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Inactivation of Dimeric D-Amino Acid Transaminase by a Normal Substrate through Formation of an Unproductive Coenzyme Adduct in One Subunit†

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Received February 21, 1992; Revised Manuscript Received April 20, 1992

ABSTRACT: D-Amino acid transaminase, which contains pyridoxal 5′-phosphate (vitamin B_6) as coenzyme, catalyzes the formation of D-alanine and D-glutamate from their corresponding α-keto acids; these D-amino acids are required for bacterial cell wall biosynthesis. Under conditions usually used for *kinetic* assay of enzyme activity, i.e., short incubation times with dilute enzyme concentrations, D-alanine behaves as one of the best substrates. However, the enzyme slowly loses activity over a period of hours when exposed to substrates, intermediates, and products at *equilibrium*. The rate of inactivation is dependent on enzyme concentration but independent of substrate concentration greater than K_m values. Continuous removal of the product pyruvate by enzymic reduction precludes the establishment of equilibrium and prevents inactivation. The formation of small but detectable amounts of a quinonoid intermediate absorbing at 493 nm is proportional to inactivation. Studies with [^{14}C]-D-alanine labeled on different carbon atoms indicate that the α-carboxyl group of the substrate is absent in the inactive enzyme; such decarboxylation is not a usual function of this enzyme. The inactive transaminase contains 1.1 mol of [^{14}C]-D-alanine-derived adduct per mole of dimeric enzyme; this finding is consistent with the 50% reduction in the fluorescence intensity at 390 nm (due to the PMP form of the coenzyme) for the inactive enzyme. Thus, inactivation of one subunit of the dimeric enzyme renders the entire molecule inactive. Inactivation may occur when a coenzyme intermediate, perhaps the ketimine, is slowly decarboxylated and then undergoes a conformational change from its catalytically competent location. Evidence indicates that acetaldehyde is the adduct attached to the coenzyme and not to the protein in the inactive enzyme. The activity and spectral properties of the native enzyme are restored upon treatment of the inactive enzyme at slightly acidic pH. These findings may have ramifications in terms of a slow decrease in enzyme function under physiological conditions.

In most *kinetic* assays used to measure the activity of enzymes with their normal substrates, the time for analysis with microgram amounts of enzyme is usually short, i.e., of the order of minutes, and reactive intermediates are not readily detectable. In enzymes with cofactors that absorb at higher wavelengths, milligram amounts of enzyme are sometimes used to detect such intermediates and to measure their turnover. Such conditions also present the opportunity to examine the integrity of the enzyme–coenzyme system upon exposure to such intermediates for extended periods. Described herein is the slow substrate-induced inactivation of D-amino acid transminase when it is permitted to remain at *equilibrium* in contact with all of the intermediates in the reaction pathway. This enzyme, which contains pyridoxal 5′-phosphate as coenzyme, catalyzes the reversible interconversion of D-alanine and α-ketoglutarate to form pyruvate and D-glutamate; these two D-amino acids are an integral part of most, if not all, bacterial cell walls. Hence, information on the inactivation of this enzyme by any type of substrate, whether it be a normal or a suicide substrate, may facilitate the development of novel antimicrobial agents that have this protein as their target (Gale et al., 1981; Manning et al., 1974).

Materials and Methods

D-Alanine, D-glutamate, D-α-aminobutyrate, yeast aldehyde dehydrogenase (EC 1.2.1.5), and bovine liver alkaline phosphatase (EC 3.1.3.1) were purchased from Sigma. The various batches of D-alanine used in this study were judged pure by amino acid analysis and by elemental analysis, which were kindly performed by Mr. Robert Buzolich. Uniformly labeled [U-^{14}C]-D-alanine and [1-^{14}C]-D-alanine were purchased from Amersham. For accurate determination of their specific ra-

† This work was supported in part by NSF Grant DMB 9106174 and by Biomedical Research Support Grant BRSG-507-RR-07065 from the National Institutes of Health to Rockefeller University. A.M.P. received a fellowship from the Comunidad de Madrid, Consejeria de Educacion.
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0006-2960/92/0431-6018$03.00/0

dioactivities, each was diluted with a weighed amount of nonradioactive D-alanine whose concentration was accurately measured by amino acid analysis.

