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REVERSIBLE MODIFICATION OF AMINO GROUPS IN ASPARTATE AMINOTRANSFERASE

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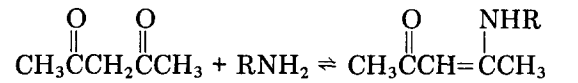
Summary

Amino groups in the pyridoxal phosphate, pyridoxamine phosphate, and apo forms of pig heart cytoplasmic aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) have been reversibly modified with 2,4-pentanedione. The rate of modification has been measured spectrophotometrically by observing the formation of the enamine produced and this rate has been compared with the rate of loss of catalytic activity for all three forms of the enzyme. Of the 21 amino groups per 46 500 molecular weight, approx. 16 can be modified in the pyridoxal phosphate form with less than a 50% change in the catalytic activity of the enzyme. A slow inactivation occurs which is probably due to reaction of 2,4-pentanedione with the enzyme-bound pyridoxal phosphate. The pyridoxamine phosphate enzyme is completely inactivated by reaction with 2,4-pentanedione. The inactivation of the pyridoxamine phosphate enzyme is not inhibited by substrate analogs. A single lysine residue in the apoenzyme reacts approx. 100 times faster with 2,4-pentanedione than do other amino groups. This lysine is believed to be lysine-258, which forms a Schiff base with pyridoxal phosphate in the holoenzyme.

Introduction

A variety of studies have demonstrated that a lysine residue is involved in the catalytic activity of aspartate aminotransferase (aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) [1,2]. In the pyridoxal-*P* form of the enzyme, this lysine residue (Lys-258 in the pig heart cytoplasmic enzyme [3]) forms an imine with the pyridoxal-*P* cofactor. The side-chain amino group of this residue is resistant to modification in the pyridoxal form of the enzyme but is very reactive in the apoenzyme. It has been suggested that this lysine may also act as a binding site for the substrate carboxyl group [4] and as a general base in the course of transamination [5,6].

We have recently shown that 2,4-pentanedione is a useful reagent for the modification of lysine and arginine residues in proteins [7]. Reaction of a protein for a short time with 2,4-pentanedione leads to the reversible formation of enamines with the amino groups present (Eqn. 1).



$$CH_3\overset{O}{\overset{\|}{C}}CH_2\overset{O}{\overset{\|}{C}}CH_3 + RNH_2 \rightleftharpoons CH_3\overset{O}{\overset{\|}{C}}CH{=}\overset{NHR}{\overset{|}{C}}CH_3 \quad (1)$$

The reaction is complete in a few hours and can easily be quantitated spectrophotometrically. Free amino groups can be regenerated either by dialysis at pH 6 or by reaction with hydroxylamine. At longer times, 2,4-pentanedione also reacts with arginine residues, but the difference between rates of lysine and arginine modification is at least a factor of 20, and lysine modification can be studied without complication due to arginine modification.

In this paper we present the results of studies of the modification of amino groups in all three forms of pig heart cytoplasmic aspartate aminotransferase, the pyridoxal-*P* form, the pyridoxamine-*P* form, and the apoenzyme, with 2,4-pentanedione. We show that most of the lysine residues in this enzyme are not essential for catalytic activity, but the lysine which forms an imine with pyridoxal-*P* has an unusually reactive amino group.

Materials and Methods

Materials. Aldrich Gold Label 2,4-pentanedione was used without further purification. Pyridoxal-*P* and pyridoxamine-*P* were obtained from Sigma. All other materials were reagent grade and were used as received. Water was purified with a Millipore Super-Q filtration system.

The α subform of pig heart cytoplasmic aspartate aminotransferase was prepared by the method of Martinez-Carrion et al. [8] except that the enzyme isolation was carried out in glutarate buffer rather than maleate, and all buffers contained 10^{-4} M dithiothreitol. Specific activities when assayed at 280 nm with 10.3 mM 2-oxoglutarate and 20.7 mM L-aspartate at pH 8.3 in 0.069 M Tris/chloride buffer [9] were 210—260 μmol oxaloacetate/min per mg (reported value 220 ± 10 μmol oxaloacetate/min per mg [10]). At pH 5.4 in 0.1 M sodium acetate the absorbance ratios $A_{430\,nm}/A_{340\,nm}$ and $A_{280\,nm}/A_{362\,nm}$ were 2.5—3.0 and 7.4, respectively (reported 3—4 and 8.0 [10]).

