The Structure and Enzyme-Coenzyme Relationship of Supernatant Aspartate Transaminase after Dye Sensitized Photooxidation*

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SUMMARY

Photooxidation of supernatant glutamate aspartate transaminase, limited to the stage during which much of the enzyme activity and only histidine residues are destroyed, does not induce gross protein structural changes as judged by ultracentrifugation, optical rotatory dispersion in the ultraviolet region, and microcomplement fixation techniques. Photooxidized apoenzyme has been shown to bind at least 90% as much coenzyme as the native enzyme and forms holopyridoxal or pyridoxamine enzyme which is very similar to the native enzyme in optical and absorption spectral properties in the visible region of the spectrum. The absorption spectrum of photooxidized apo- or holoenzyme has a new maximum at 325 mμ. Because of analogy to the absorption of the photooxidation product of a model compound, glycy1-histidyl-glycine, the new maximum in photooxidized transaminase has been assigned to the photo-product of the altered histidines in the enzyme.

Circular dichroism measurements of apoenzyme revealed positive ellipticity bands in the 280 to 300 mμ regions of the spectrum. They remain essentially unaltered after photooxidation. Binding of pyridoxal phosphate to this apoenzyme results in the appearance of the well known positive ellipticity bands in the visible region and formation of new negative ellipticity bands centered at 298 and 290 mμ in the aromatic region. Photooxidation eliminates the 290 mμ band from the pyridoxal form of the holoenzyme. The circular dichroism pattern of pyridoxamine phosphate bound to photooxidized transaminase is identical with that of native pyridoxamine holoenzyme only if this enzyme form is produced by transamination of photooxidized pyridoxal holoenzyme, not if formed by addition of pyridoxamine phosphate to photooxidized apoenzyme.

Another physical anomaly resulting from photooxidation is the shift of the bound pyridoxal phosphate pH from 6.3 to 6.8. Since controlled photooxidation results in modification of only histidyl residues, this shift in pH may be due to the modification of a histidine in the steric vicinity of the pyridoxal phosphate at the active site, resulting in a localized perturbation of the environment of the coenzyme. The role of the histidyl residue at the active site cannot be associated with coenzyme binding.

Photooxidation of proteins in the presence of dyes can result in alteration of histidyl, tryptophyl, methionyl, tyrosyl, and cysteinyl residues (3, 4). However, photooxidation of soluble pig heart aspartate transaminase (EC 2.6.1.1) in the presence of either methylene blue or rose Bengal results in the alteration of only histidyl residues, with a concomitant loss of enzymatic activity (5). Since during photooxidation the first order rate constant for the alteration of rapidly oxidized histidyl residues is identical with the first order constant for the loss of enzymatic activity, it has previously been concluded that loss of catalytic activity was due to the specific alteration of one of the transaminase's histidyl residues (5). This histidine need not necessarily be at the active site since the alteration of these protein amino acid residues could result in inactivation by changing the basic enzyme structure needed for maintenance of a catalytically efficient conformation. Indeed, methylene blue-sensitized photooxidation of dehydrogenase has been shown to cause inactivation because of gross changes in tertiary structure (6).

On the other hand, this histidine could be located at the active site and function directly or indirectly in catalysis. Histidine residues have been implicated, for example, in the catalytic mechanism of rabbit muscle aldolase (7).

It is important to distinguish between these possibilities if the role of this residue is to be understood. We have, therefore, carried out a series of studies on the structure of the photooxidized enzyme with spectral, optical, and immunochemical techniques to determine whether there are detectable differences between the structure of this enzyme and the native enzyme. Both protein structure and the enzyme-coenzyme relationship have been investigated in this work. The accompanying paper (8) deals with the assignment of a function for the active site histidyl residue in the mechanism of transamination.

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EXPERIMENTAL PROCEDURE

Materials

Soluble aspartate transaminase was prepared from pig hearts as previously described (9). Pyridoxal phosphate, cysteine sulfinate, and TEAE-cellulose were purchased from Sigma. 5'-Amino tetrazole monohydrate was purchased from Aldrich. The reagents for immunochemical studies were obtained from the Colorado Serum Company. Glycyl-histidyl-glycine was purchased from Miles Chemicals.

