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COMPOSITION OF A PHOTOSYSTEM I CHLOROPHYLL PROTEIN COMPLEX FROM *ANABAENA FLOS-AQUAE**

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SUMMARY

The use of Triton X-100 to solubilize membrane fragments from *Anabaena flos-aquae* in conjunction with DEAE cellulose chromatography allows the separation of three green fractions. Fraction 1 is detergent-solubilized chlorophyll, and Fraction 2 contains one polypeptide in the 15 kdalton area. Fraction 3, which contains most of the chlorophyll and shows *P*-700 and photosystem I activity, shows by SDS gel electrophoresis varying polypeptide profiles which reflect the presence of four fundamental bands as well as varying amounts of other polypeptides which appear to be aggregates containing the 15 kdalton polypeptide. The four fundamental bands are designated Band I at 120, Band II at 52, Band III at 46, and Band IV at 15 kdaltons. Band I obtained using 0.1 % SDS contains chlorophyll and *P*-700 associated with it. When this band is cut out and rerun, the 120 kdalton band is lost, but significant increases occur in the intensities of Bands II, III, and IV as well as other polypeptides in the 20–30 kdalton range.

The use of 1 % Triton X-100 coupled with sucrose density gradient centrifugation allows the separation of three green bands at 10, 25 and 40 % sucrose. The 10 % layer contains a major polypeptide which appears to be Band IV. The 25 and 40 % layers show essentially similar polypeptide profiles, resembling Fraction 3 in this regard, except that the 40 % layer shows a marked decrease in Band III. Treatment of the material layering at the 40 % sucrose level with a higher (4 %) concentration of Triton X-100 causes a loss (disaggregation) of the polypeptides occurring in the 60–80 kdalton region and an increase in the lower molecular weight polypeptides. Thus, aggregation of the lower molecular weight polypeptides accounts for the variability seen in the electrophoresis patterns. Possible relations of the principal polypeptides to the known photochemical functions in the original membrane are discussed.

Abbreviation: SDS, sodium dodecyl sulfate.

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INTRODUCTION

The use of detergent fractionation procedures, coupled with polyacrylamide gel electrophoresis to analyse the membrane fragments so produced, has allowed considerable progress in our understanding of the composition and organization of the photosynthetic membrane in many plant and bacterial species. As indicated in the reviews by Thornber [1] and by Boardman [2], many workers have been able to isolate membrane fragments in varying degrees of purity by use of a number of detergents, including digitonin as first shown by Boardman and Anderson [3] and Triton X-100 [4]. The particle from higher plants which has been best characterized in terms of function and protein composition is the light-harvesting chlorophyll *a*/*b*-protein, originally described by Ogawa et al. [5] and Thornber et al. [6]. For a discussion of the current state of our knowledge concerning the isolation and composition of this chlorophyll-protein complex, as well as those of photosystem I and photosystem II, the reader is referred to the review by Thornber [1].

The blue green algae contain both photosystem I and photosystem II, as in higher plants, but do not contain either chlorophyll *b* or the 22 and 24 kdalton polypeptides which are characteristic of the light harvesting chlorophyll *a*/*b*-protein [7]. The blue green algae can be fragmented by detergents to produce active chlorophyll-protein complexes, as shown by Dietrich and Thornber [8] who prepared a photosystem I particle from *Phormidium luridum*, and by Ogawa et al. [9] for *Anabaena variabilis*. Shimony et al. [10] have reported a fractionation of *Anacystis nidulans* by treatment with digitonin, but Arnon et al. [11] failed to obtain a separation of the two photosystems of *Nostoc muscorum* with digitonin. Since the phycocyanin is removed early in the purification procedures, the only pigments present in the complexes obtained with detergents from the blue green algae are carotenoids and chlorophyll *a*.

Although not investigated as extensively as higher plants, the protein composition of some blue green algal membranes has been studied [7, 12]. Examination of the photosynthetic membrane of *Anabaena variabilis* showed the presence of several polypeptides, but not the 22 and 24 kdalton species found in the light harvesting chlorophyll *a*/*b* protein of higher plants [7]. Analysis of *Anabaena flos-aquae* heterocysts showed the presence of a major band at 75 kdaltons with minor ones at 57 and 48 [12]. A recent report by Hunter and Thornber [13] concerns the polypeptides contained in a Photosystem I chlorophyll-protein complex isolated from *Phormidium luridum* by means of hydroxyapatite chromatography, using either SDS or Triton X-100 as the solubilizing detergent. When SDS was used, the resulting complex showed a green band with polypeptides at 110 kdaltons, which shifted to two bands at 46 and 48 kdaltons, after reduction with mercaptoethanol in an SDS-urea buffer system; the chlorophyll was no longer associated with these polypeptides. The present investigation concerns the polypeptides of a Photosystem I chlorophyll-protein complex isolated from *Anabaena flos-aquae* using detergent extraction and chromatography on DEAE cellulose after the method employed by Bengis and Nelson [14].

