Purification and Initial Characterization of 124-Kilodalton Phytochrome from *Avena*†

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ABSTRACT: Two procedures for the purification of 124-kdalton (kDa) phytochrome from etiolated *Avena* seedlings are described. These procedures involve combinations of existing protocols but with two key modifications aimed at precluding previously encountered proteolysis: phytochrome is maintained in its far-red absorbing form (Pfr), and the serine-protease inhibitor phenylmethanesulfonyl fluoride is included in all media until proteolysis is no longer a problem. The initial steps in both procedures are identical but are followed in one case by Affi-Gel Blue chromatography and in the other by immunoaffinity chromatography. The phytochrome preparations obtained by either procedure are >95% homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and lack detectable levels of the proteolytically degraded 118- and 114-kDa species that constitute preparations obtained by previous protocols. The spectral properties of 124-kDa phytochrome purified by the Affi-Gel Blue procedure contrast with those of the extensively characterized 118/114-kDa products in several major respects. The purified 124-kDa molecule has a Pfr absorbance maximum at 730 nm, negligible dark reversion with or without sodium dithionite, and enhanced absorbance at 730 nm of the spectrum (predominantly Pfr) obtained after saturating red irradiation relative both to the 673 nm shoulder of this spectrum and to the absorbance maximum of the Pr spectrum at 666 nm. The difference spectrum, with a spectral change ratio ($\Delta A_r/\Delta A_{fr}$) of 1.07, is indistinguishable from that determined in vivo, indicating retention of the spectral properties of the native molecule through the purification procedure. Immunoaffinity-purified 124-kDa phytochrome on the other hand is spectrally denatured and has been used here only for compositional analyses. The amino acid composition of 124- and 118/114-kDa preparations does not differ significantly on a mole percent basis, but in contrast to published data for the 118/114-kDa species, 124-kDa phytochrome has a blocked NH_2 terminus. These data provide further evidence that the proteolytic conversion of 124- to 118/114-kDa phytochrome involves removal of polypeptide segments critical to the structural and spectral integrity of the native molecule and indicate that at least part of this proteolysis involves removal of the NH_2-terminal residue(s).

Many aspects of plant growth and development are regulated by the photoreceptor phytochrome (Smith, 1975; Pratt, 1979). The molecule is a chromoprotein with two photointerconvertible forms: a red absorbing form, Pr,[1] and a far-red absorbing form, Pfr. The molecular mechanism(s) of phytochrome action is unknown but physiological evidence indicates that Pfr is the active form and Pr is the inactive form [Smith, 1975; Mohr, 1972; Pratt, 1982a; see H. Smith (1981) for recent dissenting view].

Efforts to understand this mechanism of action have focused considerable attention on the physicochemical properties of the protein and on the molecular differences between Pr and Pfr [see Pratt (1982a)]. The success of this approach clearly depends on the isolation of phytochrome in an undegraded, undenatured form. Attainment of this goal has been hindered in the past by proteolysis of the phytochrome during purification. Initially a 60-kDa chromoprotein was purified and characterized (Mumford & Jenner, 1966) but was later shown to be the proteolytic degradation product of a larger species with a monomeric molecular weight of about 120000 (Gardner et al., 1971; Rice et al., 1973). For over a decade this approximately 120-kDa species ("large" phytochrome) was widely considered to represent the undegraded photoreceptor, and its molecular properties were extensively studied [see Pratt (1982a)]. Recently, however, we have demonstrated that the 118- and 114-kDa polypeptides that actually comprise the "120"-kDa preparations from *Avena* are derived in turn from a yet larger 124-kDa monomer by posthomogenization proteolysis (Quail et al., 1981; Vierstra & Quail, 1982a,b). Several lines of evidence indicate that this 124-kDa molecule is in fact the native photoreceptor and not yet another degradation product, including its comigration on $NaDodSO_4$–polyacrylamide gel electrophoresis with the in vitro translation product of phytochrome (Bolton & Quail, 1982). Because the Pr form is highly susceptible to the proteolytic degradation of 124- to 118/114-kDa phytochrome, and because existing purification protocols stipulate rigorous maintenance of the photoreceptor in the Pr form, there is a high probability that most, if not all, of the data accumulated on the molecular properties of purified phytochrome have been obtained with partially degraded preparations [see Pratt (1979, 1982a)]. Indeed this limited proteolysis has now been shown to occur also in extracts of etiolated rye, corn, pea, and zucchini seedlings (unpublished data; Kerscher & Nowitzki, 1982), indicating the generality of the problem. The impact that this proteolysis has on the molecular properties of the photoreceptor is illustrated by the change in spectral properties that results from conversion of 124- to 118/114-kDa phytochrome in crude extracts (Vierstra & Quail, 1982b). These changes indicate that the proteolytically labile domains are involved in the maintenance of the spectral integrity of the photoreceptor.

This paper describes two relatively rapid procedures for the purification of 124-kDa phytochrome from *Avena*. These procedures are composites of previous methods developed by

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[1] Abbreviations: Pr and Pfr, red and far-red absorbing forms of phytochrome, respectively; PMSF, phenylmethanesulfonyl fluoride; FMN, flavin mononucleotide; $\Delta(\Delta A)$, difference in absorbance at 665 and 730 nm taken from the phytochrome difference spectrum (Pr − Pfr); kDa, kilodaltons; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-2498$01.50/0

Bolton (1979), Hunt & Pratt (1979), and Smith & Daniels (1981) but with two key modifications: the inclusion of phenylmethanesulfonyl fluoride (PMSF) in the media and the maintenance of phytochrome in the Pfr form throughout much of the purification. The potential value of these measures in preventing proteolysis of the molecule became obvious during our previous analytical studies (Quail et al., 1981; Vierstra & Quail, 1982a,b). These investigations demonstrated that Pfr is relatively resistant to proteolytic degradation from 124 to 118/114 kDa and that PMSF also strongly inhibits this degradation. An initial characterization of the 124-kDa molecule obtained by these procedures is also presented.

