Reactivation of Enzymes by Light-Stimulated Cleavage of Reduced Pyridoxal 5′-Phosphate–Enzyme Complexes†

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ABSTRACT: The Schiff base adduct formed between pyridoxyl 5′-phosphate and a lysyl residue at the active site of the catalytic subunit of aspartate transcarbamoylase from *Escherichia coli* can be reduced with sodium borohydride to give an inactive derivative (Greenwell, P., Jewett, S. L., and Stark, G. R. (1973), *J. Biol. Chem. 248,* 5994). This derivative was found to be light-sensitive. Under normal laboratory conditions with fluorescent illumination the initial rate of reactivation was approximately 3% per hour. Irradiation of the inactive pyridoxylated derivative with light from two 15-W fluorescent bulbs produced almost a quantitative regeneration of active enzyme. The kinetics of the process were followed by five techniques: (a) restoration of enzyme activity; (b) decrease in the maximal absorption due to the covalently attached pyridoxyl phosphate moiety; (c) release of tritium that had been introduced by reduction of the Schiff base with sodium borotritiide; (d) regeneration of the electrophoresis pattern characteristic of the unmodified protein in a polyacrylamide–urea gel; and (e) release of phosphate. All five methods yielded essentially identical rates. Pyridoxylated catalytic subunits which had their enzymic activity regenerated by light can be repyridoxylated with a consequent drop in activity. This observation, along with an amino acid analysis of the pyridoxylated enzyme before and after exposure to light, indicated that a total cleavage of the covalent modification occurred and the original lysyl residue was regenerated from the reduced Schiff base. The reaction exhibited no oxygen dependency. An action spectrum of the process indicated that maximum regeneration of activity coincided with the 326-nm peak in the pyridoxyl absorption band. A pH dependence was observed with the maximum rate occurring between pH 4.5 and 7.5. The photochemical reaction was also observed with model compounds and with two other enzymes, D-serine dehydratase and tryptophanase, both from *Escherichia coli.* Pyridoxyl 5′-phosphate was also reductively attached to poly(L-lysine), and the product underwent photodegradation. The rate of the reaction varied for the different species. Studies were also conducted on an ortho-acetylated model compound in order to determine whether the 3-hydroxyl group was involved. Acetylation resulted in a significant (~70%) decrease in the rate of amine cleavage.

The photodegradation of vitamin B-6 and many of its derivatives has been known for three decades (Schlenk et al., 1946; Meister et al., 1951; Morrison and Long, 1958; Chen, 1965; Moroyov et al., 1968; Reiber, 1972; Bayhulina et al., 1974). These light-stimulated reactions have been examined primarily in order to provide some knowledge of the bothersome contaminants produced upon exposure of the compounds to irradiation. Among the various complexes that have been studied previously is the Schiff base, pyridoxylidene-D-serine (Reiber, 1972). Little if any photodegradation occurred with this compound. With the Schiff base formed between pyridoxal 5′-phosphate and lysyl residues of proteins the bound pyridoxal phosphate acts as a photosensitizer for neighboring amino acid residues (Rippa and Pontremoli, 1969; Davis et al., 1971). Since pyridoxyl 5′-phosphate forms Schiff bases with lysyl residues of many proteins this reaction followed by reduction with sodium borohydride has been used widely for the covalent modification of proteins and the subsequent identification of reacted lysyl residues and neighboring amino acids (Fischer et al., 1959; Nolan et al., 1964; Anderson et al., 1966; Rippa et al., 1967; Piszkiewicz et al., 1970; Schnackerz and Noltmann, 1971; Raetz and Auld, 1972; Greenwell et al., 1973). For most of these enzymes, many of which have no obvious requirement for pyridoxyl 5′-phosphate as a cofactor, the reduction of the Schiff base is accompanied by the loss of photooxidizability of neighboring groups. Hence the reduced protein derivatives were thought to be relatively inert with respect to pyridoxyl cleavage through a photochemical reaction.

In the course of experiments on the reduced complex formed between pyridoxyl 5′-phosphate and the catalytic subunit of aspartate transcarbamoylase (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) from *Escherichia coli* we encountered an apparent light reactivation of the previously inactivated enzyme derivative (Gibbons et al., 1976). Hence, we have investigated the nature of this photoinduced cleavage of pyridoxamine derivatives of several enzymes and model compounds. As shown here the inactive, reduced Schiff base adducts of several enzymes are very sensitive to illumination. Whereas this photochemical cleavage of the pyridoxyl moiety from the polypeptide chains may cause difficulty in certain types of studies, it can also be exploited for other investigations since almost quantitative reactivation of inactivated enzymes can be obtained merely by exposing the derivatives to light of the appropriate wavelength.

Materials and Methods

Chemicals and Enzymes. Pyridoxamine, pyridoxamine 5′-phosphate, and carbamoyl phosphate (Sigma), pyridoxal 5′-phosphate, pyridoxine, and L-aspartic acid (Calbiochem), sodium borohydride (Metal Hydrides, Inc.), sodium borotri-

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tiide (Schwarz/Mann), dimethylamine (Eastman), acetic anhydride (Allied Chemicals), and all other standard organic and inorganic reagents were used without further purification. L-[^{14}C]Aspartic acid (New England Nuclear) was purified by the procedure of Porter et al. (1969). Reacting sodium metal with D_2O (Bio-Rad, 99.9% pure), under nitrogen in an ice bath, produced the required NaOD.