MATERIALS AND METHODS

Anabaena flos-aquae cells were grown in BG-11 medium as described earlier [12] in batches of 1.5 l under fluorescent light of $5 \cdot 10^3$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}, employing a gas atmosphere of about 5 % CO_2 in air.

Photosynthetic membrane fragments (MF) were obtained as described by Ogawa et al. [9]. They were washed three times with 0.01 M Tris · HCl buffer, pH 7.6, containing 0.001 M EDTA plus 0.1 % mercaptoethanol. Further treatment with Triton X-100 in 0.01 M Tris · HCl, pH 7.6, involved the solubilization of the MF in 1 % Triton [15] or 4 % Triton [9, 14] at a chlorophyll/Triton ratio of 160 mg/g and 40 mg/g, respectively. Separation of the chlorophyll-protein complex containing *P*-700 from other chlorophyll and membrane components was achieved by passage through a sucrose density gradient [9, 15] and/or DEAE-cellulose chromatography column [14].

SDS acrylamide electrophoresis was routinely performed according to the method of Laemmli [16] and in one case, that of Weber and Osborn [17]. Densitometer tracings were obtained at 540 nm wavelength with a Schoeffel spectrodensitometer Model SD 3000.

P-700 estimations were obtained from difference spectra at 700 nm of oxidized against reduced *P*-700 using ferricyanide as the oxidant and ascorbic acid as the reductant; $E = 64 \cdot 10^3$ mol^{-1}l^{-1}. Chlorophyll *a* was estimated from its absorbancy at 672 nm using the extinction coefficient $E = 65 \cdot 10^3$ mol^{-1}l^{-1}, or after extraction with 80 % acetone according to Vernon [18]. Photoactivity was measured by the photoreaction of diphenylcarbazone [19]. Protein determinations were made according to the method of Kalb and Bernlohr [20] after extraction of the pigments with methanol and redissolving the protein residue in 0.1 % SDS/0.01 M Tris · HCL, pH 7.6.

RESULTS

The procedure described by Bengis and Nelson [14], utilizing Triton X-100 to solubilize the photosynthetic membrane followed by chromatography on DEAE cellulose, allows the separation of a Photosystem I chlorophyll-protein complex from the photosynthetic membrane fragments prepared from *Anabaena flos-aquae* by sonic oscillation as described earlier [12]. The elution pattern from the chromatographic step is shown in Fig. 1. Fraction 1 contains solubilized chlorophyll and carotenoids. The majority of the chlorophyll occurs in Fraction 3. Fraction 2 is a lighter green. The *P*-700 activity, representative of the reaction center, is found in Fraction 3, and it is this material which has been used for further experimentation.

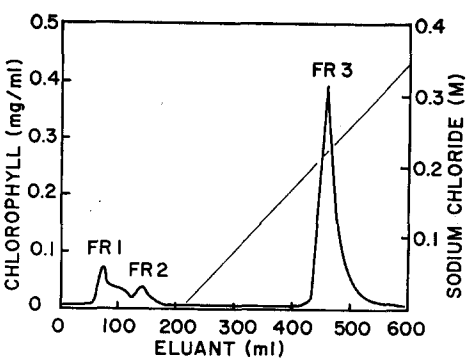

Fig. 1. Elution pattern of the three chlorophyll-containing fractions derived from *Anabaena flos-aquae* membrane by treatment with Triton X-100 followed by DEAE cellulose chromatography [14].

TABLE I

Chlorophyll/*P*-700 ratios and photochemical activities of three Photosystem I chlorophyll-protein preparations obtained by DEAE-cellulose chromatography [14]. Photosystem I activity was measured as diphenylcarbazone (DPCO) reacting, reported as μmol reacting per h per mg chlorophyll.

