States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.
Strickland, E. H., & Mercola, D. A. (1976) *Biochemistry 15*, 3875–3884.
Tager, H., Given, B., Baldwin, D., Mako, M., Markese, J., Rubenstein, A., Olefsky, J., Kobayashi, M., Kolterman, O., & Poucher, R. (1979) *Nature (London) 281*, 122–125.
Tager, H., Thamoas, N., Assoian, R., Rubenstein, A., Saekow, M., Olefsky, J., & Kaiser, E. T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3181–3185.
Torda, A. E., Sheek, R. M., & van Gunsteren, W. F. (1990) *J. Mol. Biol. 14*, 223–235.
Wang, C.-C., & Tsou, C.-L. (1986) *Biochemistry 25*, 5336–5340.
Weiss, M. A., Nguyen, D. T., Khait, I., Inouye, K., Frank, B. H., Beckage, M., O'Shea, E. K., Shoelson, S. E., Karplus, M., & Neuringer, L. J. (1989) *Biochemistry 29*, 9855–9873.
Weiss, M. A., Frank, B. H., Khait, I., Pekar, A., Heiney, R., Shoelson, S. E., & Neuringer, L. J. (1990) *Biochemistry 29*, 8389–8401.
Wollmer, A., Fleischhauer, J., Strassburger, W., Thiele, H., Bradenburg, D., Dodson, G., & Mercola, D. (1979) *Biophys. J. 20*, 233–243.
Wommer, A., Strassburger, W., Glatter, U., Dodson, G. G., & McRittel, W. (1981) *Z. Physiol. Chem. 362*, 581–591.
Wood, S. P., Blundell, T. L., Wollmer, A., Lazarus, N. R., & Neville, R. W. J. (1975) *Eur. J. Biochem. 55*, 531–542.
Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, New York.
Wuthrich, K. (1989) in *Methods of Enzymology* (Oppenheimer, N. J., & James, T. L., Eds.) Academic Press, New York.
Wuthrich, K., Wider, G., Wagner, G., & Braun, W. (1983) *J. Magn. Reson. 155*, 311.

Physicochemical Properties of Amaranthin, the Lectin from *Amaranthus caudatus* Seeds†

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Received March 8, 1990; Revised Manuscript Received July 17, 1990

ABSTRACT: Amaranthin is the lectin present in the seeds of *Amaranthus caudatus*, which specifically binds the T-disaccharide (Galβ1,3GalNAcα-*O*-). The lectin is composed of a single type of subunit with M_r = 33 000–36 000 (Rinderle et al., 1989). Equilibrium sedimentation (M_r = 62 900) and low-angle laser light scattering (M_r = 61 400) methods have been used to unambiguously establish the native multimeric structure of amaranthin as a homodimer. These absolute molecular weight methods and the calculated Stokes radius (27.2 Å) indicate that the amaranthin dimer is highly compact relative to typical globular proteins, and thus, anomalous molecular weight values are obtained when simple size exclusion chromatography is used to determine the molecular weight of amaranthin. Studies with a homobifunctional cross-linking reagent and amaranthin further support the existence of a lectin homodimer. The stoichiometry of carbohydrate binding was determined to be one T-disaccharide-binding site per amaranthin subunit ($K_a = 3.6 \times 10^5$ M^{-1}). Amaranthin exhibits hydrophobic-binding properties as indicated by binding of 8-anilino-1-naphthalenesulfonate ($K_a = 3.6 \times 10^3$ M^{-1}) and 6-toluidinyl-2-naphthalenesulfonate ($K_a = 2 \times 10^4$ M^{-1}). Serological studies suggest that amaranthin does not appear to be present in the stems or leaves of the *A. caudatus* plant, nor were there any indications for the presence of cross-reactive material.

The lectin present in the seeds of *Amaranthus caudatus*, amaranthin, is very specific for the Thomsen–Friedenreich antigen (T-antigen; Galβ1,3GalNAcα-*O*-)[1] and selected derivatives of this disaccharide (Rinderle et al., 1989). The carbohydrate-binding specificity of amaranthin was found to be significantly different from that of peanut (*Arachis hypogaea*) agglutinin, explaining differences in reactivity with glycoproteins and red blood cells for these two T-antigen-specific lectins. Suggestions that the T-antigen may be a carcinoma-associated antigen (Springer et al., 1975; Anglin et al., 1977; Springer, 1984; Yuan et al., 1986) indicate the importance of developing well-defined T-antigen probes.

A recent report concerning the isolation and characterization of amaranthin (Rinderle et al., 1989) indicated that the lectin is composed of a single type of subunit with M_r = 33 000–36 000. The native multimeric structure of amaranthin, however, could not be accurately defined since the lectin migrated with a molecular weight (M_r = 54 000) between that expected for a homodimer and that expected for a monomer

† This work was supported by Grant GM 29470 (to I.J.G.) from the National Institutes of Health.
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[1] Abbreviations: T-antigen, Galβ1,3GalNAcα-*O*-; GalNAc, 2-acetamido-2-deoxy-D-galactose; ANS, 8-anilino-1-naphthalenesulfonate; TNS, 6-toluidinyl-2-naphthalenesulfonate; DSS, disuccinimidyl suberate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 7.2; LALLS, low-angle laser light scattering.

0006-2960/90/0429-10555$02.50/0

by standard gel filtration methods. This report clearly defines the native multimeric structure, examines the hydrophobic-binding properties of amaranthin, and presents serological studies conducted to determine the distribution of amaranthin within the *A. caudatus* plant.

