Acylation of Aspartate Aminotransferase

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1. Acetylation of aspartate aminotransferase from pig heart inhibits completely the enzymic activity when the coenzyme is in the amino form (pyridoxamine phosphate) or when the coenzyme has been removed, but not when the coenzyme is in the aldehyde form (pyridoxal phosphate). 2. The group the acylation of which is responsible for the inhibition has been identified with the ϵ -amino group of a lysine residue at the coenzyme-binding site. Moreover, in the pyridoxamine-enzyme the amino group of the coenzyme is also acetylated. 3. The reactivity of the coenzyme-binding lysine residue is greatly different in the pyridoxamine-enzyme and in the apoenzyme, suggesting the possibility of an interaction of its ϵ -amino group with pyridoxamine or with other groups on the protein.

In a previous paper (Turano, Giartosio & Vecchini, 1962) the blockage of most of the amino groups of aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) from pig heart was reported. In particular, the behaviour of the pyridoxal phosphate form (PLP-enzyme*) of the holoenzyme was investigated, and it was shown that the acetylation of the freely reacting amino groups of the protein, amounting to about 80% of the total amino groups, caused essentially no decrease in the catalytic activity. Acetylation performed without protection of the thiol groups resulted in an inhibition of about 50%, due to the blockage of the more reactive thiol groups.

Acylation of the other two forms of the enzyme, i.e. the pyridoxamine phosphate holoenzyme (PMP-enzyme) and the apoenzyme, followed by a decrease in the enzymic activity, has also been reported (Torchinskii, 1963; Turano & Giartosio, 1964; Braunstein, Torchinskii, Malakhova & Sinicyna, 1965). Some aspects of this reaction, however, still await a more detailed description.

The purpose of the present paper is to report the relative rates of inhibition for the different forms of the enzyme, the effect of the acylation on the coenzyme-binding and on the reaction of the enzyme with the substrates, and the nature of the groups of the protein that are involved in the inhibition of activity.

EXPERIMENTAL

The enzyme was prepared in a highly purified form by the method of Jenkins, Yphantis &Sizer (1959) with some

modifications: after the $(\text{NH}_4)_2\text{SO}_4$ fractionation the enzyme was purified on a CM-cellulose column according to the method of Lis (1958), and then on a DEAE-cellulose column (A. J. Lawrence, personal communication), equilibrated with 20 mM-phosphate buffer, pH 7.4, and eluted with the same buffer. The enzyme was finally concentrated by acetone precipitation according to the method of Lis (1958). In some preliminary experiments the enzyme from CM-cellulose was used without further purification.

The enzyme was usually prepared in maleate buffer, which is known to block about one cysteine residue of the enzyme (Turano, Giartosio, Riva & Fasella, 1964b). When preparations obtained in succinate buffer were tested, however, no substantial difference in the behaviour towards the acylating reagents was found.

The PMP form of the enzyme was prepared according to the method of Lis, Fasella, Turano & Vecchini (1960).

The apoenzyme was prepared either by the method of Wada & Snell (1962), or by the following method, based essentially on the procedure of Scardi, Scotto, Iaccarino & Scarano (1963): the enzyme solution (5 ml.) was dialysed in the cold (2–4°) against three changes of 100 ml. each of 0.9 M-potassium phosphate buffer, pH 5.3, containing glutamate (0.1 M), then against three changes of 200 ml. each of M-phosphate buffer, pH 5.3, and finally extensively against phosphate buffer at pH 7.8, first 10 mM and then 5 mM. The last dialysis at a slightly alkaline pH was necessary to avoid denaturation of the protein in the preparation of acetylated apoenzyme. Any precipitate formed during the dialysis was removed by centrifugation.