Fraction	mg · chlorophyll	mg · protein	chlorophyll/ protein	chlorophyll/ *P*-700	DPCO activity
Membrane fragment	5.5	122	0.045	105	710
Fraction 3 (DEAE)	1.48	24	0.062	56	347
	1.9	34	0.056	36	287
	–	–	–	31	–

The composition and photochemical activities of membrane fragments and the material from Fraction 3 are reported in Table I. Fraction 3 shows an enrichment of *P*-700 and also shows a ratio of 1 mol of chlorophyll *a* for each 16 kdaltons of protein. This is a lower ratio of chlorophyll *a* to protein than reported by Thornber for *Phormidium luridum* preparations [21]. All fractions show Photosystem I photochemical activity, as measured by the photoreaction of diphenylcarbazone, although this activity does not coincide with the enrichment in *P*-700 which is observed in the purified fractions.

The polypeptides observed in Fraction 2 and Fraction 3 from DEAE chromatography [14] are shown in Fig. 2. Fraction 2, which is eluted earlier with the equilibration buffer, is light green in color, and shows the presence of a single polypeptide of

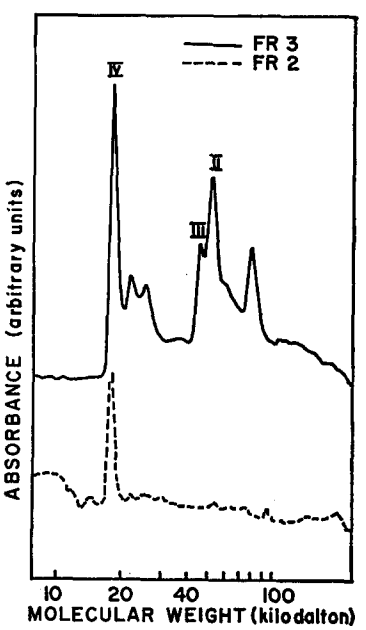

Fig. 2. SDS gel electrophoresis pattern of Fraction 2 and Fraction 3 (described in Fig. 1) after staining with Coomassie Blue.

molecular weight about 15 000. Fraction 3, which contains most of the chlorophyll *a* as well as *P*-700, shows a polypeptide pattern of seven different polypeptides. Three of these, at approx. 52, 46 and 15 kdaltons are routinely observed in preparations of this type, while the other bands observed vary in both location and intensity. The tracing shown in Fig. 2 is representative of the patterns obtained. Because their occurrence in the gel tracings is more reproducible and since they are the bands of major interest in our further considerations, we are designating the band which occurs at 120 kdaltons as band I, with band II at 52, band III at 46 and band IV at 15 kdaltons. The calculated molecular weights vary somewhat from preparation to preparation, but they generally appear at these values. There is less certainty concerning the observed molecular weight of band I, and the observed molecular weights of all bands cannot be taken as precise molecular weights, because of the nature of the SDS gel electrophoresis procedure.

Apel et al. [15] utilized a low concentration (1 %) of Triton X-100 to produce from a green alga chloroplast fragments which could be separated by sucrose density gradient centrifugation. Analysis of one of these fractions, which showed Photosystem II activity, allowed the separation of a 67 kdalton polypeptide which was an aggregated form of two smaller polypeptides of 21.5 and 23 kdaltons. We have applied this technique to the membrane fragments obtained from *Anabaena flos-aquae*. Fig. 3 shows the polypeptide patterns obtained from the fractions sedimenting at the 10, 25 and the 40 % layers. The 10 % layer shows one prominent polypeptide band at 15 kdaltons, with several minor bands at higher molecular weights. The 25 and the 40 % layers show quite similar patterns, with the exception that band III at 46 kdaltons is present only in the 25 % layer, which in the experiments of Apel et al. contained photosystem II activity [15].

The regular procedure employed in the gel electrophoresis procedure utilizes 1 % SDS with boiling to dissociate the sample. This concentration of SDS removes chlorophyll from the fragments, making it impossible to relate chlorophyll to the resolved polypeptides. Through the use of 0.1 % SDS with no boiling or incubation of the sample, some chlorophyll is solubilized but sufficient chlorophyll remains associated with the polypeptides to allow for the detection of green bands on the gel. Fig. 4 shows the tracing of such an electrophoresis pattern, and shows that chlorophyll is located in three regions of the gel. A strong, sharp band occurs at 120 kdaltons, while diffuse bands occur in the 45–60 kdalton region as well as in the 15–20 kdalton region. Because of the diffuse nature of the band in the 45–60 kdalton region, this chlorophyll does not appear to be associated with specific polypeptides and could be a detergent-chlorophyll complex of some nature. This is also true of the chlorophyll which migrates in the 15–20 kdalton range.

