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Partial Characterization of Undegraded Oat Phytochrome†

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ABSTRACT: We characterized immunoaffinity-purified, undegraded oat (*Avena sativa* L., cv. Garry) phytochrome by several physicochemical techniques. Phytochrome, of greater than 98% purity [Hunt, R. E., & Pratt, L. H. (1979) *Plant Physiol. 64*, 332-336], existed in solution as a dimer of its 118 000-dalton monomers and had a full complement of the typical amino acids with about 35% nonpolar residues, 115 carboxylic acid groups per monomer, and an average of one phosphate per monomer. Although the dimer was not held together by disulfide bridges, each monomer contained three disulfide bonds and 14 reduced cysteines out of a total of 27 cysteine–half-cystine residues. Phytochrome preparations, although very pure, exhibited heterogeneity by discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which revealed three closely spaced bands, and by nondenaturing gel electrophoresis at pH 7.0, which revealed four bands. Amino-terminal analysis indicated two residues, Lys and Ala. Manual Edman degradation yielded Leu and Ala after one round and Val and Leu after a second round. These data indicate a possible amino-terminal sequence of NH_3-Lys-Ala-Leu-Val- with some monomers not having Lys.

Phytochrome is the photoreceptor for many light-mediated developmental responses in plants (Smith, 1975). The chromoprotein exists in two photointerconvertible forms, the physiologically inactive, red-absorbing form and the physiologically active, far-red-absorbing form. An understanding of the differences between these two conformations of the chromoprotein is crucial to elucidating its mechanism of action. However, we must first investigate its fundamental physicochemical properties before we will be in a position to probe for these differences.

Physiological responses to light by oats (*Avena sativa* L.) are well documented (Smith, 1975). Nevertheless, undegraded oat phytochrome is not well characterized biochemically because of practical difficulties in obtaining purified preparations in soluble, undegraded form [cf. Pratt (1978) for a discussion]. Most reported characterizations of oat phytochrome are of questionable value since they were performed on a proteolytically degraded, apparently nonphysiological form of the molecule [e.g., see Mumford & Jenner (1966)], which was generated by the action of endogenous proteases, to which phytochrome is highly susceptible (Gardner et al., 1971).

The only undegraded phytochrome that has been significantly characterized is that purified from rye (*Secale cereale* L.; Rice et al., 1973; Rice & Briggs, 1973). However, the physiology of the oat phytochrome system is better documented than that of the rye system, we are able to obtain larger quantities of this relatively scarce pigment (about 0.1% of extractable protein under the best of conditions) from oats than from rye, and there is a large background of information concerning degraded oat phytochrome [see Pratt (1978, 1979) for reviews]. Hence, it is best to begin a characterization of undegraded oat phytochrome rather than to continue with a characterization of rye phytochrome.

Recently, we developed an immunoaffinity purification technique (Hunt & Pratt, 1979) that rapidly yields highly purified undegraded oat phytochrome in quantities sufficient for physicochemical characterization. This phytochrome, except for its reliably higher purity, is identical with conventionally purified phytochrome. We report here a partial

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physicochemical characterization of undegraded oat phytochrome.

Materials and Methods

Phytochrome. Immunoaffinity-purified phytochrome was obtained as previously described (Hunt & Pratt, 1979). Briefly, phytochrome was partially purified from extracts of 5-day-old, dark-grown oat (*A. sativa* L., cv. Garry) shoots by brushite chromatography and ammonium sulfate fractionation. Phytochrome was then adsorbed by agarose-immobilized antiphytochrome immunoglobulins and, after nonadsorbed protein was washed away, eluted with 3 M $MgCl_2$ at pH 7.5. Eluted phytochrome was precipitated with 33% saturation of ammonium sulfate, collected by centrifugation, resuspended in 0.1 M sodium phosphate, pH 7.8, and 1 mM EDTA,[1] and stored at −76 °C. A 1-mL sample of the red-absorbing form of phytochrome with an $A_{667}^{1cm} = 1.0$ and an $A_{280}^{1cm} = 1.2$ was assumed to contain 1.7 mg of phytochrome (Tobin & Briggs, 1973).