The pyridoxamine form of the enzyme was prepared by incubation of the enzyme with 0.5 mM cysteine sulfinate in 0.1 M sodium phosphate buffer, pH 7.0, followed by dialysis. The apoenzyme was prepared by incubation of holoenzyme with 0.5 mM cysteine sulfinate in 0.1 M sodium phosphate buffer, pH 7.0. After 30 min, an equal volume of 1.0 M sodium phosphate buffer, pH 3.8, was added, and the solution was allowed to stand for 10 min at 21° C. The solution was then cooled to 4° C and the apoenzyme precipitated by the addition of $(NH_4)_2SO_4$ to 70% of saturation [11]. The precipitated enzyme was centrifuged, redissolved, and dialyzed against water containing 10^{-4} M dithiothreitol. The apoenzyme was always used the same day it was prepared.

Methods. Ultraviolet spectra were recorded on a Cary 15 spectrophotometer equipped with a 0.1 absorbance slidewire. Kinetic measurements were per-

formed on a Gilford model 222 spectrophotometer equipped with a constant temperature cell compartment maintained at 25°C. Fluorescence spectra were measured on an Aminco-Bowman SPF-2 ratio-recording spectrophotofluorimeter.

Modification of aspartate aminotransferase with 2,4-pentanedione. The enzyme in the pyridoxal, pyridoxamine, or apo form (1—4 mg/ml) was incubated at 21°C in the dark with 0.5 M 2,4-pentanedione in either 0.5 M $NaHCO_3$ buffer, pH 9.0, or in 0.1 M sodium phosphate buffer, pH 7.0. At various times aliquots were withdrawn and the excess 2,4-pentanedione was removed by gel filtration on Sephadex G-25 with 0.05 M Tris/chloride buffer, pH 8.0. The time required for isolation of the modified enzyme was less than 5 min. No significant enamine hydrolysis occurs within 60 min as evidenced by constancy of both absorbance at 310 nm and activity measurements on the modified pyridoxal-*P*, pyridoxamine-*P*, and apoenzymes. The extent of amino group modification was determined spectrophotometrically [7].

Treatment of the pyridoxal-*P*, pyridoxamine-*P*, or the apo form of aspartate aminotransferase with 0.1 M hydroxylamine, pH 6.0, followed by dialysis caused no loss in catalytic activity of the enzyme and no change in the ultraviolet spectrum. Although the pyridoxal-*P* form of the enzyme reacts with hydroxylamine at pH 6, the oxime which is formed decomposes during the dialysis, regenerating the holoenzyme [12].

Titration of apoenzyme with pyridoxamine-P. Apoenzyme was modified with 0.5 M 2,4-pentanedione at pH 7.0 in 0.1 M sodium phosphate buffer for 30 min at 21°C and the enzyme was isolated by gel filtration at pH 8.0. The enzyme was placed in a fluorescence cell and aliquots of 0.1 mM pyridoxamine-*P* were added. Following each addition, the solution was incubated for 10 min in the dark, and the fluorescence emission at 390 nm (excitation wavelength 330 nm) was observed. A sharp break in the plot of fluorescence intensity versus pyridoxamine-*P* concentration is observed when the enzyme is saturated with coenzyme.

Results

Modification of the pyridoxal-P form of aspartate aminotransferase with 2,4-pentanedione.

The pyridoxal-*P* form of aspartate aminotransferase was treated with 0.5 M 2,4-pentanedione at pH 7.0 in 0.1 M sodium phosphate buffer or at pH 9.0 in 0.5 M bicarbonate buffer at 21°C. Aliquots were withdrawn periodically and subjected to gel filtration into 0.05 M Tris/chloride buffer, pH 8.0, and ultraviolet spectra and catalytic activities were determined. The ultraviolet absorbance of the protein at 310 nm increased on reaction of the protein amino groups with 2,4-pentanedione (Fig. 1). The number of amino groups modified was estimated from the change in absorbance at 310 nm [7]. Moderate changes in the environment of the enamine chromophore (0—100% dioxane/water) do not affect the extinction coefficient or λ_{max} of the model compound *N*-*n*-butyl-4-amino-3-penten-2-one, so this method should not be seriously in error for estimating the extent of modification.