The 120 kdalton band shown in Fig. 4A was cut from several gels before staining (and the material from several gels was combined) and rerun in the regular Laemmli procedure (Fig. 4B). The chlorophyll was removed by this procedure, and thus could not be allocated to any of the polypeptide bands observed. The interesting point, however, is that the original 120 kdalton band yielded a polypeptide profile which was not much different from that observed with the original Fraction 3 shown in Fig. 2, or from the original membrane fragment. Bands II, III and IV were the major ones observed, along with other bands in the 20–30 kdalton range.

Cutting the material from the gels in the 10–15 kdalton region and performing

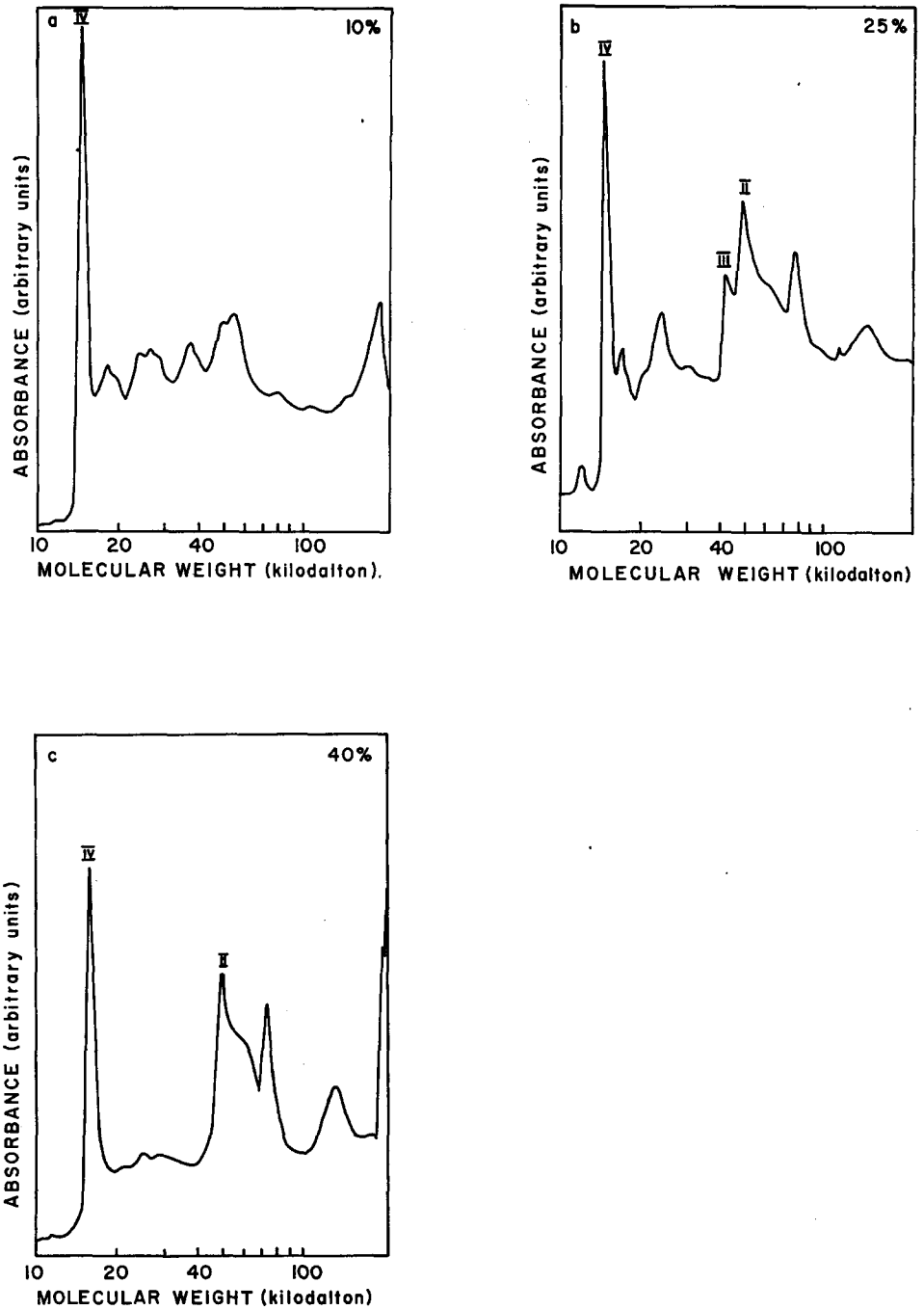

Fig. 3. SDS-gel electrophoresis patterns of the fractions obtained from membrane fragments treated with 1 % Triton X-100 according to the directions of Apel et al. [15]. The tracings are of the material sedimenting to (a) the 10 % sucrose level, (b) the 25 % sucrose level and (c) the 40 % sucrose level.