Gel Electrophoresis. Discontinuous $NaDodSO_4$ gel electrophoresis was performed as described by Laemmli (1970) with the following modifications: the running gel was 7.5% (w/v) acrylamide and 0.2% (w/v) *N,N'*-methylenebis(acrylamide); the electrode buffer was 50 mM Tris, 0.5 M glycine, and 0.1% (w/v) $NaDodSO_4$, pH 8.5; the sample buffer was 50 mM Tris-HCl, 1% (w/v) $NaDodSO_4$, 300 mM 2-mercaptoethanol, 10% (w/v) sucrose, and 0.006% (w/v) bromphenol blue, pH 6.8, at 20–25 °C. After electrophoresis, gels were soaked in trichloroacetic acid, stained, and destained as described by Weber & Osborn (1969).

Continuous $NaDodSO_4$ gel electrophoresis was performed as described by Weber & Osborn (1969) with gels of 5% (w/v) acrylamide and 0.14% (w/v) *N,N'*-methylenebis(acrylamide).

Nondenaturing, continuous gel electrophoresis was performed at pH 7.0 and 5.0. Gels of 5% (w/v) acrylamide and 0.14% (w/v) *N,N'*-methylenebis(acrylamide), made either pH 7.0 with 0.1 M sodium phosphate or pH 5.0 with 0.1 M sodium citrate, were preelectrophoresed in the same buffer at 8 mA/gel toward the anode (15 min for pH 7.0 and 5 h for pH 5.0). Samples were precipitated by ammonium sulfate and resuspended in 12.5 mM sodium phosphate, pH 7.0, or 12.5 mM sodium citrate, pH 5.0, and 45% (v/v) glycerol and were electrophoresed for 5 h at 8 mA/gel toward the anode for pH 7.0 gels and toward the cathode for pH 5.0 gels. Gels were fixed and stained as described before.

Sedimentation Equilibrium Centrifugation. Phytochrome was centrifuged in a Beckman Model E ultracentrifuge at 10800 rpm for 30 h at 4 °C. Phytochrome concentration (initially 0.3, 0.6, or 1.2 mg/mL) was determined by scanning the three double-sector windows at 280 nm with scanner optics. Molecular weight was calculated from the slope of the plot of ln A_{280} vs. the square of the distance from the center of the rotor (X^2).

Amino Acid Analysis. Amino acid analyses were performed essentially as described by Moore & Stein (1963) with a single DC 6A (Durrum Chemical, Sunnyvale, CA) column at 50 °C. Concentrations of amino acids were determined with a Spectra Physics (Model 4000) automatic integrator.

Cysteine was determined as cysteic acid by the method of Spencer & Wold (1969) and by the method of Hirs (1967). Methionine was determined as methionine sulfone by the method of Hirs (1967). Tryptophan was determined by the method of Goodwin & Morton (1946).

Cysteine–Cystine Measurements. Total cysteine was determined by the procedure of Habeeb (1975) with 120 μg of phytochrome in a 1-mL final volume. Freshly diluted 2-mercaptoethanol was used as a standard. We determined total cysteine–cystine by a scaled-down procedure adapted from Cavallini et al. (1966) using 60 μg of phytochrome and appropriate volumes of all reagents to a final volume of 0.5 mL.

COOH Measurement. Total carboxylic acid groups were determined by a modification of the procedure of Hoare & Koshland (1967). Phytochrome (240 μg) was precipitated with an equal volume of acetone and dried under a stream of N_2. Phytochrome was denatured in 7.5 M urea, pH 4.5 (1 mg/mL final concentration), and the reaction was started by addition of [^{14}C]glycine ethyl ester (1.5 mCi/mol, 0.33 M final concentration) and 1-ethyl-3-(dimethylamino)propylcarbodiimide (33 mM final concentration). After 1, 2, and 4 h, two 30-μL aliquots were placed in 150 μL of 1 M sodium acetate, pH 4.5, and were precipitated and washed 3 times with 3 mL of acetone–HCl (39:1 v/v). Washed pellets were dissolved in 400 μL of 10% (w/v) $NaDodSO_4$, and radioactivity was determined by liquid scintillation counting. Zero-time blanks were obtained from urea-denatured phytochrome in 150 μL of 1 M sodium acetate, pH 4.5, to which reactants were added. Since nonradioactive experiments indicated that the pH increased to no more than 4.65 after 4 h, pH was not adjusted during the course of the reaction.