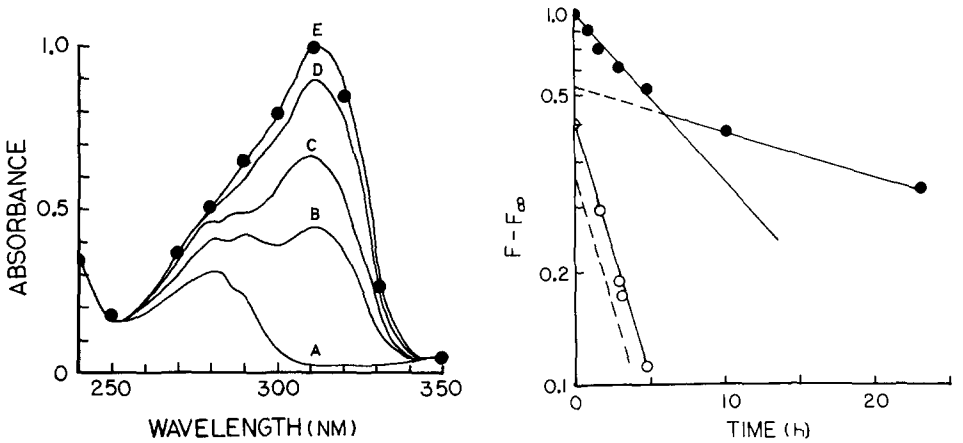


Fig. 1. Ultraviolet spectra of the pyridoxal form of aspartate aminotransferase (4.4 μM) treated for various times with 0.5 M 2,4-pentanedione in 0.1 M phosphate buffer, pH 7.0, at 21°C. Excess reagent was removed by gel filtration before spectra were measured. A, initial spectrum; no modification; B, 1.5 h; 4.1 amino groups modified; C, 2.9 h; 6.2 amino groups modified; D, 10.0 h; 9.4 amino groups modified; E, 24 h; 10.2 amino groups modified. •, calculated points for modification of 10.2 amino groups.

Fig. 2. Time course of the modification of the pyridoxal form of aspartate aminotransferase with 0.5 M 2,4-pentanedione in 0.1 M phosphate buffer, pH 7.0, at 21°C. F = fraction remaining (•) activity; (○) lysine. The lower dotted line is the calculated difference between the observed line for the fast loss of activity and the extrapolated line for the slow loss of activity (k_2). The slope of this line gives the rate constant for the fast activity loss (k_1).

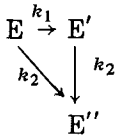
A first-order plot of the progress of modification * is linear and gives a rate constant of $5.4 \cdot 10^{-3}$ min^{-1} at pH 7.0 (Fig. 2, dotted line), essentially identical to the rate constant of $5.3 \cdot 10^{-3}$ min^{-1} for the reaction of *n*-butylamine with 2,4-pentanedione under the same conditions ** [7]. Kinetic data for the modification are summarized in Table I. At equilibrium at pH 7.0 with 0.5 M 2,4-pentanedione, 9.8 ± 0.4 of the 20 amino groups have been modified. At pH 9.0 with 0.5 M 2,4-pentanedione, the rate constant for amino group modification is approx. $7 \cdot 10^{-3}$ min^{-1}, and 16 ± 1 amino groups are modified at equilibrium. When the concentration of 2,4-pentanedione is reduced to 0.2 M at pH 9.0 16 ± 1 amino groups are still modified at equilibrium.

Although enamine formation on reaction of the pyridoxal-*P* form of aspartate aminotransferase with 2,4-pentanedione follows first-order kinetics, the loss in catalytic activity resulting from this reaction shows biphasic kinetics

* Since the equilibrium constants for enamine formation with 2,4-pentanedione and protein amino groups are not known accurately, the rate constants reported here and throughout this paper represent observed rate constants for the reaction in question. They have not been corrected for ionization of substrates or for the rate of the reverse reaction when it occurs.