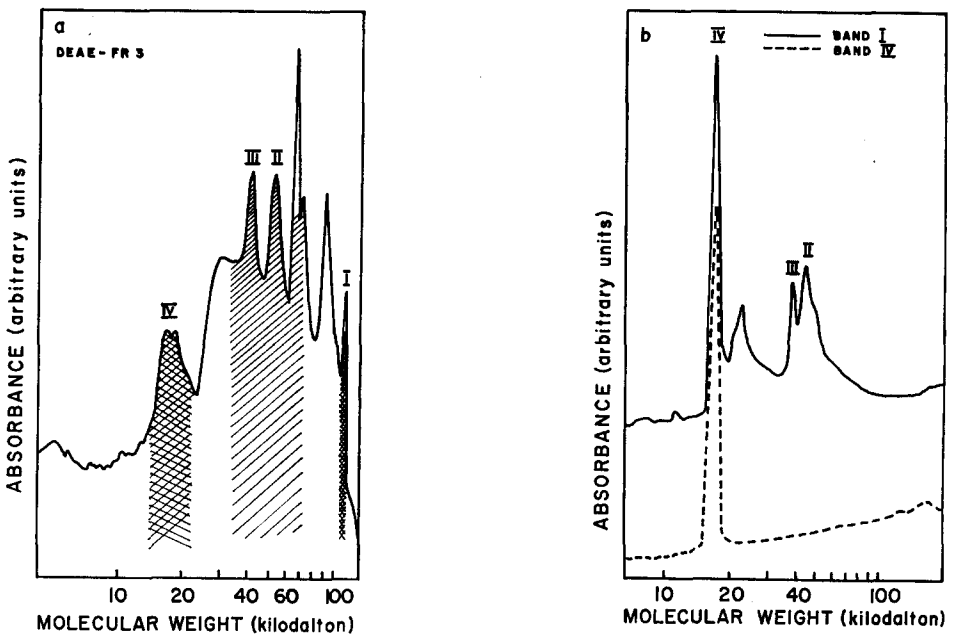

Fig. 4. Identification of chlorophyll-containing electrophoretic bands. (a) The SDS gel electrophoresis pattern obtained with Fraction 3 exposed to 0.1 % SDS with no incubation prior to running the electrophoresis pattern, and (b) the electrophoresis patterns obtained upon cutting out Band I and Band IV for a rerun in the same electrophoresis procedure. The location of the chlorophyll is indicated by the hatched areas.

an electrophoresis on this material in the usual manner produced only one sharp band corresponding to band IV.

The data presented in Fig. 4 show that the polypeptides in Fraction 3 (obtained from DEAE chromatography) exist as an aggregate of 120 kdaltons which contains chlorophyll *a* and *P*-700. Further evidence for this aggregation is shown in Fig. 5, which shows the data obtained by taking the 40 % fraction from a sucrose density gradient centrifugation according to the directions of Apel et al. [15] (using 1 % Triton X-100) and exposing this fraction to 4 % Triton. The original fraction at 40 % sucrose showed strong polypeptide bands at 64 and 70 kdaltons, while the band IV at 15 kdaltons shows a doublet at 12 and 13 kdaltons (this is occasionally observed in these preparations). The material exposed to 4 % Triton X-100, however, showed only one minor band in the 60–80 kdalton range, while showing a marked increase in the magnitude of band IV along with major bands in the 20–30 kdalton range. This represents a disaggregation of the material in the higher molecular weight bands to produce polypeptides of lower molecular weight.

For purposes of comparison, the 40 % fraction was also subjected to electrophoresis according to the Weber and Osborn method. These data (Fig. 5C) are presented to show that the Weber and Osborn method fails to show the polypeptides of lower molecular weight, and shows none of the Band IV regularly shown by the Laemmli procedure [16] reported in Fig. 5B. This is important in relating the present

