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Specific Labeling of the Active Site of Cytosolic Aspartate Aminotransferase through the Use of a Cofactor Analogue, *N*-(Bromoacetyl)pyridoxamine†

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ABSTRACT: The cofactor analogue *N*-(bromoacetyl)pyridoxamine (BAPM) has been employed to inactivate the cytosolic isozyme of apo-aspartate aminotransferase. Inactivation is the result of covalent bond formation in the (bromoacetyl)-pyridoxamine–transferase complex, via the ϵ-amino group of a lysyl residue at the active site. The stoichiometry of this inactivation is one molecule of (bromoacetyl)pyridoxamine per subunit of the transaminase dimer. Trace amounts of inorganic phosphate protect the enzyme from BAPM inactivation. In the absence of phosphate, inactivation demonstrates time, concentration, and pH dependence with an apparent p*K* for the target group of about 8.5 or higher. A tryptic peptide from the α subform has been obtained containing the carboxymethyl derivative of lysine-258, identifying this particular residue as the reactive group in the region of cofactor binding. Evidence is presented indicating that the p*K* of Lys-258 appears to be highly dependent upon the electrostatic state of neighboring groups in the active site region. Hence, experimentally obtained values vary according to the chemical nature and charge of the modifying agent or probe.

Aspartate aminotransferase (EC 2.6.1.1) is a functional dimer containing 1 mol of pyridoxal 5′-phosphate bound per subunit active site. It catalyzes the following conversion via two half-reactions:

$$\text{glutamate} + E_{\text{pyridoxal-5′-P}} \rightleftharpoons \alpha\text{-ketoglutarate} + E_{\text{pyridoxamine-5′-P}} \quad (1)$$

$$E_{\text{pyidoxamine-5′-P}} + \text{oxaloacetate} \rightleftharpoons \text{aspartate} + E_{\text{pyridoxal-5′-P}} \quad (2)$$

Two natural isozymes occur which are localized within the cytosolic and mitochondrial compartments of the cell and which presumably occur as part of the malate–aspartate shuttle which brings reducing equivalents into the mitochondrium (Dawson, 1979). The cytosolic isozyme consists of 412 amino acid residues having a tertiary structure which has been determined by X-ray crystallography to a resolution of 2.7 Å (Arnone et al., 1982).

In the absence of substrate, the cofactor is covalently bound at the active site via an internal aldimine with Lys-258. During transamination, the amino acid substrate is proposed to form a new aldimine with the aldehyde group of the pyridoxal-5′-P[1]

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[1] Abbreviations: BAPM, *N*-(bromoacetyl)pyridoxamine; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; [^{14}C]BAPM, *N*-(bromo[^{14}C]acetyl)pyridoxamine; pyridoxal-5′-P, pyridoxal 5′-phosphate; pyridoxamine-5′-P, pyridoxamine 5′-phosphate.

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by a trans-Schiffization mechanism which generates a free amino group on Lys-258.

The cofactor analogue *N*-(bromoacetyl)pyridoxamine (BAPM) has been previously shown to specifically label an active site sulfhydryl residue of bacterial tryptophan synthetase, another member of the family of B_6-requiring enzymes (Higgins & Miles, 1978). This compound should be a good alkylating agent for reactive residues situated on either the aldehyde or the amine side of the pyridoxal binding region. In the cytosolic isozyme of pig heart aspartate aminotransferase, not only Lys-258 but also Tyr-70 and Cys-390 are in the proximity to be considered candidate target groups. We describe here the use of BAPM as an analogue affinity labeling reagent which under our conditions reacts with one of these active site moieties, namely, the ϵ-amino group of Lys-258, and via covalent bond formation leads to irreversible inactivation of the enzyme. The reactivity of this lysyl residue has been previously demonstrated from active site labeling experiments, and p*K* values for this residue have been estimated both from ^{19}F nuclear magnetic resonance studies in the holoenzyme and from carbamoylation of the apoenzyme (Martinez-Carrion et al., 1976; Slebe & Martinez-Carrion, 1978). While X-ray maps can provide information concerning the accessibility of particular amino acids in the overall tertiary structure, the intrinsic properties of these groups must be studied dynamically in solution. The kinetics of BAPM inactivation as a function of pH, ionic strength, and presence of various ionic species are reported to provide further insight into the electrostatic behavior and reactivity of specific activity site amino acid residues under varying experimental conditions.