Materials and Methods

Chemicals. Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] was made according to Siegelman et al. (1965) with 1 mol of KOH to convert 1 mol of brushite to hydroxyapatite. Affi-Gel Blue (100–200 mesh) and Bio-Gel A-1.5 M (200–400 mesh) were obtained from Bio-Rad Laboratories (Richmond, CA); ammonium sulfate (ultrapure, special enzyme grade) was from Schwarz/Mann (Orangeburg, NY); FMN (commercial grade) and PMSF were from Sigma (St. Louis, MO); poly(ethylenimine) was from Eastman Kodak (Rochester, NY). PMSF was added from a freshly prepared 200 mM stock solution in 2-propanol.

Plant Material. Oat seedlings (*Avena sativa* cv. Garry, Olds Seed Co., Madison, WI) were grown for 4.5 days in the dark at 25 °C, before being harvested in dim green light and chilled to 4 °C.

Purification of 124-kDa Phytochrome. The initial steps of the two purification protocols are identical, employing the homogenization buffer and the poly(ethylenimine) and ammonium sulfate precipitation steps of Bolton (1979). Further purification then involves the use of either of two affinity columns: Affi-Gel Blue (Smith & Daniels, 1981) or antiphytochrome immunoglobulins coupled to Sepharose 4B (Hunt & Pratt, 1979).

All manipulations were performed in dim green light at 0–4 °C unless otherwise noted. Phytochrome was converted to Pfr by irradiating seedlings for 5 min with red light (λ_{max} 660 nm, I = 5 J m^{-2} s^{-1}) immediately prior to homogenization. Approximately 1 kg of seedlings was homogenized in a Waring Blendor with an extraction buffer (0.75 mL/g of tissue) containing 50% ethylene glycol, 100 mM Tris-HCl, 140 mM $(NH_4)_2SO_4$, 10 mM Na_4EDTA, and 20 mM sodium bisulfite (added fresh), final pH 8.3 (4 °C), as described by Bolton (1979) with the addition of 4 mM PMSF just prior to use. The resulting crude extract was squeezed through two layers of cheesecloth.

Nucleic acids, pectins, and acidic proteins were precipitated with the addition of 10 mL of a 10% poly(ethylenimine) solution (pH adjusted to 7.8, 4 °C, with HCl) (Jendrisak & Burgess, 1975) per L of crude extract. The extract was stirred for 15 min and centrifuged for 20 min at 13000*g*. Additional PMSF (final concentration 2 mM) was added to the supernatant and phytochrome precipitated with the addition of 250 g of $(NH_4)_2SO_4$/L of extract. The solution was stirred for 1 h and centrifuged for 30 min at 27000*g*. The resultant pellet was resuspended in a volume of 25% ethylene glycol, 50 mM Tris-HCl, 5 mM Na_4EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF, final pH 7.8 (4 °C), sufficient to give a final $(NH_4)_2SO_4$ concentration of 70 mM as determined by conductivity measurement (0.015 Ω^{-1} cm^{-1}). The solution was clarified by centrifugation at 48000*g* for 5 min and applied with phytochrome still in the Pfr form to a 2.4 × 10 cm hydroxyapatite column equilibrated in 25% ethylene glycol, 50 mM Tris-HCl, 5 mM Na_4EDTA, 70 mM $(NH_4)_2SO_4$, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C). The column was subsequently washed with 2 column volumes of 50 mM Tris-HCl, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C), followed by 5 column volumes of 5 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C). The phytochrome was eluted with 20 mM potassium phosphate 5 mM Na_4EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF, final pH 7.8 (4 °C). Subsequent steps involved either Affi-Gel Blue or immunoaffinity column chromatography.

For the Affi-Gel Blue procedure (Smith & Daniels, 1981), phytochrome-containing fractions from the hydroxyapatite column were pooled, phytochrome was converted to Pr by a 5-min far-red irradiation (λ >700 nm), and protein was precipitated by the addition of 0.65 mL of 3.3 M $(NH_4)_2SO_4$ and 50 mM Tris (Smith & Daniels, 1981) per mL of phytochrome pool. After centrifugation for 10 min at 48000*g*, the pellet was resuspended in 20 mL of 10 mM potassium phosphate, 5 mM Na_4EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF, final pH 7.8 (4 °C), clarified by centrifugation for 5 min at 48000*g*, and applied to a 1.2 × 26 cm Affi-Gel Blue column equilibrated in the same buffer without PMSF. The column was sequentially washed (flow rate = 0.6–0.7 mL min^{-1}) with 3–4 column volumes of the equilibration buffer, 4 column volumes of 1.0 M KCl, 100 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C), and 2 column volumes of 250 mM KCl, 100 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C). Phytochrome was then eluted with 10 mM FMN, 250 mM KCl, 100 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C). The phytochrome-containing fractions were pooled, 0.9 mL of 3.3 M $(NH_4)_2SO_4$ and 50 mM Tris was added per mL of pool, and the samples were centrifuged for 10 min at 48000*g*. The pellet was resuspended in 5 mL of 100 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C), clarified by centrifugation for 5 min at 48000*g*, and applied to a 2.6 × 48 cm Bio-Gel A-1.5 M column (flow rate = 0.4 mL min^{-1}) equilibrated in the same buffer. Phytochrome-containing fractions were pooled and concentrated by precipitation with 0.9 mL of 3.3 M $(NH_4)_2SO_4$ and 50 mM Tris per mL of phytochrome pool. The purified phytochrome was resuspended in 100 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C), and stored at −80 °C in the same buffer with the addition of glycerol to 5% (v/v). Repeated freezing and thawing of these preparations did not alter the spectral properties as judged by difference spectra (see below). After each freeze/thaw cycle, between 5 and 10% of the soluble phytochrome irreversibly precipitated.