** The rate of enamine formation from *n*-butylamine and 2,4 pentanedione which is used for comparison with the rate of enzyme modification is the rate constant for approach to the enamine equilibrium (which equals the rate constant for enamine formation plus the rate constant for enamine hydrolysis under the same conditions).

(Fig. 2) consistent with the scheme:



(2)

where E is native enzyme, E′ is a modified form of the enzyme retaining 50—60% of the activity of E, and E″ is a totally inactive form of the enzyme. neither E′ nor E″ is necessarily a single species; instead, each may represent a collection of closely related species with similar catalytic activities. The formation of E′ may be observed easily because rate constant k_1 is more than 10-fold greater than k_2.

Rate constant k_2 can be obtained directly from Fig. 2, and k_1 can be obtained from Fig. 2 by difference (Fig. 2, dotted line). At pH 7.0 with 0.5 M 2,4-pentanedione, the value of k_1 is $5 \cdot 10^{-3}$ min^{-1}, which is identical to the rate constant for amino group modification under the same conditions (Table I). Extrapolation of the slow process to zero time shows that enzyme E′ retains about 50—60% activity at pH 7.0. Similar results are obtained at pH 9.0 (Table I). A control sample treated in the same manner except for the exclusion of 2,4-pentanedione retains 90% activity over the course of the experiment. Inclusion of 0.5 M dioxane in the control sample has no effect on control activity.

Aspartate aminotransferase in the pyridoxal-*P* form was modified for 24 h with 0.5 M 2,4-pentanedione at pH 7.0. After gel filtration, the modified enzyme was titrated with *p*-chloromercuribenzoate at pH 7.0 [13]. In the presence of 0.5% sodium dodecyl sulfate, 3.7 ± 0.3 cysteine residues could be

TABLE I

MODIFICATION OF LYSINE IN ASPARTATE AMINOTRANSFERASE WITH 0.5 M 2,4-PENTANEDIONE AT 21°

Phosphate buffer was used at pH 7, bicarbonate at pH 9.

Enzyme form	pH	Glutarate concentration	10^3 k (min^{-1})		Modified enzyme	
			Lysine modification	Activity loss	Percent activity remaining	Number of lysines modified
Pyridoxal	7.0	0.0	5.4	5.3	60	9.8 ± 0.4
	7.0	0.1	5.4	5.4	50	9.8 ± 0.4
	9.0	0.0	7.0	5.0	60	16 ± 1
Pyridoxamine	7.0	0.0	6.2	4.8	3	10.2 ± 0.5
	7.0	0.1	6.2	3.9	5	10.2 ± 0.5
	9.0	0.0	8.0	5.0	0	16 ± 1
Apo						
(fast)	7.0	0.0	400	480	4	1.0
	7.0	0.1	96	56	8	1.0
(slow)	7.0	0.0	4.9	—	2	10.8 ± 0.5
	7.0	0.1	5.8	—	4	10.8 ± 0.5

titrated in both the modified enzyme and a control treated in the same manner except for the exclusion of 2,4-pentanedione. Although imidazole residues of histidine might add to the carbonyl of 2,4-pentanedione, a product stable to dilution would not be formed [14]. Thus, the modification reaction leading to the rapid activity loss is not the modification of cysteine or imidazole residues under the conditions of the experiment.

Preparation and properties of E′

The presence of 0.1 M sodium succinate (k_d = 18 mM [12]) does not affect k_1 at pH 7.0, although k_2 is decreased by at least a factor of 10. Because of this, enzyme form E′ could be prepared by reaction of the pyridoxal-*P* form of the enzyme with 0.5 M 2,4-pentanedione at pH 7.0 for 24 h in the presence of 0.1 M succinate. E′ could then be isolated by gel filtration. Modified enzyme E′ (10 amino groups modified) is still capable of binding succinate as determined by observing the increase at 430 nm on addition of aliquots of succinate to E′ [9]. Both the magnitude of the absorbance increase at 430 nm (relative to total enzyme concentration) and the K_d are the same for E′ and native enzyme treated in the same manner except for the exclusion of 2,4-pentanedione.