Experimental Procedures

The α subform of cytosolic aspartate aminotransferase was isolated from pig hearts as previously described (Martinez-Carrion et al., 1965). Activity was assayed spectrophotometrically by using published procedures (Slebe & Martinez-Carrion, 1978). Apoenzyme was prepared by the method of Jenkins (Jenkins & D'Ari, 1966). Phosphate used in the preparation of the apoenzyme was removed by exhaustive dialysis against buffer until all phosphate could be shown to be removed with the techniques of differential scanning calorimetry as well as ^{31}P NMR (A. Iriarte and M. Martinez-Carrion, unpublished results). Protein concentrations were calculated from the absorbance at 280 nm by using a molar absorptivity of 140 000 M^{-1} cm^{-1} for a dimer M_r 94 000 (Martinez-Carrion et al., 1967). *N*-(Bromoacetyl)pyridoxamine was synthesized as described (Higgins & Miles, 1978). The stoichiometry of inactivation was determined as described under Results. The enzyme modification reaction was performed at 37 °C under a variety of conditions including addition of various ionic species which based upon earlier observations conceivably could have affected the inactivation (Relimpio et al., 1981). At 25 °C, the reaction was very slow, and considerable BAPM hydrolysis occurred prior to inactivation (see Results). All experiments were performed at a constant enzyme concentration of 1 mg/mL in a buffer solution consisting of 0.1 M Mops, Mes, and Bicine (1:1:1) prepared at the pHs indicated in the text. Enzyme inactivation was followed by displacing BAPM from noncovalent complexes by addition of solution containing 10^{-4} M pyridoxal-5′-P in 0.05 M Hepes, pH 7.5, and subsequently assaying the reconstitutable activity. Spectra were taken in a Cary 210 spectrophotometer interfaced to an Apple II microcomputer.

Radiolabeled *N*-(bromo[^{14}C]acetyl)pyridoxamine ([^{14}C]-BAPM) was prepared as described (Higgins & Miles, 1978). The identity of the active site amino acid being modified was determined by using a conventional approach in which tryptic digestion of the BAPM–transaminase complex proceeded for 3 h at 37 °C in a 0.1 M ammonium bicarbonate solution containing 50 μg of trypsin/5 mg of enzyme. Resulting peptides were isolated on a Varian Model 5000 high-pressure liquid chromatograph equipped with a Waters MCH-10 C_{18} reverse phase column and rechromatographed until resolved when necessary. Following hydrolysis in sealed evacuated tubes with constant boiling HCl for 24–96 h at 110 °C the amino acid composition of the purified radiolabeled peptides was determined by using published procedures (Peterson, 1981). (Carboxymethyl)lysine and (carboxymethyl)cysteine from acid-hydrolyzed aspartate aminotransferase were identified by coelution on the analyzer with (carboxymethyl)lysine prepared according to published procedures (Korman & Clarke, 1956) and commercially available (carboxymethyl)-cysteine.