Activities were measured according to the method of Lis (1958), at 24°. In some experiments a triethanolamine-HCl buffer was employed (Banks, Lawrence, Thain & Vernon, 1963) for the activity measurements, instead of phosphate buffer, to catalyse more efficiently the keto-enol tautomerization of oxaloacetate. Under our experimental conditions the results obtained with the two methods agreed within the limits of the experimental error. Activity measurements of the apoenzyme were performed by incubating the reaction mixture for 10 min. with 0.3 mM-PLP before starting the

* Abbreviations: PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; FDNB: 1-fluoro-2,4-dinitrobenzene.

reaction with the addition of α -oxoglutarate. A longer incubation time did not increase the activity.

Acetylation of the enzyme. Acetylation of the different forms of the enzyme was performed with acetic anhydride as described by Turano *et al.* (1962), with sodium acetate solution or a potassium phosphate buffer to keep the pH of the reaction mixture above 7.

Acylations carried out with other reagents were performed with phosphate buffer; when succinic anhydride was used, weighed amounts of the solid were added to the reaction vessel.

In some experiments the acetylation was performed in a pH-stat (Radiometer) to keep the pH constant during the reaction, thereby allowing the reaction to be carried out at a lower ionic strength. The reaction vessel of the pH-stat unit was refrigerated at 2°; the pH, after the introduction of acetic anhydride, was maintained constant by the automatic addition of 2M-K₂CO₃-KHCO₃ buffer, pH 9.2. When the curve recording the amount of buffer added as a function of time became flat, the enzymic activity was tested on samples withdrawn from the reaction vessel before the next addition of acylating reagent.

Preparation of sodium borohydride-reduced enzyme (pyridoxyl-enzyme). Native or acetylated PLP-enzyme was dialysed in the cold first against distilled water, then for 15 min. against 500 ml. of 30 mM-NaBH₄, and finally extensively against distilled water.

Dinitrophenylation of the active apoenzyme. PLP-enzyme was first acetylated and then submitted to the treatment for the removal of the coenzyme. FDNB was added to a final concentration of 5 μ l./ml. to apoenzyme solution in 0.8 M-sodium acetate, final pH 7.4. The reaction mixture was stirred continuously and its pH was kept constant by addition of 2M-K₂CO₃-KHCO₃ buffer, pH 9.2.

After 45 min., the reaction was stopped by lowering the pH to 1 with HCl, and the unchanged FDNB and the 2,4-dinitrophenol formed were extracted with ether and discarded. The insoluble enzyme was recovered by centrifugation and washed several times with water and acetone.

Dinitrophenylation of the denatured enzyme. Acetylated holoenzyme, or apoenzyme, was denatured by heating for 5 min. in a boiling-water bath and the precipitate formed (after the addition of few drops of N-acetic acid) was collected by centrifugation and suspended in 2–3 ml. of 4% (w/v) NaHCO₃; 3 vol. of ethanol, containing 0.02 ml. of FDNB/ml., was added, and the reaction was allowed to proceed with occasional stirring for 4 hr. at room temperature in the dark. The mixture was then acidified to pH 1 with conc. HCl and a few millilitres of water were added. Ethanol, unchanged FDNB and 2,4-dinitrophenol were extracted with ether and discarded. The insoluble enzyme was recovered by centrifugation and washed several times with water and acetone.

Digestion of the protein and analysis of the peptides. The acetylated enzymes after borohydride reduction or after dinitrophenylation were submitted to chymotryptic hydrolysis according to the following procedure. If the protein was still in solution, it was first denatured by heating in a boiling-water bath for 5 min. The precipitate was suspended in 0.1 M-NH₄HCO₃ and digested with one-fortieth of its weight of chymotrypsin (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) for 16 hr. at 25°. The pH was then lowered to 5–6 with N-acetic acid, and any precipitate formed was removed by centrifugation. Usually,

only a slight turbidity developed from the digestion products of the DNP-protein. The samples were then freeze-dried.

For the analysis of DNP-peptides, the freeze-dried samples were dissolved in a minimum amount of dilute NH₃, spotted on Whatman no. 3 MM paper and subjected to a two-dimensional separation, first by chromatography in butan-1-ol-pyridine-water (6:5:5, by vol.) and then by electrophoresis in 50 mM-NH₄HCO₃ (30 v/cm. for 2 hr.) on a water-cooled brass plate.