The ultraviolet spectrum of E′ shows absorbance at 310 nm due to amino group modification but no significant change in the absorbance at 362 nm, indicating that pyridoxal-*P* is not modified or lost. Reaction of E′ with cysteine sulfinate [15] results in the conversion of more than 90% of the bound pyridoxal-*P* coenzyme into pyridoxamine-*P*. Reaction of E′ with 0.1 M hydroxylamine followed by dialysis restores 80—85% of the original activity. No evidence was found for the modification of arginine residues at pH 7.0 with 0.5 M 2,4-pentanedione, although the modification of up to one arginine residue could have gone undetected. A kinetic study of enzyme form E′ at pH 8 revealed that the K_m values for aspartate (1.5 mM) and for 2-oxoglutarate (0.12 mM) are identical to those for the native enzyme. The maximum velocity of transamination by E′ is 50% of that for native enzyme.

Structure and properties of E″

The slow inactivation (E → E″ and E′ → E″, Eqn. 2) observed during modification of the pyridoxal-*P* form of aspartate aminotransferase with 2,4-pentanedione is retarded by substrate analog inhibitors, suggesting that a process occurring near the active site is responsible for the activity loss. E″ was produced by reaction of the pyridoxal-*P* form of aspartate amino transferase with 0.5 M 2,4-pentanedione at pH 7.0 for 70 h. After isolation by gel filtration, E″ shows a decrease in absorbance in the pyridoxal-*P* imine region (362 nm) and increased absorbance at 330 nm indicative of a pyridoxal derivative that contains a tetrahedral carbon at C-4′. Treatment with hydroxylamine followed by dialysis or treatment with excess pyridoxal-*P* has no effect of the activity of E″. The rate constant for the decrease in absorbance at 362 nm on reaction with 0.5 M 2,4-pentanedione at pH 7.0 is $8 \cdot 10^{-4}$ $M^{-1} \cdot min^{-1}$, similar to the rate constant of $9 \cdot 10^{-4}$ $M^{-1} \cdot min^{-1}$ for the slow activity loss (k_2).

Modification of the pyridoxamine-P enzyme

Aspartate aminotransferase in the pyridoxamine-*P* form was modified at pH

7.0 with 0.5 M 2,4-pentanedione in the presence and absence of 0.1 M glutarate. The experimental procedure was identical to that described above for modification of the pyridoxal-*P* enzyme. Again, no significant regeneration of activity or amino groups was observed during the time required to make activity and spectral measurements on the modified enzyme.

The rate constants for modification of amino groups and for activity loss are similar to those for the pyridoxal-*P* form of the enzyme (Fig. 3 and Table I). However, in this case the activity loss and modification of amino groups both follow first-order kinetics with approximately the same rate constants. At equilibrium, the activity of the modified enzyme is approx. 3% of the activity of the native enzyme. The presence of 0.1 M glutarate, which binds at the active site of the pyridoxamine-*P* enzyme [18], has only a small effect on the rate or extent of activity loss.

After reaction of the pyridoxamine-*P* enzyme at pH 7.0 with 0.5 M 2,4-pentanedione for 24 h, 10.3 ± 0.5 amino groups are modified. This is the same within experimental error as the number modified in the pyridoxal-*P* enzyme. There is no increase in the absorbance at 362 nm due to transamination of pyridoxamine-*P* on treatment of the modified enzyme with 3 mM 2-oxoglutarate for 30 min. After treatment with 0.1 M hydroxylamine at pH 7.0, the ratio of absorbance at 330 nm to that at 280 nm is the same as in the native enzyme; thus no pyridoxamine-*P* coenzyme has been lost irreversibly from the protein during modification or isolation. The activity of the modified enzyme can be restored to greater than 90% of its original value by treatment of the inactive modified enzyme with hydroxylamine and dialysis.