Materials and Methods

Materials. [6-^{3}H]-D-Galactose (25 Ci/mmol) and [^{3}H]-GalNAc (0.8 Ci/mmol) were purchased from ICN Biomedicals, Inc. GalNAcα-*O*-CH_3 and [^{14}C]GalNAcα-*O*-CH_3 were available in our laboratory. ANS was obtained from Eastman Kodak (Rochester, NY) as the magnesium salt and TNS from Sigma Chemical Co. [^{14}C]Adenine (60 mCi/mmol) was from NEN Research Products (Boston, MA). Disuccinimidyl suberate (DSS) was from Pierce (Rockford, IL). Bovine testis β-galactosidase (Distler & Jourdian, 1973) was donated by Dr. G. W. Jourdian, University of Michigan. Sephacryl S-200 and Galβ1,3GalNAcα-*O*-benzyl were purchased from Sigma Chemical Co. New Zealand white rabbits (3–4 kg) were obtained from Langshaw (Coldwater, MI). Freund's complete and incomplete adjuvants were purchased from Difco Laboratories (Detroit, MI).

Amaranthin. Amaranthin was isolated as previously described (Rinderle et al., 1989) from *A. caudatus* seeds with Galβ1,3GalNAcα-*O*-$(CH_2)_8$CONH–Synsorb (Synsorb-T, Chembiomed Ltd., Alberta, Canada). The lectin appeared homogeneous when analyzed by polyacrylamide gel electrophoresis (PAGE) at pH 4.3 and gave a single protein band by SDS–PAGE (with or without β-mercaptoethanol) with subunit molecular weight of 33 000–36 000 (Rinderle et al., 1989).

Sedimentation Equilibrium. Amaranthin was concentrated in an ultrafiltration cell and filtered with an Acrodisc 0.2-μm filter (Gelman Sciences, Ann Arbor, MI). Protein concentration was determined to be 5.93 mg/mL of PBS according to the total tryptophan concentration as indicated for equilibrium dialysis. A weight-average molecular weight for amaranthin in PBS was determined by sedimentation equilibrium following the method of Pollet (1985). Sedimentation equilibria were generated with an air-turbine ultracentrifuge (Airfuge, Beckman Instruments, Palo Alto, CA) operated in a refrigerated cabinet thermostated at 7 ± 1 °C. A Model A100/18 rotor (Beckman Instruments) loaded with two 100-μL protein solution (5.93 mg of amaranthin/mL of PBS) columns was used. Protein concentrations of 5 mg/mL or greater provide sufficient density to prevent convective mixing during rotor deceleration (Pollet, 1985), and no additional solute was needed for the amaranthin solution. The solution columns were centrifuged at 30 000 ± 50 rpm for 24 h, after which time one of the solution columns was sacrificed for a temperature measurement and the remaining solution was fractionated into 10-μL aliquots by use of a syringe pump operated with a 100-μL gas-tight syringe. The centrifugation temperature was 13.1 and 13.5 °C for duplicate sedimentations. The aliquots were assayed for total protein content with the bicinchoninic acid protein assay (Pierce Chemical Co.) as previously described (Redinbaugh & Turley, 1986). The weight-average molecular weight for amaranthin was obtained from the measured dependence of $c(r)$ on r:

$$\frac{\mathrm{d} \ln c(r)}{\mathrm{d}r^2} = \frac{M(1 - v\rho)\omega^2}{2RT}$$

where $c(r)$ is the protein concentration at radial distance r, v is the partial specific volume, ρ is the solution density, T is the temperature, R is the molar gas constant, ω is the angular velocity, and M is the weight-average molecular weight of the macromolecule. M was calculated from the slope of the plot of ln A_{562} versus r^2. Radial positions for the 10-μL aliquots were calculated by numerical integration of the expression relating volume from the meniscus in the centrifuge tube to radial position (Pollet, 1985). The amino acid composition of amaranthin was used to calculate a v value of 0.727 mL/g at 20 °C (Cohen & Edsall, 1943), and a temperature-corrected value (Pollet et al.; 1979) of 0.720 mL/g for 13 °C was used for all molecular weight calculations. The solution density, ρ, was considered to be 1.0086 g/mL (13.5 °C) and 1.0087 g/mL (13.1 °C) as measured for bovine serum albumin (5.93 mg/mL of PBS) on a digital density meter (Mettler/Parr).

Size Exclusion Chromatography/Low-Angle Laser Light Scattering. Molecular weight distribution averages for amaranthin were determined by size exclusion chromatography/low-angle laser light scattering (SEC/LALLS) similarly to the method described by Maezawa and Takagi (1983). The SEC/LALLS system consisted of a Model 590 pump, a Model 712B WISP autosampler (Waters Associates), a Superose 6 size exclusion chromatography column (Pharmacia), a KMX-6 low-angle laser light scattering (LALLS) detector (LDC/Milton Roy), and a Model 410 differential refractive index (DRI) detector (Waters Associates). Elution of the protein from the size exclusion column was sequentially monitored by the LALLS detector and the DRI detector. Light scattering intensities for 100 μL of injected protein solution (5.93 mg/mL PBS) were converted to Rayleigh ratios, and a weight-average molecular weight was calculated from each chromatographic data point. The concentration of protein for each data point (c_i) was determined from the normalized peak heights of the DRI detector, the flow rate, and the injected mass:

$$c_i = (h_i m_t)/(\mathrm{fr} T_i)$$

where h_i is the normalized DRI response for point i, m_t is total mass injected, fr is the flow rate, and T_i is the time interval between data points. A dn/dc value for amaranthin was determined to be 0.226 mL/g by a method previously described for the SEC/LALLS analysis of polysaccharide molecular weights (Yu & Rollings, 1987). Data acquisition and reduction were performed on a Micro pdp 11/23+ computer (Digital Equipment Corp.) with the program MOLWT3 (LDC/Milton Roy).