Protein Purification. Wild-type D-amino acid transaminase (EC 2.6.1.21) from a thermostable *Bacillus* was purified to homogeneity as described previously (Martinez del Pozo et al., 1989; Merola et al., 1989) except that a larger DEAE-Sephadex column (2 × 32 cm) was employed. This column, equilibrated in 50 mM potassium phosphate, pH 7.6, containing 0.01% 2-mercaptoethanol, 50 μM PLP,[1] and 0.2 mM EDTA, was eluted with a 1-L gradient of 0–0.2 M KCl. These modifications resulted in a 2–3-fold higher yield of enzyme. Before use, the purified protein was dialyzed extensively to remove excess PLP.

Spectroscopic Characterization. Absorption spectra were recorded at 25 °C and at a speed of 0.5 nm/s in a Cary 15 or in a Cary 2200 UV–vis spectrophotometer in cells with an optical path of 1 cm. The molar extinction coefficient of dimeric wild-type D-amino acid transminase at 280 nm is calculated to be 57 410 by amino acid analysis after acid hydrolysis. Fluorescence spectra were measured at 25 °C in a Hitachi–Perkin-Elmer MPF2A spectrofluorometer at 1 nm/s scanning speeds with slit widths of 7 and 5 nm for the excitation and emission wavelengths, respectively. The absorbances of the samples at the corresponding excitation wavelengths were always lower than 0.05 to avoid inner filter effects on the readings.

Enzyme Reactions. Enzyme activity was measured by determining the rate of pyruvate production from D-alanine and α-ketoglutarate at pH 7.2 and 37 °C either by an assay employing NADH and lactate dehydrogenase or with salicylaldehyde, as described previously (Martinez-Carrion & Jenkins, 1965; Jones et al., 1985). One unit of enzyme activity is defined as the amount of protein that catalyzes the formation of 1 μmol of α-keto acid/min (Martinez del Pozo et al., 1989a,b). Specific activity is defined as units per milligram of protein. The enzyme is a dimer with a subunit molecular weight of 32 226 (Tanizawa et al., 1989a,b), and it contains 1 mol of PLP/mol of subunit as determined by the method of Wada and Snell (1961); each subunit is catalytically active (Martinez del Pozo et al., 1989b). Experiments were carried out in tubes wrapped in aluminum foil to prevent any light-induced destruction of PLP.

Assays with aldehyde dehydrogenase required fresh solutions of enzyme (10 μg/mL) in 0.1 M potassium phosphate, pH 7.5, at 25 °C; the increase in absorbance at 340 nm due to NAD reduction was measured on a Cary 2200 instrument at the 0.05 absorbance scale. Treatment of the coenzyme adduct with alkaline phosphatase (300 μg/mL) was performed in 0.1 M NH_4HCO_3, pH 11, for 100 min at 37 °C.

Proteolytic Digestions. The inactivated enzyme was treated with 10% TPCK-treated trypsin (Sigma fraction XIII) in 100 mM NH_4HCO_3, pH 8.0, containing 2 M urea. Digestions were also performed with endoproteinase Glu-C or endoproteinase Arg-C (both from Boehringer-Mannheim) at 10% of the weight of the transaminase in 50–100 mM NH_4HCO_3, pH 8, containing 1 M urea. After 24 h, each digest was lyophilized and the residue was dissolved in 0.1% TFA. After adjustment to pH 1–2 with acetic acid, each digest was applied to several types of HPLC columns—TSK ODS-120A (0.46 × 25 cm), TSK 80TM (0.46 × 25 cm), TSK DEAE-5PW (0.75 × 7.5 cm), TSK SP-5-PW (0.75 × 7.5 cm) and Vydac C_{18} reverse phase (0.46 × 15 cm)—either on a Shimadzu HPLC or on a Beckman HPLC. The columns were eluted with a linear gradient from 0.1% TFA to 0.1% TFA/50% 1-propanol. The radiolabeled component whose location was determined in an LKB Model 1218 scintillation counter was hydrolyzed in 6 N HCl for 18 h prior to amino acid analysis.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