Reaction of apo-aspartate aminotransferase with 2,4 pentanedione

In contrast to the pyridoxal-*P* and pyridoxamine-*P* forms of aspartate aminotransferase, the reaction of the apoenzyme with 0.5 M 2,4-pentanedione results

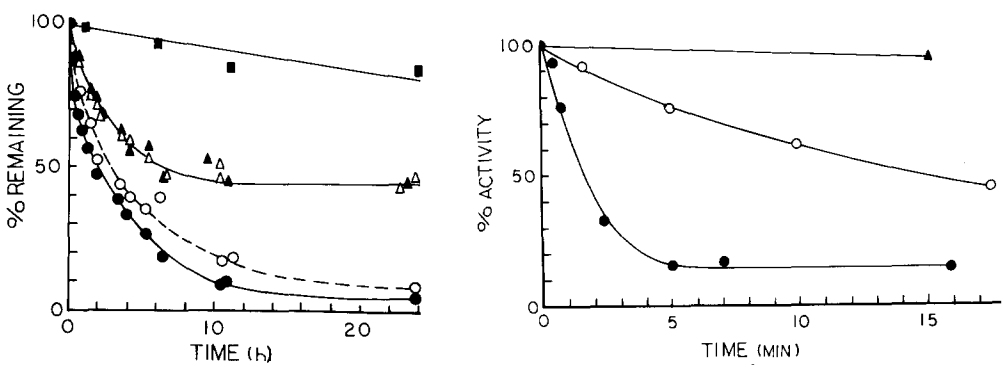


Fig. 3. Time course of the modification of the pyridoxamine form of aspartate amino transferase with 0.5 M 2,4-pentanedione in 0.1 M phosphate buffer, pH 7.0, at 21°C. ○, remaining activity; ●, remaining activity for modification conducted in the presence of 0.1 M glutarate; △, remaining amino groups; ▲, remaining amino groups in the presence of 0.1 M glutarate; ■, remaining activity of a control sample not treated with 2,4-pentanedione.

Fig. 4. Time course of the modification of apo-aspartate aminotransferase with 0.5 M 2,4-pentanedione in 0.1 M phosphate buffer, pH 7.0, at 21°C. ●, remaining activity; ○, remaining activity for modification conducted in the presence of 0.1 M glutarate; ▲, remaining activity of a control sample not treated with 2,4-pentanedione. 4% residual activity due to holo-pyridoxal-*P* enzyme has been subtracted.

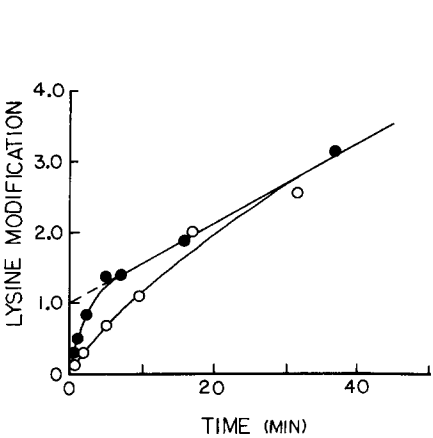
in inactivation of the enzyme within 5 min (Fig. 4). The isolation and assay procedure was similar to that used for the modification of the other enzyme forms except that the apoenzyme was incubated for 10 min with $5 \cdot 10^{-4}$ M pyridoxal-*P* before assay. Again, no significant regeneration of free amino groups occurs over the time period required to perform the assay and record spectral data.

The rapid loss in activity is associated with an increase in absorbance of the protein at 310 nm. The magnitude of the rapid change in absorbance corresponds to the modification of one amino group per 46 500 daltons (Fig. 5). The rate constant for the modification of this amino group is very similar to the rate constant for inactivation (Table I).

Both the activity loss and the change in absorbance at 310 nm are inhibited by glutarate. The inhibition constant for glutarate calculated from the inhibition of the activity loss by 0.1 M glutarate is approx. 12 mM. This is similar to the dissociation constant of 12 mM determined for dissociation of succinate from the apoenzyme [18].

After a 10 min incubation of the apoenzyme with 0.5 M 2,4-pentanedion, approx. 12% of the original activity remains. 4% of this residual activity represents activity due to holoenzyme in the preparation. The remaining 8% activity may be due to the presence of unmodified enzyme at equilibrium. However, activity due to transamination by modified enzyme where the pyridoxal cofactor is bound as the free aldehyde cannot be excluded.