Dynamic Light Scattering. A Stokes radius (R_s) for amaranthin was determined by dynamic light scattering (Bloomfield, 1985). The dynamic light scattering (DLS) instrument consisted of the KMX-6 LALLS photometer operated with a 128-channel BI2030AT digital correlator (Brookhaven Instruments Co.). DLS measurements for the protein solution (5.93 mg/mL of PBS) were made at 22 ± 1 °C with a scattering angle of 4.5°, a correlator sampling time of 200 μs, and a duration of 10 min. The protein solution was filtered into the light scattering cell through a 0.22-μm poly(vinylidene fluoride) membrane filter. Measured and computed base lines of the autocorrelation function agreed to within 0.1%. Autocorrelation functions were analyzed with standard DLS software (Brookhaven Instruments Co.) which employed single-exponential fitting and cumulants analysis routines.

DLS measurements of R_s were also obtained for BSA (6 mg/mL of PBS) to verify instrumental accuracy. Since approximately 10 wt % of BSA contained dimer and higher aggregates, the SEC/LALLS system was used to isolate unimeric BSA in the light scattering cell prior to DLS measurements. Digital correlator and LALLS photometer instrument settings used for BSA were identical with those for

amaranthin with the exception of sampling time and duration, which were 250 μs and 5 min, respectively.

Synthesis of Radiolabeled T-Disaccharide. [6-^{3}H]-Galβ1,3GalNAcα-*O*-CH_3 was prepared by the method of Flowers and Shapiro (1965) with [6-^{3}H]Gal and GalNAcα-*O*-CH_3 as starting reagents. The tritium-labeled T-disaccharide was cleaved completely to [6-^{3}H]Gal and GalNAcα-*O*-CH_3 by bovine testis β-galactosidase (Distler & Jourdian, 1973) but remained unaffected by coffee bean α-galactosidase. Neutral and amino sugar analysis (Perini & Peters, 1982) revealed a ratio of Gal:GalNAc equal to 0.92:1 and a specific activity of 2.3×10^9 dpm/μmol of disaccharide.

Equilibrium Dialysis. Equilibrium dialysis was performed to multichamber dialysis cells (Technilab Instruments, Inc., Pequannock, NJ). Chambers on one side of the dialysis membrane contained 20 μM amaranthin (assuming subunit molecular weight of 33 000) and chambers on the opposite side contained [6-^{3}H]Galβ1,3GalNAcα-*O*-CH_3 (10–200 μM) in 150 μL of PBS. Dialysis cells were rotated at 4 °C for 3 days. Aliquots (100 μL) from each side were removed for liquid scintillation counting. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard and converted to amaranthin concentration according to the spectrophotometric tryptophan determination of Edelhoch (1967). By use of the tyrosine content determined from the amino acid composition of amaranthin (Rinderle et al., 1989), a ratio of $[\text{amaranthin}]^{\text{Lowry}}/[\text{amaranthin}]^{\text{Trp}} = 1.64$ was calculated. The results were analyzed according to the method of Scatchard (1949).

Hydrophobic Ligand Binding. Fluorescence titrations were done as previously described by Roberts and Goldstein (1982) on a SLM spectrofluorometer equipped with SPC-822/823 data acquisition electronics, a SMC-220 monochromator controller, and a LH-450 xenon arc lamp (SLM Instruments, Inc., Urbana, IL).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) in the presence of 0.1% SDS on an 11% slab gel. Protein samples were prepared with buffer containing 2% SDS and 5% β-mercaptoethanol and heated to 100 °C for 5 min. Protein bands were visualized by silver staining (Morrissey, 1981).

Cross-Linking of Amaranthin with DSS. Concentrated stock solutions of disuccinimidyl suberate (DSS) were prepared in dimethyl sulfoxide. Small aliquots of the stock solution were added to indicated amounts of amaranthin in PBS to a final concentration of 1 mM. At indicated times, aliquots (90 μL) of the reaction mixture were removed and added to 10 μL of 1 M ethanolamine to quench the cross-linking reaction. Equivalent amounts of total protein for each time point were then analyzed by SDS–PAGE on an 11% polyacrylamide slab gel.

Polyclonal Antibodies against Amaranthin. Preimmune serum was drawn from each of three New Zealand white rabbits. The rabbits were each injected intradermally, at multiple sites in their backs, with 70 μg of amaranthin emulsified in Freund's complete adjuvant (1:1). Two weeks later each rabbit was injected with 70 μg of amaranthin in Freund's incomplete adjuvant (1:1). Seven days after the second immunization, 10 mL of blood was drawn from each rabbit, and the blood was allowed to clot. Serum was collected by centrifugation and stored at 4 °C in the presence of 0.05% sodium azide.

Agar Gel Diffusion. Agarose double diffusion was conducted according to Ouchterlony (Ouchterlony, 1958, 1962; Clausen, 1971) with 0.85% Ionagar (Oxo Limited, Chicago Heights, IL) in phosphate-buffered saline, pH 7.2. After 72 h at 4 °C, plates were washed in 0.9% NaCl by repeated flooding over a period of 4 days and stained for 2 h in 0.5% amido black, 5% $HgCl_2$, and 5% acetic acid. Excess stain was removed from the gel by immersion in 2% acetic acid for 24 h, with stirring.

Growth Conditions and Care of A. caudatus Plants. A. caudatus plants were grown at the University of Michigan Matthaei Botanical Gardens under the care of Jim Dickinson. All stages of plant growth occurred in the research greenhouse with no particular attention given to lighting conditions. Seeds were germinated in clay pots in soil consisting of one part each of peat moss, processed pine bark, Perlite, and sand. Plants were transferred to larger pots as needed in the same soil. Watering schedule was once daily throughout all stages of plant life. The soil was supplemented with Uni-mix II (Peters) micronutrients containing sequestered iron. Peters 20.20.20 (N.P.K) fertilizer was applied once each week after the plants developed leaves.