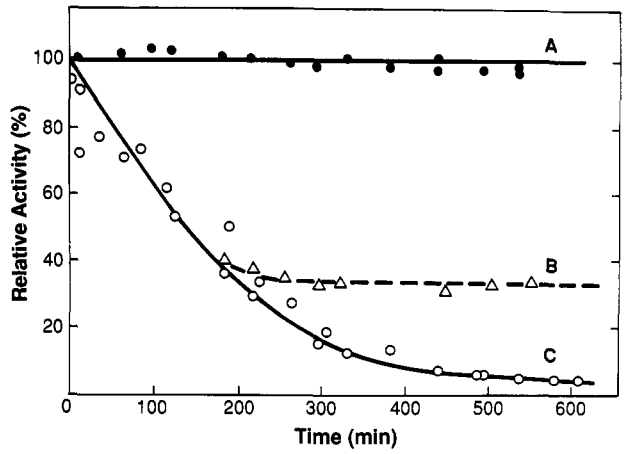

FIGURE 1: Inactivation of high concentrations of D-amino acid transaminase by D-alanine. Line A (●), enzyme alone (3.4 mg/mL) or enzyme (3.4 mg/mL) and D-alanine (0.1 M) in the presence of lactate dehydrogenase (0.2 mg/mL) and NADH (60 μM); line C (○), enzyme (3.4 mg/mL) and 0.1 M D-alanine; line B (△), concentrated enzyme diluted 10-fold at 180 min with the same D-alanine concentration maintained (0.1 M). The buffer was 0.1 M Tris, pH 7.5, and 2 mM EDTA.

Other Analytical Procedures. SDS–PAGE electrophoresis of samples that had been previously reduced with 2-mercaptoethanol was carried out according to Laemmli (1970). A Beckman Paragon electrophoresis system was used for electrophoresis of native proteins. Gel filtration in Sephadex G-25 (column size 2 × 40 cm, either in 0.05 M potassium phosphate, pH 7.4, and 0.2 mM EDTA or in 5 mM NH_4HCO_3) was employed to separate the enzyme from substrate and products. Gel filtration in Sephadex G-100 (1 × 70 cm in 5 mM NH_4HCO_3, pH 8) was also employed in some experiments. Amino acid analysis, which was used to establish the purity of the amino acid substrates, to measure specific radioactivity, and to calculate protein concentrations after acid hydrolysis, was performed either with a Beckman Model 6300 amino acid analyzer with System Gold enhancement or with the original amino acid analyzer (Spackman et al., 1958) from which fractions could be collected.

RESULTS

Inactivation of D-Amino Acid Transaminase by D-Alanine. While monitoring the formation of intermediates in the catalytic pathway of D-amino acid transaminase with relatively large (i.e., milligram) amounts of enzyme in the presence of D-alanine, we previously reported the slow formation of a small amount of a quinonoid intermediate absorbing at 493 nm (Martinez del Pozo et al., 1989b). We now find that during the turnover of this quinonoid, whose concentration can be measured on a conventional spectrophotometer, the enzyme slowly became inactivated (Figure 1, line C). If, during the course of this inactivation, the enzyme concentration was rapidly reduced by a factor of 10 but the same D-alanine concentration was maintained, further inactivation was prevented but not reversed (Figure 1, line B).

The rate of inactivation was proportional to enzyme concentration (Figure 2, right) and independent of D-alanine concentration as long as it was greater than its K_m value. D-Alanine very rapidly shifts the PLP form of the enzyme into

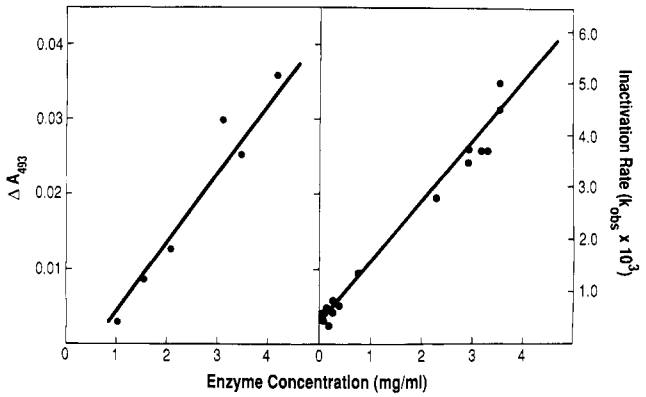

FIGURE 2: (Right) Dependence of inactivation on enzyme concentration. Varying concentrations of enzyme were mixed with 0.1 M D-alanine in 0.1 M Tris, pH 7.5, and 2 mM EDTA. The inactivation rate constants calculated from the graph of ln % activity vs time were plotted at different protein concentrations. (Left) Relationship between enzyme concentration and formation of the absorbance band at 493 nm. Varying concentrations of enzyme were mixed with 0.1 M D-alanine in 0.1 M Tris, pH 7.5, and the spectrum was recorded immediately. The maximum increase in absorbance at 493 nm was plotted against enzyme concentration.