Poly(L-lysine) was purchased from Sigma and had an approximate molecular weight of 30 000. The catalytic subunit of aspartate transcarbamoylase was prepared by the method of Kirschner (1971). D-Serine dehydratase (Dowhan and Snell, 1970) and tryptophanase (Morino and Snell, 1967), both from *E. coli*, were kindly supplied by Dr. E. E. Snell.

Protein Concentrations. Protein concentrations were determined by employing the published extinction coefficients at 280 nm: catalytic subunit (Greenwell et al., 1973); D-serine dehydratase (Dowhan and Snell, 1970); and tryptophanase (Morino and Snell, 1967). The modified proteins were assumed to have unaltered extinction coefficients.

Enzyme Activity. The catalytic subunit was assayed at pH 7 (30 °C) by either the method of Porter et al. (1969) or by the colorimetric procedure of Prescott and Jones (1969). Dr. Snell and coworkers performed the assays of D-serine dehydratase and tryptophanase according to their previously reported procedures.

Pyridoxylation and Repyridoxylation. Reaction of catalytic subunit with pyridoxal 5′-phosphate and sodium borohydride was accomplished by the method of Greenwell et al. (1973). Tritium incorporation was achieved using 5 MCi of sodium borotritiide diluted in 0.5 mL of 1 M sodium borohydride (20 mM triethanolamine acetate buffer, pH 8 and 0 °C). The protein concentration in these reduction mixtures was approximately 10 mg/mL, and either 1.5 or 5.0 equiv of pyridoxal 5′-phosphate was present per active site. Light-reacted samples were repyridoxylated by the procedure described above. The amount of added pyridoxal 5′-phosphate corresponded to the number of regenerated active sites estimated from activity measurements. Pyridoxylation of poly(L-lysine), D-serine dehydratase, and tryptophanase was also conducted by the procedure of Greenwell et al. (1973). A 1:1 molar ratio of pyridoxal 5′-phosphate to polymer was used for the poly(L-lysine). For both D-serine dehydratase and tryptophanase 1.1 equiv of pyridoxyl 5′-phosphate was added per active site. After modification, all the species were dialyzed against several changes of either 50 mM imidazole acetate–0.2 mM EDTA[1] (pH 7) or 40 mM potassium phosphate–0.2 mM EDTA (pH 7) buffer. Vessels containing pyridoxylated samples were covered with aluminum foil in order to protect the samples from exposure to light during all manipulations other than the deliberate irradiation described below.

Illumination Conditions. Two procedures were used to irradiate the pyridoxylated samples: (a) the unstirred solution was placed in a 10 × 75 mm Pyrex culture tube and positioned 10 cm in front of two 15-W fluorescent bulbs; and (b) the sample with mixing by manual inversion every 2 min was illuminated in a 1-cm quartz cuvette (1-mL volume) with light of variable wavelength in a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer with a band width of 40 nm. Most studies were conducted with samples at a concentration of 0.5 mg/mL of buffer; those experiments with samples intended for either nuclear magnetic resonance (NMR) or amino acid analysis were performed at approximately 80 mg/mL. Unless noted otherwise, the standard buffer used in these experiments was 50 mM imidazole acetate–0.2 mM EDTA (pH 7).

Determination of the Extent of Photodegradation in Macromolecule–Pyridoxyl Derivatives; Enzyme Activity. Aliquots of each irradiated sample were removed at set times and stored in the dark until assayed. All assays were performed immediately following the removal of the last aliquot. Enzymatic activities are reported relative to the values obtained for the enzymes that were treated with sodium borohydride alone.

Tritium Loss. Pyridoxylated samples of catalytic subunit prepared by reduction of the Schiff base with sodium borotritiide were subjected to irradiation as described above. Appropriate aliquots (at different times) were removed and the protein precipitated by 10% trichloroacetic acid was collected on Millipore H A 0.45-μm filters. The loss of tritium was determined from the radioactivity measured in a Unilux II (Nuclear-Chicago) scintillation counter with aquasol (New England Nuclear) as the scintillation cocktail. A small amount (~20%) of the total tritium incorporation occurred merely by treating the protein with sodium borotritiide in the absence of pyridoxal 5′-phosphate. Appropriate corrections were made for this incorporation in the determination of the loss of tritium due to irradiation of the reduced Schiff base.

Effect of Oxygen on Photochemical Cleavage. In order to evaluate the possible role of oxygen in the photodegradation process two parallel experiments were conducted. With one set of solutions no special efforts were made to exclude oxygen. For the second, deoxygenation was accomplished by repeated evacuation of the samples in a desiccator followed by the introduction of water-saturated N_2. During irradiation a stream of N_2 (water-saturated) was passed over the surface of the stirred deoxygenated solution. Aliquots were removed as rapidly as possible and the protein precipitated immediately.