For the analysis of the pyridoxyl-peptides obtained from the NaBH₄-treated enzymes, a chromatographic separation was first carried out on a Whatman no. 3 MM paper with the solvent pyridine-3-methylbutan-1-ol-water (7:7:6, by vol.). The fluorescent spots were detected with the aid of a Mineralight ultraviolet lamp. The fluorescent zones were cut, placed as a bridge between two strips of Whatman no. 3 MM paper wetted with pyridine-acetic acid buffer, pH 6.4 (Ingram, 1958), and submitted to electrophoretic separation on a cooled plate under a potential gradient of 30 v/cm.

Chromatographic identification of N'-acetyl-PMP. PMP (30 mg.) in 1 ml. of 0.2 N-NaOH was treated with 0.05 ml. of acetic anhydride in five portions, each portion being followed by 0.1 ml. of 2 N-NaOH. After 2 hr. at room temperature, the reaction mixture was placed on an Amberlite IRC-50 (H⁺ form) column (1 cm. $\times$ 50 cm.). The fluorescent fractions obtained by washing the column with water were spotted on Whatman no. 4 paper and chromatographed with the solvent 2-methylpropan-2-ol-89% formic acid-water (14:3:3, by vol.) (Peterson & Sober, 1954). The first fractions gave two violet fluorescent spots, with R_F 0.60 and R_F 0.23. The last fractions from the column gave only the second spot. The spot with the higher R_F value did not react with ninhydrin, but reacted with diazotized *p*-aminoacetophenone (Siliprandi, Siliprandi & Lis, 1954); the other spot reacted with both reagents. On the basis of the behaviour on the ion-exchange column, on paper chromatography and towards the specific reagents, the fluorescent spot with R_F 0.60 was attributed to N'-acetyl-PMP, the other spot to unchanged PMP.

RESULTS

Acetylation of PMP-enzyme. The effect of acetic anhydride and other acylating reagents on the PMP form of the enzyme is shown in Table 1. For comparison, the effect on PLP-enzyme is also reported, and the data show that the PMP-enzyme is not particularly sensitive to acylation, but is inhibited, at least in our experimental conditions, only slightly more than the PLP form.

In spite of the relatively high concentration of buffer used in the acetylation reaction, the pH decreased as the amount of added reagent increased; therefore, to perform a more extensive acetylation, the reaction was carried out at constant pH in a pH-stat; the effect of subsequent additions of acetic anhydride to the PLP and PMP forms of the enzyme is shown in Fig. 1. Both the PLP and PMP forms of the enzyme were inhibited by 30–50% after the first addition of reagent, but only with the PMP-enzyme did the addition of a greater quantity of reagent progressively increase the inhibition.

Table 1. *Effect of acylating reagents on the activity of the PLP and PMP forms of the holoenzyme*

The reaction was performed in 0.5M-K₂HPO₄. Enzyme concentrations were in the range 65–150 µg./ml. Activities were determined 10–30 min. after the addition of the reagent, except when succinic anhydride was used, in which case the activity was determined after 4–20 hr.

Reagent	Amount of reagent added (mg./ml. of solution)	Inhibition (%)	
		PLP-enzyme	PMP-enzyme
Acetic anhydride	2.7	21	25
	5.3	26	30
	8.0	27	32
Succinic anhydride	2.5	21	27
	5.0	28	37
	8.3	16	19
Acetyl chloride	14	22	33

Fig. 1. Effect of increasing amounts of acetic anhydride on PLP-enzyme and PMP-enzyme. The reaction was performed in a pH-stat. Activities were measured 10 min. after each addition of the reagent. ●, PLP-enzyme in m-sodium acetate, pH 7.4; ■, PLP-enzyme in 0.3M-potassium phosphate buffer, pH 7.8; ○, PMP-enzyme in m-sodium acetate, pH 7.4; □, PMP-enzyme in 0.3M-potassium phosphate buffer, pH 7.8. The numbers in parentheses show the percentages of residual free amino groups.

This behaviour was more evident when the acylation was performed in sodium acetate than in phosphate.