the ketimine and the PMP form (both of which absorb near 330 nm) prior to the slow inactivation process. Inactivation was also found to occur in the reverse reaction, i.e, when pyruvate was added to the PMP form of the enzyme. However, inactivation was barely evident when pyruvate formed from D-alanine was continuously removed from the equilibrium mixture, as shown in Figure 1 (line A), by addition of lactate dehydrogenase and NADH. The k_{obs} for inactivation is 4.0 $\times 10^{-3}$ min^{-1} when pyruvate is permitted to remain and 9.7 $\times 10^{-5}$ min^{-1} when pyruvate is removed by enzymic reduction. The small absorbance band at 493 nm (see below) also disappeared upon removal of pyruvate. The addition of α-ketoglutarate (0.1 M) enhanced the inactivation about 2-fold to give a rate constant of 8.3 $\times 10^{-3}$ min^{-1}. Thus, equilibrium conditions are conductive to enzyme inactivation. These events are depicted in eq 1.

$$\text{D-Ala} + \underset{\substack{(A_{420})\\(\text{internal aldimine})}}{\text{E–PLP}} \rightleftarrows \rightleftarrows \underset{(A_{493})}{\text{quinonoid intermediate}} \rightleftarrows \rightleftarrows \underset{\substack{(A_{330})\\(\text{ketimine})}}{\text{E–PMP}} = \text{Pyr} \rightarrow \rightarrow \rightarrow \text{E (inactive)} \quad (1)$$

In this enzyme the internal aldimine form of the enzyme (E–PLP) contains the coenzyme linked to Lys-145 as a Schiff base. Upon addition of D-alanine, a series of intermediates rapidly form, beginning with the transfer of PLP to the substrate to generate the external aldimine (not shown in eq 1) and progressing through the quinonoid intermediate and then to coenzyme PMP covalently linked to pyruvate (ketimine structure). The hydrolysis of this intermediate into E–PMP and free pyruvate completes the half-reaction of transamination. D-Alanine rapidly promotes the equilibrium formation of both the ketimine and the PMP forms of the enzyme, both of which absorb near 330 nm, before inactivation commences.

Relationship between Enzyme Inactivation and Equilibrium Conditions. The behavior of one intermediate that absorbs at 493 nm was monitored at equilibrium (Figure 2, left). At low concentrations of enzyme (<1 mg/mL), the absorbance band at 493 nm was barely detectable and there was little inactivation of the enzyme. At higher concentrations (3–4 mg/mL), there were small but measurable increases in the 493-nm absorbance band and significant inactivation of the enzyme. The data suggest that, in the presence of both substrates, there is an opportunity for equilibrium to be established and the quinonoid intermediate can be replenished as enzyme inactivation proceeds. However, it should not be construed that the quinonoid itself leads to inactivation; no evidence is available to answer that question. The presence of the quinonoid merely reflects the pool of intermediates whose turnover is conveniently measured because of its characteristic absorbance at 493 nm. When the enzyme was completely inactivated, the 493-nm absorbance band was lost and only the coenzyme absorbance band at 330 nm remained (eq 1).

D-Glutamate or D-α-aminobutyrate, which are both good substrates for D-amino acid transaminase, also led to slow inactivation of the enzyme. Three separate preparations of D-alanine that were shown to be pure by amino acid analysis were equally effective in promoting the inactivation, which has been consistently observed in many experiments. There is no measurable consumption of D-alanine during the inactivation since the recovery of D-alanine by amino acid analysis was complete within experimental error; the small amount of D-alanine used in a single turnover is not enough to be measured by amino acid analysis in the presence of such an excess of the substrate. The inactivation was readily detected either by the lactate dehydrogenase assay or by the salicylaldehyde assay and was not reversed by dialysis against neutral buffers containing PLP. There was no change in pH during the 4–6-h inactivation period nor was there any denaturation of the enzyme as ascertained by the absence of any changes in the circular dichroism spectrum in the far-ultraviolet. There was no adventitious cleavage, cross-linking, or aggregation of the enzyme since both the active and the inactive enzymes behaved identically by gel electrophoresis either in the native or in the denatured state. Furthermore, gel filtration of the inactive enzyme under nondenaturing conditions showed only a single protein component with a molecular weight identical to that of the native enzyme, i.e., 64 000.

Coenzyme Binding in the Inactive Enzyme. With some PLP enzymes, loss of activity in the presence of normal substrates is due to the dissociation of the coenzyme PMP from the enzyme after the covalent internal aldimine bond has been transferred to the substrate (Novogrodsky & Meister, 1964). In such cases, full activity is restored by addition of fresh PLP. The possibility that the inactivation induced by D-alanine occurred by this mechanism was evaluated by subjecting the inactive enzyme to gel filtration on Sephadex G-25 at high ionic strength (0.5 M potassium phosphate); 90% of the absorbance of the coenzyme adduct was retained by the inactive enzyme after this treatment. These results indicate that the inactivation was not due to dissociation of the coenzyme adduct from the enzyme.

