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EFFECT OF DETERGENTS ON THE RELIABILITY OF A CHEMICAL ASSAY FOR *P*-700

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Summary

A chemical assay for *P*-700 was developed using 0.36 mM potassium ferricyanide as oxidant and 1.6 mM sodium ascorbate as reductant. The major difference from other chemical assays for *P*-700 is procedural. The method is designed to take advantage of the availability of microprocessor-linked spectrophotometers to obtain greater accuracy by minimizing the spectral changes due to irreversibly oxidized antenna chlorophyll molecules. The value measured for the *P*-700 concentration in a sample of chloroplasts was not changed by the presence of EDTA, Mg^{2+} or sucrose in the assayed solution. Similarly, half of the detergents tested (Triton X-100, Nonidet P-40, digitonin, Deriphat 160, Miranol S2M-SF and Miranol M2M) did not alter the value when added to the chloroplasts. The remainder of the detergents examined caused a significant decrease or increase in the value for *P*-700 content. Sodium dodecyl sulfate, of particular interest due to its widespread use, caused a doubling in the amount of apparent *P*-700. This effect may be due to this detergent and some others enabling an additional long wavelength form of chlorophyll, possibly an intermediary electron acceptor in Photosystem I, to be chemically oxidized and reduced under the assay conditions.

Introduction

An accurate determination of *P*-700 content in biological samples is essential for plant scientists of diverse interests, from those interested in learning more about the biochemistry and biophysics of Photosystem I's primary photochemical act to those interested in studying the effects of environmental conditions on the composition of the photosynthetic apparatus [1,2]. The methods available for quantitating this dimeric chlorophyll *a* species use a measure-

ment of the reversible absorbance decrease having a minimum around 700 nm when *P*-700 is oxidized by light (photochemical assay) or by redox chemicals (chemical assay) [3]. Attempts to isolate *P*-700 in a highly enriched form have been hindered in recent years because the usual photochemical assay for *P*-700, using conventional spectrophotometers, has not detected the *P*-700 present in material treated with some detergents (e.g. sodium dodecyl sulfate) [4,5]. That *P*-700 was still functionally active in such material was recently observed by Mathis et al. [6] using single short flashes of light for activation and a more rapidly responding absorbance detecting system. Apparently recombination of the separated changes between P^+-700 and the reduced primary electron acceptor occurs so rapidly in material from which secondary electron acceptors have been removed (e.g. by treatment with sodium dodecyl sulfate) that conventional spectrophotometer recorders cannot respond fast enough to detect the absorbance changes due to photooxidation and re-reduction of *P*-700. Most research groups do not have access to apparatus capable of detecting these rapid absorbance changes and therefore they must resort to a chemical assay for *P*-700 content under these circumstances.

A major problem with the chemical assay is that the oxidants most commonly used (e.g. potassium ferricyanide) cause irreversible oxidation of antenna chlorophyll molecules with absorbance maxima close to that of *P*-700. Such absorbance changes are often large enough to contribute to those at 700 nm, making accurate quantitation of *P*-700 virtually impossible. These factors are less of a problem in material from which much of the antenna chlorophyll has been removed but they still remain a major obstacle in making determinations on material from leaf extracts in which antenna chlorophyll/*P*-700 ratios may be 400 to 1000/1.

With the availability of moderately priced microprocessor-interfaced spectrophotometers, we decided to reexamine the chemical assay, taking advantage of the computerized instrumentation to minimize the contribution from oxidation of non-*P*-700 species to the absorbance change at 700 nm. This paper describes the assay. While developing this assay it was discovered that some commonly used detergents markedly affect the values obtained.

