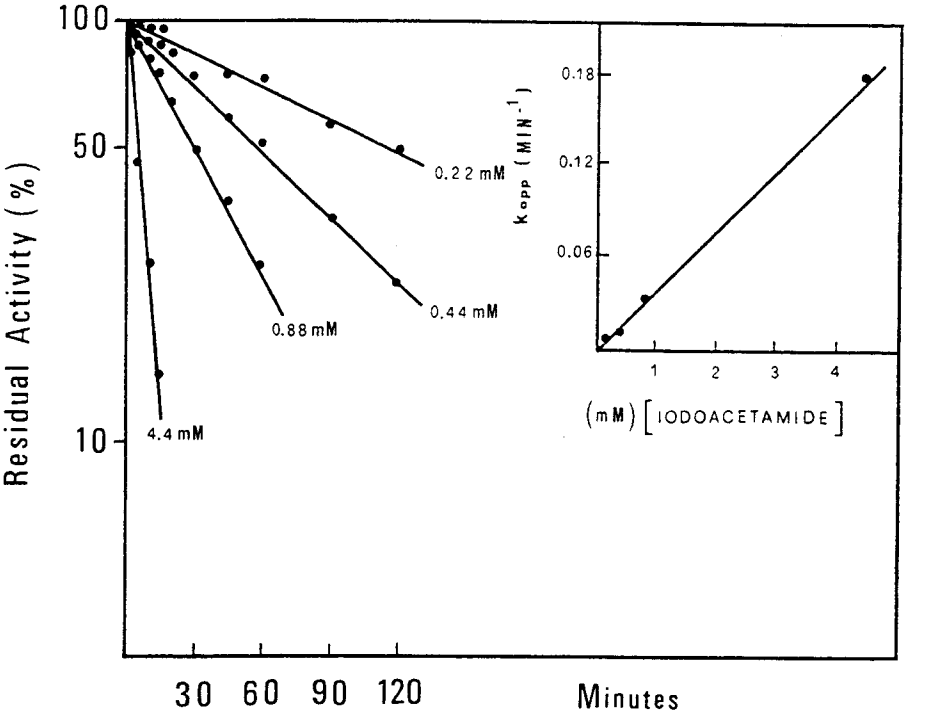

FIGURE 1 Inactivation of Dopa decarboxylase by iodoacetamide. The enzyme (10 μM) was incubated in 100 mM potassium phosphate buffer pH 6.8, at 25 °C. At intervals aliquots were removed for measurement of the residual enzyme activity. Iodoacetamide concentration is indicated on the slopes. Apparent first-order rate constants were determined from the slopes of the semilog plots. (Inset): plot of apparent first-order rate constants obtained at various concentrations of iodoacetamide as the function of reagent concentration.

identical to those of the corresponding unmodified species. When chromatographed on a Ultro Pac TSK-G3000 SW column, the modified enzyme was eluted as a single symmetrical peak at the position expected for the native enzyme (data not shown). These results indicate that the inactivation of the enzyme by iodoacetamide does not involve release of bound pyridoxal-P or dissociation or aggregation of the enzyme. Although holoDopa decarboxylase is fully protected against inactivation by the susbstrate L-5HTP, the enzyme, once inactivated by iodoacetamide, still retained the capacity to form Schiff's base intermediates, as shown by spectrophotometric studies. In fact, both the native and inhibited enzymes show an absorption increase at 420 nm upon the addition of L-5HTP or L-Dopa in 100 mM potassium phosphate buffer, pH 6.8. However, for the native enzyme the absorption decreases during the decarboxylation, whereas this is not the case for the modified enzyme. Also, the maximal change induced by the addition of L-Dopa to the inactivated enzyme was apparently equal to that of the unmodified enzyme, while the maximal change induced by the addition of L-5HTP was about 50–60% of that found with the native enzyme.