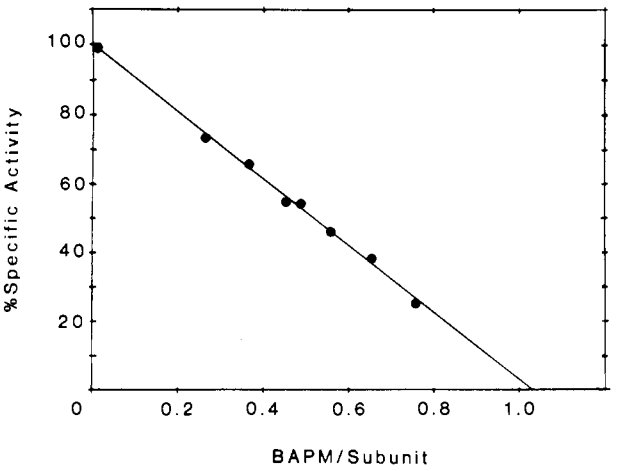

FIGURE 1: Stoichiometry of *N*-(bromoacetyl)pyridoxamine inactivation of cytosolic aspartate aminotransferase. Radiolabeled BAPM at a concentration of 2 mM was allowed to react with the transaminase at pH 8.0 over a period of time during which aliquots of equal volume were removed. Nonreacted BAPM was inactivated with 1% mercaptoethanol in 0.1 M Hepes and 0.1 M KCl, pH 8.0, buffer following which the mixture was dialyzed extensively to remove all traces of noncovalently bound affinity label. The enzyme concentration in the final mixture was then determined by its absorbance at 280 nm, the activity remaining assayed as described under Experimental Procedures and the associated radioactivity per enzyme subunit calculated. Although not shown, monitoring of the increase in absorbance at 330 nm due to bound BAPM gives similar results in which a single molecule of the cofactor analogue per subunit appears to be responsible for inactivation.

Results

Stoichiometry of N-(Bromoacetyl)pyridoxamine Inactivation. As predicted from the model where

$$E + I \rightleftharpoons E{\cdot}I \rightarrow E{-}I$$

the reaction between apo-aspartate aminotransferase and BAPM irreversibly inactivates the enzyme, and residual activity at any time point is inversely proportional to the moles of BAPM incorporated per mole of active site. The number of molecules of BAPM incorporated per subunit of transaminase was determined by measuring the residual activity following incorporation of [^{14}C]BAPM of known specific radioactivity as shown in Figure 1. Only one molecule of affinity label per subunit is required to inactivate the enzyme. Prolonged exposure to BAPM was shown, however, to lead to labeling of a pair of nonessential surface sulfhydryls (Cys-45 and -82; see section on peptide mapping), although this modification appears unrelated to the loss of activity and occurs

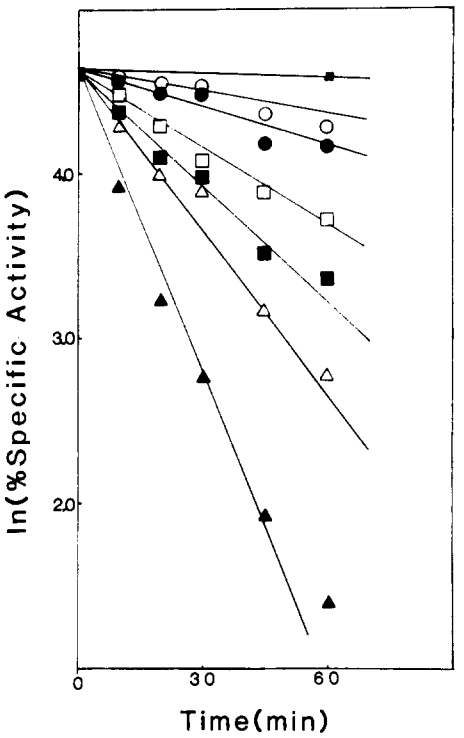

FIGURE 2: Concentration dependence of inactivation. A constant amount of enzyme was incubated with increasing concentrations of BAPM and the residual activity assayed following quenching of the reaction with pyridoxal 5′-phosphate as described under Experimental Procedures. (×) Apoenzyme alone; (○) 0.5 mM BAPM; (●) 1 mM BAPM; (□) 2 mM BAPM; (■) 3 mM BAPM; (△) 4 mM BAPM; (▲) 20 mM BAPM. The pH in all reactions was 9.0; data represent an individual experiment. Reaction rates were estimated from initial slopes because the hydrolysis of BAPM in aqueous solution led to a rate decrease over the time course of the experiments (see text for details).

much more slowly. Only after extensive labeling was allowed to occur was an overall stoichiometry approaching a value of two BAPM per subunit found.

