Photoactivation of Urocanase in *Pseudomonas putida*. Purification of Inactive Enzyme*

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ABSTRACT: Urocanase from stored cells of *Pseudomonas putida* grown on L-histidine had low activity and was termed inactive. The inactive urocanase was purified about 60-fold by protamine fractionation, gel filtration, and DEAE-cellulose chromatography. Inactive urocanase could be photoactivated to active urocanase at all steps of the purification by irradiation with near-ultraviolet light. The K_m of both forms was 0.05 mM. The pH for optimum enzyme activity of the active form was 7.4–7.5. The capacity for photoactivation of inactive urocanase was more heat stable than the catalytic activity of photoactivated urocanase. The molecular weight of the inactive enzyme was approximately 100,000 based on Sephadex gel filtration. The purified enzyme exhibited only the ultraviolet absorption band of a simple protein. The two forms did not behave differently when subjected to gel electrophoresis or gel filtration. The evidence suggests that the enzyme is activated by a minor alteration of the protein by a photochemical reaction related to the active site. The unknown photoreceptor remained associated with the enzyme during purification.

Urocanase catalyzes the formation of imidazolonepropionic acid from urocanic acid. This enzyme, the second in the pathway of histidine catabolism, has been investigated in various microorganisms (Chasin and Magasanik, 1968; Lessie and Neidhardt, 1967; Magasanik *et al.*, 1965; Tabor, 1955). The native bacterial enzyme has been purified by George and Phillips (1970) and the liver enzyme by Swaine (1969).

Hug and Hunter (1970) observed that washed cells of *Pseudomonas putida* grown on histidine slowly lost urocanase activity when stored at 8°. Enzyme in extracts prepared from such cells had low activity (3–10% of initial activity) and is referred to as "inactive." Irradiation of cells or extracts by near-ultraviolet light restored urocanase activity. Aged intact cells exhibited enhanced oxygen consumption on urocanate and histidine after irradiation (Hug and Hunter, 1970).

Studies of the photoactivation have revealed that the enzyme urocanase seems to be activated by light. After the inactive enzyme has been irradiated, we term it "active" or "photoactivated." We refer to the enzyme contained in extracts of freshly harvested cells by the term "native" enzyme. The activation by light was independent of temperature and could be accomplished even after separation by gel electrophoresis (Hug *et al.*, 1971).

Because of the importance of light in regulation of functions in many organisms (phototaxis, phototropism, photoperiodism, etc.) and because of the possible role of an enzyme responsive to light in these functions, we decided to attempt to purify inactive urocanase. The purification of the inactive enzyme and some properties and comparisons of the two states of the enzyme are reported here.

Methods and Materials

Organism. Pseudomonas putida A.3.12 was grown aerobically and washed as previously described (Hug *et al.*, 1968) in a medium containing 0.2% L-histidine (Tabor and Mehler, 1955). The cells were harvested near the end of the logarithmic phase of growth and suspended in 0.01 M potassium phosphate buffer (pH 7.0) at a concentration of 100 mg/ml (wet weight).

Urocanase Assay. Urocanase was assayed by monitoring the change of absorbance at 277 nm with a Beckman DU spectrophotometer connected to a recorder. The cuvet contained 100 μmoles of potassium phosphate buffer (pH 7.4), 0.2 μmole of urocanate, and enzyme in a volume of 3.0 ml. The cuvet holder was surrounded with circulating water at 30° and the reaction mixture was incubated in a 30° water bath prior to addition of urocanate which initiated the assay reaction. The assay was modified from that of Tabor and Mehler (1955).

A unit of enzyme is defined as the activity which consumes 1 μmole of urocanate/min under the assay conditions. Specific activity is expressed as units of enzyme activity per milligram of protein.