The Affi-Gel Blue was regenerated by washing the column with 10 column volumes of distilled H_2O to remove the FMN, 4 column volumes of 6 M guanidine hydrochloride, and finally 8–10 column volumes of distilled H_2O. The column was then repacked and equilibrated in the starting buffer.

To obtain purified 124-kDa phytochrome by the immunoaffinity column procedure of Hunt & Pratt (1979), phytochrome-containing fractions from the hydroxyapatite column were pooled and directly applied as Pfr to an 8-mL column of antiphytochrome immunoglobulins covalently coupled to CNBr-activated Sepharose 4B. Phytochrome was eluted as Pr or Pfr with 3 M $MgCl_2$ (pH 7.6, adjusted with Tris base) as described previously (Hunt & Pratt, 1979).

Table I: Purification of 124-kDa Phytochrome from 1 kg of Etiolated *Avena* Seedlings Using the Affi-Gel Blue Procedure

	total phytochrome [$\Delta(\Delta A)$][a]	total protein (mg)	yield (%)	sp act. [$\Delta(\Delta A)$ mg^{-1}]	fold purifcn
crude extract[b]	20.9	6820	100	0.00306	1.0
supernatant after poly(ethylenimine) precipitation	17.7	5770	85	0.00307	1.0
ammonium sulfate precipitate	12.8	385	61	0.0333	11
hydroxyapatite pool[c]	5.8	20.4	28	0.283	93
Affi-Gel Blue pool[c]	3.9	8.0	19	0.49	163
Bio-Gel A-1.5 M pool[c]	2.7	4.1	13	0.67	220[d]

[a] $\Delta(\Delta A)$ refers to the difference in absorbance at 665 and 730 nm taken from phytochrome difference spectrum (Pr − Pfr) in a 1-cm cuvette. [b] Filtered crude extract was clarified for 2 min at 8000*g* in a Beckman microfuge prior to assay. [c] Values measured after concentration by ammonium sulfate precipitation. [d] Phytochrome preparation has an A_{666}/A_{280} of 0.94.

Purification of 118/114-kDa Phytochrome. A phytochrome preparation containing polypeptides of 118 and 114 kDa was prepared as described by Hunt & Pratt (1979).

Protein Determination. Total protein was determined according to the method of Lowry et al. (1951) with bovine serum albumin as a standard. Because poly(ethylenimine) strongly interferes with the Lowry procedure (Jendrisak & Burgess, 1975), protein aliquots (10 and 100 μL) were first precipitated with 0.5 mL of 10% (w/v) trichloroacetic acid and redissolved in 0.6 mL of 0.1 N NaOH prior to the assay.

NH_2-Terminal Amino Acid Analysis. Phytochrome purified by the Affi-Gel Blue procedure was desalted on a 2.5 × 37 cm Sephadex G-25 column (Pharmacia) equilibrated with 50 mM ammonium carbonate (pH 7.8 with acetic acid) and exhaustively lyophilized. The protein was then subjected to automated Edman degradation using a Beckman 890C protein sequencer in collaboration with Dr. Mark Hermodson, Department of Biochemistry, Purdue University.

Amino Acid Analysis. Amino acid analyses were performed as described by Moore (1972) with a Durrum D-500 amino acid analyzer (Durrum Chemical, Sunnyvale, CA). Cysteine and methionine were determined as cysteic acid and methionine sulfone respectively after performic acid oxidation according to the method of Moore (1963). Tryptophan content was measured spectrophotometrically (Goodwin & Morton, 1946).

Spectroscopy. Phytochrome spectra were recorded with a Perkin-Elmer Model 557 spectrophotometer (Norwalk, CT) with the cuvette chamber cooled to 3 °C. Rates of phytochrome dark reversion (Pfr to Pr) at 3 °C with or without the addition of sodium dithionite were determined as described by Pike & Briggs (1972a). Sodium dithionite was added to a final concentration of 5 mM immediately after a 0.5 M stock solution was prepared.

Results and Discussion

Initial Steps Common to Both Purification Protocols. Proteolysis of phytochrome can be very rapid in crude extracts of *Avena*, being detectable within 30 min at 2 °C with the molecule as Pr (Vierstra & Quail, 1982b). Of the various conditions tested only extraction of the photoreceptor as Pfr in a homogenization buffer containing a high concentration of PMSF (4 mM), 50% ethylene glycol, and 140 mM $(NH_4)_2SO_4$ was found to afford complete protection of 124-kDa *Avena* phytochrome against proteolysis in the crude extract. Preparations extracted as Pfr with PMSF in buffers that did not include 50% (v/v) ethylene glycol and 140 mM $(NH_4)_2SO_4$ contained up to 20% 118/114-kDa degradation products within 1 h of extraction as determined by rapid immunoprecipitation and $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown). Previous data suggest that the EDTA and sodium bisulfite in the homogenization buffer might also contribute to the inhibition of phytochrome degrading proteases (Pike & Briggs, 1972b; Vierstra & Quail, 1982a). Additional PMSF is introduced at later stages of the purification because of its instability in aqueous buffers (James, 1978) and because of the possibility that proteases initially protected from PMSF by bound polypeptide inhibitors might become active subsequently during the purification (Pringle, 1975).

Although poly(ethylenimine) precipitation does not yield a significant increase in specific activity (Table I), this step facilitates clarification of the crude extract at low *g* forces and improves the efficiency with which phytochrome is resolubilized at the subsequent ammonium sulfate step. Between 70 and 80% of the phytochrome from the poly(ethylenimine) step was recovered after ammonium sulfate precipitation whether phytochrome was in the Pr or Pfr form. This result contrasts with the report of Briggs et al. (1968) that Pfr is less stable than Pr during ammonium sulfate precipitation.