Moreover, as reported for the native enzyme, the addition of D-Dopa to the iodoacetamide modified enzyme results in a concentration-dependent increase in

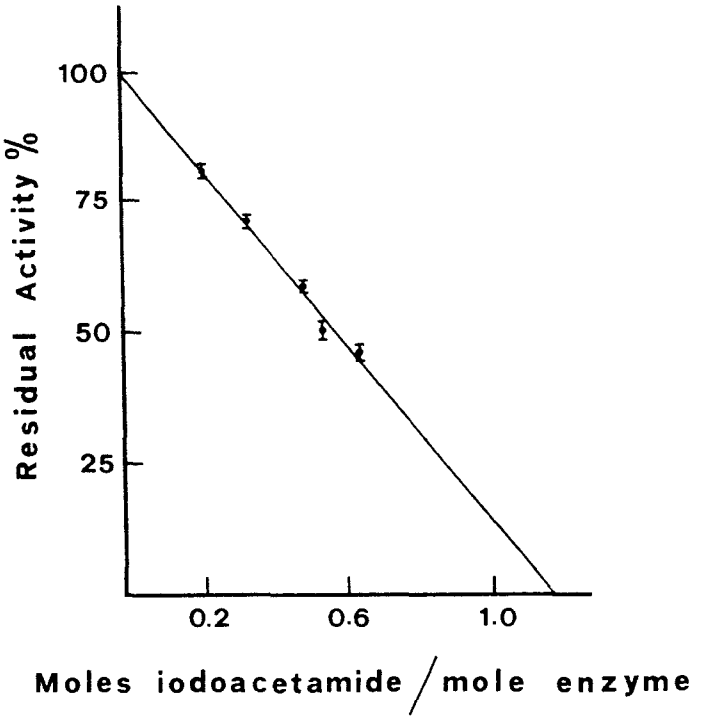

FIGURE 2 Relationship between residual enzyme activity and incorporation of iodo(1-^{14}C)acetamide into Dopa decarboxylase. Enzyme (10 mol) was treated with 0.5 mM iodo(1-^{14}C)acetamide in 100 mM potassium phosphate buffer, pH 6.8, at 25 °C. Aliquots were withdrawn for determination of enzyme activity and ^{14}C-incorporation. The data points represent the average of three determinations. The bars represent the standard error. The line is from linear least-squares regression.

absorbance at 420 nm. The maximum change in absorbance and the dissociation constant (derived by plotting the observed absorbances, corrected for changes in volume and for the absorbance of the solution before the addition of amino acid, as a function of D-Dopa concentration) are similar to those measured for the native enzyme[12].

Localization of the ^{14}C-labeled site in the fragments obtained by limited tryptic proteolysis.

As previously reported for the native enzyme[13], limited trypsin treatment (as described under Materials and Methods) of iodo(1-^{14}C)acetamide-modified Dopa decarboxylase yields two fragments (M_r = 38,000 and 14,000) which have been separated under denaturing conditions on a reverse-phase HPLC column. The smaller fragment corresponds to the COOH-terminal third of the enzyme and the larger fragment, which contains the lysine residue which binds pyridoxal-P, corresponds to the amino terminal two thirds of the enzyme. Radioactivity measurements of the eluant from the reverse-phase HPLC column show that the ^{14}C label was associated with only the