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Near-Ultraviolet Irradiation. Ultraviolet irradiation (8000 ergs/cm² per sec for 15 min) was given by two 15-W General Electric black light lamps (filtered, maximum T at 356 nm) as previously described (Hug *et al.*, 1971), except that in this series samples were irradiated at room temperature, not in an ice bath. A sample of the enzyme preparation suitable for the assay (3.5–800 μg of protein depending on the step of purification) was diluted in potassium phosphate buffer, pH 7.4 (100 μmoles), to 2.9 ml in a 10-ml beaker and was placed 10 cm beneath the filter of a Chromatovue, "long-ultraviolet" lamp assembly obtained from Ultraviolet Products, Inc., San Gabriel, Calif. The irradiated mixture was transferred to a silica cuvet, urocanate was added and the urocanase was assayed. For irradiation by sunlight (3.1×10^4 ergs/cm² per sec of near-ultraviolet radiation measured by

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TABLE I: Summary of Inactive Urocanase Purification.

Purification Step	Total Protein (mg)	Not Irradiated				Irradiated			
		Total Units	Sp Act.	Yield (%)	Purifcn (X)	Total Units	Sp Act.	Yield (%)	Purifcn (X)
1. Crude[a]	160	0.568	0.00356			4.96	0.0310		
2. Protamine (supernatant)	91.5	0.522	0.00570	91.9	1.6	4.76	0.0520	96.0	1.7
3. Protamine (elution)	29.0	0.354	0.0121	62.3	3.4	3.54	0.122	71.4	3.9
4. Sephadex G-200	12.8	0.420	0.0328	73.9	9.2	2.86	0.223	57.7	7.2
5. DEAE-cellulose	1.2	0.277	0.231	48.8	65	2.15	1.79	43.3	58

[a] The actual data of fractions 1, 2, and 3 have been mutliplied by the factor 0.4 in order to normalize the table. A 4-ml sample from the 10-ml fraction 3 was filtered on the Sephadex G-200 column in actual practice (see text).

long-wave ultraviolet Meter, J221, Ultraviolet Products, Inc.), the beaker was wrapped in aluminum foil and the filter, when required (Table II), was placed on the beaker. The dark control was kept completely wrapped until the assay was started.

Polyacrylamide Gel Electrophoresis. The enzyme was subjected to polyacrylamide gel electrophoresis at pH 8.3 as described by Abadi (1969). The gel slab was stained with aniline blue black (1% in 7% acetic acid). In this method, various samples of enzyme can be placed in adjacent channels and electrophoresced simultaneously so that each sample is subjected to the same conditions.

Molecular Weight by Gel Filtration. The molecular weight of the purified urocanase was estimated by Sephadex gel filtration. A column of Sephadex G-200 (2.5 × 38 cm) was equilibrated with 0.1 M Tris-HCl buffer (pH 8.0), containing 1 M NaCl. Gel filtration (descending) was performed with the same buffer at 5–8° with a flow rate of 15 ml/hr. Fractions of 1.4 ml were collected. Blue Dextran and proteins for calibration were obtained in kit form from Pharmacia Fine Chemicals, Piscataway, N. J. The procedure utilized is given in the manufacturer's manual on protein molecular weight determination.

Chemicals. Urocanic acid was obtained from Calbiochem, Los Angeles, Calif. L-Histidine was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Protamine sulfate was obtained from Eli Lilly and Co., Indianapolis, Ind. Other chemicals were reagent grade.

Results

Enzyme Purification. STORAGE AND DISRUPTION OF CELLS. Cell suspensions were stored at 8° for 2–4 weeks. During this time the urocanase activity decreased by about 90–97%. This was an easy reliable method to obtain the inactive enzyme (Hug and Hunter, 1970) which was purified in the steps to follow. Cells were disrupted by treatment of 50 ml of cell suspension for 15 min with the Biosonic ultrasonic probe (Bronwill Scientific) with intensity set at 70. The cell-free crude extract (fraction 1 of Table I) was decanted after centrifugation.

PROTAMINE SULFATE TREATMENT. A 1% (w/v) solution of protamine sulfate equal to 0.17 volume of enzyme solution was added dropwise with constant stirring to remove nucleic acid. The mixture was stirred for 30 min, centrifuged, and the precipitate was discarded. Nearly 100% of the activity in the crude extract was recovered in the supernatant fraction (fraction 2 of Table I).