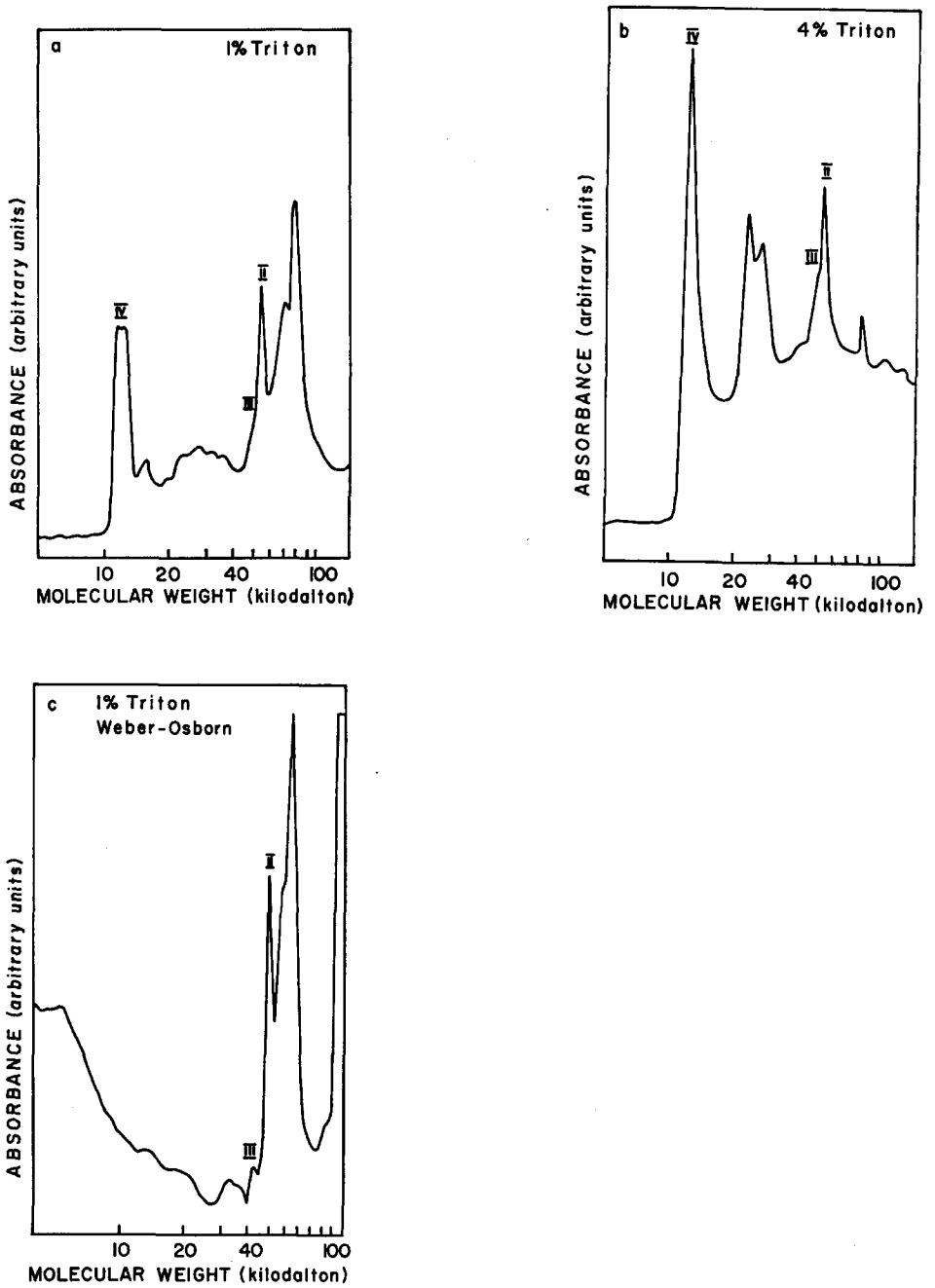

Fig. 5. Effect of detergent concentration upon the electrophoretic patterns obtained with the 40 % sucrose fraction of Fig. 3. (a) The original 40 % fraction obtained by the use of 1 % Triton X-100, (b) the same fraction exposed to 4 % Triton X-100 and chromatographed on DEAE cellulose before electrophoresis, and (c) the original 40 % fraction examined by the use of the Weber and Osborn method of SDS gel electrophoresis [17].

data to our earlier work, which utilized the Weber and Osborn method of gel electrophoresis [12].

Since the original photosynthetic membrane fragments have been extensively washed in EDTA and mercaptoethanol followed by Triton treatment and chromatography, it is not expected that there would be any algal coupling factor or carboxydismutase contaminating the preparations. This is supported by the fact that the membrane fragments are completely inactive in terms of phenazine methosulfate-mediated cyclic phosphorylation as well as carboxydismutase activity (data not shown).