Polyacrylamide–Urea Gel Electrophoresis. Electrophoresis was conducted according to the method of Jovin (1973). Gels (5 cm × 0.4 cm) contained 4.8% acrylamide and 8 M urea, and 250 V was applied for 1 h and 45 min at 2 mA/gel. Samples were dissolved in 8 M urea–10 mM 2-mercaptoethanol. Staining was performed according to Diezel et al. (1972).

Absorption Measurements. A Cary 14 spectrophotometer was used to record the spectra with 1-cm pathlength cells. The baseline was assumed to be the absorbance remaining after extensive illumination produced no further detectable change in the spectrum; this curve was recorded before any significant dark reaction occurred. Photodegradation products generated by the irradiation and not covalently attached to the protein were removed by rapid dialysis.

Action Spectrum. Samples of pyridoxylated catalytic subunit were exposed for 4 min to various fixed wavelengths of light between 260 and 395 nm using a Hitachi Perkin-Elmer fluorimeter light source. The measured change in the residual absorbance at 326 nm as a function of the wavelength of the incident light yielded the action spectrum for the destruction of the pyridoxylated protein.

Phosphate Release. Samples of the reduced pyridoxal 5′-phosphate derivative of catalytic subunit in the reaction buffer (40 mM potassium phosphate–0.2 mM EDTA (pH 7)) were irradiated and then exhaustively dialyzed against twice-distilled H_2O. The quantity of phosphate remaining per chain was determined by the technique of Ames and Dubin (1960). The ascorbic–molybdate mixture was prepared fresh daily.

Urea-Denatured Pyridoxylated Catalytic Subunit. A solution that contained 0.5 mg of pyridoxylated catalytic subunit

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; DMPMP, *N′,N′*-dimethylpyridoxamine 5′-phosphate; DMAPMP, *N′,N′*-dimethyl-3-*O*-acetylpyridoxamine 5′-phosphate.

per mL of buffer (50 mM imidazole acetate–0.2 mM EDTA (pH 7)) containing 8 M urea was exposed to the fluorescent lamp for a total of 48 h. At recorded intervals aliquots were withdrawn and analyzed by electrophoresis on polyacrylamide–urea gels.

Amino Acid Analysis. Amino acid analyses were performed with a Beckman 120-B amino acid analyzer. Each of three samples was hydrolyzed with 6 N HCl at 110 °C for 24 h in a sealed vessel.

Photodegradation of Pyridoxamine and Pyridoxamine 5′-Phosphate; Absorption Measurements. Pyridoxyl concentrations were 0.5 mg/mL and the buffer was 50 mM imidazole acetate–0.2 mM EDTA (pH 7). The samples were all too concentrated to have their spectra recorded directly in a 1-cm pathlength cell (in a Cary 14 spectrophotometer with a 0–1:1–2 slidewire). Therefore, each aliquot was diluted by a factor that gave an absorption maximum of 1.5 OD (optical density) unit for the preirradiated material. The variable-wavelength light source was used for the irradiation.

pH Dependence of Photodegradation. Pyridoxamine 5′-phosphate or pyridoxamine was dissolved in 50 mM imidazole acetate–0.2 mM EDTA buffer. Each solution was then divided into 13 samples and the pH adjusted so that the samples ranged from pH 1 to 13 in 1-unit steps. The amine concentration was approximately 0.05 mg/mL. At this concentration the change in pH was no more than 0.1 for any sample after a 4-min exposure to light of wavelength 333 nm. At pH 4 and below, the decrease in the adsorbance at 326 nm was so extensive that the light-induced lowering in the newly produced 294-nm peak was used to calculate the photodegradation rates.

Analysis of the Photoreaction Products. Following illumination at 333 nm sufficient to cause at least a 50% decrease in the absorbance at 326 nm, the amine samples were applied to long and short columns of a Beckman 120-B amino acid analyzer. Identification of some of the peaks was achieved by comparison with unreacted pyridoxamine (or its 5′-phosphate derivative) and ammonia.

In the NMR investigations, pyridoxamine (80 mg/mL) was used in order to avoid unnecessary signal splitting due to the phosphorus. Since pH (and presumably pD) but not buffer composition appeared to influence the photoreaction, a solution of pyridoxamine was prepared in D_2O and adjusted to pD 7 with NaOD. Half of this sample was irradiated at 333 nm and then both halves were evaporated four times and redissolved in D_2O to eliminate most of the HDO produced. The final solutions were adjusted to pD 7. Just before recording the NMR spectra (in a Varian T-60), the probe was cooled in an ice bath to shift the peak of the remaining HDO further downfield, away from the signals of interest. An external tetramethylsilane standard was used.