PBS Extracts of A. caudatus Stems and Leaves. Thirty-five grams of *A. caudatus* leaves was collected from two of the plants at the age of 7 months. The green leaves were immediately placed in extraction buffer (0.1 M phosphate, pH 7.2, 0.15 M ascorbic acid, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride) at 4 °C and homogenized in a blender. The leaf homogenate was stirred at 4 °C overnight in the extraction buffer and centrifuged, and the supernatant fluid was passed through cheesecloth for further purification. The resulting solution was made 80% saturated with $(NH_4)_2SO_4$; the precipitated protein was redissolved in PBS (50 mL) and dialyzed extensively against PBS. The dialyzed sample was centrifuged to remove insoluble material and stored at 4 °C.

Thirty-five grams of stems from *A. caudatus* was obtained from regions throughout two of the 7 month old plants. The stems were placed in extraction buffer, homogenized, extracted, and concentrated as described above for the leaf extract sample.

Results

Native Molecular Weight Determinations. The results of duplicate sedimentation equilibrium analyses of amaranthin (5.93 mg/mL) are shown in Figure 1. Slopes obtained from linear regression analysis of the data yielded an average molecular weight of 62 900.

An elution profile from the SEC/LALLS system is shown in Figure 2. Separate experiments utilizing amaranthin concentrations of 5.9×10^{-4}, 0.059, and 5.9 mg/mL gave identical results. Both the DRI detector and the LALLS detector show a major peak corresponding to a molecular weight of 61 400 (average of two determinations). A weight-average molecular weight, i.e., considering all species under the area of the eluted peak (Figure 2), gave a molecular weight of 58 100. This discrepancy probably results from lower molecular weight components in the lectin solution. Evidence for at least one lower molecular weight species was found in DRI and LALLS chromatograms (Figure 2); a weak shoulder was observed at approximately 95.5 min for each of the detector chromatograms. The amount or size of this lower molecular weight component could not be accurately determined; however, it probably represents no more than 5% of the total mass. It is not known whether this minor, low molecular weight species represents amaranthin monomer or a contaminant(s) associated with the affinity-purified lectin sample.

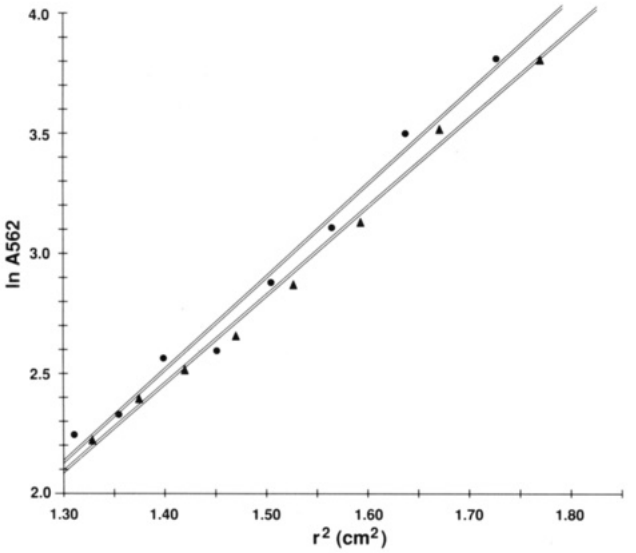

FIGURE 1: Sedimentation equilibrium data for amaranthin (5.93 mg/mL) in PBS. Two data sets, one represented by solid circles and the other represented by solid triangles, are shown. Straight lines represent the linear regression equations for the data sets. Average molecular weight is 62 900.

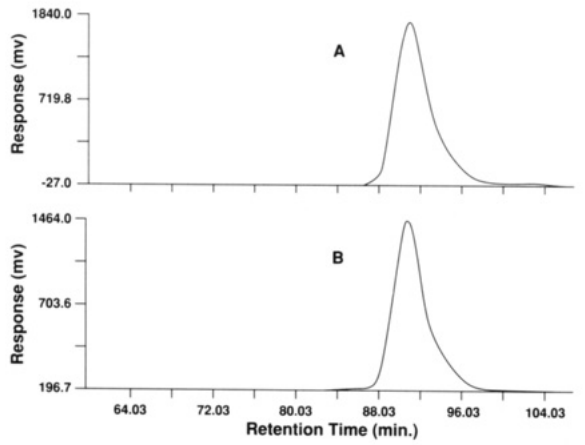

FIGURE 2: Size exclusion chromatography/low-angle laser light scattering chromatograms for amaranthin in PBS. Chromatogram A corresponds to the DRI detector. Chromatogram B corresponds to the LALLS detector. Peak at 92.4 min corresponds to molecular weight of 61 400 (average of two determinations).

On the basis of the molecular weight determinations of BSA using sedimentation equilibrium and SEC/LALLS, reported molecular weight averages for amaranthin are expected to be accurate to within 5% of the true values. Duplicate molecular weight determinations, for both techniques, for amaranthin and BSA differed by less than 1%.

Stokes Radius Determination. An R_s value of 27.2 Å was calculated for amaranthin from a single-exponential fit to the autocorrelation data (data not shown). Application of the method of cumulants (Koppel, 1972) to the same data yielded a small polydispersity (defined as the square of the relative standard deviation in the inverse hydrodynamic diameter) of 0.04, which indicated that the amaranthin solution was nearly monodisperse.

On the basis of the good agreement between the measured R_s for BSA (34.0 Å) and a literature R_s for BSA (35.2 Å) calculated from a previously reported DLS determination (Rimai et al., 1970) of the BSA diffusion consant, the R_s for amaranthin is expected to be accurate to within 5% of the true value. Duplicate R_s determinations for amaranthin and BSA differed by less than 3%.