Methods

Photooxidation

Aspartate transaminase was photooxidized as previously indicated (5) and the methylene blue was removed with Norit or by passage through a column, 2 x 25 cm, of Sephadex G-25 equilibrated with 0.05 M potassium phosphate, pH 7.5. Rose Bengal was removed by passage through a TEAE-cellulose column, 1 x 15 cm, equilibrated with 0.05 M Tris-HCl buffer, pH 8.7, which absorbed the dye but not the protein. Throughout this work, enzyme with 20% residual activity or greater was used because photooxidation to this extent resulted in the modification of only histidyl residues (5). With further photooxidation, other amino acids, notably methionine, cysteine, and tryptophan, began to be altered. Glycyl-histidyl-glycine, acetyl histidine, and imidazole were photooxidized under identical conditions.

Spectrophotometric Studies

All absorption spectra were recorded with a Cary model 15 recording spectrophotometer equipped with a 0- to 0.1 absorbance unit expanded scale slidewire. The pH values were determined with a Corning model 12 pH meter equipped with a Sargent glass combination electrode at 25°C.

Optical Rotatory Dispersion and Circular Dichroism

These measurements were made with a Cary model 60 spectropolarimeter with a circular dichroism attachment. Silica cells with light path ranging from 1 to 10 mm were used. The slit width was programmed so that the dynode voltage did not exceed 0.25 volt with a 1-cm cell containing distilled water. Scan speeds of 40 to 80 A per min were used, with time constant settings varying from 3 to 10. Recorded base lines were subtracted from dispersion or ellipticity bands. Different enzymes preparations yielded comparable results. Protein concentrations, which varied between 0.1 and 10 mg per ml, were determined from the absorbance at 280 mp.

The specific rotation, [α], was calculated from the equation

\[ [\alpha] = 100 \frac{\alpha}{l c} \]

where \( l \) is the cell length in decimeters, \( c \) is the protein concentration in grams per 100 ml, and \( \alpha \) is the observed rotation, always corrected for the solvent blank. The \( \theta \) values were recorded directly from the instrument. Molecular ellipticity \( [\theta] \) is given as degree square centimeter per dmole.

Preparation of Apoenzyme

Removal of pyridoxal phosphate was accomplished by adding to 1 ml of enzyme (5 to 10 mg) in 0.05 M potassium phosphate buffer, pH 7.5, a molar excess of cysteine sulfinate followed by 1 ml of 1 M monobasic potassium phosphate. The mixture was then incubated at 25°C for 40 min and solid, finely ground ammonium sulfate added to 70% saturation. The precipitate was collected by centrifugation and recovered in 1 ml of 0.005 M potassium phosphate buffer, pH 7.5. The entire procedure was repeated and the enzyme was dialyzed against 0.1 M potassium phosphate buffer, pH 7.5, to remove ammonium sulfate. This procedure gives over 85% recovery of enzyme that is 95% free of pyridoxal phosphate. In all instances, the specific activity can be restored by the addition of 10 \( \mu \) m pyridoxal phosphate and removal of the excess unbound coenzyme by dialysis against water.

Pyridoxal Phosphate Content

The pyridoxal phosphate content of aspartate transaminase was determined either spectrophotometrically in sodium acetate, pH 5.1, with an extinction coefficient of 8050 at 430 mp, or by the phenyl hydrazone method of Wada and Snell (10).

Recombination of Apoenzyme with Coenzyme

Pyridoxal Phosphate—Samples containing 1 to 2 mg of native or photooxidized apoenzyme in 0.05 M Tris-HCl, pH 8.2, were titrated with 2-\( \mu \)l aliquots of 6.9 \times 10^{-4} M pyridoxyl phosphate solution. The resultant increase in optical density at 360 mp, due to bound pyridoxal phosphate, was followed spectrophotometrically.

Pyridoxamine Phosphate—Samples, 1.5 ml, of 3.6 \times 10^{-7} M pyridoxamine phosphate in 0.05 M Tris-HCl, pH 7.5, were titrated with 10-\( \mu \)l aliquots of 6.2 \times 10^{-4} M native or photooxidized enzyme solution. After incubation for 60 min at 25°C in the dark, the fluorescence at 405 mp was measured in a Turner fluorimeter.