Preparation of N′,N′-Dimethylpyridoxamine 5′-Phosphate (DMPMP).[1] Two equivalents of dimethylamine was added to a solution of 250 mg of pyridoxal 5′-phosphate. This step and all subsequent reactions were conducted in covered vessels to exclude light. The solution was cooled to 0 °C in an ice bath, after 15 min sodium borohydride (1 M) was added at a 25 M excess relative to pyridoxal 5′-phosphate, and the mixture was slowly stirred for 30 min. After the solvent was evaporated under reduced pressure, the product was resuspended in methanol and the evaporation process repeated. The final material was colorless and very hydroscopic. Therefore, it was stored in a vacuum desiccator over Drierite and H_2SO_4. The aldehyde was apparently converted quantitatively to *N′,N′*-dimethylpyridoxamine 5′-phosphate as judged by the following: (a) the characteristic 388-nm absorbance peak of the aldehyde was totally missing in the spectrum, and the absorbance ratio for the 327 and 254-nm maxima was 1.76 which compares favorably to 1.80 for pyridoxamine 5′-phosphate, and (b) the NMR spectrum showed no trace of the original aldehyde proton in the final product and a new peak was recorded at 4.20 ppm (assigned to the 4′ protons).

Preparation of N′,N′-Dimethyl-3-O-acetylpyridoxamine 5′-Phosphate (DMAPMP). The acetylation solution was composed of equal portions of acetic anhydride and pyridine. DMPMP (200 mg) was dissolved in this solution and stirred for 24 h at room temperature. The solvent was immediately evaporated in a desiccator containing concentrated H_2SO_4 and NaOH pellets. The absorption spectrum of the product had one maximum at 273 nm in 50 mM imidazole acetate (pH 7) buffer. A spectrum with a single absorbance maximum has been observed for a similar compound, 3-*O*-methylpyridoxamine 5′-phosphate (Pocker and Fischer, 1969).

Photodegradation of N′,N′-Dimethyl-3-O-acetylpyridoxamine 5′-Phosphate. Illumination was conducted with the variable-wavelength light source at either 273 or 333 nm. Only the light at 273 nm caused a reaction with the pure DMAPMP, and absorption measurements at the 273-nm peak were used to follow the reaction. The observed rate of decrease in the 273-nm peak was complicated because of the hydrolysis of the acetyl derivative. In 50 mM imidazole acetate (pH 7) buffer, the hydrolysis rate constant was approximately 6×10^{-5} s^{-1}. This value was determined by following the rate at which the 326-nm peak reappeared in the absence of light. The actual rate of photodegradation was corrected accordingly.

Results

Stoichiometry of Reduction of Schiff Bases. Reduction of the Schiff base adduct formed between the catalytic subunit of aspartate transcarbamoylase and 1.5 equiv of pyridoxal 5′-phosphate per catalytic chain yielded a derivative with 3.6 pyridoxyls per subunit or 1.2 per catalytic chain. A 5 M excess in pyridoxal 5′-phosphate resulted in modification of approximately three groups per chain. A more complete investigation of this reductive modification was conducted previously by Greenwell et al. (1973). (Unless specifically noted, all studies were conducted with the derivative having 1.2 pyridoxyl residues per chain.) Spectral analysis indicated that a singly modified derivative was achieved with a 1.1 M excess of pyridoxal 5′-phosphate to the active sites in either tryptophanase or D-serine dehydratase. Equal concentrations of pyridoxal 5′-phosphate and poly(L-lysine) gave, upon reduction, one pyridoxyl per polymer chain, based on an average molecular weight of 30 000 for the polymer.

Photodegradation of Reductively Pyridoxylated Catalytic Subunit of Aspartate Transcarbamoylase. The residual enzymic activity in the reduced Schiff base–catalytic subunit complex was about 2%. Irradiation of the derivative with two 15-W fluorescent lights at a distance of 10 cm for 48 h produced a 66.5% regeneration of enzymatic activity at pH 7. The time course of reactivation is shown in Figure 1. Each experimental point represents the maximal velocity (V_{max}) expressed as a percentage of the enzymic activity (23.8 μmol of carbamoyl aspartate h^{-1} μg^{-1}) of the unmodified catalytic subunit. Reactivation studies conducted under normal laboratory conditions with fluorescent lighting showed that the rate of recovery of enzyme activity was approximately 3% per h.

When the Schiff base was reduced with sodium borotritiide the derivative was radioactive due to the incorporation of tritium into the newly formed amine linkage. Light exposure of this derivative caused a release of the covalently bound tritium (Figure 1). A logarithmic plot of the remaining radioactivity vs. time of illumination yielded a reasonably straight line in-