Labeling of the Enzyme by [^{14}C]-D-Alanine. During the inactivation of the enzyme, there was progressive incorporation of uniformly labeled [^{14}C]-D-alanine into the protein as determined by gel filtration on Sephadex G-100 (Figure 3). This incorporation parallels the loss in enzyme activity. However, when the inactivation was performed in duplicate with [^{14}C]-D-alanine labeled only on the α-carboxyl group, there was no incorporation of label into the inactive protein. With uniformly labeled [U-^{14}C]-D-alanine, the completely inactivated enzyme contained 1.1 mol of adduct/mol of dimeric enzyme, taking into account the decarboxylation that occurred during the process of inactivation. These studies were performed with [^{14}C]-D-alanine whose final specific radioactivity was accurately determined by amino acid analysis after a

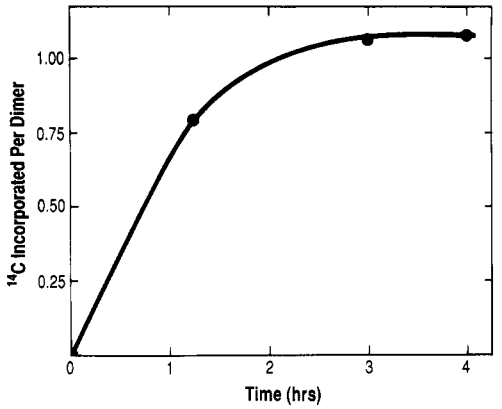

FIGURE 3: Incorporation of [^{14}C]-D-alanine during inactivation. At the indicated times during inactivation of D-amino acid transaminase (2.0 mg/mL) with uniformly labeled 0.1 M [^{14}C]-D-alanine in 0.1 M Hepes, pH 7.5, samples were removed and applied to Sephadex G-100. The protein peak was collected, and the amount of incorporation was determined. A separate experiment with 39 μM enzyme subunit showed 70% inactivation under the same conditions; this value correlates with the 0.77 mol of ^{14}C adduct incorporated per dimer.

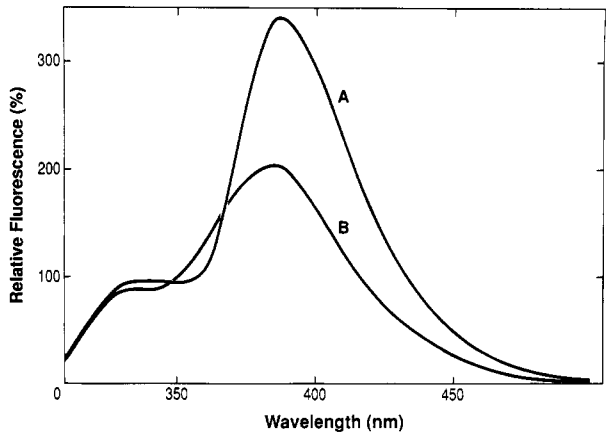

FIGURE 4: Changes in coenzyme fluorescence upon inactivation. The enzyme (3.4 mg/mL) and D-alanine (0.1 M) were incubated in 0.1 M Tris, pH 7.5, and 2 mM EDTA. At various times during the inactivation, the samples were diluted to 25 μg/mL, and the emission fluorescence intensity spectrum was recorded; the excitation wavelength was 295 nm. Curves: (A) active enzyme; (B) inactive enzyme.

weighed amount of unlabeled D-alanine was added to the labeled compound. The rate of inactivation under these experimental conditions in Hepes buffer is more rapid than in the experiment described in Figure 1 where Tris buffer was used. We ascribe these differences in inactivation rate to the nucleophilicity of the two buffers.

Changes in Coenzyme Fluorescence. Both subunits of D-amino acid transaminase are catalytically competent since there is formation of 1 equiv of pyruvate for each subunit (Martinez del Pozo, 1989b). Like L-aspartate transaminase (Churchich & Farrelly, 1969), the PMP and the ketimine forms of D-amino acid transaminase exhibit fluorescence at 390 nm due to the conformational change that occurs when E–PLP is converted to E–PMP (eq 1). After complete inactivation, the enzyme had lost about 50% of the fluorescence at 390 nm, suggesting that the coenzyme adduct formed by decarboxylation was present in only one subunit and it became reoriented into an environment not conducive with fluorescence (Figure 4). These results, when taken together with the value of 1 mol of the [^{14}C]-D-alanine-derived moiety incorporated per mole of inactive dimeric enzyme, are consistent with a model in which inactivation of only one subunit leads to total inactivation of the enzyme.