By using a relatively high concentration of extensively acetylated enzymes it was shown that the acetylated PMP-enzyme is unable to react with the keto acid substrate to form the PLP-enzyme (Lis *et al.* 1960; Jenkins & Sizer, 1957); by contrast the PLP-enzyme reacts completely with glutamate to give PMP and α -oxoglutarate (Fig. 2). The inhibition of the PMP-enzyme is not due to loss of coenzyme, since prolonged dialysis or passage through a 15 cm.-long column of Sephadex G-25

Fig. 2. Absorption spectra of extensively acetylated PLP-enzyme and PMP-enzymes, before and after the addition of substrates. The enzyme solutions were in m-sodium acetate, pH 7.4. (a) Acetylated PLP-enzyme (1.3 mg./ml.): —, without substrate; ----, with L-glutamate (0.1 M). (b) Acetylated PMP-enzyme (1.3 mg./ml.): —, without substrate; ----, with α -oxoglutarate (12 mM) (the extinction due to α -oxoglutarate has been subtracted from the total extinction).

failed to modify the E_{333} value of the enzyme due to bound coenzyme.

Considering the stability of the bond between PMP and the acetylated enzyme it seems improbable that the inhibition is due to an extensive blockage of thiol groups, which is known to cause a pronounced increase in the dissociation constant of the PMP-enzyme (Turano, Giartosio & Fasella, 1964a). This view is further confirmed by the results of the *p*-chloromercuribenzoate titration of normal and acetylated enzymes by the method of Boyer (1954), showing that about the same number of thiol groups has been acetylated in the PLP-enzyme and PMP-enzyme, as shown in Table 2.

Table 2. *Effect of acetylation on the thiol content of PLP-enzyme and PMP-enzyme*

Experimental conditions were as described in Fig. 1. The total amount of acetic anhydride added was 9–10 mg./ml. of solution. Thiol groups were determined on urea-denatured enzyme.

	Residual activity (%)	Residual free SH groups
Enzyme before acetylation	(100)	3.8
Acetylated PLP-enzyme*	64	3.0
Acetylated PLP-enzyme†	68	2.7
Acetylated PMP-enzyme*	12	2.7
Acetylated PMP-enzyme†	38	2.1

* Acetylation performed in the presence of α -sodium acetate, pH 7.4.

† Acetylation performed in the presence of 0.3 M-potassium phosphate buffer, pH 7.8.

Table 3. *Effect of acetic anhydride on the activity of the apoenzyme*

Enzyme concentrations were in the range 0.1–0.6 mg./ml. Activities were determined after 10 min. incubation of the enzyme with 0.16 M-PLP.

Type of apoenzyme tested	Amount of reagent added (mg./ml. of solution)	Reaction medium	Inhibition (%)
Normal	0.54	0.5 M-K ₂ HPO ₄	96
apoenzyme	1.1	0.5 M-K ₂ HPO ₄	97
	0.36	M-Sodium acetate	95
Apoenzyme obtained	5.4	Half-saturated sodium acetate	97
from acetylated	0.67	0.9 M-Sodium acetate	95
holoenzyme	0.25	0.5 M-K ₂ HPO ₄	92

The thiol content reported here is different from the one reported previously, both for the untreated and the acetylated enzyme (Turano *et al.* 1962, 1964a). However, the present values are referred to a lower molecular weight [47 000 (Martinez-Carrion *et al.* 1967) instead of 58 000 (Jenkins *et al.* 1959)] and were obtained from an enzyme treated with maleate during the purification procedure. As already stated, maleate blocks about one thiol group of the native enzyme (Turano *et al.* 1964b).

The involvement of a phenolic hydroxyl group also seems to be excluded by the fact that acetyl-imidazole, a good acetylating reagent for tyrosine residues (Simpson, Riordan & Vallee, 1963), fails to inhibit the aminotransferase (Turano, Giartosio, Riva, Baroncelli & Bossa, 1966).