Amino-Terminal Analysis. We manually determined the amino-terminal sequence of phytochrome as described by Weiner et al. (1972), using Edman degradation and dansylation. Phytochrome (1.4 mg) was precipitated with an equal volume of acetone, collected by centrifugation, and resuspended in 440 μL of 0.5 M $NaHCO_3$, pH 9.8, and 1% (w/v) $NaDodSO_4$. The mixture was heated to 100 °C to dissolve phytochrome, and 200 μg of phytochrome was removed for amino-terminal analysis. The remainder was subjected to one round of manual Edman degradation, 600 μg of phytochrome was removed for dansylation, and the other 600 μg was subjected to a second round of degradation and dansylation. Dansyl amino acids were identified as described by Weiner et al. (1972).

Phosphate Content. Covalently bound phosphate was determined as described by Chen et al. (1956) with 240 μg of phytochrome in a final volume of 0.5 mL and KH_2PO_4 as a standard.

Each assay was performed at least twice with independent preparations, except for the carboxylic acid determination, which was replicated with different aliquots of the same preparation. Except for variation noted in the amino acid analyses, all replicate determinations gave identical results.

Results

Molecular Weight. Continuous $NaDodSO_4$ gel electrophoresis indicated that immunoaffinity-purified phytochrome was over 98% homogeneous (Figure 1a; Hunt & Pratt, 1979) and had a monomer weight of 118000 (average of 30 measurements taken from linear regressions of standards as in Figure 1a). Sedimentation equilibrium centrifugation of phytochrome at 0.3 mg/mL yielded a linear plot of ln A_{280} vs. X^2 (Figure 2a) and a calculated molecular weight of 233000, indicating that phytochrome is a dimer in solution. At higher concentrations (0.6 and 1.2 mg/mL) the plots showed curvature (Figure 2b,c), with the higher concentration sample indicating more heterogeneity than the lower one. The upper limiting slopes to the curves yielded molecular weights

[1] Abbreviations used: EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; Mops, 4-morpholinepropanesulfonic acid.

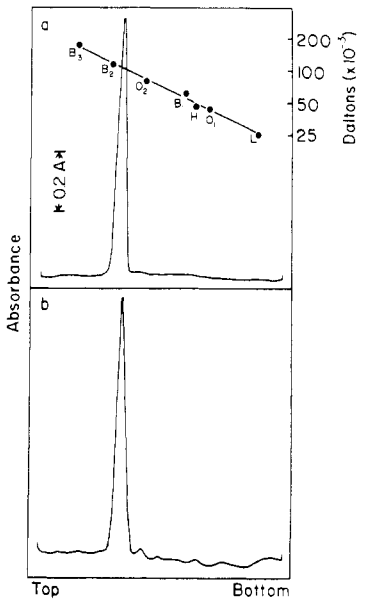

FIGURE 1: Absorbance scans of continuous $NaDodSO_4$–polyacrylamide gels (5-mm diameter) after electrophoresis of (a) 30 μg of immunoaffinity-purified phytochrome and (b) 22 μg of the same preparation after sedimentation equilibrium centrifugation. Size standards, run with each experiment, are as follows: O_1 and O_2, ovalbumin monomer (45 000 daltons) and dimer, respectively; H and L, bovine immunoglobulin G heavy (50 000 daltons) and light (25 000 daltons) chains, respectively; B_1, B_2, and B_3, bovine serum albumin monomer (66 000 daltons), dimer, and trimer, respectively.

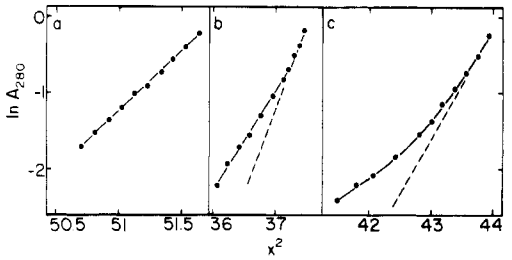

FIGURE 2: Plots of ln A_{280} vs. the square of the distance from the center of the rotor (X^2) derived from data obtained from equilibrium centrifugation of immunoaffinity-purified oat phytochrome at concentrations of (a) 0.3, (b) 0.6, and (c) 1.2 mg/mL. Dashed lines are the estimated upper limiting slopes of the curved lines.

of 220 000 and 260 000 for the 0.6 and 1.2 mg/mL samples, respectively. Therefore, the observed heterogeneity might result from peptides of molecular weight lower than undegraded phytochrome. After centrifugation, the phytochrome sample showed by $NaDodSO_4$ gel electrophoresis (Figure 1b) a minor degradation product of 100 000 daltons which contains about 3% of the total Coomassie blue stain. The observed heterogeneity might be accounted for by this contaminant plus the 18 000 daltons that is the difference between the 118 000-dalton monomer and the 100 000-dalton degradation product and that is not accounted for in the gel scan.