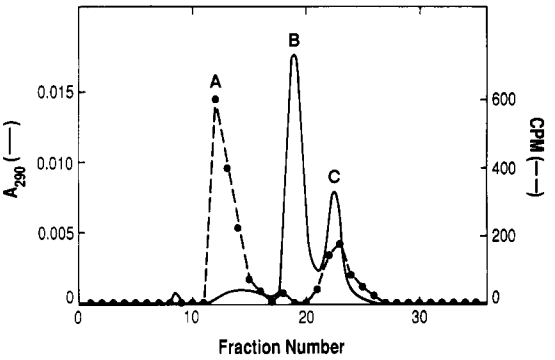

FIGURE 5: Isolation of the inactive adduct released by trichloroacetic acid. The inactive enzyme was denatured with 10% trichloroacetic acid as described in the text. After removal of the precipitated protein by centrifugation, the trichloroacetic acid in the supernatant was extracted with diethyl ether and then subjected to HPLC on a TSK ODS-120A (0.46 × 25 cm) column as described in the text.

Absence of Covalent Linkage between the Coenzyme Adduct and the Protein. Proteolytic digestion by trypsin, endoproteinase Glu-C, or endoproteinase Arg-C, as well as precipitation of the protein with trichloroacetic acid, promoted release of the inactive coenzyme adduct from the protein. This radiolabeled component eluted in the same position from the several types of HPLC columns described under Materials and Methods. Amino acid analysis of this labeled component after acid hydrolysis did not indicate significant amounts of amino acids even though detectable amounts should have been present based on the amount of coenzyme present, as calculated from its 290-nm absorbance. The identity of this radiolabeled component was further investigated as described next.

Nature of the Inactive Coenzyme Adduct. The absence of linkage to the protein as well as several other findings described above suggested that the inactive adduct may be a derivative of the coenzyme formed upon decarboxylation of substrate, intermediate, or product linked to the coenzyme. Evidence for this mechanism was obtained by recovery of the labeled coenzyme adduct after rapid denaturation of the inactive enzyme with 10% trichloroacetic acid; three components were found by HPLC (Figure 5). Since component C contains both the coenzyme absorbance and the radiolabel, it is considered to be the intact coenzyme adduct released from the inactive enzyme upon denaturation. This possibility was addressed by several approaches. Hence, the synthetic 2-carbon adduct with coenzyme (in this case, PLP reduced with $NaCNBH_3$ in the presence of ethylamine, which gives the same product as reduction of the PMP–acetaldehyde adduct) cochromatographs with component C. Component B, which has no radiolabel, has been identified as PLP by several lines of evidence. First, it has the characteristic spectrum of PLP and not any of the other forms of vitamin B_6. Second, its chromatographic behavior of HPLC is identical to that of authentic PLP, and third, upon treatment with alkaline phosphatase, it moves to a unique position on HPLC, identical to that of free pyridoxal. Rechromatography of isolated component C gives rise, in part, to components A and B, suggesting that these are the major parts of the inactive coenzyme adduct formed by hydrolysis of component C.

Component A, which contains radiolabel but no coenzyme absorbance, has been identified as acetaldehyde by several lines of evidence even though the facile reaction of acetaldehyde with the functional groups of proteins as well as its volatility posed some experimental problems. First, authentic acetaldehyde both labeled and unlabeled cochromatographs with component A during HPLC. Second, in different trichloroacetic acid extracts of inactive enzyme, the released component

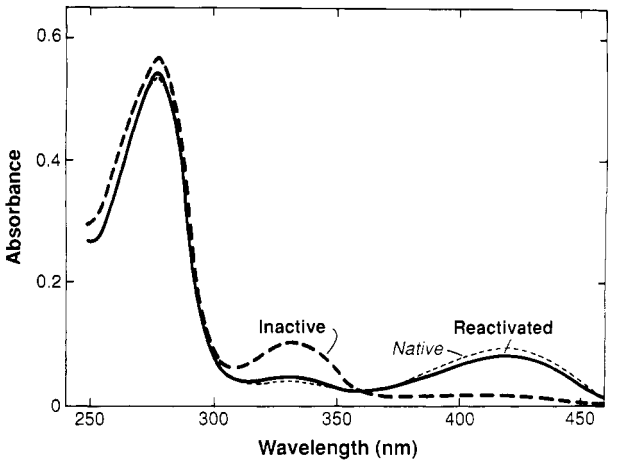

FIGURE 6: Spectra of the native, the inactive, and the reactivated enzymes. The enzyme was inactivated and then reactivated as described in the text.