The other functional protein groups that are likely to be involved in the acylation reaction under the experimental conditions used are the amino groups. From the data reported in Fig. 1, 78–85% of the protein amino groups are acetylated, but this extensive acetylation does not appear to be in itself the cause of the inhibition: in fact, though the PLP-enzyme and the PMP-enzyme are acetylated to about the same extent, only the PMP form undergoes a nearly complete inhibition. The small difference in the number of acetylated amino groups between the PLP-enzyme and the PMP-enzyme amounts to about 10% of the total protein amino groups (Fig. 1), i.e., according to the amino acid composition of the aminotransferase (Martinez-Carrion *et al.* 1967), to about two amino groups. The inhibition therefore could be caused by the blockage of one or few specific amino groups, not available in the PLP-enzyme but free to react in the PMP-enzyme. The identification of these amino groups involved in the inhibition is reported below.

Acetylation of the apoenzyme. The apoenzyme is

easily inactivated by reaction with acetic anhydride (Table 3), as already reported by Torchinskii (1963) and Turano & Giartosio (1964).

In view of the decreased stability of the apoenzyme relative to the holoenzyme, this inactivation might have been due to non-specific denaturation after the extensive chemical modification. That this is not so is shown by the fact that when acetylated PLP-enzyme, with about 70% of its original activity left, was submitted to the treatment for the removal of the coenzyme, the apoenzyme obtained in this way would bind the coenzyme again to give active holoenzyme; acetylation of this apoenzyme led to complete inhibition (Table 3). Thus the inhibition of the apoenzyme appears to be due to the blockage of one or few specific groups, free to react only after the removal of the coenzyme. Apoenzyme, in contrast with PMP-enzyme and PLP-enzyme, is very sensitive to acetic anhydride, since even small amounts of reagent gave a nearly complete inhibition of the enzymic activity.

In the spectrum of normal apoenzyme, preincubated with PLP, a peak at 362 m μ appears, characteristic of the enzyme-bound PLP, whereas in the spectrum of acetic anhydride-treated apoenzyme, preincubated with PLP, a peak at 390 m μ appears, characteristic of free PLP. Apoenzyme therefore appears to be completely inactivated by acetic anhydride, since this reagent inhibits the recombination with the coenzyme; the small residual activity of the apoenzyme after acetylation (Table 3) is to be attributed to the presence of some holoenzyme. The thiol content, determined in 8 M-urea, before and after acetylation of the apoenzyme (prepared from an acetylated holoenzyme as explained above) was 2.3 and 2.5 respectively.

Scheme 1. Procedure for the preparation and analysis of modified aminotransferase

Fig. 3. Chromatographic and electrophoretic separation of the yellow DNP-peptides obtained by dinitrophenylation of the acetylated and denatured PLP-enzyme (see Scheme 1 procedure A).

Fig. 4. Chromatographic and electrophoretic separation of the yellow DNP-peptides obtained by dinitrophenylation of acetic anhydride-treated and denatured apoenzyme (see Scheme 1, procedure B). The apoenzyme was treated with 0.25 mg. of acetic anhydride/ml. in 1.5 M-sodium acetate. The DNP-peptides from the acetylated PMP-enzyme (see Scheme 1, procedure C) gave essentially the same pattern.

Moreover, enzymic activity could not be restored by treatment with hydroxylamine in the pH range 7–9, followed by dialysis. This rules out the possibility that a labile acetyl group is responsible for the inactivation of the apoenzyme: an amino group is therefore probably involved in this inactivation.

Identification of the sites involved in the inhibition. To detect amino and thiol groups of the protein that do not react with acetic anhydride, the procedure described in Scheme 1 (procedure A) was followed. The amino and thiol groups that were not acetylated were converted into their corresponding yellow DNP derivative; the protein was then partially hydrolysed with chymotrypsin, and the resulting

Fig. 5. Chromatographic and electrophoretic separation of the yellow DNP-peptides obtained by dinitrophenylation of acetylated, reduced and denatured PLP-enzyme (see Scheme 1, procedure D).

peptides were analysed by chromatography and electrophoresis. The peptides carrying amino or thiol groups unavailable to the acetylating reagent appeared as yellow spots. By comparing the peptide 'maps' obtained in this way (Figs. 3 and 4) from the PLP-enzyme, the apoenzyme and the PMP-enzyme, it was shown (see Scheme 1, procedures A, B and C) that only one yellow spot (spot no. 1) is missing from the peptide 'maps' derived from the acetylated apoenzyme or the acetylated PMP-enzyme, i.e. from completely inhibited enzymes. This suggests that the same group is involved in the inhibition of the two forms of the enzyme; this must be an amino group since, as shown above, thiol groups are not involved.