Ultracentrifugation

The enzyme solutions used for these studies were in 0.1 M potassium phosphate buffer, pH 7.5, at concentrations of 3 to 6 mg of protein per ml. The sedimentation velocities of proteins were determined with a Spinco model E analytical ultracentrifuge equipped with a phase plate as a schlieren diaphragm. The sedimentation coefficient at 20°C in water was calculated as described by Schachman (11).

Immunochemistry

Female California White rabbits were injected intramuscularly with 10 mg of soluble aspartate transaminase with complete Freund’s adjuvant. After 21 days, 10-ml blood samples were taken. The serum showed antigenic value. One month later, they were reinjected intraperitoneally with 5 mg of aspartate transaminase in Freund’s adjuvant. After 1 additional month the rabbits were bled totally by heart puncture and the serum was prepared. The serum of the first bleeding was used for pilot experiments and that of the second bleeding for the studies reported in this work. Double diffusion experiments were done on microscope slides following the Ouchterlony technique (12). The complement fixation studies were performed by using the microcomplement technique of Wasserman and Levine (13).

Determination of Histidine Content

The histidine content of native and photooxidized enzyme was determined after alkali denaturation in 0.5 N NaOH with the
5-diazo-lH-tetrazole method of Sokolovsky and Vallee (14). This method also yields the number of tyrosine residues.

RESULTS

Histidine and Tyrosine Content—Table I shows the histidine and tyrosine content of native and photooxidized enzyme, determined by our previously reported total amino acid analysis (5) and by using the diazonium-lH-tetrazole method. Both methods yield the same number of histidine residues for the native enzyme, but the colorimetric method does not disclose all of the tyrosines observed by amino acid analysis.

Photooxidation does not destroy any of the tyrosine residues; however, when only 20% residual activity remains, 4 to 6 histidine residues have been destroyed.

Absorption Spectral Properties of Photooxidized Enzyme—The absorption spectra of the native and photooxidized aspartate transaminase after removal of methylene blue or rose Bengal are shown in Fig. 1. At low pH, native enzyme absorbs light with a maximum at 430 mμ. Photooxidized enzyme also exhibits an absorption maximum at this wave length, although it is decreased somewhat in magnitude. At high pH, native enzyme absorbs at 360 mμ as does photooxidized enzyme. However, the 360 mμ peak in photooxidized enzyme is obscured by a new absorption maximum at 325 mμ. The ultraviolet absorption spectra are very similar, with some discrepancy in the depth of the 250 mμ absorption minimum.

The most striking change, however, is the appearance of a new absorption maximum at 325 mμ which increases proportionally with the extent of photooxidation, regardless of the dye used. This absorbance is not due to a derivative of pyridoxal phosphate or the formation of pyridoxal phosphate dye complex, because photooxidation of the apoenzyme also leads to a protein with absorptivity at 325 mμ (Fig. 2). Since we have shown that only histidine residues are altered by photooxidation (9), it was of interest to determine whether model compounds of histidine also showed abnormal absorbances after photooxidation. Free histidine and imidazole were destroyed by photooxidation, but the spectrum of the photoproduct(s) did not resemble that of the photooxidized enzyme (Fig. 3). Acetyl histidine's photoproduct showed new absorption at 320 mμ, but the spectrum of photoxidized gycyl-glycine most nearly resembled the photooxidized enzyme (Fig. 3), having distinct absorption maxima at 316 mμ and 245 mμ, which increased as photooxidation progressed. Apparently, the new absorption maximum in the photooxidized enzyme is due to this photoproduct of the histidine residue.

Although the spectra of native and photooxidized enzyme are qualitatively the same in regard to the bound coenzyme, they quantitatively differ in the visible region of the spectrum. We have, therefore, investigated the binding of the coenzymes pyridoxal phosphate and pyridoxamine phosphate to the photooxidized enzyme.

Table I

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Histidine</th>
<th>Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino acid analysis</td>
<td>Dinitro</td>
</tr>
<tr>
<td>Native</td>
<td>16b</td>
<td>16.5</td>
</tr>
<tr>
<td>Photooxidized</td>
<td>10.4b</td>
<td>12.1</td>
</tr>
</tbody>
</table>

a Diazonium lH tetrazole method (12).
b From the total amino acid composition of native and photooxidized enzymes (3).