Hydroxyapatite chromatography (Figure 1, upper panel) typically resulted in an approximately 11-fold purification of the phytochrome. The hydroxyapatite pool after concentration by ammonium sulfate precipitation had A_{666}/A_{280} ratios ranging from 0.3 to 0.4, indicating that these preparations were roughly 30–40% phytochrome [see Pratt (1982b)]. Generally about 50% of the phytochrome applied to the column was eluted with 20 mM potassium phosphate with no additional phytochrome eluting at higher concentrations. This step is useful in washing phytochrome free of ethylene glycol prior to elution. We assume that the ethylene glycol would interfere with the subsequent binding of phytochrome to Affi-Gel Blue since W. O. Smith (1981) has demonstrated that phytochrome already bound to Affi-Gel Blue is released by this solvent. Because Pr elutes more efficiently than Pfr from Affi-Gel Blue with FMN (W. O. Smith, 1981), phytochrome in the hydroxyapatite pool is converted to Pr before addition to the Affi-Gel Blue column.

Affi-Gel Blue Chromatography. Several modifications of the original procedure of Smith & Daniels (1981) were required to purify 124-kDa *Avena* phytochrome further. When the hydroxyapatite pool was applied to the Affi-Gel Blue column in the 10 mM potassium phosphate buffer indicated (Figure 1) and the column immediately washed with several column volumes of the same buffer, the phytochrome bound while many contaminating proteins washed through (Figure 1, lower panel). The 30-min incubation of the sample with Affi-Gel Blue as previously recommended (Smith & Daniels, 1981) was found to be unnecessary for complete binding of phytochrome. In contrast, when the hydroxyapatite pool was applied in a 100 mM potassium phosphate buffer and the column immediately washed with 0.5 M KCl according to the

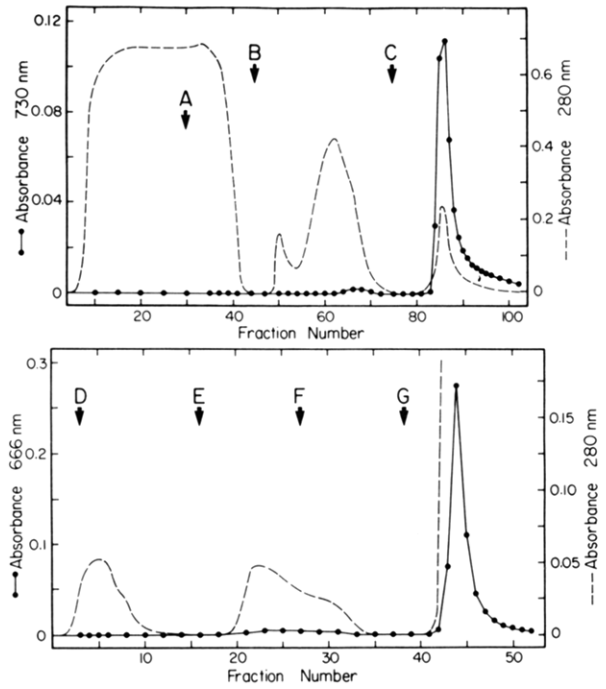

FIGURE 1: (Upper panel) Hydroxyapatite chromatography of Pfr following ammonium sulfate fractionation. The sample was applied in 25% ethylene glycol, 50 mM Tris-HCl, 70 mM ammonium sulfate, 5 mM Na_4EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF, final pH 7.8 (4 °C), to a 2.4 × 10 cm hydroxyapatite column equilibrated in the same buffer. The column was washed sequentially with (A) 50 mM Tris-HCl, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C), and (B) 5 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C). Phytochrome was eluted with (C) 20 mM potassium phosphate, 5 mM Na_4EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF, final pH 7.8 (4 °C). Each fraction contained 8 mL. The flow rate was maintained at 1.3 mL min^{-1}. (Lower panel) Affi-Gel Blue affinity chromatography of Pr following hydroxyapatite chromatography. The sample, in 10 mM potassium phosphate, 5 mM Na_4EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF, final pH 7.8 (4 °C), was applied to a 1.3 × 26 cm Affi-Gel Blue column equilibrated in the same buffer without PMSF. The column was washed with the equilibrium buffer (D) followed by (E) 1.0 M KCl, 100 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C), and (F) 250 mM KCl, 100 mM potassium phosphate, 5 mM Na_4-EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C). Phytochrome was eluted with (G) 10 mM FMN, 250 mM KCl, 100 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C). Each fraction contained 8 mL. The flow rate was maintained at 0.7 mL min^{-1}.

recommended procedure (Smith & Daniels, 1981), these contaminating proteins bound to the column and eluted along with phytochrome at the FMN step. In the present procedure, additional contaminants were removed prior to FMN elution by washing the column with 1 M KCl after first washing with 4 column volumes of the 10 mM potassium phosphate application buffer (Figure 1, lower panel).

In contrast to rye and 118/114-kDa *Avena* phytochrome, we have observed that 124-kDa *Avena* phytochrome elutes inefficiently from the Affi-Gel Blue column with the recommended 10 mM FMN in 100 mM potassium phosphate (Smith & Daniels, 1981), usually requiring 8–10 column volumes to release 50% of that initially bound. Efficient elution (i.e., elution in 1–2 column volumes of 70–80% of that initially bound) was achieved by addition of ≥250 mM KCl to the FMN. Concentrations of KCl up to 2 M did not further enhance this efficiency. We have also found that preincubation of the column with FMN prior to elution as prescribed by Smith & Daniels (1981) is not needed for efficient elution. The present elution conditions release only phytochrome and

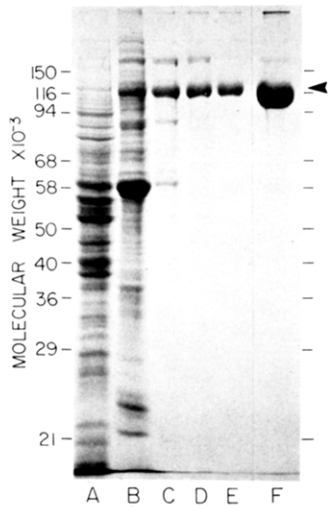

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of aliquots of phytochrome-containing fractions at various stages of purification by the Affi-Gel Blue affinity procedure. Gels containing 10% acrylamide were prepared according to Laemmli (1970) and stained with Coomassie Blue R: lane A, supernatant after poly(ethylenimine) precipitation; lane B, resuspended ammonium sulfate precipitate; lane C, hydroxyapatite pool; lane D, Affi-Gel Blue pool; lanes E and F, Bio-Gel A-1.5 M pool. Lanes C–E contain 2 μg of phytochrome, and lane F contains 10 μg of phytochrome as determined by the absorbance at 666 nm. The positions of the protein molecular weight markers used for calibration are shown to the left [see Vierstra & Quail (1982a)]. The arrow to the right indicates the position of the phytochrome band.