The same yellow spot (no. 1) was missing (Fig. 5) when an acetylated PLP-enzyme was reduced with sodium borohydride before the denaturation and the FDNB treatment (Scheme 1, procedure D). Since sodium borohydride is known to reduce and stabilize the aldimine bond between the PLP and a lysine ϵ -amino group (Fischer & Krebs, 1959; Turano, Fasella, Vecchini & Giartosio, 1961), spot no. 1 should be given by a peptide containing the lysine residue that binds the coenzyme.

The disappearance of this spot on acetylation of the PMP-enzyme or of the apoenzyme therefore shows that the inhibition of these two forms of the enzyme is accompanied by acetylation occurring at one of the coenzyme-binding sites.

Further proof of the identity of spot no. 1 was obtained by elution of the spot from the chromatogram, hydrolysis in 6N-hydrochloric acid of the eluate for 16 hr. at 110° and qualitative analysis of the amino acids present in the hydrolysate. All the amino acids that according to Hughes, Jenkins & Fischer (1962) are present in the pyridoxyl-peptide obtained by chymotryptic hydrolysis of the reduced aminotransferase were present in the

Fig. 6. Chromatographic and electrophoretic separation of the yellow DNP-peptides obtained from active apoenzyme dinitrophenylated in mild conditions (see Scheme 1, procedure E).

hydrolysate of spot no. 1, i.e. alanine, aspartic acid, glutamic acid, glycine, isoleucine, phenylalanine, serine, threonine and valine; free lysine was absent, but its yellow ϵ -DNP derivative was present; traces of proline were also found, which can be attributed to contamination.

The lysine residue (or residues) of this peptide appears to be, in the apoenzyme, very reactive also towards other types of reagents besides acetic anhydride, as shown by the fact that, when an active apoenzyme was treated with FDNB without a preliminary denaturation and under mild conditions, i.e. in water at pH 7.4, at room temperature for 45 min. (see Scheme 1, procedure E), the peptide 'map' revealed the presence of only one intense yellow spot, corresponding to spot no. 1 (Fig. 6).

To investigate the occurrence of acetylation at the amino group of PMP, an acetylated PMP-enzyme was resolved by heating at 100° for 5 min., and dialysed against several small portions of water. The diffusate was collected, concentrated by freeze-drying and analysed by paper chromatography as described in the Experimental section. Most of the coenzyme was transformed into a substance that behaved on the chromatogram as *N*'-acetyl-PMP.

The possibility exists that some acetyl group is introduced in the proximity of the coenzyme-binding lysine group on acetylation of the PLP-enzyme, particularly since it has been suggested that a second lysine residue could be near (Hughes *et al.* 1962) or even adjacent (Polyanovskii & Keil, 1963) to the first one. To check this possibility a normal and an acetylated PLP-enzyme preparation were treated with sodium borohydride, denatured and digested with chymotrypsin. This treatment is known (Turano *et al.* 1961; Hughes *et al.* 1962) to yield a peptide with a pyridoxyl group attached in a stable way, easily detected by its violet fluorescence.

No difference was found between the fluorescent pyridoxyl-peptides from the normal and the acetylated holoenzyme when they were analysed by chromatography in pyridine-3-methylbutan-1-ol-water (7:7:6, by vol.) or by electrophoresis (pyridine-acetic acid buffer, pH 6.4).