The second of these alternatives seems more likely since the modified inactive apoenzyme binds 85—90% of the amount of pyridoxamine-*P* bound by a control sample treated in the same manner except for exclusion of 2,4-pentanedione (Fig. 6). Addition of excess pyridoxal-*P* to the inactivated apoenzyme causes no increase in absorbance at 362 nm due to pyridoxal-*P* imine forma-



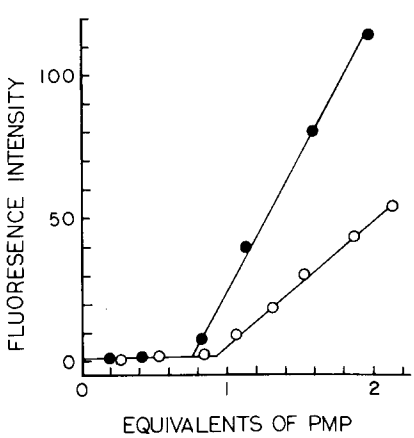


Fig. 5. Time course of enamine formation on reaction of apo-aspartate aminotransferase with 0.5 M 2,4-pentanedione in 0.1 M phosphate buffer, pH 7.0, at 21°C. •, number of lysines modified; ○, number of lysines modified in the presence of 0.1 M glutarate.

Fig. 6. Fluorescence titration of apo-aspartate aminotransferase with pyridoxamine-*P* (PMP). ○, native enzyme 2.9 μM; •, enzyme 4.2 μM modified for 30 min with 0.5 M 2,4-pentanedione in 0.1 M phosphate buffer, pH 7.0, at 21°C.

tion. Thus, the modified enzyme either does not bind pyridoxal-*P* or binds pyridoxal-*P* without formation of the imine with Lys-258.

The activity loss on modification of the apoenzyme with 2,4-pentanedione is not due to thermal denaturation. Control samples lose no activity when treated in the same manner except for the exclusion of 2,4-pentanedione. In addition, treatment of the modified enzyme with hydroxylamine and dialysis restored 80—90% of the original activity.

Following the rapid modification of one reactive amino group, the remaining amino groups react at approximately the same rate as in the pyridoxal-*P* or pyridoxamine-*P* forms of the enzyme (Table I). After reaction for 24 h at pH 7.0 with 0.5 M 2,4-pentanedione, 10.8 ± 0.5 amino groups are modified.

Discussion

2,4-Pentanedione is a useful reagent for modifying amino groups in proteins because the reaction is readily reversible under mild conditions. At pH 7 modification of arginine residues by this reagent occurs at a negligible rate, and the reaction seems to be totally specific for amino groups. All three forms of aspartate aminotransferase, the pyridoxal enzyme, the pyridoxamine enzyme, and the apoenzyme, react readily with 2,4-pentanedione. The rate constant for modification of the majority of amino groups is the same in all three forms of the enzyme and is similar to the rate of reaction of 2,4-pentanedione with *n*-butylamine under similar conditions.

Except for the irreversible reaction of the enzyme-bound pyridoxal-*P* coenzyme with 2,4-pentanedione, modification of aspartate aminotransferase with 2,4-pentanedione can be reversed by treatment with hydroxylamine and dialysis. This reversal of amino group modification restores essentially complete activity to all forms of the modified enzyme, demonstrating that activity loss on modification is due to modification of amino groups and not to irreversible denaturation of the enzyme.

Extent of modification

At pH 9.0, both the pyridoxal-*P* and pyridoxamine-*P* forms of aspartate aminotransferase have about 16 amino groups which are susceptible to modification by 2,4-pentanedione out of a total of 21 amino groups per subunit (20 lysine amino groups and one N-terminal amino group) [3]. The extent of amino group modification is independent of the concentration of 2,4-pentanedione over the range 0.2—0.5 M reagent, suggesting that under these conditions the modification equilibrium lies completely on the side of modified amino groups. This conclusion is also consistent with the extent of modification predicted from the equilibrium constant calculated from modification experiments conducted at pH 7. It is unlikely that the assumed extinction coefficient for the enamine formed by reaction of lysine amino groups with 2,4-pentanedione is seriously in error because this extinction coefficient is quite independent of the environment of the chromophore.