PROTAMINE SULFATE FRACTIONATION. An additional volume of 1% (w/v) protamine sulfate equal to 0.08 volume of the protamine supernatant fraction was added dropwise with stirring and the mixture was stirred for 30 min. After centrifugation, the supernatant fraction was discarded and the precipitate was stirred with 8 ml of 0.25 M potassium phosphate buffer (pH 7.5), for 15 min and the resulting suspension was centrifuged and decanted. The precipitate was discarded and the supernatant fluid was diluted to 10 ml with the same buffer. The protamine steps were monitored (enzyme activity) for each batch and slight adjustments were sometimes made in the protamine addition. Only 4 ml of this 10-ml fraction was processed through the next steps at one time. Protamine fractionation of an enzyme has been described by Berg (1956).

GEL FILTRATION. The protamine fraction (4 ml), fraction 3 of Table I, was subjected to gel filtration through a Sephadex G-200 column (2.5 × 38 cm) which had been equilibrated with 0.1 M Tris buffer (pH 7.5). It was filtered by upward flow with the same buffer at the rate of 15 ml/hr. Fractions of 1.3 ml were collected and analyzed for urocanase activity and protein (absorbance at 230 nm). The most active fractions usually found between fraction 55 and 85 were combined (fraction 4 of Table I). The combined fractions (about 40 ml) were concentrated by pressure filtration through a Diaflo membrane (XM-50) to 3–5 ml (Amicon Corp.).

CHROMATOGRAPHY ON DIETHYLAMINOETHYLCELLULOSE. The concentrated fraction was adsorbed to a column of diethylaminoethylcellulose (1.5 × 22.5 cm) which had been equilibrated with 0.1 mM potassium phosphate buffer (pH 7.5). The enzyme was eluted with 100 ml of a concentration gradient of 0.1 to 1.0 M potassium phosphate buffer (Figure 1). The mixing chamber contained 50 ml and the reservoir contained 100 ml. The rate of flow was 30 ml/hr. The fractions (2.1 ml) were analyzed for enzyme activity and protein. The active fractions were eluted from about 42 to 56 ml, combined (fraction 5 of Table I) and stored at 8° in darkness.

COMMENTS ON PURIFICATION. Table I summarizes the results of a typical purification. All centrifugations were carried out at 4° for 15 min at 12,000*g*. All steps in the purification were performed in a dark cold room or a dimly illuminated laboratory (black shades drawn at windows, room lights off, less than 1 ft-candle) at 0–5°. The enzyme was assayed in samples of each fraction before and after the standard 15-min

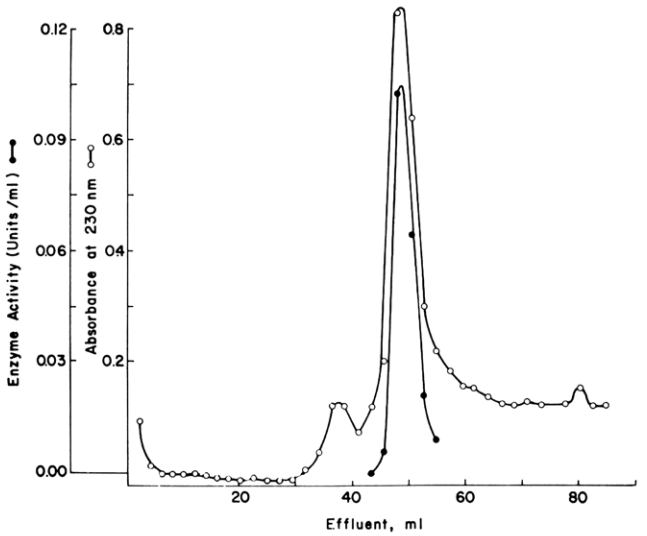

FIGURE 1: Chromatography of inactive urocanase on a DEAE-cellulose column. The sample applied was the combined active fractions of the gel filtration. Protein (○) was detected by absorbance at 230 nm. Enzyme activity (●) was determined after dilution in assay buffer, and irradiation for 15 min as described in Materials and Methods.

near-ultraviolet irradiation. This simple and effective purification has been carried out 12 times.