Fig. 1. Absorption spectra of native and photooxidized aspartate transaminase. Solid line, native enzyme, 0.5 mg per ml in 0.1 M acetaete, pH 5.3. Dashed line, photooxidized enzyme, increases given in minutes by the numbers in the figure. Dotted line, native enzyme, 0.5 mg per ml in 0.1 M Tris, pH 8.2. Dashed and dotted line, 0.5 mg per ml in 0.1 M Tris, pH 8.7, enzyme photooxidized for 40 min to 20% residual activity.

Fig. 2. Absorption spectra of holoenzyme and apoenzyme before and after photooxidation, 0.1 M acetate, pH 5.3. 1, native holotransaminase; 2, native apotransaminase; 3, photooxidized apotransaminase or photooxidized holotransaminase after removal of pyridoxal phosphate; 4, photooxidized apoenzyme after addition of pyridoxal phosphate, pH 5.3. Protein concentrations, 5.6 mg per ml.
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Fig. 3. Absorption spectra of photooxidized model compounds. 1, photooxidized glycy1-histidi1-glycine (5 mg); 2, photooxidized acet1yl histidine (10 mg); 3, photooxidized imidazole (10 mg). Photooxidation in 0.1 M potassium phosphate, pH 7.5, for 60 min.

Table II

Coenzyme content of native and photooxidized supernatant glutamate aspartate transaminase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Coenzyme content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Holoenzyme&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Native</td>
<td>2.00 2.20 2.00 2.23</td>
</tr>
<tr>
<td>Photooxidized</td>
<td>1.80 2.05 1.75 2.00</td>
</tr>
</tbody>
</table>

<sup>a</sup> Spectrophotometric method at 430 nm.

<sup>b</sup> Spectrophotometric titration with pyridoxal phosphate at 360 nm.

<sup>c</sup> Spectrofluorometric titration with pyridoxamine phosphate at 405 nm. See text for details.

Table II shows that photooxidation of the phosphopyridoxal form of aspartate transaminase results in some destruction of the pyridoxal phosphate. The resultant inactivation cannot be due to this alone, since enzyme which has only 20% residual activity contains 90% as much pyridoxal phosphate as native enzyme, as determined spectrophotometrically and by the phenyl hydrazone method.

Previous studies have shown that apoenzyme is photooxidized at the same rate as holoenzyme (5). Fig. 4 and Table II show the results of titrating native and photooxidized apoenzyme with pyridoxamine phosphate. It can be seen that photooxidized enzyme binds at least 90% as much pyridoxamine phosphate as the native enzyme. The degree of fluorescence quenching upon binding of the coenzyme appears to be identical for the two enzymes. Fig. 5 and Table II show the results of titrating native and photooxidized enzyme with pyridoxal phosphate. Again, photooxidized enzyme binds at least 90% as much coenzyme as native enzyme. It is also apparent that the extinction coefficients at 430 nm in acid buffers or 360 nm in basic buffers have not been altered by photooxidation.

Fig. 4. Spectrofluorometric titration of native and photooxidized apoenzyme with pyridoxamine phosphate. Circles, native enzyme; triangles, photooxidized enzyme with 20% residual activity. Details in text.

Aspartate transaminase in the native state is known to be a pH indicator which is colorless (absorbance at 362 nm) at high pH and yellow (absorbance at 430 nm) at low pH. This shift with pH is characterized by a sharp isosbestic point, suggesting that the yellow color is produced by the addition of a proton to the enzyme chromophore. The pK of the dissociation of this proton was determined to be 6.3 (15). Since we observed that the photooxidized holoenzyme absorption spectrum at pH 8.6 possessed an unexpected amount of 430 nm absorbing material which disappeared when the pH of the solution was raised, a possible shift of the pK of the chromophoric group was suspected. If the dissociation constant of the chromophoric group is studied with the photooxidized enzyme by following the difference spectra of the enzyme in buffers at various pH values against that of the enzyme in acetate buffer, pH 4.86, it will be noticed that there is also a proton dissociation with the subsequent spectral shift of the maximum at 430 nm to 360 nm (Fig. 6). However, when these results are plotted as shown in the inset of Fig. 6, it is clear that photooxidation increases the value of the pK of the dissociation of the chromophore by 0.4 pH unit compared to that obtained by the same method with native enzyme. If calculations are based on the assumption that this specific preparation contained 80%...
Acid dissociation constant of the chromophore. The absorption spectra of aspartate transaminase solutions in the sample beam at the pH indicated in the figure were recorded against identical enzyme concentrations in the reference beam in 0.1 M sodium acetate, pH 4.86. The buffers used were sodium acetate below pH 6; potassium phosphate for pH 6 to 7.5; Tris-HCl for pH 7 to 8.2; and sodium tetraborate at pH 9.1. Inset shows a plot of the difference in absorbance at the absorption maxima with respect to pH from values obtained in similar difference spectra. Dotted line, the native enzyme; solid line, the theoretical calculation for the photooxidized enzyme; open and full circles, experimental data of the decrease in absorbance at 430 nm or the increase at 360 nm with increases in pH in the photooxidized enzyme.