Thus, most amino groups in aspartate aminotransferase are readily modified by 2,4-pentanedione and this modification causes only a small change in the activity of the enzyme. However, a few lysine amino groups in the holoenzyme

are not suceptible to modification by 2,4-pentanedione. Similar results were reported by Turano and co-workers [2], who reported that several (perhaps as many as six) amino groups in the pyridoxal form of the enzyme were resistant to reaction with acetic anhydride.

Pyridoxal-P enzyme

At both pH 7 and pH 9 modification of the pyridoxal-*P* form of the enzyme results in the rapid loss of about 40% of the original catalytic activity of the enzyme. This activity loss occurs at approximately the same rate as the modification of amino groups and is reversible on treatment with hydroxylamine. The modified enzyme (E′ in Eqn. 2) binds the inhibitor succinate and has K_m values for both aspartate and 2-oxoglutarate identical to those of the native enzyme. At pH 9 after the modification of all 16 available amino groups the enzyme still retains more than 50% of its original activity. Spectrophotometric observation of the coenzyme chromophore following addition of amino acid substrates shows that all enzyme molecules are transaminated.

Thus, we can find no evidence for a functional role of amino groups in the action of aspartate aminotransferase except for the amino group involved in Schiff base formation with pyridoxal-*P*. A similar conclusion was reached by Turano et al. [2] who showed that extensive modification of the enzyme with acetic anhydride causes little change in activity. Slebe and Martinez-Carrion [22] showed that carbamylation of all available amino groups in the enzyme caused no loss of activity. However, it should be noted that to date no one has been able to modify all 20 lysine amino groups, and the possibility remains that one or more of the inaccessible amino groups might be involved in binding or catalysis.

The second stage of the modification of the pyridoxal enzyme by 2,4-pentanedione is more problematical. The spectral change which occurs on formation of E″ (Eqn. 2) indicates that the coenzyme is being modified, and the spectral properties of the modified product are the same as those of the product of reaction of free pyridoxal-*P* with 2,4-pentanedione (Gilbert, H.F. and O'Leary, M.H., unpublished), but lack of material has prevented a complete characterization of this product.

Pyridoxamine-P enzyme

The pyridoxamine-*P* form of aspartate aminotransferase is almost completely inactivated at pH 7 by reaction with 2,4-pentanedione, and only about 10 amino groups are modified under these conditions. The ultraviolet spectrum of the coenzyme isolated following modification indicates that the amino group of the coenzyme has been modified. We assume that the complete inactivation which occurs on modification of this form of the enzyme is due to coenzyme modification, rather than to any change in the properties of enzyme amino groups.

It is interesting that the activity loss resulting from reaction of pyridoxamine-*P* with 2,4 pentanedione is not inhibited by glutarate, even though glutarate is a competitive inhibitor of transamination and binds to the pyridoxamine-*P* enzyme [12,18]. This must mean that glutarate must bind in such a way as not to block access of the modifying reagent to the coenzyme amino

group. This fact makes it unlikely that this amino group serves as a binding site for a substrate or inhibitor carboxyl group.

Apoenzyme

Removal of the coenzyme from aspartate aminotransferase exposes a new amino group which is kinetically about 100-fold more reactive than other amino groups. Modification of this amino group causes complete or nearly complete activity loss. The modified enzyme is able to bind pyridoxamine-*P*, but no Schiff base is formed on addition of pyridoxal-*P*. Thus the highly reactive amino group appears to be that of lysine-258, which forms a Schiff base with pyridoxal-*P* in the unmodified enzyme. The high reactivity of this particular amino group has also been observed in modification of the apoenzyme with acetic anhydride [2] and with cyanate [22]. Whether this reactivity is due to the abnormal pK_a of the group [22] or due to factors peculiar to its environment has not been determined.

Glutarate inhibits modification of this reactive amino group, presumably by occupying the substrate binding site. The inhibition constant for glutarate with the apoenzyme is significantly greater than that with the holoenzyme. It is interesting that glutarate inhibits modification of this amino group in the apoenzyme but does not inhibit modification of the coenzyme group in the pyridoxamine-*P* enzyme.

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