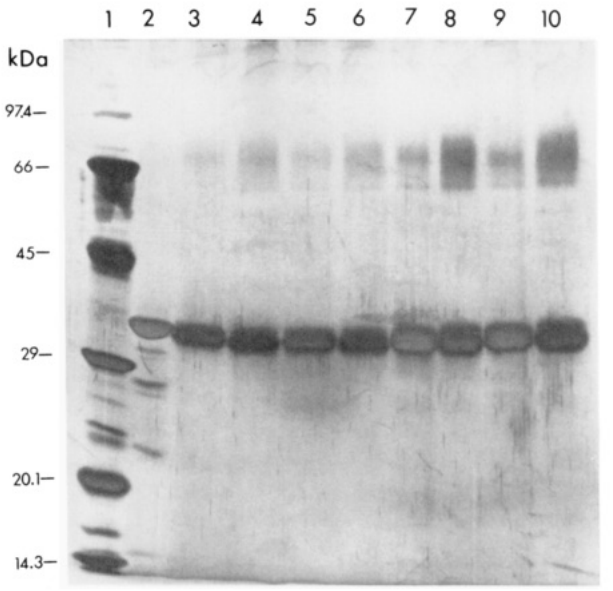

FIGURE 3: Analysis of DSS cross-linked amaranthin by SDS–PAGE. (Lane 1) Molecular weight standards: Phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. (Lane 2) Amaranthin, no treatment. (Lanes 3–6) Amaranthin, cross-linking concentration 0.082 mg/mL. (Lanes 7–10) Amaranthin, cross-linking concentration 0.245 mg/mL. (Lanes 5–6 and 9–10) Cross-linking in the presence of 1 mM Galβ1,3GalNAcα-*O*-benzyl. (Lanes 3, 5, 7, and 9) After 30 min of cross-linking time. (Lanes 4, 6, 8, and 10) After 120 min of cross-linking time. Seven micrograms of total protein was applied to each lane.

Cross-Linking of Amaranthin with DSS. Figure 3 shows the results of a cross-linking experiment for various concentrations of amaranthin in the presence or absence of 1 mM Galβ1,3GalNAcα-*O*-benzyl. DSS concentration was 1 mM for all samples. Lane 2 represents native amaranthin (i.e., not exposed to DSS), giving M_r = 34 100. Lane 2 also indicates that the native sample contained several smaller molecular weight components. These contaminating components represent only a very small percentage of the total protein (less than 5%) and are visible because of the large amount of protein applied to the gel and prolonged silver stain developing time. Lanes 3 and 4 illustrate that for 0.082 mg of amaranthin/mL there was a small amount of a species with $M_r \simeq$ 70 000 after 30 min (lane 3) and a significant amount of this species after 2 h of cross-linking time (lane 4). Similarly, lanes 7 and 8 show the appearance of a cross-linked amaranthin species with $M_r \simeq$ 70 000 for 0.245 mg of amaranthin/mL. Again, increased amounts of the cross-linked species were observed for a longer DSS reaction time. Lanes 5–6 and 9–10 are identical with lanes 3–4 and 7–8, respectively, except the DSS reaction mixtures contained 1 mM Galβ1,3GalNAcα-*O*-benzyl. It is evident that in the presence of greater than a 100-fold excess of haptenic disaccharide, relative to the amaranthin K_d for Galβ1,3GalNAcα-*O*- (see below), the amount of the cross-linked $M_r \simeq$ 70 000 species is unchanged. This would suggest that ligand binding does not stabilize the aggregated state and/or ligand does not have to be bound for aggregation of amaranthin to occur. The $M_r \simeq$ 70 000 species is most likely a cross-linked dimer of two amaranthin subunits.

For an amaranthin concentration of 0.245 mg/mL (Figure 3; lanes 7–10), formation of a species with M_r = 61 000 was also observed. The appearance of this smaller aggregate is probably a cross-linked dimer that has also undergone intrachain DSS cross-linking. Intrachain cross-links would not allow the protein to be fully extended for the SDS–PAGE analysis, and thus, these protein molecules would appear

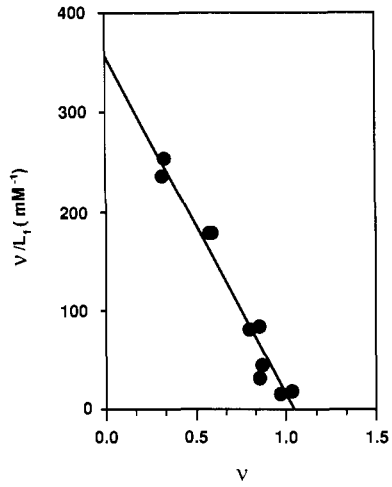

FIGURE 4: Equilibrium dialysis results for amaranthin (M_r = 33 000; subunit) and tritium-labeled Galβ1,3GalNAcα-*O*-CH_3. After 3 days at 4 °C aliquots were removed and counted to determine the amount of tritium-labeled ligand. Results are expressed according to Scatchard (1949). $K_a = 3.6 \times 10^5$ M^{-1}. 1.04 carbohydrate-binding sites per subunit.

somewhat smaller than similar molecules without intrachain linkages. It is unlikely that the $M_r \simeq 61\,000$ band is due to cross-linking of the low molecular weight contaminants (lane 2). It is apparent that for 30 min of cross-linking time (lane 7) these contaminants are no longer visible; however, for 120 min of reaction time (lane 8) there is a significant increase in the amount of $M_r \simeq 61\,000$ band, and therefore, it must be amaranthin subunit specific.

Ovalbumin, which does not aggregate to form higher multimeric species (Andrews, 1964), at total lysine concentrations equal to that of amaranthin, showed no cross-linking of monomers under identical reaction conditions (data not shown). This result serves as a control to illustrate that the observed cross-linking of amaranthin subunits was due to specific association of lectin subunits and was not due simply to random subunit–subunit contacts at the given protein concentrations.