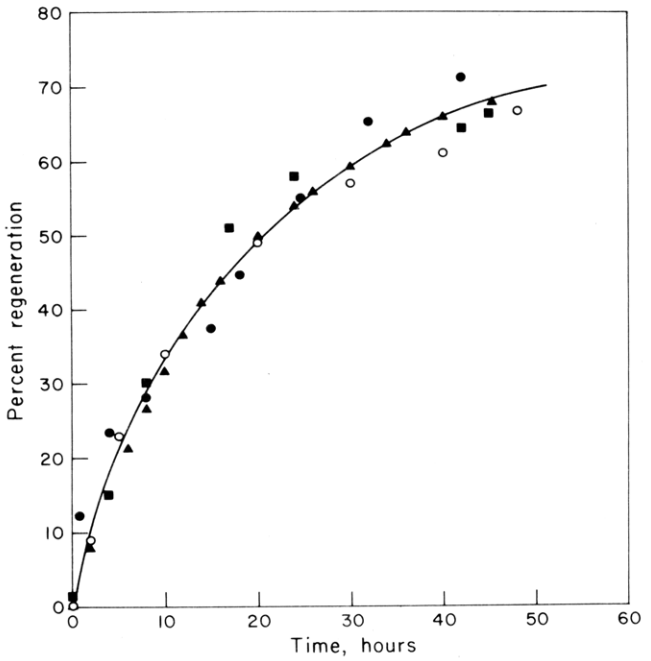

FIGURE 1: Regeneration of the properties of native catalytic subunit as a function of time of irradiation of the pyridoxylated derivative. Solutions of the pyridoxylated protein at 0.5 mg/mL in 50 mM imidazole acetate buffer containing 0.2 mM EDTA at pH 7 were exposed to irradiation from two 15-W fluorescent bulbs for various lengths of time as indicated on the abscissa. After irradiation, measurements were made of the regeneration of enzyme activity (■), the decrease in light absorption at 326 nm (▲), the decrease in amount of ^3H bound to the acid-precipitated protein (●), and the formation of unmodified protein evaluated from densitometer tracings of the electrophoresis patterns of polyacrylamide gels containing urea (○). These data were then normalized relative to results with the catalytic subunit that had been treated only with sodium borohydride. The ordinate represents this normalized percent regeneration of the properties of the native protein.

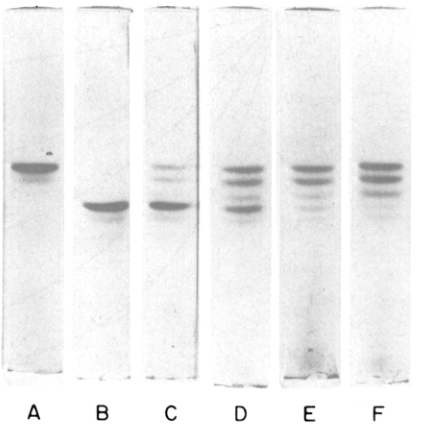

FIGURE 2: Effect of irradiation on the electrophoretic pattern of pyridoxylated catalytic subunit. Electrophoresis in polyacrylamide gels containing urea was performed by the procedure of Jovin (1973) as described under Materials and Methods. For all experiments 1.5 μg of protein was applied to each gel. The patterns in A and B represent the unmodified and pyridoxylated catalytic subunits, respectively. The remaining samples illustrate the cleavage of the pyridoxyl moiety due to irradiation for various times of illumination with two 15-W fluorescent bulbs. The times were 2.4 h for C; 14.7 h for D; 23.2 h for E; and 47.7 h for F.

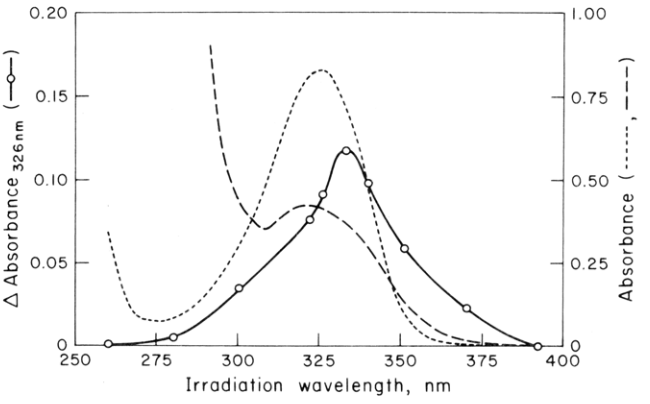

FIGURE 3: Action spectrum for the cleavage of the pyridoxylated catalytic subunit. Samples containing 0.5 mg/mL of pyridoxylated protein in 50 mM imidazole acetate buffer containing 0.2 mM EDTA at pH 7 were irradiated for 4 min at the wavelengths indicated on the abscissa. Then the absorbance was measured at 326 nm and the decrease compared to that of the unilluminated sample ($\Delta Absorbance_{326\ nm}$) is plotted on the ordinate (○). Experiments were performed with a Hitachi Perkin-Elmer MPF-2A fluorimeter. For comparative purposes the absorption spectra of pyridoxamine 5′-phosphate (- - -) and of the pyridoxylated catalytic subunit (– –) are also presented.

dicative of a first-order process. In samples saturated with either nitrogen or oxygen, the rates of decrease in radioactivity bound to the protein were identical.

As seen in Figure 2 electrophoresis in polyacrylamide–urea gels readily distinguished native catalytic chains (sample A) from the reductively pyridoxylated species (sample B). The regeneration of the catalytic subunit pattern due to irradiation is illustrated by the remaining patterns in Figure 2 (samples C to F). The newly produced bands were assumed to represent native catalytic subunits, and the amount of protein in these bands is plotted in Figure 1. When the protein derivative obtained using 5 equiv of pyridoxal 5′-phosphate per catalytic site was exposed to light, the covalently linked pyridoxyl moieties were cleaved at approximately the same rate as in the 1.5-equiv derivative.

Figure 1 also shows the kinetics of the decrease in the absorbance at 326 nm as the pyridoxyl group is cleaved from the protein in the course of the irradiation. In addition, analysis of the irradiated reduced pyridoxylated sample revealed a loss of covalently bound phosphate. After a 25-h exposure to light, the average amount of phosphate remaining on the protein was reduced to 39%. As shown in Figure 1 this release of phosphate is consistent with the time course of the reaction as measured by the restoration of activity, release of tritium, and decrease in absorbance at 326 nm.