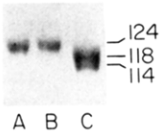

FIGURE 3: High-resolution comparison of the electrophoretic mobilities on $NaDodSO_4$–polyacrylamide gel electrophoresis of various phytochrome preparations. Gels were prepared with 6% acrylamide according to Laemmli (1970) and stained with Coomassie Blue R: lane A, 1 μg of 124-kDa phytochrome purified by the Affi-Gel Blue affinity procedure (see Figure 2 and text); lane B, 1 μg of 124-kDa phytochrome purified by the immunoaffinity column procedure (see Figure 4 and text); lane C, 1.5 μg of 118/114-kDa phytochrome purified by the immunoaffinity column procedure according to Hunt & Pratt (1979). Estimated molecular weights of the phytochrome bands are shown to the right.

a doublet of 180–190 kDa from the column (Figure 2D). Subsequent gel filtration on Bio-Gel A-1.5 M separates phytochrome from FMN and from the contaminating doublet (Figure 2E,F) which elutes ahead of phytochrome as an aggregate of at least 500 kDa.

This modified Affi-Gel Blue procedure permits 124-kDa phytochrome to be purified approximately 220-fold in 2 days with yields between 10 and 15% (Table I). These preparations routinely have A_{666}/A_{280} ratios between 0.90 and 0.94 and are >95% homogeneous as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2F). Electrophoresis on lower porosity gels (6% acrylamide) demonstrates that the phytochrome consists of a single polypeptide of 124 kDa (Figure 3A). Pooling only the peak phytochrome fractions from the Bio-Gel column has yielded 124-kDa phytochrome preparations with A_{666}/A_{280} ratios up to 0.97. The phytochrome obtained when this procedure was used has been found to be free from proteolytic degradation as Pr for at least 24 h at 4 °C or several hours at room temperature. Longer times have not been tested.

Immunoaffinity Chromatography. Approximately 20% of the phytochrome from the hydroxyapatite pool was recovered

Table II: Amino Acid Composition of *Avena* Phytochrome[a]

residue	hydrolysis time (h)[b] 24	48	72	average or extrapolated residue no.	nearest-integer residue no. this study, 124 kDa	Hunt & Pratt (1980),[g] 120 kDa	Roux et al. (1982),[g] 120 kDa
Asx	112.9	114.3	112.8	113.3	113	114	112
Thr	46.8	45.2	45.6	45.9	46	36	43
Ser	87.1	79.5	82.7	83.1	83	70	77
Glx	126.1	126.0	127.1	126.4	126	117	118
Pro	47.3	50.8	47.2	48.4	48	43	43
Gly	78.4	76.1	81.4	78.6	79	69	75
Ala	97.6	95.9	95.5	96.3	96	90	91
Val	70.2	74.3	76.1	76.1[c]	76	76	78
Met	33.5	34.2	33.5	33.7[d]	34	25	30
Ile	47.5	50.8	49.7	49.7[c]	50	49	51
Leu	123.7	127.1	122.2	124.3	124	114	111
Tyr	17.8	19.6	19.9	19.1	19	22	20
Phe	44.0	45.3	43.2	44.1	44	43	42
Lys	65.3	65.1	65.5	65.3	65	62	61
His	37.0	34.8	37.1	36.3	36	33	32
Arg	58.7	58.5	56.5	57.9	58	49	48
Cys				19.7[e]	20	26	15
Trp				10.2[f]	10	8	9
total residues					1127	1046	1053
molecular weight					124534	115833	116360

[a] Phytochrome used in the present study was purified by the immunoaffinity column method (A_{666}/A_{280} = 0.96). Residues are expressed per 124-kDa monomer. [b] Residues after 24-h hydrolysis are the average of three determinations of two different phytochrome preparations. Residues after 48- and 72-h hydrolysis are single determinations. [c] Determination after 72 h used due to slow release during hydrolysis. [d] Average of unmodified samples with two different performic acid oxidized samples. [e] Average of two different samples after performic acid oxidation. [f] Determined spectrophotometrically from two different samples. [g] Recalculated to represent preparations containing equimolar amounts of the 118- and 114-kDa species with an average molecular mass of 116 kDa.

from the immunoaffinity column upon elution with 3 M $MgCl_2$ whether applied as Pr or Pfr. The molecule purified in this manner is a single polypeptide of 124 kDa (Figure 3B), has A_{666}/A_{280} ratios up to 0.96, and is >95% homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4D). Phytochrome is more rapidly purified by this method than by the Affi-Gel Blue procedure (1 day vs. 2 days), but elution from the immunoaffinity column with 3 M $MgCl_2$ alters the spectral properties of the molecule (see below), making it useful only for chemical characterizations and antibody production.

NH_2-Terminal Amino Acid Sequencing. Repetitive Edman degradation of 33 nmol of Affi-Gel Blue purified phytochrome yielded no detectable amino acid derivatives for at least 10 cycles. This result indicates the strong likelihood that 124-kDa phytochrome contains a blocked NH_2 terminus. Direct confirmation of this conclusion will require isolation of the modified residue. In contrast to these results, Hunt & Pratt (1980) have reported an NH_2-terminal amino acid sequence of NH_2-Lys-Ala-Leu-Val for immunopurified 118/114-kDa *Avena* phytochrome with about 40% of the polypeptides lacking the terminal lysine. Rice & Briggs (1973) have also reported free NH_2 termini for both oat and rye phytochrome. Together these data indicate that the proteolytic conversion of 124-kDa to 118/114-kDa phytochrome involves, at least in part, cleavage of residue(s) from the NH_2 terminus.