DISCUSSION

The data obtained with the various factions obtained from *Anabaena flos-aquae* show a variability in the overall polypeptide pattern observed, but these become understandable when the tendency of the smaller molecular weight polypeptides toward aggregation is understood. Those bands which we have designated as bands I–IV are routinely observed, while the other bands are variable in both appearance and magnitude. The data of Fig. 4 show that band I, at 120 kdaltons, is an aggregate of a number of smaller molecular weight polypeptides. Since this band contains associated chlorophyll *a* and *P*-700, it contains the photochemically active chlorophyll-protein complex of Photosystem I. Therefore, one or more of the polypeptides which are produced when the material in band I is disaggregated must be related to the reaction center of Photosystem I.

Band IV is routinely observed in all preparations examined with the Laemmli technique, but not when the Weber and Osborn method is used. It is also the only significant polypeptide observed in Fraction 2 from DEAE chromatography [14] and in the 10 % layer obtained by the Apel procedure [15]. A 15 kdalton band, which we assume to be the same as band IV, is also produced when band I is disaggregated, or when the 40 % sucrose fraction is further dissociated with 4 % Triton X-100. The magnitude of this band in the disaggregated band I shows it is a major component. Because of the nature of the experimental procedures, in which chlorophyll is lost during the electrophoresis procedure, it is not possible to determine if the band IV polypeptide is associated with chlorophyll in the native membrane. It is interesting to note, however, that the light-harvesting polypeptide of photosynthetic bacteria is a 12 kdalton polypeptide which is readily aggregated [22]. An 11 kdalton polypeptide which readily aggregates has been isolated from higher plants, and antibody studies show that it is closely associated with Photosystem II [23].

The polypeptide bands represented by bands II and III are more stable and are not influenced greatly by the procedures which lead to disaggregation of the higher molecular weight polypeptides. It is natural to consider them as candidates for the polypeptides associated with Photosystem I reaction centers and perhaps also Photosystem II. Nothing definitive in this regard can be deduced from these experiments, however, since the chlorophyll is removed and photochemical activities destroyed by the electrophoresis procedure used. It is interesting, however, that the 25 % sucrose fraction obtained by the Apel procedure contains a prominent band at 46 kdaltons, which is missing in the 40 % sucrose layer. The experiments of Apel et al. [15] demonstrated that with *Acetabularia mediterranea*, the 25 % sucrose layer contained Photosystem II activity, but this was not present in the 40 % layer. Coupled to this is the

association of a 47 kdalton polypeptide with a functional Photosystem II in *Chlamydomonas reinhardii* [24]. Also, active Photosystem II reaction center preparations from spinach show the presence of a major band at 44 [7] and 46 kdaltons [25]. Therefore, there is evidence relating a polypeptide in the 46 kdalton range with the reaction center of Photosystem II, and our data are consistent with this. This 46 kdalton polypeptide also appears in the Fraction 3 prepared by DEAE chromatography, but we have no data concerning the purity of this fraction so far as Photosystem I chlorophyll-protein complex is concerned, since the sonication procedure used to prepare the membrane fragments destroys Photosystem II activity.

The very interesting data obtained by Hunter and Thornber [13] and Malkin et al. [26] on the polypeptides from a *P*-700-chlorophyll-protein complex from *Phormidium luridum* need to be considered and related to the data we have obtained. When this complex of 110 kdaltons is obtained through the use of SDS, it produces only two bands at 46 and 48 kdaltons after reduction with mercaptoethanol [13]. The original 110 kdalton complex shows *P*-700 activity at 300 K, but the derived polypeptides have lost their chlorophyll and an exact identity of these polypeptides with the *P*-700 reaction center cannot be made. One of them must contain the reaction center *P*-700, however, in the 110 000 dalton complex and thus represent the Photosystem I reaction center. There is some similarity to the 46 and the 52 kdalton polypeptides we have observed, since these are the more stable ones observed, and our band I appears to be an aggregate of these two polypeptides plus varying amounts of the 15 kdalton polypeptide.

When the complex from *Phormidium* is prepared with Triton X-100 [13] it contains several smaller molecular weight polypeptides which would make it somewhat similar to our preparations. Such preparations show a light-induced EPR signal relating to the reduction of an iron compound at 15 K [26] and the authors relate this to the reduction of an iron-sulfur center in one of the low molecular weight polypeptides which are present in the preparations made with Triton. The preparations made with SDS do not show this signal. Whether the 15 kdalton polypeptide observed in our experiments plays this role is not known but this must be considered.