Properties of the Purified Enzyme. PURITY OF THE FINAL PREPARATION. The crude extract revealed 15 or more protein bands by gel electrophoresis of which urocanase was the major one (by inspection). Purification steps, 1–5, were monitored by this method and in each succeeding step fewer bands were detected. When samples of the purified inactive urocanase usually containing about 35 μg of protein were subjected to polyacrylamide gel electrophoresis, a single protein band was detected after staining (Figure 2B). The stained protein band coincided with the locus of urocanase activity as detected by an activity stain (Roth and Hug, 1971). When purified inactive urocanase was rechromatographed on the DEAE-cellulose column, a single protein peak coincided with the single enzyme activity peak (data not shown).

ENZYME STABILITY. Fraction 3 (Table I) obtained by protamine fractionation retained 96% of its activity for 7 days and 53% of its activity after 21 days at −20°. After 21 days at 8°, 80% of its activity was retained. The final preparation, fraction 5 of Table I, when stored at −20° retained 92 and 70% of its activity for 7 and 21 days, respectively. When stored at 8°, fraction 5 retained 100 and 80% of its activity for 7 and 21 days, respectively. Additions of EDTA, albumin, and glycerol had no effect. These stability data were obtained after irradiation of the stored inactive enzyme.

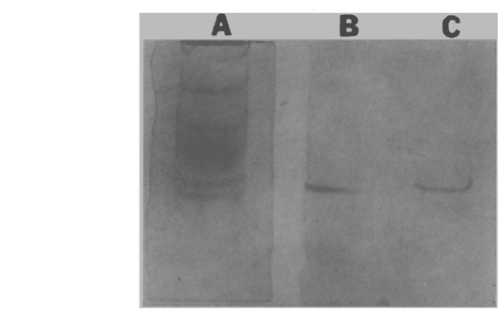

FIGURE 2: Polyacrylamide gel electrophoresis of purified inactive urocanase and photoactivated urocanase. (A) Crude extract; (B) inactive, fraction 5 of Table I; (C) same fraction irradiated as described in Materials and Methods.

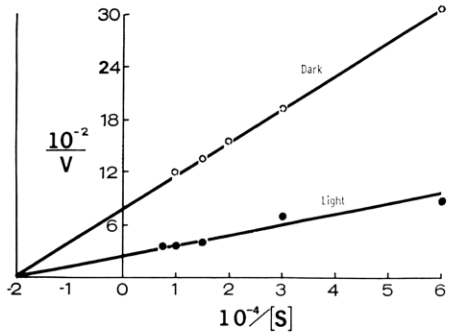

FIGURE 3: Lineweaver–Burk plot of inactive and active urocanase. The urocanase assay described in Materials and Methods was used except the substrate was varied as indicated. The activity of the non-irradiated enzyme (open circles, labeled "Dark") is compared to the activity of irradiated enzyme (closed circles, labeled "Light") at various substrate concentrations. Each point represents the average of two rate determinations. The enzyme was fraction 5 of Table I.

MICHAELIS CONSTANT. The Michaelis constant of the purified nonactivated and photoactivated enzyme was determined from a double-reciprocal plot of the activity and substrate concentration (Figure 3). The values of V_{max} were different for the two forms. The K_m for both forms was 0.05 mM (average of two determinations, each assay in duplicate). The native enzyme as measured in crude extract had the same K_m (not illustrated, also see Hug *et al.*, 1968).

EFFECT OF pH ON ACTIVITY. Purified, photoactivated urocanase exhibited activity over a broad pH range with a maximum at pH 7.4–7.5 as shown in Figure 4. This experiment was carried out so that pH was not adjusted until after irradiation. The native enzyme as measured in crude extracts in our laboratory (data not shown) has about the same pH activity profile.