Spectral Properties of BAPM-Inactivated Enzyme. The BAPM–aspartate aminotransferase complex showed absorption spectra which were indistinguishable from the pyridoxamine form of the enzyme with maxima at 280 and 330 nm.

Effect of Concentration and pH. The inactivation of aspartate aminotransferase by BAPM was found to be pH dependent under pseudo-first-order conditions. Nonetheless, as shown in Figure 2, concentrations of BAPM below 5 mM demonstrated nonlinear kinetics of inactivation which linearized when the concentration of inactivator was increased to 20 mM. This result is attributed to the instability and spontaneous hydrolysis of BAPM in aqueous solution which reduces the effective concentration of inactivator over the time intervals used in these experiments. Elevation of the concentration of BAPM overcomes this effect by increasing the rate of inactivation. Because of the problem with BAPM hydrolysis, initial rates were estimated from the curves and these values used in kinetic plots. Only at high BAPM concentrations (where inactivation preceded hydrolysis), was it possible to completely inactivate the enzyme. The apoenzyme alone was stable under these conditions over the time course of these experiments.

Inactivation with BAPM shows remarkable pH dependence. At near neutral pH, the rate of inactivation is very slow at all concentrations. Increasing the pH, however, leads to faster inactivation which does not plateau before the apoenzyme begins to denature above pH 9.5 (see Figure 3). Unfortunately, the denaturation of the apoenzyme at this pH does

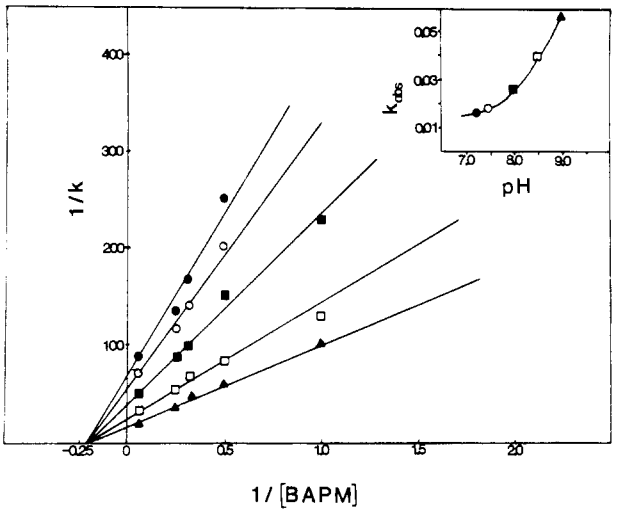

FIGURE 3: Effect of pH on the rate of BAPM inactivation. The effect of pH on inactivation by varying BAPM concentrations was determined and the initial rate of inactivation estimated from the 50% inactivation ($t_{1/2}$ in minutes); data not shown. (●) pH 7.25; (○) pH 7.5; (■) pH 8.0; (□) pH 8.5; (▲) pH 9.0. The inset shows the observed rate constant, K_{obsd}, from the reciprocal plot as a function of pH. Symbols are the same as those in the figure.

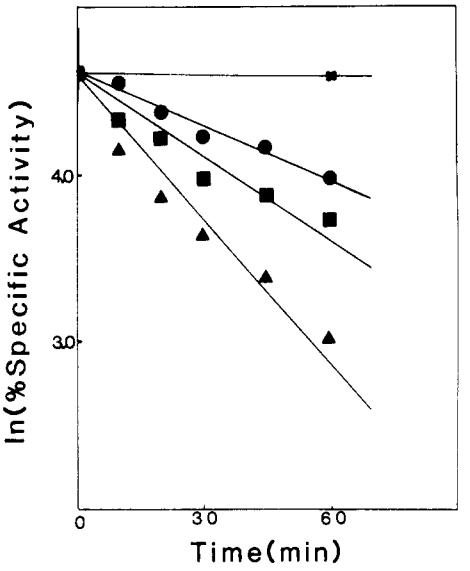

FIGURE 4: Effect of phosphate on BAPM modification. The ability of P_i to inhibit BAPM inactivation of aspartate aminotransferase was demonstrated by adding stoichiometric (■) or a 10-fold excess (●) of P_i per subunit to the inactivation mixture containing 4 mM BAPM, pH 9.0. Parallel reactions were run to compare the stability of the unmodified apoenzyme (×) or BAPM inactivation in a phosphate-free control (▲).