Amino Acid Analysis. The amino acid composition of 124-kDa *Avena* phytochrome purified by the immunoaffinity column procedure is similar on a mole percent basis to that for "120"-kDa *Avena* phytochrome preparations determined by us in this study (data not shown) and by others previously (Hunt & Pratt, 1980; Roux et al., 1982) (Table II). This result is not unanticipated given the relatively large size of the phytochrome monomer. Although the number of amino acids removed by the proteolysis is substantial (about 50 and 90 for the 118- and 114-kDa polypeptides respectively), this represents only 5 and 8%, respectively, of the total monomer. Thus even substantial bias toward any individual or class of amino acid in the cleavable segments would not be readily detectable within the limits of accuracy of the analysis. On the basis of the polarity index of Capaldi & Vanderkooi (1972), 124-kDa phytochrome can be described as a typical water-soluble protein consisting of 46.8% polar residues.

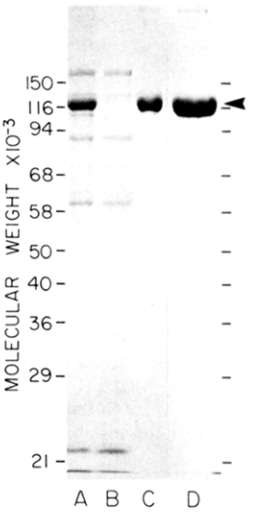

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of aliquots of phytochrome-containing fractions at various stages of purification using the immunoaffinity column procedure. Gels containing 10% acrylamide were prepared according to Laemmli (1970) and stained with Coomassie Blue R: lane A, hydroxyapatite pool; lane B, proteins from the hydroxyapatite pool not adsorbed to the immunoaffinity column; lanes C and D, proteins from the hydroxyapatite pool adsorbed to the immunoaffinity column and released by 3 M $MgCl_2$. Lanes A and C contain 2 μg of phytochrome, and lane D contains 8 μg of phytochrome as determined by the absorbance at 666 nm. The positions of the protein molecular weight markers used for calibration are shown to the left [see Vierstra & Quail, 1982a,b)]. The arrow to the right indicates the position of the phytochrome band.

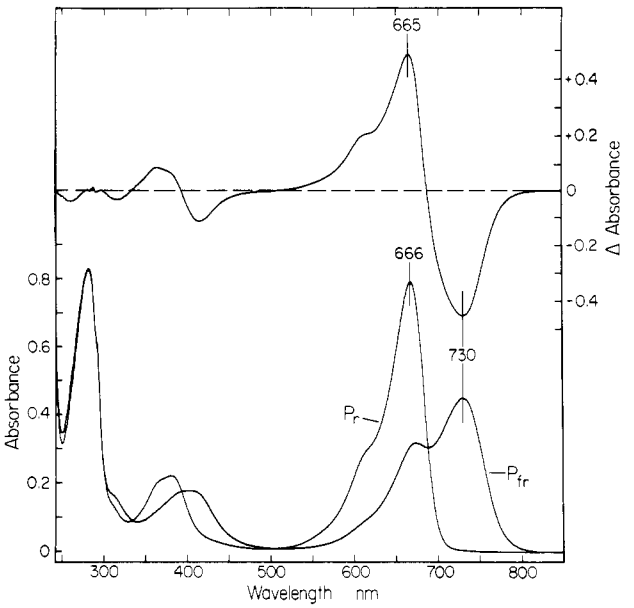

FIGURE 5: Absorbance and difference spectra of 124-kDa phytochrome (A_{666}/A_{280} = 0.97) purified by the Affi-Gel Blue affinity procedure in 100 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C). Absorbance spectra were measured at 3 °C after saturating red (Pfr) and far-red (Pr) irradiation. The difference spectrum was determined by subtracting the spectrum of Pfr from that of Pr.

Spectral Properties. The absorbance spectra of 124-kDa phytochrome purified by the Affi-Gel Blue procedure display maxima for Pr at 666, 379, and 280 nm and for the spectrum obtained after saturating red irradiation at 730, 673, about 400, and 280 nm (Figure 5). Cycling of Pr through Pfr caused only a 1–2% irreversible loss of absorbance at 666 nm. The difference spectrum (Pr – Pfr) exhibits prominent maxima and minima at 730, 665, 414, and 363 nm and has a spectral change ratio ($\Delta A_r/\Delta A_{fr}$) of 1.06–1.07. The difference spectrum of the purified molecule is thus similar to that observed in vivo and is identical with that measured in crude extracts immediately after extraction (Vierstra & Quail, 1982b), indicating that the spectral properties of native phytochrome are preserved with this isolation procedure.

Comparison of the absorbance spectra of purified 124- and 118/114-kDa phytochrome confirms and extends conclusions drawn from previous studies with crude and partially purified preparations (Vierstra & Quail, 1982b). Proteolytic degradation to the 118/114-kDa species causes the absorbance maxima for Pfr in both the far-red and blue regions of the spectrum to shift to shorter wavelengths, i.e., from 730 to 724 nm and from 400 to 390 nm (Figure 5; Hunt & Pratt, 1979; Vierstra & Quail, 1982b). In addition, the absorbance of Pfr at 730 nm for the 124-kDa molecule is substantially greater than at the comparable far-red maximum, 724 nm, for the 118/114-kDa species. Thus the Pfr absorbance at 730 nm relative to the shoulder at 673 nm (A_{730}/A_{673}) is 1.45 for 124-kDa phytochrome (Figure 5) compared to only 0.92–1.13 (A_{724}/A_{673}) for the 118/114-kDa preparations (Pratt, 1982b; Roux et al., 1982; Vierstra & Quail, 1982b). Similarly, the peak far-red absorbance of Pfr divided by the peak red absorbance of Pr is 0.58 for the 124-kDa species and only 0.48–0.43 for the degraded species. This result would suggest an increase in Pfr absorbance relative to Pr of 20–30% for the 124-kDa molecule. Such changes could reflect an increase in the percent Pfr at photostationary state [reported previously to be 75% Pfr and 25% Pr for 118/114-kDa *Avena* phytochrome after saturating red irradiation (Pratt, 1975)] and/or an increase in the absolute extinction of Pfr. The report of Yamamoto & Smith (1981) that partially purified rye phytochrome with a Pfr absorbance maximum at 730 nm has a photostationary state of 84% Pfr is consistent with the former possibility.