HEAT STABILITY. The purified enzyme was heated at 50°

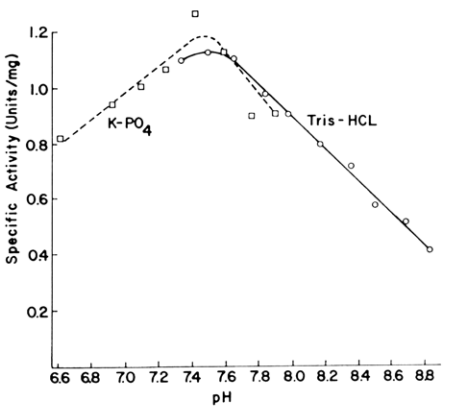

FIGURE 4: Effect of pH on the enzyme activity of light-activated urocanase. The standard urocanase assay was used as described under Materials and Methods with 1.8 μg of urocanase (fraction 5 of Table I) in each reaction mixture. The enzyme was diluted to 2.5 ml in water and irradiated 15 min as described in the text; then buffer and substrate were added for the assay. The pH of the complete reaction mixture was measured immediately after the assay. Each point represents the average of two assays. Potassium phosphate buffer (100 μmoles) was added to each reaction mixture for pH 6.6–7.9 (□) and Tris-HCl buffer (100 μmoles) was used for pH 7.3–8.8 (○).

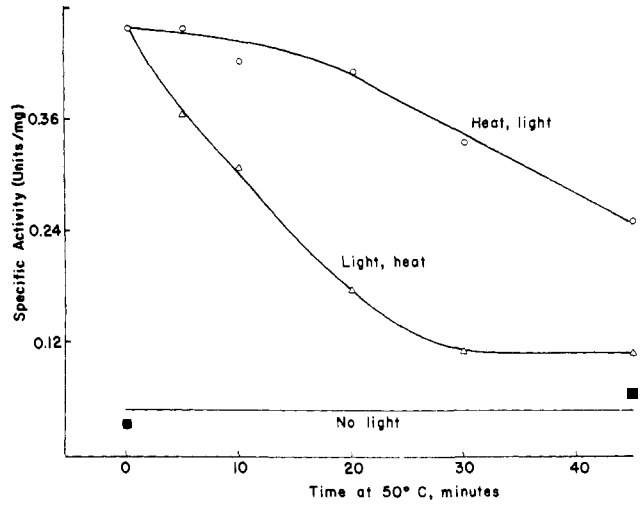

FIGURE 5: Heat stability of inactive urocanase and photoactivated urocanase. A series of test tubes containing the enzyme diluted to 3.0 ml in standard assay buffer were incubated in a 50° water bath. Irradiation, as described in Materials and Methods was performed either before heating (△) or after heating (○). After these treatments, the tubes were incubated at 30° for 2 min to adjust them to assay temperature. Each reaction mixture contained 11 μg of urocanase. Dark controls (■) were wrapped in aluminum foil.

for various time periods. When irradiation preceded heating (the photoactivated enzyme was heated), the enzyme activity was relatively heat labile (Figure 5). When irradiation followed the heat treatment (that is, the inactive enzyme was heated), the capacity for photoactivation was much more stable. The low residual activity of the inactive enzyme (dark control) exhibited remarkable heat stability but this activity was completely destroyed by 5 min at 100°. When we repeated the experiment or when we carried out the same type of experiment using slightly different conditions (45° with fraction 5, or 50° with fraction 1), the results were essentially similar to those illustrated in Figure 5.

MOLECULAR WEIGHT ESTIMATION. Gel filtration was used to estimate the molecular weight of inactive urocanase. The estimated molecular weights were 91,000 and 108,000 based on the two peaks in Sephadex G-200. The reason for the appearance of two peaks is unknown but they consistently appeared whenever Sephadex G-200 filtration was

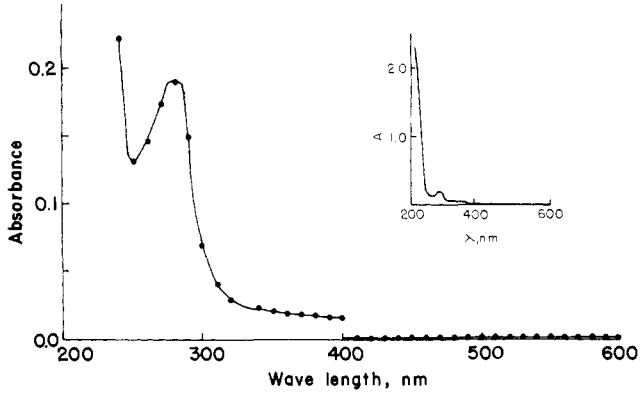

FIGURE 6: Absorption spectrum of inactive urocanase at pH 7.5. The protein concentration was 0.18 mg/ml (fraction 5 of Table I) in 0.2 M potassium phosphate buffer. Absorption spectra from several batches of purified enzyme with protein concentrations up to 2.4× the one given above revealed no other absorption bands.