create uncertainty in the estimation of the pK value of the reactive moiety. Nonetheless, by fitting the rate data for the pH dependence of the reaction to a theoretical curve, we estimate the pK of the target group at the cofactor binding site to be no lower than 8.5. The reciprocal plot for BAPM inactivation gives a constant K_d for BAPM of 5 mM at all pHs for the cytosolic isozyme, indicating a relatively low affinity and pH independence of binding for this unphosphorylated affinity label prior to covalent bond formation within the complex. The low affinity allows the reaction to be effectively quenched with pyridoxal-5′-P which readily displaces BAPM from noncovalent complexes.

Effect of Ions. The effect of ionic strength on the rate of BAPM modification was determined by varying the concentration of salt present in the reaction mixture. At concen-

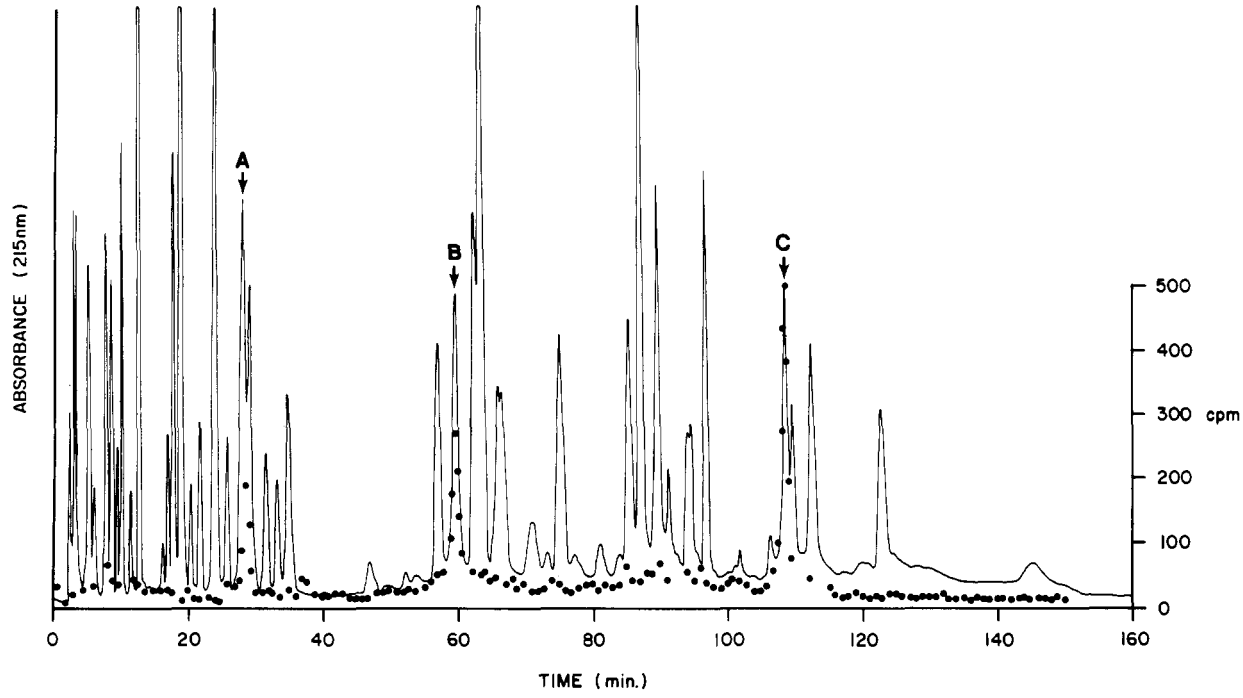

FIGURE 5: Sites of [^{14}C]BAPM incorporation identified from labeled tryptic peptides. Extensively modified and trypsinized aspartate aminotransferase (see Experimental Procedures) was eluted from a Waters MCH-10 C_{18} reverse phase column with a buffer consisting of 3.7% H_3PO_4 (—). The radiolabeled peptides were located (···) and rechromatographed, and the amino acid composition was determined. Identification was made by comparison to the composition of tryptic peptides determined from the sequence. In each case, the carboxymethylated derivative of the labeled residue could be seen in the analyzer. The labeled peptides were identified as those containing (A) Cys-82, (B) Cys-45, and (C) Lys-258.