An earlier study conducted in our laboratory [12] on heterocysts from *Anabaena flos-aquae* showed the presence of one predominant polypeptide band at 75 kdaltons, with a shoulder at 57 kdaltons. Our present understanding of the nature of the Weber and Osborn procedure used in those experiments is that under such conditions none of the 15 kdalton band (band IV) is observed, and instead aggregates of this material are observed. We conclude this is the case with the heterocysts, and the 75 kdalton band represents an aggregated form of smaller molecular weight polypeptides.

When the procedure of Apel et al. utilizing 1 % Triton X-100 to solubilize the membrane [15] is applied to *Anabaena flos-aquae*, the same three bands at 10, 25 and 40 % sucrose during density gradient centrifugation are also produced. Earlier experiments with *Anabaena variabilis* [9] utilized 5 % Triton X-100 to solubilize the membrane, and the major green band was observed in the 8 % sucrose layer. In the absence of Triton treatment the membrane fragments sediment to the bottom of the tube, and in some cases using the Apel procedure, we observe significant amounts of green material at the bottom of the centrifuge tube. Accordingly, it appears that the different densities of the membrane fragments represent varying amounts of Triton X-100

complexed to the membrane fragments and with 1 % Triton there is sufficient difference between certain areas of the membrane fragments to allow differential amounts of Triton to be absorbed, resulting in separation during a subsequent centrifugation. The polypeptide patterns of the 25 and 40 % layers are not much different, however, and if the 25 % band obtained with *Anabaena flos-aquae* does contain a polypeptide derived from Photosystem II reaction center (at 46 kdaltons) this appears to be associated with other polypeptides which would be related to Photosystem I. Because of the lack of detectable Photosystem II activity in the membrane fragments, it is not possible to draw any definite conclusions concerning Photosystem II polypeptides in our preparations. This emphasizes the need to obtain separate chlorophyll-protein complexes of the two photosystems in active form before definite information concerning the specific polypeptides related to both photosystems can be obtained.

The reaction center obtained from *Rhodopseudomonas spheroides* R-26 [27] shows the presence of three polypeptides in equimolar amounts, at 21, 24 and 28 kdaltons. The two lower molecular weight species are aggregated under some conditions to produce a larger active complex. This indicates that the 28 kdalton polypeptide is not essential for activity, and also would place the aggregated form of the two lighter species in the 45 kdalton range, which would be close to the polypeptide species observed by Hunter and Thornber [13], as well as those of our studies. The Photosystem I complex obtained by Bengis and Nelson from Swiss chard shows one band at 70 kdaltons. Considering the data of Apel et al. [15] and the known tendency of the individual polypeptides of photosynthetic membranes to aggregate, it may be that the 70 kdalton polypeptide described by Bengis and Nelson is an aggregated form, and fundamental units of all photosynthetic membranes will be found in the 20–50 kdalton range.

The Photosystem I chlorophyll-protein complex obtained from *Phormidium luridum* is less complex than our Fraction 3 complex in terms of polypeptide composition [13]. Malkin et al. [26] report a more complex polypeptide pattern if Triton X-100 is used to fragment the membrane. Since SDS inactivates the photochemical activity of chlorophyll-protein complexes other than that associated with Photosystem I [1], it is necessary to use more gentle detergents to obtain information about Photosystem II complexes. The experimental approach of Apel et al. [15], using Triton for the isolation of Photosystem II complex from a green alga was not too conclusive in our experiments, primarily because we have no way of determining if the 25 % sucrose fraction is actually a Photosystem II complex. This further emphasizes the need to obtain active Photosystem II complexes before the polypeptide composition is studied.

A recent report by Acker et al. [28] is of interest, since it indicates that polypeptides in the 20–30 kdalton range should be considered as the basic polypeptide units in the photosynthetic complexes of higher plants. Using flashing light to develop the photosynthetic capability of zea mays leaves, coupled with the incorporation of δ[^{3}H]aminolevulinic acid into chlorophyll, it was shown that the radioactivity was located in two bands at 21 and 29 kdaltons. If these do represent the basic polypeptide units of the chlorophyll-protein complexes of plant photosynthetic membranes (and this would closely resemble the situation in the photosynthetic bacteria), the higher molecular weight polypeptides that are obtained by fractionation of fully developed leaves and algae would be aggregates of these basic units. Our data would be consistent with this concept, since we generally observe polypeptides in the 20–30 kdalton region,

and the intensities of the bands are variable. This would not negate our suggestion that bands II and III observed in our studies are related to Photosystem I and Photosystem II, since these bands could be stable aggregates of smaller molecular weight polypeptides.

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