Materials and methods

All of the material used for the reported experiments was prepared from young leaves of tobacco (*Nicotiana tabacum*) although similar results were observed with barley (*Hordeum vulgare*) or maize (*Zea mays*). All plants were grown in a greenhouse in a soil-vermiculite mixture. Leaves were ground with a mortar and pestle in 50 mM *N*-tris-(hydroxymethyl)-methylglycine (pH 8.0) (Calbiochem, La Jolla, CA) and filtered through Miracloth (Chicopee Mills, Inc., Milltown, NJ). The filtrate was centrifuged at $250 \times g$ for 10 min to remove large debris. The supernatant was then centrifuged at $18\,000 \times g$ for 10 min to pellet most of the chlorophyll-containing membranes. These were resuspended in 50 mM *N*-tris-(hydroxymethyl)methylglycine (pH 8.0) with the aid of a glass homogenizer. Values for chlorophyll/*P*-700 ratios obtained with these samples were similar to the values obtained using washed thylakoid membranes [8] made from the same plants. The purification of a *P*-700-enriched particle

from a Triton X-100 extract has been described previously [7]. The chlorophyll concentrations of the various samples were determined spectrophotometrically [9] following extraction into 80% (v/v) acetone.

The spectrophotometer used for these data was an Aminco DW-2 with a MIDAN microprocessor data analyzer (American Instrument Co., Silver Spring, MD); a Perkin-Elmer Model 557 microprocessor-linked spectrophotometer has also been used successfully. The instrument was set in the split beam mode with a bandpass of 3.0 nm. The standard assay solution contained the following: 50 mM *N*-tris-(hydroxymethyl)methylglycine (pH 8.0), 0.1% Nonidet P-40 (Particle Data Laboratories Ltd., Elmhurst, IL), 1 μM 5-methylphenazonium methosulfate and the chlorophyll-containing sample under investigation. Identical solutions were placed in the sample and reference cuvettes and allowed to equilibrate to the temperature of the cuvette chamber. A freshly prepared solution (0.18 M) of $K_3Fe(CN)_6$ (Baker Chemical Division, Allied Chemical and Dye Corp., New York, NY) was added to the sample cuvette such that the final concentration was 0.36 mM. After 90 s of oxidation a freshly prepared solution (0.8 M) of sodium ascorbate was added to the sample cuvette to give a final concentration of 1.6 mM. Sixty seconds later the reference cuvette was made 0.36 mM in $K_3Fe(CN)_6$. After 70 s of oxidation, the 650 to 750 nm difference spectrum between the solutions in the two cuvettes was placed into the memory of the microprocessor at a speed of 5 nm/s. When the reference cuvette had been allowed to incubate with the oxidant for 90 s, it was made 1.6 mM in sodium ascorbate and an equivalent volume of buffer was added to the sample cuvette. Two minutes after reduction of the reference cuvette the difference spectrum of the two cuvettes was again recorded. The previously stored spectrum was subtracted from the latter spectrum, and the resulting spectrum plotted. The difference between the absorbance minimum around 700 nm and the isosbestic point (725 nm) [3] was determined, and an extinction coefficient of 64 000 $M^{-1} \cdot cm^{-1}$ [10] was used to calculate the *P*-700 concentration of the assayed solution.

Sodium dodecyl sulfate was purchased from Pierce, Rockford, IL. The Tris salt of dodecyl sulfate was prepared by passing sodium dodecyl sulfate through Amberlite IR-120 (H^+ form) and neutralizing with solid Tris base. Deriphat 160 (a generous gift of the Henkel Corporation, Minneapolis, MN) was neutralized to pH 8 with added Amberlite IR-120 (H^+ form). Digitonin was purchased from Matheson, Coleman and Bell, Norwood, OH. *N*-Lauroylsarcosine, sodium ascorbate and Tris were purchased from Sigma Chemical Co., St. Louis, MO. Miranol detergents were the generous gift of the Miranol Chemical Company, Irvington, NJ. Sodium decyl sulfate was purchased from Eastman Kodak Co., Rochester, NY. Lauryldimethylamine oxide was the generous gift of the Onyx Chemical Co., Jersey City, NJ. All detergent solutions were prepared on a percent (w/v) basis. Sucrose, $MgSO_4$, acetone and Amberlite IR-120 were purchased from Mallinckrodt Chemical Works, St. Louis, MO. EDTA and 5-methylphenazonium methosulfate were purchased from J.T. Baker Chemical Co., Phillipsburg, NJ.