The rate of decrease in the absorbance at 326 nm was dependent upon the wavelength of the incident light. The action spectrum in Figure 3 indicates that the photoinduced destruction occurs at its greatest rate with light having a wavelength of 333 nm. This maximum in the action spectrum coincides approximately with the maxima in absorbance for both pyridoxal 5′-phosphate and the reduced catalytic subunit–pyridoxal 5′-phosphate complex.

Denaturation of the reduced catalytic subunit–pyridoxal 5′-phosphate complex with 8 M urea prior to the irradiation had no effect on the rate of the regeneration of nonpyridoxylated chains. Irradiation of samples in the presence of 8 M urea had little influence on the electrophoresis pattern. Densitometer scannings of the gels did indicate the presence of a number of very faint new bands. These may be attributed to the conversion of urea into cyanate and ammonia with a subsequent carbamoylation of the free amino groups in the protein.

Reduced pyridoxylated catalytic subunit that had been irradiated for a sufficient period of time to regenerate 70% of the original enzymatic activity was mixed with a quantity of pyridoxal 5′-phosphate that corresponded to the concentration

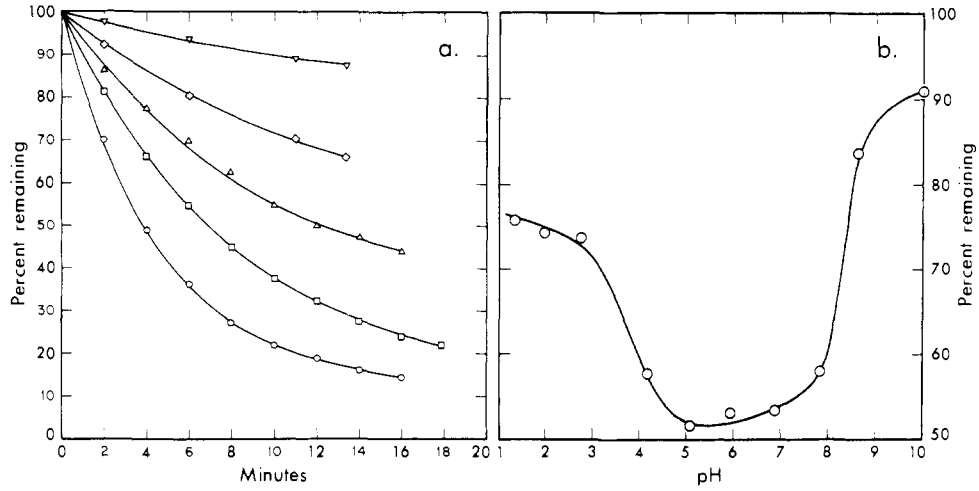

FIGURE 4: (a) Effect of irradiation of various pyridoxyl containing compounds. For pyridoxylated tryptophanase (▽) and D-serine dehydratase (◇) the photodegradation was measured from densitometer tracings of the electrophoresis patterns in polyacrylamide gels containing urea. The cleavage of the pyridoxylated poly(L-lysine) (□) and pyridoxamine or pyridoxamine 5′-phosphate (○) was followed by the decrease in the absorption of light due to the pyridoxyl group. For the catalytic subunit of aspartate transcarbamoylase (△) both procedures were employed. Irradiation was performed with a Hitachi Perkin-Elmer MPF-2A fluorimeter with light at 333 nm. Times of illumination are indicated on the abscissa and the ordinate represents the percent of pyridoxyl-containing compound remaining. (b) Effect of pH on the photodegradation of pyridoxamine. Samples contained 0.05 mg/mL pyridoxamine dissolved in 50 mM imidazole acetate–0.2 mM EDTA. Each sample was adjusted to the appropriate pH before irradiation with light of wavelength 333 nm for 4 min.

TABLE I: Effect of Irradiation on the Amino Acid Composition of Pyridoxylated Catalytic Subunit of Aspartate Transcarbamoylase.

	Residues/catalytic chain[a]		
Amino acid	Native catalytic subunit	Pyridoxylated catalytic subunit	Irradiated pyridoxylated catalytic subunit[b]
Lysine	14.4	12.7	13.3
Histidine	9.8	9.8	10.0
Tyrosine	6.6	6.4	6.7

[a] Values are reported as moles of amino acid residue per mole of catalytic polypeptide chain. [b] The pyridoxylated protein in 50 mM imidazole acetate buffer containing 0.2 mM EDTA at pH 7 was irradiated for 20 h with light from two 15-W fluorescent bulbs. This illumination resulted in approximately 50% regeneration of the enzyme activity of the native catalytic subunit.

of specific pyridoxyl binding sites that were presumably regenerated by the illumination. Upon sodium borohydride reduction of this solution, the amount of enzymatic activity decreased to less than 0.5%. The illumination–reduction cycle was repeated with this repyridoxylated derivative, and the enzymatic activity once again increased upon irradiation. As in the previous cycle the enzyme activity decreased upon reduction with fresh pyridoxal 5′-phosphate.