A has been found to be a substrate for the enzyme aldehyde dehydrogenase, which oxidizes acetaldehyde to acetate and NADH. For example, after precipitation of 97 nmol of dimeric inactive enzyme with trichloroacetic acid, 120 nmol of acetaldehyde was recovered by the aldehyde dehydrogenase assay. This value of 1.2 mol of acetaldehyde/mol of inactive dimeric enzyme is close to the 1.1 value of the [^{14}C]-D-alanine-derived adduct found in Figure 3.

Reactivation of the Inactive Enzyme. In order to determine whether the structure of the inactive coenzyme adduct with its absorbance at 330 nm was related to the compound described by Likos et al. (1982) for L-aspartate transaminase inactivated by a different mechanism, the inactive D-amino acid transaminase was treated at pH 11 for 0.5 h and then subjected to gel filtration on Sephadex G-25. However, unlike the adduct which was detached from L-aspartate transaminase by this treatment, no such compound was released from the inactive D-amino acid transaminase; the ^{14}C-label derived from D-alanine together with the coenzyme absorbance remained attached to the protein. Upon adjustment to pH 5, 96% of the original enzyme activity was regained, as determined with the lactate dehydrogenase assay in three separate experiments. Furthermore, the spectrum of the active wild-type enzyme with a coenzyme absorption band at 420 nm was restored (Figure 6). Thus, whereas the inactive enzyme has a coenzyme absorbance at 330 nm but very little absorbance at 420 nm, the native, wild-type enzyme has an absorption maximum at 420 nm due to the coenzyme structure as the internal aldimine; the A_{420}/A_{280} ratio of the native, untreated active enzyme is 0.19 (Martinez del Pozo et al., 1989a). The spectrum of the reactivated enzyme closely resembled that of the active, wild-type enzyme with a A_{420}/A_{280} ratio of 0.16 (Figure 6).

The ^{14}C-labeled D-alanine moiety released from the protein during reactivation at pH 5 is acetaldehyde since it coelutes with component A of Figure 5. Thus, when the coenzyme re-forms the active enzyme at low pH, the inactive adduct is expelled from the protein and the coenzyme re-forms the internal aldimine between PLP and Lys-145, as judged from the spectrum (Figure 6). Since D-glutamate and D-α-aminobutyrate also slowly inhibit the enzyme under these experimental conditions, it is likely that the slow decarboxylation of their corresponding α-keto acids may also inhibit the enzyme by this mechanism. Therefore, it appears that the inactivation is not related to any special properties of acetaldehyde but rather to this side reaction, which is undergone by this and perhaps by other transaminases. Indeed, exogenous acetaldehyde (5 mM), when added to the PMP form of the enzyme for a period of 3 h, does not cause any inactivation. It probably adds to external amino groups on the protein before it has a chance to penetrate the active site.

Discussion

The inactivation of D-alanine does not appear to proceed by any of the known pathways for inactivation by enzyme-activated inhibitors (suicide substrates), such as β-chloroalanine (Soper & Manning, 1985) or serine *O*-sulfate (Likos et al., 1982), which contain good leaving groups on their side chains. Inactivation of a decarboxylase caused by a slow transamination event has been reported for glutamate decarboxylase (Sukhareva & Braunstein, 1971). Some other decarboxylases also catalyze transamination (Novogrodsky & Meister, 1964), but examples of transaminases that catalyze decarboxylation are rare. For example, chemically modified L-aspartate transaminase, dimethylated at its active site Lys-258, catalyzes slow enzymic turnover and decarboxylation of oxaloacetate (A. Iriarte et al., personal communication). D-Alanine-induced inactivation takes place not only with the wild-type enzyme but also within K145N active site mutant D-amino acid transaminase (Yoshimura et al., unpublished results). The inactivation described in this paper bears some similarity to the inactivation of β-lactamases (Fisher et al., 1978; Frere et al., 1982; Waley, 1991), but there are some obvious differences as well.