inactive and 20% active transaminase and the active enzyme still retained a chromophore pK of 6.3, it follows that a theoretical curve assigning a pK of 6.8 to the 80% inactive transaminase will fit the experimental data as represented by the solid lines of the inset of Fig. 6.

Optical Rotatory Dispersion and Circular Dichroism Studies of Photooxidized Enzyme—Optical rotatory dispersion measurements in the 300 to 220 nm region of the spectrum are shown in Fig. 7 where it can be seen that the depth of the trough at 232 nm, as well as the cross-over point at 222 nm, remained unchanged for the enzyme in either its holo or apo forms after photooxidation. From these results we concluded that the enzyme's average total of secondary and tertiary structure remains largely unaffected by photooxidation.

The complexity of the dispersion curves of aspartate transaminase in the visible and near ultraviolet regions of the spectrum is consistent with a system with multiple optically active transitions. Such spectra are difficult to interpret since a single asymmetrical center may contribute to the optical rotation over a wide spectral region. For this reason we have chosen, rather, to compare the circular dichroism spectra of the enzyme prior to and after photooxidation, since ellipticity bands are restricted to a relatively small wave length range.

Fig. 7. Optical rotatory dispersion and circular dichroism spectra of native and photooxidized pyridoxamine enzyme. A, optical rotatory dispersion of the native (solid line) and photooxidized enzyme (broken line). Enzyme concentration, 1.23 mg per ml. The rotatory dispersion curve is identical for the apo and holo forms of both the native and photooxidized transaminase. B, circular dichroism. 1, native pyridoxamine enzyme prepared either by reaction with a tenfold molar excess of cysteine sulfinate in 0.04 M sodium pyrophosphate, pH 8.8, or by reconstitution of buffer. Excess cysteine sulfinate or pyridoxamine-phosphate was removed by dialysis against pyrophosphate buffer for 4 hours. 2, photooxidized pyridoxamine enzyme prepared by reaction of photooxidized pyridoxal enzyme (35% residual activity) with a 10-fold molar excess of cysteine sulfinate as described for native enzyme. 3, photooxidized pyridoxamine enzyme prepared by recombination of photooxidized apoenzyme (20% residual activity) with excess pyridoxamine phosphate, as described for native enzyme. All spectra were normalized to an enzyme concentration of 5.3 x 10⁻⁶ M. Spectra were taken of 3.0-ml samples in cuvettes with 1.0-cm path length. 8, given in millidegrees.

Fig. 8. Circular dichroism (1.23 mg per ml) of aspartate transaminase. The solid line represents the native enzyme, the broken line, the enzyme photooxidized to 20% residual activity. A, apo-enzyme, pH 7.5; B, holoenzyme, pH 8.1; C, holoenzyme, pH 9.

Holoaspartate transaminase has positive optically active transitions in the regions of the bound chromophore absorption bands (16). One negative dichroic band in the 295 to 300 nm region, which is presumably due to an interaction of the pyridoxal phosphate with aromatic residues in the protein was reported recently (17). We have measured the circular dichroism spectra in the 550 to 250 nm region in the native and photooxidized enzyme in their holo and apo forms at different pH values (Fig. 8). Under conditions of small slit width, in addition to the previously reported peaks at 380 nm (high pH) and 430 nm (low pH), the
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Table III

Optical rotatory dispersion and dichroicity of aspartate transaminase

All calculations are based on a molecule with two identical subunits with one pyridoxal phosphate per 50,000 molecular weight.