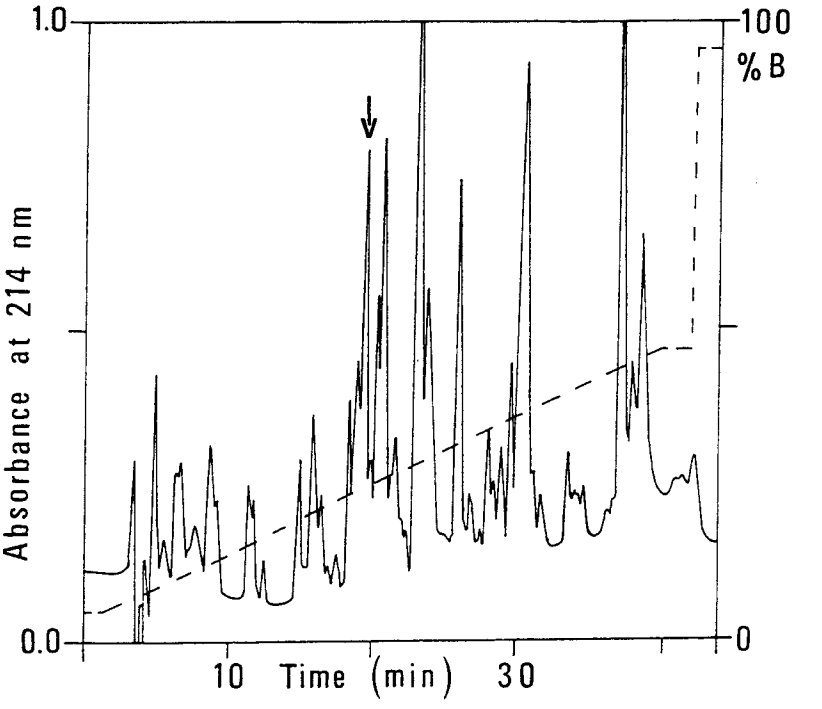

FIGURE 3 Reverse phase high performance liquid chromatography of the tryptic digest of the radioactive fraction obtained by cyanogen bromide cleavage of the labeled protein. Conditions of analysis are reported in the text. (—) Absorbance at 214 nm; (- - -) % of solvent B in the gradient elution. The radioactive fraction is indicated by the arrow.

smaller peptide fragment (data not shown). Thus, the ^{14}C-labeled site of modification in pig kidney Dopa decarboxylase appears to be on the smaller fragment.

Isolation and sequence determination of the peptide containing ^{14}C carboxyamidomethylcysteine.

The elution pattern of the cyanogen bromide fragments obtained from iodo(1-^{14}C)acetamide-modified Dopa decarboxylase after all cysteine residues had been blocked with unlabeled iodoacetic acid (as described under Materials and Methods) shows only one major radioactive peak (data not shown). The contents of tubes containing radioactivity were pooled, concentrated and digested with trypsin. Fractionation of the resulting peptides on a HPLC reverse-phase column yields a single radioactive peak as displayed in Figure 3. The (^{14}C)-containing peak was subjected to sequence analysis by automated Edman degradation. The sequence of a heptapeptide containing the carboxyamidomethyl group was found to be: Ile-His-Leu-Val-Pro-(Cam)Cys-Arg-. Moreover, the carboxyamidomethylcysteine released at cycle 6 contained radioactivity. This clearly indicates this residue as the reactive cysteine modified by iodo(1-^{14}C)acetamide of pig kidney Dopa decarboxylase.

DISCUSSION

Iodoacetamide rapidly inactivates pig kidney Dopa decarboyxylase at pH 6.8 in a pseudo-first order fashion. Reaction order of approximately one with respect to iodoacetamide (inset of Figure 1) and incorporation of the ^{14}C-label from iodo(1-^{14}C)acetamide into the enzyme clearly indicate that the incorporation of 1 mol of the reagent per mol of enzyme dimer is responsible for the inactivation process. Sequence analysis of the isolated iodo(1-^{14}C)acetamide modified peptide indicates cysteine as the modification target in Dopa decarboxylase. Alkylation of the cysteinyl residue does not result either in the alteration of the molecular weight of the enzyme or in the release of the bound pyridozal-P. Therefore, this amino acid residue could be considered essential since covalent modification of this moiety abolishes enzymatic acitivity. As Dopa decarboxylase is a homodimeric enzyme, it is difficult to understand why only one cysteine residue appears to be essential for activity. Moreover, previous work has indicated that a single residue of arginine[4], histidine[5] or pyridoxal-P binding lysine[14] per enzyme dimer is involved in enzyme activity. All these findings can be explained by assuming that the minimal activity unit of the enzyme is the monomer. Alternatively, it might be hypothesized that the two subunits of the enzyme are asymmetrically arranged and that they are not functionally identical. This could imply that the enzyme must be in the dimeric state to be active. If this were the case, the two subunits should be arranged in a way to contribute asymmetrically to the formation of a single active site per dimeric enzyme. Anyhow, on the basis of present experimental data, we cannot assign a definite role to this residue in the mechanism of action of the enzyme. In clarifying this issue, information concerning the spectral binding properties of iodoacetamide-modified enzyme and the determinating of the amino acid sequence of the radioactive cysteinl heptapeptide were of great help.