TABLE II: Effect of Filtered Sunlight on Photoactivation of Purified Urocanase.

Exptl Condn	Filter	Approx Wavelength (nm) for Transmittance of 1%	Approx Wavelength (nm) for Transmittance of >50%	Activation, -fold
No light	None			1.0
Sunlight[a]	None			6.6
Sunlight	Corning 0-53[b]	278	310–1700	6.2
Sunlight	Oriel G-772-3900[c]	370	390–2400	1.3
Sunlight	Oriel G-774-355, band pass[d]	300, 390	340–370	3.3

[a] Sunlight, 4 min, 6–9–70, 1:30 CDT. [b] Maximum, 92% T, 370–1700 nm. [c] Maximum, >85% T, 430–2400 nm. [d] Maximum, 62% T, 355 nm.

employed (purification; comparison, Figure 7; and molecular weight estimation).

ABSORPTION SPECTRUM. The absorption spectrum of purified inactive urocanase was typical of a simple protein (Figure 6). It had no absorption bands in the visible range and an ultraviolet absorption maximum near 280 nm (pH 7.5). Preparations of purified urocanase (fraction 5 of Table I) are colorless. The discontinuity at 400 nm is due to a change in the light source of the instrument.

EFFECTIVE WAVELENGTHS IN SUNLIGHT. If photoactivation of urocanase has a physiological role, the light source of interest is the sun. Purified inactive urocanase was photoactivated by sunlight which contains near-ultraviolet, visible, and infrared light. When sunlight was passed through a cut-off filter (390 nm), which transmitted visible and infrared light, but not ultraviolet light, photoactivation was prevented. The filters that transmitted wavelengths in the near-ultraviolet region did permit photoactivation. The data (Table II) indicate that near-ultraviolet light from approximately 290–370 nm is primarily responsible for photoactivation of urocanase by sunlight. The influence on urocanase activity of ultraviolet light from 200 to 290 nm (not present in sunlight) has not been investigated. However, irradiation by a germicidal lamp (254 nm) did result in photoactivation of inactive urocanase. Only a careful study of the action spectrum with monochromatic light will reveal the relative effectiveness of various wavelengths which will give us clues to the chromophore.

COMPARISON OF INACTIVE AND ACTIVE ENZYMES. When the purified inactive enzyme was compared to a light-treated enzyme in separate runs through a Sephadex G-200 column, they both exhibited two peaks and the elution volumes of these were the same (Figure 7). When the purified inactive and photoactivated enzymes were electrophoresced side by side on a polyacrylamide gel slab, the two had the same mobility (Figure 2).

Discussion

Inactive urocanase was purified about 60-fold from *P. putida*. When the purified enzyme was subjected to polyacrylamide gel electrophoresis, a single band was detected, and DEAE-cellulose column chromatography revealed

one peak. The specific activity after photoactivation (active enzyme) was similar to crystalline native enzyme (George and Phillips, 1970) although the conditions of the assay were somewhat different. It is clear that the two peaks we observed on Sephadex G-200 do not represent the inactive and active forms of the enzyme. They are not converted by light from one into the other (Figure 7).

For any photochemical reaction to occur, a molecule must absorb photons. Since light activates the enzyme and since the photoreceptor was apparently not separated from the enzyme by the purification, we suspected that some absorption bands of the photoreceptor might be present. We have presented the spectrum of purified inactive urocanase from 220 to 600 nm. However, the spectrum revealed no absorption bands other than the usual 280-nm absorption band and the peptide bond absorption band in the 200-nm region. George and Phillips (1970) demonstrated that urocanase contains α-ketobutyrate as a prosthetic group. If this group is the chromophore, it has a very low molecular extinction coefficient in the near-ultraviolet region and would not be detected by the absorption spectrum. Perhaps the chromophore absorbs in the 280-nm region and is masked by, or identified with the 280-nm band of protein. The spectrum of the cat liver urocanase purified by Swaine (1969) and the native urocanase purified by George and Phillips (1970) also had no absorption bands due to chromophore groups.