<table>
<thead>
<tr>
<th></th>
<th>[α]290</th>
<th>[α]280</th>
<th>ε290</th>
<th>ε280</th>
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<tr>
<td>Native holoenzyme, pH 5.1</td>
<td>5960</td>
<td>82.5</td>
<td>8000</td>
<td>0.0970</td>
</tr>
<tr>
<td>Photooxidized holoenzyme, pH 5.1</td>
<td>6040</td>
<td>55.5</td>
<td>5700</td>
<td>0.0970</td>
</tr>
<tr>
<td>Native apoenzyme</td>
<td>6040</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photooxidized apoenzyme</td>
<td>6100</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* [α] was calculated from [α] values with the mean residue molecular weight from the amino acid composition.

** [α] refers to degrees centimeter square per dmole.

*** ε is absorbance per mole of bound pyridoxal phosphate, as $\times 10^{-5}$ cm$^{-1}$.

... aromatic absorption region of the holoenzyme is a composite of several transitions with well defined negative extrema at 298, 290, 280, and 275 μm at pH 5.1. If the pH is raised to 9.0, the 290 μm dichroic band diminishes in amplitude in favor of a positive dichroic band at 285 μm (Fig. 8); in fact, it begins to resemble the spectrum of the apoenzyme where there are no negative bands, but a wide complex positive band with a distinctive maximum at 285 μm and shoulders at 292 and 260 μm.

The photooxidized enzyme, having modified histidines only, has different circular dichroism spectrum. The positive ellipticity bands at 430 and 360 μm, due to bound pyridoxal phosphate, are still present but with lower amplitudes. This can be explained by the concomitant decrease in the prosthetic group absorptivities due to the partial destruction of the coenzyme upon photooxidation (Fig. 8 and Table III). In the case of the pyridoxamine enzyme formed by the reconstitution of photooxidized apoenzyme with pyridoxamine phosphate, although the coenzyme is bound as shown in Fig. 4, the resultant holoenzyme has a greatly reduced ellipticity at 333 μm (Fig. 7). The amount of this ellipticity is consistent with a mixture of native enyzme with normal ellipticity and photooxidized enzyme which completely lacks dichroicity. Apparently, this reconstituted enzyme has the coenzyme bound in a different manner than the native enzyme. If the pyridoxamine enzyme is prepared from photooxidized pyridoxal enzyme by reaction with cysteine sulfinate (8), a pyridoxamine enzyme is formed exhibiting a dichroic maximum at 333 μm, which approximates that of the native enzyme (Fig. 7). Evidently, under these conditions, the coenzyme-enzyme complex retains the normal conformation when converted from the pyridoxal to the pyridoxamine form; the slight decrease in the amplitude of this maximum could result from the effects ascribed to the histidine photoprocesses.

In the aromatic absorption region, the ellipticity bands undergo more significant alterations. A new wide negative dichroic band appears above 300 μm, in addition to a positive band at 290 μm. These new bands are in the region where the absorption spectrum shows abnormalities after photooxidation and are tentatively ascribed to asymmetrical environments of the photooxidation product of the histidine residues. One of the characteristic ellipticity bands, due to the coupling of pyridoxal phosphate with the protein (290 μm), disappears with photooxidation (Fig. 8). It appears that the photooxidized histidine has affected the asymmetrical environment of an aromatic amino acid at the active site or, possibly, the coenzyme itself. Because of this phenomenon and the above-mentioned observation that photooxidation of the histidine residues results in a change in the pK of the bound prosthetic group, we feel that the photooxidizable histidine is near the bound pyridoxal phosphate. Therefore, the histidine destruction results in a perturbation of the protein properties of the chromophoric coenzyme. The nature of this perturbation is not intense enough to produce a change in the over-all protein tertiary structure since it does not result in a rotation change at 232 μm in either the holo or apoenzymes (Table III).

The sedimentation coefficient of the photooxidized enzyme is 5.5 for both native and photooxidized aspartate aminotransferase.

Immunological Properties of Photooxidized Enzyme—Subtle differences in protein structure can normally be detected by immunological techniques (18). Both the standard Ouchterlony double diffusion technique and the more sensitive quantitative microcomplement technique of Wasserman and Levine (13) failed to reveal any significant change in the immunochemical properties of the enzyme with photooxidation to 20% residual activity with a variety of enzyme preparations and photooxidation samples (Fig. 9). The photooxidized enzyme remains immunochemically identical with the native enzyme for as long as 1 week if the sample is kept refrigerated at 5°C. However, after several weeks of storage, we observed loss of ability to fix complement.