The finding that adduct formation occurs with only one subunit and thereby renders the entire molecule inactive is supported by three different experimental observations: by the incorporation of about one [^{14}C]-D-alanine-derived moiety per dimeric enzyme (Figure 3), by the 50% decrease in the PMP coenzyme fluorescence (Figure 4), and by the aldehyde dehydrogenase assay indicating about 1 mol of acetaldehyde per dimeric inactive enzyme. The results may indicate that the active site of each subunit is made up of segments contributed from different subunits, as in L-asparate transaminase (Arnone et al., 1985).

The inactivation could arise from the inability of the inactive adduct to be displaced from the coenzyme by normal substrates. A likely candidate that undergoes the decarboxylation is the ketimine form of the enzyme which absorbs at 330 nm, E–PMP = Pyr (eq 1). The resultant acetaldehyde–PMP adduct may move to a catalytically incompetent position after decarboxylation since the α-COOH group is needed to maintain the proper position of substrate at the active site. This explanation is consistent with the loss of 50% of the fluorescence in this inactive form of the enzyme. In order for acetaldehyde to be an inactivator through the unproductive linkage with the coenzyme PMP, it must be generated at the active site since added acetaldehyde is not an inactivator.

The events leading to inactivation were revealed under conditions to which enzymes are not usually subjected in a laboratory setting, i.e., the exposure of relatively high concentrations of enzyme to a substrate for relatively long periods of time, i.e., at *equilibrium*. Hence, the short time periods usually used to measure *kinetic* velocities of enzyme reactions are not conducive to detect inactivation of the type described here, especially with low concentration of enzyme. Furthermore, if the assay itself involves the removal of one of the products, i.e., by enzymic reduction of pyruvate to lactate in the presence of NADH, inactivation would be precluded, as found in this investigation. Since the intracellular concentration of some enzymes may be of the same order of magnitude as that used in the present study and if intermediates on the reaction pathway persist, inactivation events of the type

described here may have some physiological relevance, i.e., the possible substrate-induced loss in catalytic efficiency of enzymes in cells.

ACKNOWLEDGMENTS

We are grateful to Adelaide Acquaviva for preparation of the manuscript and to Maria Pospischil and Edward Yatco for their assistance with some of the experiments.

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Labeling the (Ca^{2+}–Mg^{2+})-ATPase of Sarcoplasmic Reticulum with 4-(Bromomethyl)-6,7-dimethoxycoumarin: Detection of Conformational Changes†

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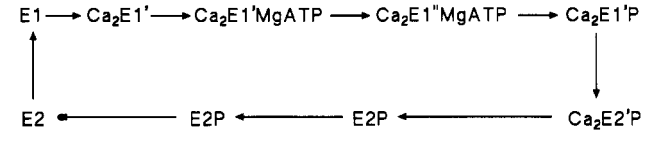
Received November 27, 1991; Revised Manuscript Received March 19, 1992

ABSTRACT: The (Ca^{2+}–Mg^{2+})-ATPase of sarcoplasmic reticulum was labeled with 4-(bromomethyl)-6,7-dimethoxycoumarin. It was shown that a single cysteine residue (Cys-344) was labeled on the ATPase, with a 25% reduction in steady-state ATPase activity and no reduction in the steady-state rate of hydrolysis of *p*-nitrophenyl phosphate. The fluorescence intensity of the labeled ATPase was sensitive to pH, consistent with an effect of protonation of a residue of p*K* 6.8. Fluorescence changes were observed on binding Mg^{2+}, consistent with binding to a single site of K_d 4 mM. Comparable changes in fluorescence intensity were observed on binding ADP in the presence of Ca^{2+}. Binding of AMP-PCP produced larger fluorescence changes, comparable to those observed on phosphorylation with ATP or acetyl phosphate. Phosphorylation with P_i also resulted in fluorescence changes; the effect of pH on the fluorescence changes was greater than that on the level of phosphorylation measured directly using [^{32}P]P_i. It is suggested that different conformational states of the phosphorylated ATPase are obtained at steady state in the presence of Ca^{2+} and ATP and at equilibrium in the presence of P_i and absence of Ca^{2+}.

One approach to the study of enzyme function is to attach a fluorescent probe to a particular residue in the enzyme and to use the spectral properties of the probe to report on changes in its environment during the reaction cycle of the enzyme. This approach has been used to study the function of the (Ca^{2+}–Mg^{2+})-ATPase from skeletal muscle sarcoplasmic reticulum (SR),[1] the major problem being that of ensuring labeling at a single, specific residue on the ATPase. Studies have

Scheme I



† We thank the SERC and the Wessex Medical Trust for financial support.

0006-2960/92/0431-6023$03.00/0