The ultraviolet difference spectrum of the purified 124-kDa molecule displays minima and maxima at 260, 280, 287, 297, and 316 nm (Figure 5) in close agreement with regard to peak heights and positions with those of 118/114-kDa *Avena* (R. D. Vierstra and P. H. Quail, unpublished results), 120-kDa rye (Tobin & Briggs, 1973), and 60-kDa *Avena* (Hopkins & Butler, 1970; Pratt & Butler, 1970) phytochrome. If the spectral changes between 275 and 310 nm reflect changes in exposure of aromatic amino acids as suggested (Tobin & Briggs, 1973), then the present data indicate that the observed proteolysis does not significantly alter the environment of the pertinent residues.

In contrast to the species obtained by the Affi-Gel Blue procedure, 124-kDa phytochrome purified by immunoaffinity chromatography has spectral properties considerably altered from those observed in vivo. The absorbance of Pfr is substantially reduced relative to Pr resulting in a $\Delta A_r/\Delta A_{fr}$ ratio of greater than 2.0 after elution from the immunoaffinity column with 3 M $MgCl_2$ in either spectral form (data not shown). Subsequent cycling of Pr through Pfr results in successive 20–30% losses of absorbance at 666 nm indicative of spectral denaturation. The 124-kDa phytochrome purified by the immunoaffinity procedure was thus deemed unsuitable for spectral studies. We have not tested for alternative conditions that would release spectrally undenatured 124-kDa phytochrome from the immunoaffinity column, although such conditions may exist. R. E. Hunt and L. H. Pratt (personal communication) have observed no such deleterious effects on the spectral properties of 118/114-kDa phytochrome during immunopurification, indicating that there are differences in sensitivity of the native and degraded molecules to denaturation. Moreover, whereas the $\Delta A_r/\Delta A_{fr}$ ratio of 2.0 reported by Pratt & Cundiff (1975) for 120-kDa phytochrome could be reduced to 1.35 by addition of EDTA or 2-mercaptoethanol, these compounds do not reverse the denaturation of the 124-kDa molecule.

Dark Reversion. It is well established that the Pfr form of large rye and *Avena* phytochrome reverts nonphotochemically to Pr in vitro (Pike & Briggs, 1972a; Hahn & Song, 1981; Song et al., 1981). The process is temperature dependent and is stimulated by reductants including sodium dithionite. However, because monocotyledonous phytochrome does not dark revert in vivo (Pike & Briggs, 1972a), a discrepancy exists. The data below indicate that, as with the aberrant spectral properties discussed above, in vitro dark reversion of *Avena* phytochrome is an artifact of the proteolytic degradation of the 124-kDa molecule. Affi-Gel Blue purified 124-kDa phytochrome exhibits negligible dark reversion at 3 °C in the absence or presence of sodium dithionite (Figure 6A). In the absence of dithionite, no dark reversion of 124-kDa phytochrome is detectable after 2 h, and only 1–2% occurs after 18 h at 3 °C. On the other hand, 118/114-kDa phytochrome exhibits dark reversion at 3 °C at a rate that is strongly stimulated by sodium dithionite (Figure 6C). Comparison of the dark reversion of 124-kDa phytochrome before and after the Affi-Gel Blue column step indicates that the slow rate of dark reversion in the purified preparation in the presence of dithionite is not the result of prior exposure of the phytochrome to lumichrome. Lumichrome, found as a contaminant in commercial sources of FMN, has been previously shown to

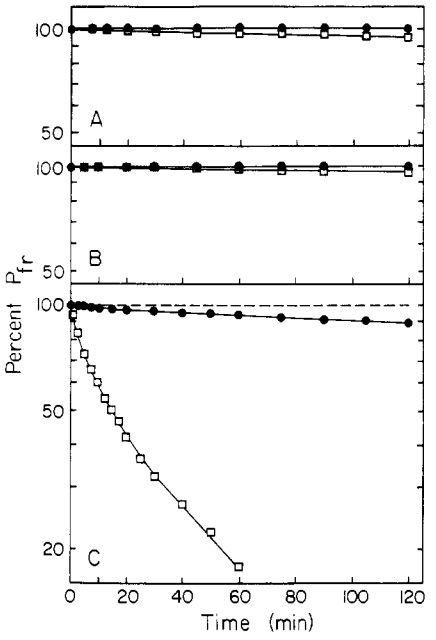

FIGURE 6: In vitro dark reversion of phytochrome at 3 °C in the absence (●) and presence (□) of 5 mM sodium dithionite: (A) 124-kDa phytochrome (A_{666}/A_{280} = 0.92) purified by the Affi-Gel Blue affinity procedure (see Figure 3, lane A); (B) 124-kDa phytochrome obtained after hydroxyapatite chromatography (A_{666}/A_{280} = 0.18); (C) 118/114-kDa phytochrome (A_{666}/A_{280} = 0.83) isolated by the immunoaffinity column procedure of Hunt & Pratt (1979) (see Figure 3, lane C). All samples were dissolved in 100 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, final pH 7.8 (4 °C).

prevent the dithionite-accelerated dark reversion in presumptive 118/114-kDa *Avena* phytochrome preparations purified by Affi-Gel Blue chromatography (Song et al., 1981). The small amount of dark reversion (~7% after 2 h at 3 °C) that does occur with 124-kDa phytochrome in the presence of sodium dithionite may represent small amounts of 118/114-kDa species not detected by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3A) or an inherently slower rate of reversion of the undegraded molecule.