Amino Acid Analysis. Amino acid analyses of undegraded phytochrome (Table I) indicated that the molecule possesses all of the typical residues. The number of residues obtained per monomer agreed within one to four residues for the duplicate analyses except for threonine (34–41 residues) and methionine (19–29 residues). Cysteine, determined by the method of Spencer & Wold (1969), varied from 25 to 34 residues in duplicate assays. Cysteine and methionine were reassayed after performic acid oxidation, giving 23 residues of cysteine as cysteic acid and 30 residues of methionine as methionine sulfone. Data for cysteine and methionine are

Table I: Amino Acid Analysis of Undegraded Phytochrome[a]

residue	hydrolysis time (h) 24	48	72	av or extrapolated residue no.	residues, nearest integer
Lys	65.2	64.4	63.1	64.2	64
His	33.0	36.0	34.0	34.3	34
Arg	49.9	51.3	50.9	50.7	51
Asp	118.8	121.1	114.9	118.3	118
Thr	36.1	35.1	33.4	37.6	38[b]
Ser	71.5	66.2	65.0	72.7	73[b]
Glu	121.4	121.7	122.6	121.9	122
Pro	46.5	43.4	45.1	45.0	45
Gly	73.5	71.8	71.1	72.1	72
Ala	94.2	92.7	92.1	93.0	93
Val	64.1	72.5	78.7	78.7	79[c]
Met	28.0	23.0	27.3	26.1	26[d]
Ile	42.7	50.4	51.1	51.1	51[c]
Leu	118.8	119.1	118.6	118.8	119
Tyr	23.0	22.6	21.8	22.5	23
Phe	45.8	45.1	44.4	45.1	45
Cys				27.3	27[e]
Trp				7.9	8[f]
total					1088

[a] Residues are expressed per 118 000-dalton monomer and are the average of at least two determinations. [b] Extrapolated to zero time. [c] 72-h determination used because of slow release due to bulky side group. [d] Average of two unmodified samples and one performic acid oxidized sample. [e] Average of two samples hydrolyzed in the presence of dimethyl sulfoxide and one performic acid oxidized sample. [f] Determined spectrophotometrically.

expressed as the averages of all determinations.

On the basis of the tyrosine and tryptophan content of phytochrome (Table I) and the extinction coefficients of these two amino acids (Goodwin & Morton, 1946), we calculated that a 1 mg/mL solution should have an $A_{280}^{1cm} = 0.63$. This value agrees well with that obtained for undegraded rye phytochrome ($A_{280}^{1cm} = 0.70$) based upon a Lowry assay for protein using bovine serum albumin as a standard (Tobin & Briggs, 1973).

Cysteine–Cystine Residues. Undegraded phytochrome was purified by the immunoaffinity technique except that 2-mercaptoethanol was omitted from the initial extraction and brushite column buffers. This phytochrome, which was never exposed to added reductant, and that extracted in the presence of 2-mercaptoethanol have identical mobilities by continuous $NaDodSO_4$ gel electrophoresis with 2-mercaptoethanol omitted from the sample-denaturing buffer (data not shown), indicating that the monomers are not held together by disulfide bonds.

Total cysteine content of $NaDodSO_4$-denatured phytochrome isolated with 2-mercaptoethanol indicated that 14 out of a possible 27 cysteine–half-cystine residues per monomer were reduced. Phytochrome isolated without 2-mercaptoethanol had only 10 reduced cysteine residues. Neither preparation showed an increase in total cysteine residues after mild treatment with 2-mercaptoethanol, indicating that no residues were partially oxidized.

COOH Content. Phytochrome contained an average of 115 carboxylic acid groups per 118 000-dalton monomer. By subtraction of one for the carboxy-terminal end, this result indicates that 114 of the 240 Asx and Glx (Table I) are the acids and the balance, presumably, are the amines. The accuracy of this assay was demonstrated by our finding in a parallel assay that bovine serum albumin contained 97 mol of the expected 101 mol of carboxylic acid per mol of protein (Dayøff, 1976).