trations up to 0.4 M KCl, no effect on the rates of enzyme inactivation could be seen. The K_d in the presence of 0.1 M KCl was also identical with that observed in its absence. In contrast, as shown in Figure 4, the presence of phosphate ion inhibits the reaction between BAPM and apoenzyme, a protection phenomenon in which no other ion seems to be as effective. Presence of bound phosphate on the apoenzyme can be directly demonstrated either by differential scanning calorimetry (Relimpio et al., 1981) or by ^{31}P NMR (Mattingly et al., 1982). Since inorganic phosphate is used in the preparation of the apoenzyme to displace the pyridoxamine-5′-P from its binding site, exhaustive removal of bound phosphate is required (see Experimental Procedures) before BAPM labeling experiments are carried out.

Identification of the Active Site Peptide. The peptide containing the specific residue which is modified by BAPM leading to enzyme inactivation was identified from a tryptic digest of the modified enzyme (see Figure 5). The radiolabeled peptide was identified by amino acid analysis and corresponded to the active site tryptic peptide containing Lys-258 (Table I). Additionally, the presence of (carboxymethyl)lysine could be directly identified by coelution from the analyzer with (carboxymethyl)lysine synthesized in our laboratory. When the inactivated enzyme was hydrolyzed immediately following incubation with BAPM, no (carboxymethyl)cysteine was detectable in the amino acid analyzer. Coupled with the observed stoichiometry of inactivation, these results suggest the modification of this single essential lysyl residue with BAPM is responsible for inactivation of aspartate aminotransferase, even though tryptic peptides obtained after prolonged exposure to BAPM show incorporation of BAPM occurring at two sulfhydryls (Cys-45 and -82) away from the active site (Table I). This modification is apparently unrelated to the loss of activity as indicated from the 1:1 stoichiometry between BAPM incorporation at Lys-258 and enzyme inactivation.

Table I: Amino Acid Compositions of Radiolabeled Peptides[a]

amino acid	A_{calcd}[b]	A_{obsd}	B_{calcd}	B_{obsd}	C_{calcd}	C_{obsd}
Ala	1	0.8			1	0.99
Asx			2	1.9	2	2.3
Glx			1	0.9	4	3.2
Cys	1	0[c]	1	0[c]	1	0.9
Thr	1	0.95[e]	1	0.8[e]		
Ser	1	0.5			3	0.6
Pro			1	ND[d]		
Leu			1	0.85	2	1.85
Ile						0.5
Lys					1	0[c]
Phe					5	3.8
Tyr					2	1.2
Val			3	2.7	1	1.0
Trp			1	ND		
Gly					2	1.97
Arg	1	0.99	1	0.95	1	0.95

[a] Values (nanomoles recovered) are uncorrected for losses during hydrolysis. [b] Based upon primary sequence of tryptic peptides containing Cys-82 (A), Cys-45 (B), and Lys-258 (C). [c] Appear as carboxymethyl derivative. [d] Not determined. [e] N-Terminal amino acid detected by manual Edman degradation (Tarr, 1977).