A report on the complete gene-deduced sequence of Drosophila Dopa decarboxylase[7] has revealed a complete homology between a 7-amino acid sequence around the pyridoxal-P-binding site of pig kidney Dopa decarboxylase[14] and a 7-amino acid stretch of Drosophila enzyme. Furthermore, we have recently observed[13] that the *N*-terminal sequence of the smaller fragment derived from limited trypsin treatment of pig kidney Dopa decarboxylase exhibits a 58% sequence homology with a 50 amino acid stretch of Drosophila Dopa decarboxylase. Considering the alignment of the above mentioned partial sequence of the two enzymes, the pig kidney cysteinyl heptapeptide has been aligned with a 7-amino acid stretch of Drosophila enzyme, located at about one-third from the C-terminal end. This is consistent with the finding that the ^{14}C-labeled modification site in pig kidney enzyme has been located, by means of limited tryptic proteolysis, on the C-terminal third of the enzyme. A comparison of the peptide regions of the two enzymes shows identical sequences except for two replacements. One concerns the conservative substitution of arginine of pig kidney enzyme by lysine in Drosophila enzyme and the other, the non conservative substitution of cysteine of pig kidney enzyme by alanine in Drosophila enzyme. This latter substitution deserves a special note and gives rise to the following considerations. The Dopa decarboxylase enzymes in insects and mammals are remarkably similar in subunit structure, molecular weight, kinetic properties and substrate specificities[15]. Therefore, the possibility that the modifiable cysteinyl residue of pig kidney enzyme is directly involved in the catalytic function of the enzyme seems rather unlikely in that its replacement by alanine in Drosophila enzyme does not appear to alter significantly its catalytic characteristics. Nevertheless, since modification of the

cysteinyl residue in pig kidney Dopa decarboxylase causes the complete loss of enzymatic activity, this might suggest that introduction of the acetamidic group into the residue affects the local conformation required for catalysis.

Iodoacetamide inactivation of pig kidney Dopa decarboxylase does not prevent the binding of L-5HTP or L-Dopa to the enzyme, as shown by the spectral change induced by the addition of these compounds. Nevertheless, the modification of the cysteinyl residue will result in the loss of catalytic activity. Conceivably, L-amino acid substrates cannot bind in the same manner as in unmodified enzyme. It might be inferred that they are able to form a Schiff base with the aldehydic group of the coenzyme, but their positioning at the active site does not result in the correct configuration for catalysis.

In addition, the inactivated enzyme binds a substrate analog, D-Dopa, with a dissociation constant quite similar to that of the unmodified enzyme. Taken together, these data support the hypothesis that the iodoacetamide modification apparently preserves the three-dimensional structure of the substrate binding site and that the modifiable cysteine is not directly involved in the substrate or substrate analog binding, regardless of its location. At present it is not possible to state whether this modifiable cysteine is located inside or outside the active centre. In fact, although our protection studies suggest that the iodoacetamide-modified residue is within the active site, the possibility that a ligand-induced conformational change decreases the reactivity of a distant residue, whose modification does not impair substrate binding, cannot be rigorously excluded. Chemical modification of this residue could then cause inactivation either by partially interfering with the conformational change induced by substrate or by preventing the correct alignment of the catalytic residues. Alternatively, chemical modification may induce a conformational change of the enzyme in such a way that it distorts the active site and perturbs the proper stereochemistry of the functional groups at the active site.

In addition, the different extent of protection exerted by L-5HTP or D-Dopa against iodoacetamide inactivation should also be considered. We have already suggested[12] the existence of two binding subsites at the active site of pig kidney Dopa decarboxylase. These sites, each specific for either catechol or indole-related structures, would partially overlap. Furthermore it might also be speculated that the modifiable cysteine residue has a differential accessibility to the chemical modifier in the D-Dopa and L-5HTP-enzyme intermediate complexes. This idea is further substantiated by the finding that the maximal changes in absorbance at 420 nm observed upon addition of L-Dopa or L-5HTP to the modified enzyme are 100% and 50–60%, respectively, of the absorbance changes obserbed with the control. Taken together, these data suggest that the modifiable sulfhydryl group is located within the active site.

Despite the fact that a definite relationship between the specific chemical modification exerted by iodoacetamide and change in catalytic activity of pig kidney decarboxylase has been established, the overall conclusion of this study is that the modifiable reactive cysteine is not a functional residue. This correlation cannot be easily explained. At present, it seems reasonable to suggest that the loss of pig kidney decarboxylase activity might be attributable to the placement of steric bulk on a cysteine residue at, or near, the active site or in a site of strategic importance to the maintenance of the correct topography of the active site.

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