Another enzymatic reaction which responds to photons is the cleavage of pyrimidine dimers in DNA by the photoreactivating enzyme. The DNA-photoreactivating enzyme of blue-green algae, which uses light as a cofactor to cleave carbon–carbon bonds, was purified 3760-fold by Saito and Werbin (1970), and absorbed weakly at 418 nm. They attributed this band to enzyme impurities rather than to a chromophore of the enzyme. Muhammed (1966) also found no chromophore in the yeast photoreactivating enzyme which he purified 3000-fold.

Exhaustive dialysis, gel electrophoresis (Hug *et al.*, 1971) and the purification of inactive urocanase did not separate the photoreceptor from the enzyme. Therefore, we conclude that the unknown photoreceptor is closely associated with the enzyme.

Comparison of the characteristics of the inactive enzyme and the active enzyme might suggest the nature of the change which occurs upon photoactivation. We detected no difference in the two forms by polyacrylamide gel electrophoresis (Figure 2) and gel filtration (Figure 7). We conclude that light activation produces no substantial change in the net charge, size, or shape of the enzyme. The inactive enzyme, photoactivated enzyme, and the native enzyme had the same K_m (0.05 mM). These findings suggest that photoactivation does not change the affinity of the enzyme for substrate but does affect the rate of conversion of urocanate into the product.

Differences in the two forms of enzyme were demonstrated by heat stability (Figure 5) and kinetic studies (Figure 3). The inactive enzyme exhibited heat stability in its low residual activity. The capacity for photoactivation was relatively more stable to heat than the catalytic activity of the activated enzyme. This demonstrated that the heat treatment which decreased the activity of the active enzyme did not denature the protein because the capacity to be photoactivated survived the same heat treatment (Figure 5). Conversion of the enzyme by light may involve only a minor change which is associated with the catalytic site and is due to a photochemical reaction.

It is likely that the native enzyme is induced in histidine-grown cells and during storage is converted to an inactive form. Light changes the inactive enzyme to photoactivated enzyme, as manifested by increased activity. Whether the photoactivated enzyme differs from the native enzyme cannot be answered at this time.

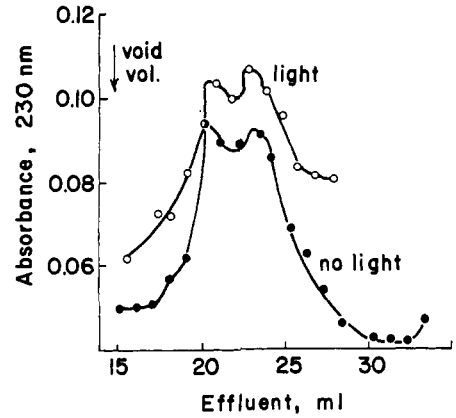

FIGURE 7: Comparison of purified inactive and photoactivated urocanase by gel filtration through Sephadex G-200. Column dimensions, 1.5 × 26.5 cm; buffer, 0.1 M potassium phosphate, pH 7.5; flow rate, 10 ml/hr; fraction volume, 1 ml; temperature, 4°; descending technique in darkness; sample, approximately 50 μg of urocanase from fraction 5 of Table I. The nonirradiated sample (●) was shielded from light. The irradiated sample (○) was irradiated 15 min as described in Materials and Methods. Similar results were obtained when this experiment was repeated with urocanase purified in another batch.

We have speculated on a possible role of urocanase in photoregulation of histidine catabolism (Hug and Hunter, 1970). The purification of inactive urocanase is a vital step in the exploration of this phenomenon. Although the physiological role of this photoactivation is unknown and may in fact not exist, we suggest that such changes in enzyme activity upon exposure to light might be related to some of the responses of photobiology. The photoactivation of urocanase provides a unique system useful for the study of the nondestructive interaction of light with an enzyme.