Discussion

Cofactor analogues which serve as affinity labels can be employed to probe the chemical environment in the region of the cofactor binding site. A number of such analogues of pyridoxal-5′-P have been devised for labeling of apo-B_6 enzymes, all of which take advantage of the existence of an empty pyridoxal binding site which directs the modifying agents to a specific region within the enzyme's active site. Although much has been learned concerning the chemical influences on pyridoxal-5′-P by the surrounding active site amino acids, many fundamental questions remain unresolved, one of the most important being how the various B_6 enzymes provide within their unique amino acid sequences the precise envi-

ronment necessary for their versatile catalytic functions. This is especially relevant in an enzyme such as aspartate aminotransferase which occurs as two structurally distinctive isozymes which demonstrate similar overall folding on X-ray maps and catalyze the same reaction while differing in p*I* (Braunstein, 1962), having only 48% sequence homology (Kagamiyama et al., 1980), and demonstrating a number of differing reactivities to a variety of modifying agents (Metzler et al., 1982). Both of the isozymes can be inactivated by BAPM (Farach et al., 1981), yet the chemical differences at the active site make it likely that the inactivation of the mitochondrial isozyme shows unique features from that reported here for the cytosolic form.

Because it is at the chemical site of catalysis, the cofactor binding region of aspartate aminotransferase must contain the moieties which are responsible for the actual interconversions of keto and amino acids in this enzyme. It is consequently of importance to understand the precise chemical environment which engineers these interconversions, including the ionization properties of local groups on the protein as well as the perturbations of these properties under varying experimental conditions.

We have successfully labeled the active site peptide of apo-aspartate aminotransferase with the affinity label BAPM and have shown that covalent incorporation of this label leads to enzyme inactivation even when the cofactor is restored. Under conditions where only specific affinity-directed labeling occurs, the sole amino acid undergoing modification by BAPM is the active site Lys-258 which contains a free amino group in the apoenzyme. Using this same reagent as a cofactor binding site affinity label of tryptophan synthetase, Higgins and Miles specifically modified the sulfhydryl of the active site cysteine, leading to inactivation of their enzyme (Higgins & Miles, 1978). While the active site of aspartate aminotransferase contains a highly reactive cysteinyl residue (Cys-390) which could conceivably react with BAPM, inactivation of aspartate aminotransferase does not correlate with appearance of (carboxymethyl)cysteine in the amino acid analyzer. Furthermore, our previous modification of Cys-390 with 3-bromo-1,1,1-trifluoropropanone only led to 30–40% inactivation (Critz & Martinez-Carrion, 1977). We feel that this indicates that Cys-390, while probably chemically susceptible to reaction with BAPM, must lie too far away from the cofactor binding site to react with BAPM. This is consistent with findings from X-ray crystallography of the holoenzyme where Cys-390 and the bound cofactor are located somewhat distantly. Such a conclusion assumes that BAPM binds to a conformation similar to the pyridoxamine form of the enzyme which is consistent with the spectral indentities of the complexes formed. Nonaffinity-directed reaction of BAPM with two reactive surface sulfhydryls does nonetheless occur very slowly. Both Cys-45 and Cys-82 can be covalently modified over long incubation times with excess BAPM as in the case of treatment with 3-bromo-1,1,1-trifluoropropanone (Critz & Martinez-Carrion, 1977).

Lys-258 reacts readily with a number of other modifying agents, all of which lead to inactivation of the cytosolic enzyme (Morino et al., 1974; Tanase & Morino, 1976; Gehring et al., 1977). Another derivative of the coenzyme, 4′-*N*-(2,4-dinitro-5-fluorophenyl)pyridoxamine-5′-P also reacts covalently with Lys-258 (Riva et al., 1980). Although the enzyme has a much higher affinity for this reagent due to the presence of the cofactor phosphate group (in contrast to our unphosphorylated analogue), little binding to Lys-258 is observed without the cofactor phosphate group.