Phosphate Content. Analysis of 2 nmol of phytochrome monomer, resuspended after purification in 25 mM Mops–Tris and 5 mM EDTA, pH 7.5, indicated the presence of 1 mol

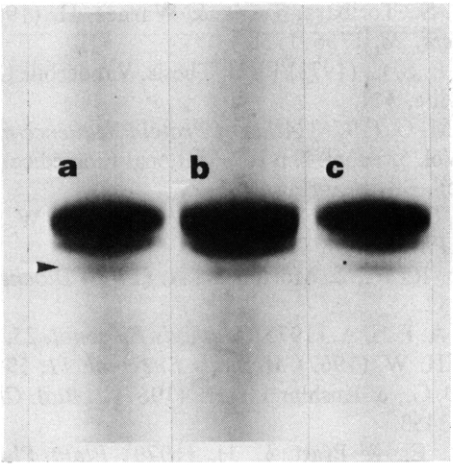

FIGURE 3: Three-band pattern observed on discontinuous NaDod-SO_4–polyacrylamide gels (5-mm diameter) after electrophoresis of 1 μg of immunoaffinity-purified phytochrome. (a) Untreated, (b) carboxymethylated, and (c) untreated phytochrome, electrophoresed in a gel that contained 0.1 mM 2-mercaptoethanol. Arrow indicates band not previously reported by Pratt (1979).

of phosphate per mol of phytochrome monomer. Controls showed that the chromatography procedure used during immunoaffinity purification of phytochrome removed all extraneous phosphate from immunoglobulin G that was initially dissolved in 0.1 M sodium phosphate, pH 7.8, and that the assay accurately quantified phosphate on phosvitin.

Amino-Terminal Sequence Analysis. Dansylated, intact phytochrome yielded two amino-terminal residues, bis(Lys) and Ala. The ratio of the two residues, by use of [^{14}C]dansyl chloride, was 60% Lys and 40% Ala. Since these results indicated that the dimer may be composed of subunits of two dissimilar primary structures, we performed two rounds of manual Edman degradation. The first round yielded Ala (42%) and Leu (58%) as the penultimate residues. The second round yielded Val (48%) and Leu (52%). These data yield a sequence of NH_3-(Lys,Ala)-(Ala,Leu)-(Leu,Val)-. If we assume that the quantitation is not precise, then the sequence condenses to NH_3-Lys-Ala-Leu-Val- with some monomers not possessing Lys. A parallel experiment with insulin using [^{14}C]dansyl chloride yielded 45% Gly and 55% Phe, indicating that the quantitation for phytochrome might well have been inaccurate. Degradation of chicken egg white lysozyme yielded the expected amino-terminal sequence of NH_3-Lys-Val-Phe-Gly-Arg-, indicating that our application of the method was reliable.

Heterogeneity of Purified Phytochrome. Discontinuous NaDodSO_4 gel electrophoresis of small amounts of phytochrome revealed the existence of three very closely spaced bands (Figure 3a), two which were reported by Pratt (1979) and one faint band which was previously unreported (arrow). These three bands might have resulted from partial oxidation of cysteine during electrophoresis (Ramachandran & Colman, 1978). However, electrophoresis of carboxymethylated phytochrome prepared as described by Sela et al. (1959) gave the same pattern as unmodified phytochrome (Figure 3b), as did electrophoresis of unmodified phytochrome in a gel containing 0.1 mM 2-mercaptoethanol (Figure 3c). Therefore, the approximate 3000-dalton difference between the two most distant bands cannot be due to partial oxidation of cysteine during electrophoresis but must reflect another type of heterogeneity.

Phytochrome exhibits four bands by nondenaturing gel electrophoresis at pH 7.0 and an indication of two bands (shoulder on front side of peak) at pH 5.0 (Figure 4), further indicating heterogeneity in the highly purified preparation.

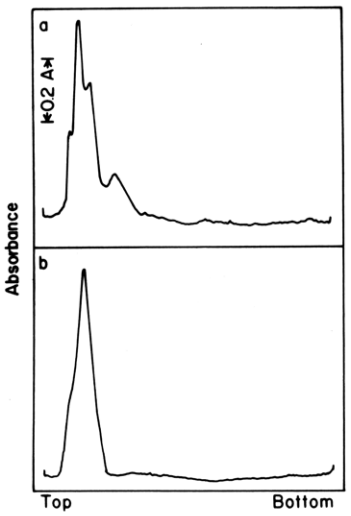

FIGURE 4: Absorbance scans of continuous nondenaturing polyacrylamide gels (5-mm diameter) after electrophoresis at (a) pH 7.0 or (b) pH 5.0 of 17 μg of immunoaffinity-purified oat phytochrome.