Discussion

No appreciable changes in the aspartate transaminase tertiary or quaternary structure occur upon photooxidation. The results of the microcomplement fixation and ultraviolet optical rotatory dispersion studies indicate a preservation of conformation. The sedimentation values agree well with our previous concept of quaternary structure and protein shape retention based on Sephadex G-200 chromatography (5). The appearance of a new absorption maximum at 325 μm and the increase in the 280 μm
minimum may be explained even though very little data exist on the ultraviolet absorption properties of photooxidized proteins and the identity of the histidine photoproduct has not yet been established. Because of the absorption spectrum of the model compounds, particularly glycyl-histidyl-glycine, it is likely that these new maxima are due to the photoproduct of histidine or, perhaps, to the interaction of this product with some other amino acid residues in the polypeptide chain. Because the spectral changes were observed when enzyme was photooxidized in the presence of either methylene blue or rose Bengal, we doubt the existence of an enzyme dye complex.

Photooxidation does not cause the resolution of either pyridoxal or pyridoxamine enzyme. Since the reconstituted photooxidized pyridoxal enzyme is essentially identical with the native enzyme in optical and absorption spectral properties, and since this form can be converted to the pyridoxamine form which is also very similar to the native enzyme, we concluded that the critical histidine does not play an essential role in coenzyme binding.

When photooxidized apoenzyme is reconstituted with pyridoxamine phosphate, a pyridoxamine enzyme is produced which is different from both native pyridoxamine enzyme and pyridoxal enzyme formed from photooxidized pyridoxal enzyme. Although the same amount of pyridoxamine phosphate is bound, and this is apparently at the active site, substrate may be bound (8), the coenzyme binding differs. This difference is reflected in the lack of dichroicity and the failure to carry out the back half-reaction (8). These observations are consistent with an altered conformation of the active site of the photooxidized apoenzyme which prevents the proper binding of pyridoxamine phosphate. This must be a secondary effect since inactivation does not depend upon the production of this altered pyridoxamine enzyme.

The results in Fig. 8 show new details in the protein-pyridoxal phosphate electronic transitions. The most dramatic are those changes in the 290 to 300 nm region in the form of two ellipticity bands when the apoenzyme is converted to holoenzyme. Changes of a similar nature have been observed by Myer (19) in cytochrome c in the ferricytochrome to ferrocyanochrome equilibrium and have been interpreted as the change of 1 ligand amino acid residue to the porphyrin to another or just a displacement in the plane of the heme iron. In the case of cytochrome c, a tryptophan residue was believed to be responsible for the new negative ellipticity bands. In the case of aspartate transaminase, these peaks may be due to a tyrosine residue since there is an observable pH effect. However, tryptophan cannot be disregarded at this point. Whatever the amino acid residue is, it exists in a different asymmetrical environment when pyridoxal phosphate binds to the protein. When the enzyme is photooxidized, the histidyl residue is modified, and this results in an environmental change or a displacement of either the aromatic amino acid residue or the pyridoxal phosphate, resulting in the disappearance of the 290 nm ellipticity band. This relative alteration of a histidine residue near pyridoxal phosphate with a subsequent perturbation of the environment of the coenzyme would be the best interpretation of the change in pK of the pyridoxal phosphate, also. Whether this is the histidine residue which can be cross-linked to the active site lysine residue by reaction with the bifunctional reagent 3-bromo propionyl bromide remains to be investigated (20). But it is evident that the proximity of this histidine to the active site lysine residue is not in the primary sequence since the chymotryptic peptide containing the pyridoxyl lysine, as isolated by Hughes, Jenkins, and Fischer (21) from aspartate transaminase, does not contain histidines. Therefore, it must be a three-dimensional tertiary structure proximity.

We believe that the data presented can best be reconciled with the concept that a histidine most accessible to photooxidation is at the active site near pyridoxal phosphate. However, it is apparently not essential to the maintenance of over-all protein tertiary structure or coenzyme binding. Inactivation due to destruction of this histidine probably results from a defect in the mechanism of transamination, either because of failure to bind substrate or failure to convert these to products.

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The Structure and Enzyme-Coenzyme Relationship of Supernatant Aspartate Transaminase after Dye Sensitized Photooxidation
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