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Circular Dichroism of Native and Illuminated Bovine Visual Pigment$_{500}$ at 77°K in the 620- to 320-nm Region*

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ABSTRACT: Absorption and circular dichroism spectra of bovine digitonin- and cetyltrimethylammonium bromide (CTAB)–visual pigment$_{500}$ complexes were measured at 77°K in glycerol–water mixtures in the 620- to 320-nm range. Upon cooling from 298°K, the absorption maxima shifted to the red and increased by a factor of 1.15, while the area under the absorption peak increased by 16% in the digitonin and by 5% in the CTAB–pigment. The small change in absorption was attributed mostly to the effect of volume changes upon cooling. Cooling of native CTAB–visual pigment from 298 to 77°K resulted in the disappearance of the circular dichroism positive peak at 490 nm. The positive peak at 340 nm was unchanged. Due to poor glass and accompanying uncertainty in the base line of digitonin–visual pigment at 77°K it was impossible to ascertain whether the circular dichroism band at 490 nm disappeared upon cooling.

Illumination at 500 nm of either CTAB–or digitonin–visual pigment at 77°K produced a new circular dichroism spectrum with a positive peak at approximately 560 nm and a negative peak at approximately 480 nm. The circular dichroic band at 340 nm was only minimally affected by illumination. This new spectrum, which had very large circular dichroism signals compared to the native pigment signals, was called state I. Continuous illumination of state I progressively increased the circular dichroism signals to a maximum after which the spectrum changed. When the illuminated visual pigment was kept in the dark the circular dichroism spectrum of state I was unchanged after 30 min. The circular dichroism spectrum of state I could be produced, at different rates, by illumination at any wavelength between approximately 400 and 560 nm. State I was always the first circular dichroic spectrum produced by illuminating visual pigment at 77°K. Illumination of state I at 560 nm or longer illumination at 500 nm produced a different circular dichroism spectrum. This spectrum had a negative peak at approximately 540 nm and a positive peak at approximately 460 nm. The peak at 340 nm was only minimally changed. This circular dichroism spectrum was called state II. When the illuminated visual pigment was kept in the dark at 77°K the spectrum of state II was unchanged after 30 min. The circular dichroism spectra of states I and II could be reversibly transformed, one into the other, by proper illumination. States I and II had an isosbestic point at approximately 512 to 518 nm, depending on the concentration of glycerol in the medium. The circular dichroism spectrum of native digitonin–or CTAB–visual pigment did not pass through this isosbestic point, and once illuminated, the circular dichroism spectra of both states I and II were always different from that of native pigment. No illumination conditions were found that could produce the native circular dichroism spectrum from either states I or II. From these experiments it was concluded that illumination of native visual pigment at 77°K produces two new states which exhibit very large circular dichroism signals. These states are always different from the native visual pigment, are interconvertible by illumination, and cannot be made to regenerate native visual pigment by illumination.

When native bovine visual pigment$_{500}$ is illuminated it undergoes a series of transformations which result, ultimately, in the dissociation of the chromophore, as *all-trans*-retinal, from the apoprotein. Several spectroscopically defined intermediates in this process have been recognized (for reviews, see Wald, 1968, Abrahamson and Ostroy, 1967, and Morton and Pitt, 1969). These intermediates of illuminated visual pigment, which are unstable at room temperature, were all found by low-temperature absorption spectroscopy. Despite a great amount of research in this field, little information is available about the structure of these intermediates. For instance, it is not known whether the various spectroscopic intermediates reflect changes in the structure or conformation of the chromophore, of the apoprotein, or both. This paucity of structural information stems from the difficulty of relating absorption spectroscopy data to molecular architecture in the absence of a model system for correlating the visual pigment spectrum with its structure.

Since circular dichroism is an expression of molecular geometry, it can yield additional structural information not obtainable from absorption spectroscopy. Several studies on the circular dichroism spectra of visual pigment$_{500}$ have been reported (Crescitelli *et al.*, 1966; Takezaki and Kito, 1967; Kito *et al.*, 1968; Shichi *et al.*, 1969; Shichi, 1970). These studies show that native bovine, frog, and squid visual pigments, when measured at room temperature,

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