Modification of Lys-258 with BAPM obeys pseudo-first-order kinetics which indicate a p*K* of this group in the active site of the apoenzyme not lower than 8.5. This value is high relative to previously observed p*K* values for the residue, presumably Lys-258, which perturbs the enzyme-bound ^{19}F-labeled probe at the active site of the holoenzyme (Martinez-Carrion et al., 1976) and that carbamoylated by potassium cyanate treatment (Slebe & Martinez-Carrion, 1978). Furthermore, BAPM modification of Lys-258 does not seem to be influenced by ionic strength, which contrasts with our previous findings for the carbamoylated ϵ-amino group where elevation of the ionic strength increased the observed p*K* of ionization (Slebe & Martinez-Carrion, 1978). We feel that the differences in the observed p*K* of Lys-258 obtained by using these unique approaches probably reflect the sensitivity of the ionization to external influences rather than errors in experimental methodology. This is even more likely when one considers that in one case (Slebe & Martinez-Carrion, 1978) the amino group itself was carbamoylated, while in the other two experiments (Martinez-Carrion et al., 1976; this work) probes with very different chemical properties were employed. The presence or absence of the cofactor phosphate would be expected to influence the chemical environment of the cofactor binding region. BAPM, although lacking the phosphate group of our earlier probe, does contain a pyridinyl structure which could conceivably neutralize an active site residue such as that of Asp-222. In fact, calorimetric studies have suggested that, at least with respect to the thermodynamics of inactivation, these various states of the enzyme are indeed different (Relimpio et al., 1981; unpublished results). All of these differences in ionization behavior under varying conditions at the active site, therefore, serve to emphasize the dynamic nature of the catalytic system, a feature which should be remembered when static pictures of the active site or p*K* values obtained by using a single approach are considered. As we have demonstrated with the cytosolic isozyme of aspartate aminotransferase, p*K* values determined from the pH dependency of modification do not necessarily reflect a "true" p*K* value of a residue in the enzyme. Rather, the observed p*K* can be strongly affected by the nature and charge of the modifying agent.

A curious result which we obtained was the extensive protection from BAPM modification afforded to the apoenzyme by inorganic phosphate which apparently is a specific ion effect. Phosphorus NMR experiments have furthermore shown the presence of phosphate in its binding site on the apoenzyme which can be displaced by both pyridoxal-5′-P and pyridoxamine-5′-P (Mattingly et al., 1982). A role of phosphate as a stabilizer of the apoenzyme has also been postulated as a result of differential scanning calorimetry studies in which it was shown that the enthalpies, T_m, and the difference in free energy are all increased in the presence of phosphate (Relimpio et al., 1981). For reasons which are as yet unclear, the presence of P_i on the apoenzyme prevents the covalent inactivation of aspartate aminotransferase by BAPM. This is difficult to explain since BAPM lacks the phosphate group and is thereby probably explained by an alteration in the conformation in the presence of P_i which prevents BAPM from binding. The ability of P_i to peculiarly inhibit the pyridoxamine transaminase activity of cytosolic but not mitochondrial aspartate aminotransferase was previously seen (Wada & Morino, 1964) which could potentially have a similar explanation to our results with a pyridoxamine derivative.

Structural changes which prevent BAPM inactivation are also hinted at by the observation (data not shown) that aging

of enzyme preparations has a profound effect on susceptibility to BAPM inactivation, with older preparations gaining resistance. It is not known at this time whether this is related to enzymic breakdown to lower subforms or merely reflects an alteration in the overall structure which somehow affords protection from inactivation. It is known that the differential scanning calorimetry thermograms for preparations of α, β, and γ subforms of cytosolic aspartate aminotransferase are very distinct, reflecting the large degree of structural heterogeneity between the subforms, and furthermore that aging of preparations leads to thermograms closely resembling those of the other subforms. This suggests that the aging effect may well translate into a differing susceptibility of the enzymic subforms to BAPM inactivation.

The features of BAPM modification show a high degree of sensitivity which can be exploited to provide data concerning the chemical behavior of an important active site residue under various experimental conditions and in various forms of a single enzyme. We feel that the use of cofactor analogues of vitamin B_6 such as the one described here can be used to provide much needed information concerning exploration of the intrinsic physical properties of the cofactor binding regions of the entire family of B_6 enzymes.

Acknowledgments

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Registry No. BAPM, 54522-09-7; pyridoxal-5′-P, 54-47-7; pyridoxamine-5′-P, 529-96-4; aspartate aminotransferase, 9000-97-9; lysine, 56-87-1.

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