The reversed mobility between pH 5.0 and pH 7.0 gels confirms an earlier report (Hunt & Pratt, 1979) that the p*I* of oat phytochrome is in the range of 5.8–6.4.

Discussion

These data represent the first significant characterization of undegraded, undenatured, highly purified oat phytochrome. Immunoaffinity-purified oat phytochrome exists in solution as a 233 000-dalton dimer (Figure 2) of its 118 000-dalton monomers (Figure 1). The amino acid analysis is typical of a water-soluble protein with a hydrophobic core, indicating about 35% nonpolar residues. The monomer has a probable amino-terminal sequence of NH_3-Lys-Ala-Leu-Val-. Our observation that, on average, each monomer contains 1 mol of phosphate quantifies an earlier report by Quail et al. (1978) which indicated that phytochrome is a phosphoprotein.

Although our phytochrome preparations are very pure [Figures 1 and 2a; see Hunt & Pratt (1979) for a discussion], they nevertheless exhibit heterogeneity by isoelectric focusing (Hunt & Pratt, 1979), by discontinuous NaDodSO_4 gel electrophoresis (Figure 3), and by nondenaturing gel electrophoresis (Figure 4). At least four explanations could account for this heterogeneity. First, genetic polymorphism in the approximately 25 000 oat shoots per kg extracted could, for example, account for the amino-terminal sequence data as well as the other observations. Second, differential post-translational modification is possible since phytochrome contains both phosphate residues and an unknown number of linear tetrapyrrole chromophores (Pratt, 1979). Third, differential in vitro modification during extraction and purification might occur. Fourth, the phytochrome dimer might be composed of monomers that, although similar in size, represent quite different gene products. However, we consider this last possibility unlikely because of the peptide map data obtained for undegraded oat phytochrome by Kidd et al. (1978), who used a method that selectively visualizes only Tyr-containing peptides. Since an average undegraded phytochrome monomer contains 23 Tyr residues (Table I), one would expect to identify close to 23 peptides (considering the possible presence of two Tyr in one peptide) if the two subunits were identical and close to 46 if they were different. Since 19 major peptides were identified, we conclude that the two subunits are probably identical or nearly so.

Although much of the information presented here is new, it is possible to compare some of our results with those obtained

for undegraded rye phytochrome, the only other undegraded phytochrome that has been significantly characterized. A monomer size of 120 000 daltons was reported for rye phytochrome (Rice et al., 1973), agreeing well with the 118 000 daltons reported here (Figure 1). On the basis of sedimentation velocity (Gardner et al., 1971) and gel exclusion measurements (Rice & Briggs, 1973), which indicated estimated molecular weights of 180 000 and 375 000, respectively, and upon preliminary unpublished sedimentation equilibrium data [Gardner, personal communication in Rice & Briggs (1973)], it was argued that rye phytochrome was a dimer in solution, again in good agreement with the more direct molecular weight measurement reported here. However, our amino acid analysis (Table I) is significantly different than that reported for rye phytochrome (Rice & Briggs, 1973), with 10 of 17 residues reported (Trp was not reported for rye) differing by more than 10%. The amino-terminal residues reported for rye phytochrome (Rice & Briggs, 1973), Asp and Glu with traces of Ser and Gly, are also different from those reported here (Lys and Ala) for oat phytochrome. These differences in primary structure are consistent with the observation that rye phytochrome exhibits reduced activity by microcomplement fixation assay when compared to oat phytochrome and when using anti oat phytochrome serum (Pratt, 1973).

Little information concerning undegraded oat phytochrome was available prior to this report. The 118 000 monomer weight (Figure 1) agrees well with the less carefully measured value of 120 000 reported earlier (Cundiff, 1973; Pratt, 1978; Stoker et al., 1978) by using incompletely purified samples. Cundiff (1973) had reported a preliminary sedimentation equilibrium measurement of 246 000 daltons in the presence of the protease inhibitor phenylmethanesulfonyl fluoride, again in good agreement with the value of 233 000 daltons reported here (Figure 2). Our amino acid analysis (Table I) agrees well with the only one reported previously (Stoker et al., 1978), which utilized samples removed from $NaDodSO_4$–polyacrylamide gels. Although 5 of the 17 residues reported (Trp was not reported by Stoker et al.) differed by more than 10%, 3 of these (Cys, Met, and Thr) differed by at least this much in our replicate determinations. Hence, much if not all of the disagreement can be attributed to experimental variability.

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