DISCUSSION

The data reported show that aspartate aminotransferase without the coenzyme or with the coenzyme in the amino form is completely inhibited by acetylation, as appears from kinetic and spectral measurements: in the apoenzyme the inhibition is to be ascribed mainly to the impairment of coenzyme-binding, whereas in the amino holoenzyme the inhibition is due to the blockage of some groups at the active site. Particularly important appear to be the spectral data obtained from the acetylated PMP-enzyme, which show the complete inability of the modified enzyme to react with the keto acid substrates; the acetylated PLP-enzyme, instead, which is about 30% inhibited according to kinetic analysis, appears to react completely with the amino acid substrates according to spectral measurements (Fig. 2).

The inhibition of the PMP-enzyme and of the apoenzyme is accompanied by acetylation of an amino group at the PLP-binding site of the protein: whereas in the PLP-enzyme this amino group is masked by its participation in an aldimine bond with the coenzyme, in the apoenzyme and in the PMP-enzyme it can react; in the latter form of the enzyme, moreover, the amino group of the coenzyme can also react. There is, however, a great difference between apoenzyme and PMP-enzyme in the reactivity towards acetic anhydride: the former is very sensitive to the reagent, whereas the latter reacts only slowly. The reaction with the PMP-enzyme also seems to be dependent on the nature of the acylating reagent, since succinic anhydride, as reported by Braunstein *et al.* (1965), does not react with the coenzyme amino group. The poor reactivity of the amino form of the enzyme could originate either from steric reasons or from the interaction of the amino groups involved with neighbouring groups.

On the other hand, the fact that the amino group of the enzyme reacts at all is a strong argument against the hypothesis of Evangelopoulos & Sizer (1963), who suggest that a real PMP form of the enzyme does not exist, but that PMP can be liberated only by drastic treatment of an amino acid-aminotransferase complex.

Up to now we have referred to the amino group of the lysine residue that binds PLP in the holoenzyme; however, the presence of a second lysine residue near

(Hughes *et al.* 1962) or even adjacent (Polyanovskii & Keil, 1963) to the first one has been suggested, though the existence of this second lysine residue has been questioned (Laurson & Westheimer, 1966). Our data do not permit any conclusion on the existence of the second lysine residue; if it exists it shows, in our experiments, the same behaviour as the coenzyme-binding lysine residue: the identity of the chymotryptic pyridoxyl-peptides and the absence of free lysine from the hydrolysate of the DNP-peptide from spot no. 1 would indicate that both lysine residues were masked in the holoenzyme; the appearance of spot no. 1 after the dinitrophenylation of the active apoenzyme in mild conditions would indicate that both lysine residues are reactive in the apoenzyme.

The problem of the existence of this second lysine residue clearly deserves careful investigation, since its presence near the active site and its reactivity in the apoenzyme might suggest a role for it in the enzymic function, possibly in the coenzyme-binding. The phosphate group of PLP or PMP, in fact, is probably bound to the protein through a positive charge; our experiments show that the only amino groups masked in the holoenzyme and freely reacting in the apoenzyme are those of the lysine residue that forms the aldimine bond in the PLP-enzyme and possibly of a second lysine residue situated nearby. Therefore, if a positive charge is truly required for the binding of the phosphate group, it should be contributed by an arginine residue, or by a lysine residue that is masked even in the apoenzyme, or by that particular lysine residue the existence of which remains to be ascertained.

The removal of the coenzyme from the holoenzyme is accompanied by a small but definite change in conformation of the protein, according to optical-rotatory-dispersion (Fasella & Hammes, 1964; Torchinskii & Koreneva, 1963) and deuterium-exchange (Abaturov, Polyanovskii, Torchinskii & Varshavskii, 1966) data, in the sense of a less ordered structure of the apoenzyme. According to our experiments, this conformational change does not unmask thiol or amino groups that are masked in the holoenzyme, apart from the amino group (or groups) that are 'buried' in the holoenzyme by the presence of the coenzyme. This fact leads us to hope that the same technique used in this work can be usefully applied to detect residues other than lysine contributing to the binding of the coenzyme in aspartate aminotransferase.

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