Equilibrium Dialysis. The Scatchard plot of the equilibrium dialysis data revealed that amaranthin has one (n = 1.04) carbohydrate-binding site per subunit (M_r = 33 000) with $K_a = 3.6 \times 10^5$ M^{-1} (Figure 4). The amaranthin used for equilibrium dialysis appeared to be completely active since all protein bound to, and was eluted from, the Synsorb-T affinity column. Protein samples from the dialysis cells were analyzed at 72 h and found to contain lectin concentrations identical with starting conditions. Equilibration time for diffusion of the disaccharide was determined to be less than 48 h, indicating that equilibrium was established for the 72-h time points used.

Equilibrium dialysis was also carried out with radiolabeled GalNAcα-*O*-CH_3 and GalNAc for amaranthin concentrations as high as 4.4 mg/mL of PBS. All experiments with these ligands failed to give significant differences in the amount of ligand in the protein versus nonprotein dialysis chambers at equilibrium.

Hydrophobic Binding Properties of Amaranthin. ANS (8-anilino-1-naphthalenesulfonate) and TNS (6-toluidinyl-2-naphthalenesulfonate) showed fluorescence enhancement with amaranthin similar to that of previous reports given for other lectins that bind these hydrophobic ligands (Roberts & Goldstein, 1983a). Fluorescence enhancement titrations of amaranthin (1 mg/mL of PBS) were used to determine the affinity constants for ANS and TNS. For low lectin concentrations, Scatchard plots can be created from corrected

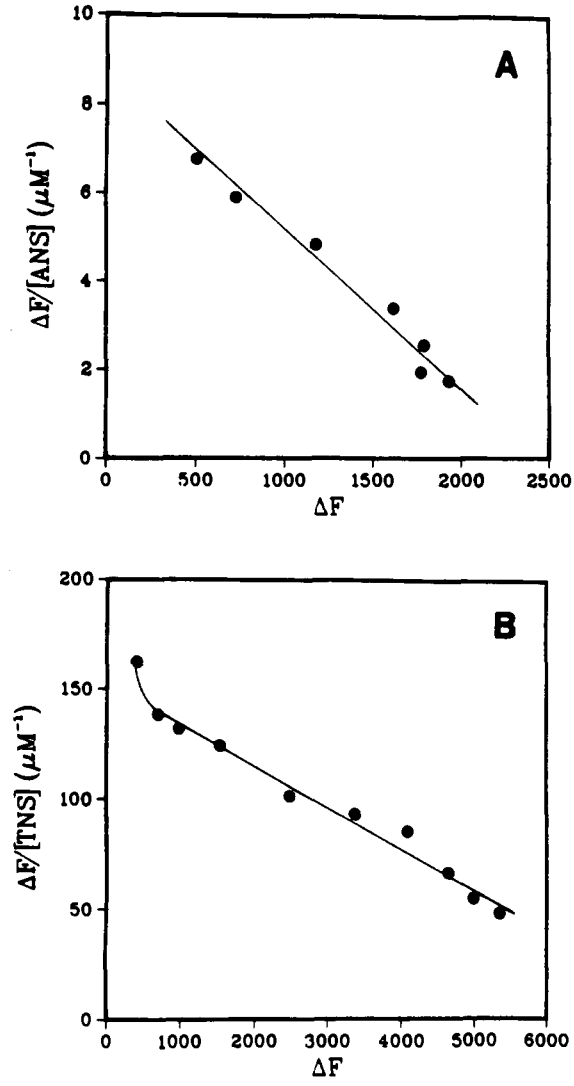

FIGURE 5: Fluorescence enhancement titrations of ANS and TNS binding to amaranthin (1 mg/mL of PBS). Small aliquots of concentrated TNS (1 mM) and ANS (10 mM) solutions were added to 2 mL of amaranthin solution. Parallel titrations were done with 2 mL of PBS to correct for the fluorescence of free ligand. Binding data are presented as Scatchard plots according to Glaudemans and Jolley (1980). (A) ANS titration, 25–1100 μM; excitation at 420 nm and emission at 480 nm. (B) TNS titration, 2.5–111 μM; excitation at 350 nm and emission at 412 nm.

fluorescence enhancement to follow bound ligand, and $[\text{ligand}]_{\text{total}}$ can be used as an approximation of $[\text{ligand}]_{\text{free}}$ (Glaudemans & Jolley, 1980; Roberts & Goldstein, 1982). Figure 5 represents the Scatchard plots obtained under these conditions for the binding of ANS and TNS to amaranthin. Binding data for ANS indicate no site heterogeneity or site–site interactions, giving $K_a = 3.6 \times 10^3$ M^{-1}. The Scatchard plot for TNS showed slight curvature at the lowest ligand concentrations. It is difficult to deduce whether the nonlinearity is due to site heterogeneity or a sensitivity of measurements, since the change in fluorescence at this concentration is very small. The linear region of the TNS data yielded $K_a = 2 \times 10^4$ M^{-1}.

Identical titrations were also conducted with ANS and TNS for amaranthin in the presence of 290 μM Galβ1,3GalNAcα-*O*-CH_3. These titration curves indicated that hydrophobic ligand binding is unaffected by carbohydrate ligand binding. The corresponding amaranthin binding constants in the presence of disaccharide were $K_a = 1.8 \times 10^4$ M^{-1} for TNS and $K_a = 3.7 \times 10^3$ M^{-1} for ANS.

Equilibrium dialysis of amaranthin (1.5 mg/mL of PBS) and [^{14}C]adenine (10–800 μM) suggested that adenine does not bind to amaranthin as has been reported for some legume lectins (Maliarik & Goldstein, 1988; Roberts & Goldstein, 1983b; Roberts et al., 1986).