Reduction of the Schiff base adduct formed between pyridoxal 5′-phosphate and catalytic subunit results in the loss of about one lysyl residue as determined by amino acid analysis (Table I). No significant loss was observed for any other amino acid. Illumination of the sample caused an increase in the number of lysyl residues which correlated well with the 50% increase in observed activity. The data are consistent with the regeneration of a free lysyl residue. As shown in Table I, irradiation produced no alteration in the amounts of histidine and tyrosine which are known to be photooxidized upon irradiation of the Schiff base of some proteins. This observation is in accord with the results of Greenwell et al. (1973) for catalytic subunit, in that reduction of the amine linkage prevents the photosensitization of neighboring amino acid residues.

Photodegradation of Reductively Pyridoxylated Tryptophanase, D-Serine Dehydratase, and Poly(L-lysine). Irradiation of either tryptophanase or D-serine dehydratase that had been reductively bound to pyridoxal 5′-phosphate resulted in a cleavage of the associated amine linkage. Unlike the catalytic subunit of aspartate transcarbamoylase, both of these enzymes require pyridoxal 5′-phosphate for enzymatic activity. Densitometer tracings of the electrophoresis patterns of polyacrylamide–urea gels indicated that the tryptophanase and D-serine dehydratase derivatives decompose at approximately one-fourth and two-thirds, respectively, the observed rate for the modified catalytic subunit (Figure 4a). For both of these enzymes the regeneration of the unmodified gel patterns was accompanied by an increase in enzymatic activity when the samples were assayed in the presence of pyridoxal 5′-phosphate. After 12-min exposure to light at 333 nm, the gel patterns indicated that approximately 31% cleavage had occurred in the D-serine dehydratase derivative whereas the increase in enzymatic activity was 19%. The results for the recovery of enzymatic activity of tryptophanase were somewhat ambiguous since the reactivation process did not depend solely upon illumination. Sodium borohydride treated tryptophanase regenerates activity (on addition of pyridoxal 5′-phosphate) even though stored in total darkness. This reactivation has been ascribed to slow air oxidation.[2] Therefore, it is difficult to evaluate the exact contribution of the photochemical process to the reactivation of the enzyme, but it appears that light does increase the rate of regeneration of activity.

Reductively pyridoxylated poly(L-lysine) was cleaved about 35% faster than the catalytic subunit of aspartate transcarbamoylase (Figure 4a). The reaction was followed by measuring the decrease in the absorbance at 322 nm.

Photodegradation of Pyridoxamine and Pyridoxamine 5′-Phosphate. Irradiation of samples of either pyridoxamine or pyridoxamine 5′-phosphate at a concentration of about 1.5×10^{-4} M in imidazole acetate buffer at pH 7 caused a marked

[2] Dr. E. E. Snell, personal communication.

change in their absorption spectra. Prior to illumination their spectra, characterized by maxima at 253 and 326 nm, were virtually identical. After irradiation for 24 h with the fluorescent light source or for less than 1 h with the high-intensity source at a wavelength of 333 nm the absorbance at 326 nm was decreased to less than 5% of the initial value. The destruction of the absorbing group was the same for both compounds and the time dependence of the initial phase is shown in Figure 4a. In addition the absorption at 253 nm diminished and the spectra developed a broad shoulder beginning at about 279 nm and rising slowly at lower wavelengths. When the solutions of the irradiated samples were stored in the dark a further reaction occurred. As had been described earlier by Reiber (1972), this dark reaction is characterized by the gradual appearance of an absorption maximum at 255 nm and an increase of 5–10% in the absorption at 326 nm.

The rate of photodegradation with light of different wavelengths was a function of pH with a maximum rate between pH 5 and 7 (Figure 4b). In this pH range both the 3-hydroxyl and the ring nitrogen are in their charged forms and the rate was almost independent of pH. Above pH 9 the rate was less than 10% of the maximum velocity, and below pH 3 the rate decreased to less than 25%. From these changes in rate with pH, values of 4.0 and 8.3 for pK_a were estimated. These values agree very well with the corresponding pK_a values for the 3-hydroxyl and the ring nitrogen determined by Metzler and Snell (1955). Protonation of the phosphate moiety, in the case of pyridoxamine 5′-phosphate, had no significant influence on the rate of the photodegradation. Likewise, the protonation of the amine (pK_a = 10.9) had no effect on the reaction.

An irradiated pyridoxamine solution contained at least seven ninhydrin positive components, two of which were positively identified as the starting amine and ammonia. The NMR spectrum of a photoreacted pyridoxamine solution showed that the four characteristic peaks of pyridoxamine (Korytnyk and Ahrens, 1970) were split into doublets, and that a fifth peak (corresponding to two protons in area and possibly arising from the 4-CH_2 group in pyridoxine) appeared at 4.7 ppm (relative to tetramethylsilane). In addition to the five peaks there was a very broad sixth peak that occurs in the region (1.1 ppm) where one might expect ring cleavage products to appear.