Conclusions

The procedures described here enable 124-kDa phytochrome from *Avena* to be purified to >95% homogeneity, free of detectable levels of the 118/114-kDa degradation products, and in yields of 10–15%. The spectral properties of the Affi-Gel Blue purified 124-kDa molecule closely resemble those measured in vivo in all respects thus far examined but differ substantially from those determined for the 118/114-kDa species. These data indicate that the phytochrome purified by this procedure represents the native molecule and confirm that the proteolytically cleaved polypeptide segments play a crucial role in maintaining the photoreceptor's spectral and structural integrity. The loss of the blocked NH_2 terminus upon proteolytic conversion of the 124- to the 118/114-kDa species indicates that at least part of this critical polypeptide domain is located at the NH_2 terminus of the molecule. Moreover, the differential accessibility of the proteolytically sensitive site(s) in the Pr and Pfr forms indicates that this domain is involved in a change in molecular topology upon phototransformation. Whether this change represents an alteration of protein conformation, chromophore orientation, or both remains to be determined. The present data underscore the need to further reexamine the properties of the native photoreceptor. For example, it might be anticipated that other spectral properties of 124-kDa phytochrome such as percent Pfr at photoequilibrium in red light, quantum yields, extinction coefficients, and the kinetics of phototransformation will differ from those previously recorded for 118/114-kDa phytochrome.

Acknowledgments

We are grateful to L. H. Pratt for assistance in initiating the immunopurification procedures, W. R. Briggs in whose laboratory much of the antiphytochrome immunoglobulin production was carried out, Mark Hermodson for performing the NH_2-terminal amino acid sequence analysis, D. Omilianowski for performing the amino acid analysis, R. Burgess for the gift of RNA polymerase, and G. Bolton, K. Dunham, H. Hershey, and S. Daniels for helpful suggestions.

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Activity and Conformational Changes in Chloroplast Coupling Factor Induced by Ion Binding: Formation of a Magnesium–Enzyme–Phosphate Complex†

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ABSTRACT: The effects of ions on the conformation and activity of chloroplast coupling factor (CF_1) were studied by using tryptophan as an intrinsic fluorescence probe in CF_1. The fluorescence of tryptophan decreased when $MgCl_2$ or sodium phosphate was added to the protein. The decrease indicated that Mg^{2+} and inorganic phosphate (P_i) bound directly to the protein. The decrease saturated at 1.2 mM Mg^{2+} and at 0.8 mM P_i, although P_i showed evidence of a higher affinity site saturating around 80 μM. The decrease in fluorescence could also be observed when P_i was added after addition of Mg^{2+}, which indicated that a ternary complex of Mg^{2+}–CF_1–P_i formed. If the reverse addition sequence was used, a ternary complex was not observed. The free energy of dissociation for each ion added singly was 4.6, 6.2, and 4.8 kcal/mol for Mg^{2+}, P_i (high-affinity site), and P_i (low-affinity site), respectively. The magnitude of these free energies and the presence of a ternary complex of Mg^{2+}, P_i, and CF_1 may be significant for the mechanism of photophosphorylation. The incubation of CF_1 in Mg^{2+} above 5 mM increased the polarization of CF_1 (tryptophan) fluorescence and changed the circular dichroism spectrum. These spectroscopic changes show that Mg^{2+} binds further at concentrations above the initial saturating concentration of 1.2 mM and that CF_1 changes conformation in response to these higher concentrations. Chloroplasts lost phosphorylating activity and CF_1 lost Ca^{2+}-ATPase activity when the preparations were preincubated at these high Mg^{2+} concentrations but then removed from the preincubating media and assayed under uniform conditions. Thus, the additional binding and altered conformation were inhibitory for CF_1 and chloroplast activities. The results indicate that Mg^{2+} binds directly to CF_1 and may have two roles: a catalytic one associated with the formation of a Mg^{2+}–CF_1–P_i complex and a regulatory one at high Mg^{2+} concentrations. The regulatory one resembles the effects in vivo caused by dehydration of leaf tissue, which increases the concentration of cellular ions, and this suggests that both the catalytic and the regulatory roles of Mg^{2+} could be important in vivo.

The synthesis of ATP, which is central to metabolic energy conversion in biological organisms, is associated with a transmembrane gradient in electrochemical potential created by proton movement. Although the dissipation of the gradient results in ATP synthesis, the molecular mechanism is poorly understood and of great interest. Several theories have been advanced (Weber, 1972; Boyer, 1977; Racker, 1977; Penefsky, 1977; Kasahara & Penefsky, 1978), all of which require the binding of ADP and inorganic phosphate (P_i)[1] to the coupling protein (ATP synthetase) complex in the membrane. In addition, certain theories suggest that other ions, particularly Mg^{2+}, may be required to bind to the protein (Weber, 1972; Racker, 1977).

Weber (1972) pointed out that, on a theoretical basis, the binding of ions to the coupling protein could provide sufficient free energy for the transfer of P_i to ADP. Racker (1977) postulated that, by analogy with the Na^+–K^+–ATPases and Ca^{2+}-ATPases, the ATP synthetases may form a magnesium acyl phosphate intermediate and that the role of protons may be to dissociate Mg^{2+} from the coupling protein with the release of ATP. Evidence for the formation of a phosphoenzyme intermediate in ADP–ATP interconversions was first found with Na^+–K^+- and Ca^{2+}-ATPases when the enzymes

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[1] Abbreviations: CF_1, chloroplast coupling factor protein (ATP synthetase when attached to the membrane); PMS, phenazine methosulfate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/83/0422-2505$01.50/0