A. caudatus Leaf and Stem Extracts. Concentrated *A. caudatus* stem (A_{280} = 0.97) and leaf (A_{280} = 1.5) extracts did not form a precipitate with asialoglycophorin and did not agglutinate red blood cells. These samples were also analyzed for cross-reacting proteins by agarose gel diffusion with polyclonal amaranthin antiserum. The antiserum reacted strongly with purified lectin and seed extract; however, neither the stem nor leaf extract gave a precipitation band (data not shown). These results suggest that the *A. caudatus* seed lectin does not appear to be present in the stems or leaves of the plant, nor were they any indications for the presence of cross-reactive material.

Discussion

The use of absolute molecular weight methods unambiguously established the native molecular weight and multimeric structure of amaranthin in PBS. A comparison of sedimentation equilibrium (M_r = 62 900) and SEC/LALLS (M_r = 61 400) molecular weight data with the SDS–PAGE molecular weight (33 000–36 000; Rinderle et al., 1989) suggests that amaranthin occurs as a homodimer in PBS solution. No evidence of self-association to multimers beyond a dimer was observed in LALLS and DRI chromatograms of amaranthin (Figure 2). Since the molecular weight of the lectin was unchanged from 0.59 μg/mL to 5.9 mg/mL, it is apparent that the equilibrium constant for dimer dissociation is very small. Thus, the small amount of low molecular weight material observed as a weak shoulder for SEC/LALLS is most likely due to contaminants associated with the amaranthin sample rather than monomeric lectin.

It is interesting to note that the amaranthin dimer, M_r = 62 150 (average of SEC/LALLS and sedimentation equilibrium), had a significantly smaller hydrodynamic volume than did the M_r 67 000 BSA monomer as measured by SEC retention time. This is consistent with the molecular weight determinations of amaranthin by Sephadex G-200 in PBS and conventional gel filtration methods (Rinderle et al., 1989); this method gave the native molecular weight of amaranthin as 54 000. However, the LALLS molecular weight obtained for amaranthin indicates that the molecular weight of the lectin can not be readily defined by relative elution volumes on a gel permeation column compared to standards such as BSA. These results suggest that the amaranthin dimer must be highly compact relative to typical globular proteins.

Further support for the small apparent hydrodynamic volume of the amaranthin dimer is indicated by comparing the lectin R_s value determined by dynamic light scattering against typical R_s versus molecular weight trends for standard proteins. It was observed that the R_s for the amaranthin dimer corresponded to a molecular weight 40% too small compared to the linear relationship between log R_s and log molecular weight for proteins of molecular weight of between 10 000 and 100 000. The reasons for this deviation are not apparent from the present data. One possibility to be considered is the effect of molecular shape. For example, if unimeric amaranthin assumes a highly elongated prolate ellipsoidal shape, dimerization of such molecules through alignment along major axes would produce a dimer with an effective spherical diameter greater than the diameter for the unimer but smaller than the sum of the individual unimer diameters. Other hypothetical shapes could also account for the observed molecular weight–molecular size behavior, but final resolution of this question can only be provided by a detailed three-dimensional structure of amaranthin.

The DSS cross-linking experiments offer additional evidence for the support of an amaranthin homodimer in PBS solution. The protein concentration range used for the cross-linking overlaps with the range used for the SEC/LALLS study. It is not possible from the cross-linking experiments to determine what percentage of the lectin is in dimeric form since the efficiency of cross-linking is unknown. Conditions were not found for which 100% of amaranthin could be trapped as dimeric cross-linked aggregates. Figure 3 illustrates typical cross-linking results, giving a maximum of 10–20% dimeric species as indicated by densitometry (data not shown). The efficiency of DSS cross-linking is a difficult problem to address since there are distance requirements between two intersubunit ϵ-NH_2 groups of lysine which must be satisfied because of the linker arm for DSS (11.4 Å). Furthermore, since an ϵ-NH_2-lysyl residue must react from each subunit, there will be cases where water hydrolyzes the unreacted succinimidyl before interaction with a second lysine or, alternatively, the unreacted succinimidyl may react with an intrachain ϵ-NH_2-lysyl residue. A similar low level ($\simeq$10%) of DSS cross-linking was reported for the disulfide-linked ricin A and B subunits (Montesano et al., 1982). Glutaraldehyde cross-linking of soybean agglutinin illustrated varying degrees of polymerization, including unmodified agglu ith an overall low efficiency (Lotan et al., 1973). Tl amaranthin cross-linking results appear consistent evious reports.

Equilibrium dialysis with the T-disaccharide as binding ligand gave one carbohydrate-binding site per subunit (M_r = 33 000); thus, the native homodimer is divalent for saccharide binding. Multimeric lectins with one carbohydrate-binding site per subunit are of common occurrence [cf. Goldstein and Poretz (1986)]. It is interesting to note that binding of monosaccharides to amaranthin could not be detected by equilibrium dialysis. *N*-Acetylgalactosamine and GalNAcα-*O*-CH_3 were 255- and 80-fold, respectively, less effective than Galβ1,3GalNAcα-*O*-CH_3 as inhibitors of amaranthin precipitation (Rinderle et al., 1989). The equilibrium dialysis results with these monosaccharides further illustrate the preference of amaranthin for the T-disaccharide over monosaccharides.

Amaranthin binds ANS and TNS with affinities very similar to those of other lectins (Roberts & Goldstein, 1983a). It is interesting that these putative hydrophobic-binding sites appear to be conserved in lectins even when taxonomically as distant as, for example, *A. caudatus* and the legume plants (Roberts & Goldstein, 1983a; Edelman & Wang, 1978). Amaranthin does not appear to have a metal cation requirement for carbohydrate binding (Rinderle et al., 1989) or to display adenine-binding properties as previously described for some legume lectins [cf. Roberts and Goldstein (1983b), Roberts et al. (1986), and Maliarik and Goldstein (1988)].