Attempt to Eliminate Photodegradation. Elimination of the 3-hydroxyl group by acetylation reduced the rate at which the photoinduced reaction occurred. Illumination of a solution of DMAPMP at 273 nm (the maximum in the absorption spectrum for this compound) caused cleavage at a rate that was one-half that observed for the compound containing no acetyl group (i.e., DMPMP). Under the conditions used in this experiment (imidazole acetate buffer, pH 7), the observed kinetics for the decrease in the 273-nm peak involved two contributing reactions, one representing photodegradation and the second stemming from the light-independent hydrolysis of the acetyl group. Since this hydrolysis is rapid and comparable to the light-dependent process, the measured rate of photodegradation, 50% that of the unmodified species, is only approximate and could be as low as 20–30% of the unmodified compound.

Discussion

As shown by the results presented above, the reduced Schiff base formed between pyridoxal 5′-phosphate and the catalytic subunit of aspartate transcarbamoylase is sensitive to light. The destruction of the pyridoxylated derivative followed approximately first-order kinetics. Apparently the photosensitivity does not require a specific environment for the pyridoxyl moiety since photodegradation occurred in both singly and triply modified catalytic chains at nearly the same rate. Moreover, the derivative of the urea-denatured protein (presumably dissociated into single polypeptide chains) was as sensitive to light as the pyridoxylated catalytic subunit which exists as a folded trimer. Although the process could implicate neighboring amino acid side chains, their contribution must be slight since derivatives involving different lysyl residues were degraded at the same rate as the protein modified at the active sites. Hence, it is unlikely that the local environments of the different pyridoxyl groups are identical. In this regard it is significant that pyridoxamine itself is light sensitive. Also, the same process was observed with two enzymes, D-serine dehydratase and tryptophanase, which require pyridoxal 5′-phosphate as a cofactor and with the polypeptide, polylysine.

The results with the pyridoxylated catalytic subunit indicate that the photodegradation leads to the formation of the native protein and the total removal of the pyridoxyl moiety. Enzymic activity was largely restored; the electrophoretic pattern of the derivative in polyacrylamide gels containing urea was converted toward that of the unmodified protein; the contribution of the pyridoxyl group to the absorption spectrum was lost; phosphate bound to the protein was removed; tritium introduced in the reductively formed amine linkage was eliminated; an unmodified lysyl residue was formed; and the protein could be repyridoxylated with concurrent loss in enzyme activity. However, the fate of the pyridoxyl moiety is still unclear. For pyridoxamine it appears that multiple products result from irradiation. Ammonia was lost and various fragments were produced as detected by the amino acid analyzer and NMR. Clearly the pathways leading to the destruction of the pyridoxyl groups are complicated and no attempt is made here to elucidate either the photochemical mechanism or the nature of the final products. Our concern is primarily with the protein.

Although the nature of the final products resulting from destruction of the pyridoxyl moiety is not clear, it is evident that cleavage occurs and that the lysyl amino group is regenerated. Moreover the process occurs even in deoxygenated solutions. There are at least two ways of reducing its occurrence. Preventing exposure of the solutions to light is possible but inconvenient. Much more preferable would be chemical modification of the pyridoxyl moiety so as to decrease the photosensitivity. Elimination of the 3-hydroxyl would tend to reduce the number of stabilizing resonant forms of the pyridoxyl group. Acetylation proved partially successful in preventing the light-induced reaction, but this derivative is easily hydrolyzed. Methylation may prove a more effective method of preventing photodegradation. As noted by Pocker and Fischer (1969), O-methylated pyridoxamine 5′-phosphate is much less sensitive to light-induced breakdown than is pyridoxamine 5′-phosphate. Obviously the modification of the pyridoxyl group aimed at decreasing its sensitivity to light must not interfere with its reactivity toward amino groups to form a Schiff base.

The general occurrence of this photoreaction with vastly different enzymes and a simple polymer illustrates the importance of considering the risks in experiments using pyridoxylated protein derivatives. Difficulties would probably arise in the use of pyridoxal 5′-phosphate as a label for determining the sequence of amino acids around a reactive lysyl residue. Indeed bond cleavage may be responsible for the loss of pyridoxyl groups from peptides during the purification and sequencing procedures (Nolan et al., 1964). An even greater potential hazard exists in studies involving Schiff base formation and reduction as a means of specifically inactivating enzymes containing reactive lysyl groups at their active sites

(Greenwell et al., 1973; Gibbons et al., 1976). A small but significant regeneration in enzymic activity due to exposure of the preparation to light, if not recognized, could influence drastically the interpretation of experimental data.

Although this light-induced cleavage of pyridoxylated proteins represents an annoying side reaction for most studies, it seems clear that it can be exploited in a very useful way for other investigations. To a first approximation the pyridoxylation of proteins can be considered a reversible process in which the modified protein is reverted to the native state under mild and controllable conditions. It may be feasible to modify a specific lysyl residue by pyridoxylation followed by a different alteration of other less reactive lysyl groups. Then the desired amino group can be regenerated by irradiation. The incorporation of a pyridoxyl group also could be used as a temporary handle in isolation techniques and in hybridization experiments. For these types of studies derivatives even more light-sensitive than pyridoxamine 5′-phosphate might be desirable.

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