Despite the abundance of information concerning plant lectin carbohydrate specificity and protein structure, their biological function or functions are still unclear (Etzler, 1986). The localization of lectins in plants is fundamental information for the evaluation of possible lectin functions. Lectins have been detected in seeds, stems, leaves, and roots of plants. The serological studies regarding the distribution of amaranthin illustrate that the lectin occurs only in seed extracts and not in *A. caudatus* stems or leaves. It is important to note that our analysis was conducted with mature plant tissues since the

appearance of numerous lectins in plant tissues has been found to be dependent on the developmental stage of the plant [cf. Etzler (1986) for review]. Given our lack of functional understanding, it would appear important to analyze all possible aspects, carbohydrate- and noncarbohydrate-binding properties, as well as tissue distribution of these fascinating proteins.

ACKNOWLEDGMENTS

We thank Dr. Jules A. Shafer for helpful discussion about the multimeric structure of amaranthin and Dr. J. K. Welply for initiating our collaboration.

REFERENCES

Andrews, P. (1964) *Biochem. J. 91*, 222–233.
Anglin, J. H., Jr., Lerner, M. P., & Nordquist, R. E. (1977) *Nature (London) 269*, 254–255.
Bloomfield, V. A. (1985) in *Dynamic Light Scattering, Applications of Photon Correlation Spectroscopy* (Pecora, R., Ed.) pp 363–406, Plenum Press, New York.
Clausen, J. (1971) in *Laboratory Techniques in Biochemistry and Molecular Biology; Immunochemical Techniques for the Identification and Estimation of Macromolecules* (Work, T. S., & Work, E., Eds.) North-Holland, Amsterdam.
Cohen, E. J., & Edsall, J. T. (1943) in *Proteins, Amino Acids and Peptides*, p 370, Van Nostrand Reinhold, Princeton, NJ.
Distler, J. J., & Jourdian, G. W. (1973) *J. Biol. Chem. 248*, 6772–6780.
Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.
Edelman, G. M., & Wang, J. L. (1978) *J. Biol. Chem. 253*, 3016–3022.
Etzler, M. E. (1986) in *The Lectins: Properties, Functions, and Applications in Biology and Medicine* (Liener, I. E., Sharon, N., & Goldstein, I. J., Eds.) pp 371–435, Academic Press, Orlando, FL.
Flowers, H. M., & Shapiro, D. (1965) *J. Org. Chem. 30*, 2041–2043.
Glaudemans, C. P. J., & Jolley, M. E. (1980) *Methods Carbohydr. Chem. 8*, 145–149.
Goldstein, I. J., & Poretz, R. D. (1986) in *The Lectins: Properties, Functions, and Applications in Biology and Medicine* (Liener, I. E., Sharon, N., & Goldstein, I. J., Eds.) pp 33–247, Academic Press, Orlando, FL.
Koppel, D. E. (1972) *J. Chem. Phys. 57*, 4814–4820.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lotan, R., Lis, H., Rosenwasser, A., Novogrodsky, A., & Sharon, N. (1973) *Biochem. Biophys. Res. Commun. 55*, 1347–1355.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maezawa, S., & Takagi, T. (1983) *J. Chromatogr. 280*, 124–130.
Maliarik, M. J., & Goldstein, I. J. (1988) *J. Biol. Chem. 263*, 11274–11279.
Montesano, L., Cawley, D., & Herschman, H. R. (1982) *Biochem. Biophys. Res. Commun. 109*, 7–13.
Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.
Ouchterlony, O. (1958) *Prog. Allergy 5*, 1–78.
Ouchterlony, O. (1962) *Prog. Allergy 6*, 30–154.
Perini, F., & Peters, B. P. (1982) *Anal. Biochem. 123*, 357–363.
Pollet, R. J. (1985) *Methods Enzymol. 117*, 3–27.
Pollet, R. J., Haase, B. A., & Standaert, M. L. (1979) *J. Biol. Chem. 254*, 30–33.
Redinbaugh, M. G., & Turley, R. B. (1986) *Anal. Biochem. 153*, 267–271.
Rimai, L., Hickmott, J. T., Cole, T., & Carew, E. B. (1970) *Biophys. J. 10*, 20–37.
Rinderle, S. J., Goldstein, I. J., Ratcliffe, R. M., & Matta, K. L. (1989) *J. Biol. Chem. 264*, 16123–16131.
Roberts, D. D., & Goldstein, I. J. (1982) *J. Biol. Chem. 257*, 11274–11277.
Roberts, D. D., & Goldstein, I. J. (1983a) *Arch. Biochem. Biophys. 224*, 479–484.
Roberts, D. D., & Goldstein, I. J. (1983b) *J. Biol. Chem. 258*, 13820–13824.
Roberts, D. D., Arunjan, P., Townsend, L. B., & Goldstein, I. J. (1986) *Phytochemistry 25*, 589–593.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Springer, G. F. (1984) *Science 224*, 1198–1206.
Springer, G. F., Desai, P. R., & Banatwala, I. (1975) *J. Natl. Cancer Inst. 54*, 335–339.
Travers, R. C., & Church, F. C. (1985) *Int. J. Pept. Protein Res. 26*, 539–549.
Yu, L.-P., & Rollings, J. E. (1987) *J. Appl. Polym. Sci. 33*, 1909–1921.
Yuan, M., Itzkowitz, S. H., Boland, C. R., Kim, Y. D., Tomita, J. T., Palekar, A., Bennington, J. L., Trump, B. F., & Kim, Y. S. (1986) *Cancer Res. 46*, 4841–4847.