Results

An example of the *P*-700 difference spectrum obtained with this assay is presented in Fig. 1. This procedure [(reduced minus reduced) minus (reduced minus oxidized)] is equivalent to the conventional chemical (oxidized minus reduced) assay method except that absorbance changes due to the oxidation of chlorophyll species other than *P*-700 are minimized (see Discussion). The measured ratio of *P*-700 to total chlorophyll was linear over a wide range of total chlorophyll concentrations. Chlorophyll-containing membranes, exhibiting a chlorophyll/*P*-700 ratio of 460 (within the generally accepted ratio for green plants), gave linear results at total chlorophyll concentrations up to 50 μM (Fig. 2). Increasing the concentrations of $K_3Fe(CN)_6$ or sodium ascorbate, or doubling the times of oxidation or reduction, did not increase the value obtained in this concentration range. The concentration of 5-methylphenazonium methosulfate employed (1 μM) ensured complete reduction within the 2-min time period.

Temperature equilibration of the sample was also found to be important. Changes in temperature between the first and second spectra resulted in absorbance peak shifts centered near the red wavelength absorbance maximum (about 678 nm) which were sometimes large enough to interfere with the *P*-700 determination. Allowing the test solutions to reach the temperature of the cuvette chamber alleviated this problem.

In some of the diverse studies requiring measurements of *P*-700 concentration it would be desirable for the assay to give data on the chlorophyll/*P*-700 ratio of plant material without extensive preparation. However, extracts of whole plants not fractionated by centrifugation gave poor results, probably due to the redox-buffering capacity of the extract. Therefore the simple centrifugation regime described in the Methods section was developed.

It has been reported that incubation of chlorophyll-containing membranes at 55°C for 5 min had no effect on the *P*-700 activity [3]. We confirmed this ob-

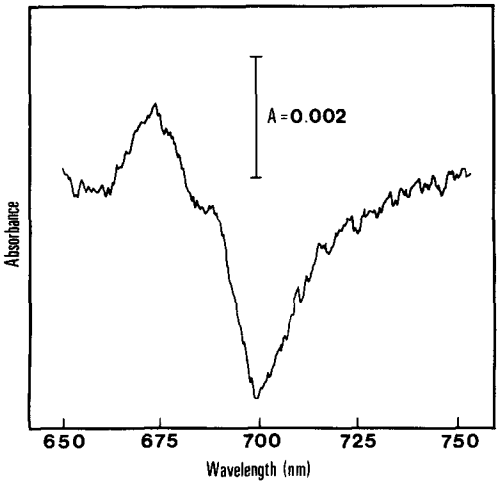

Fig. 1. Representative spectrum obtained from the chemical assay for *P*-700. The standard assay components were used. The chlorophyll concentration of the tobacco-derived sample was 23 μM.

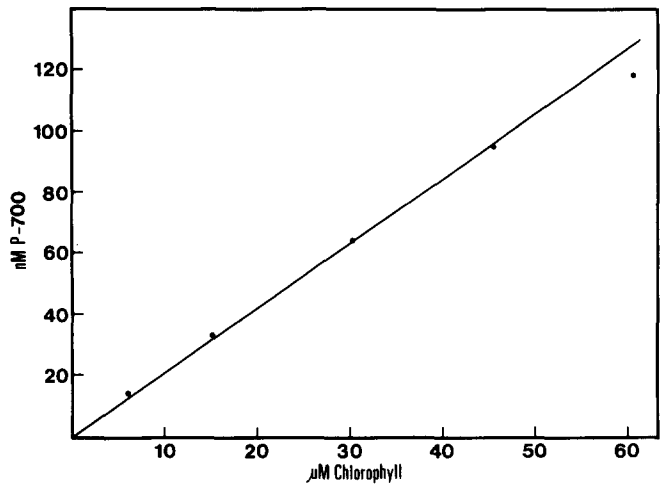

Fig. 2. Linearity of the amount of *P*-700 detected vs. concentration of chlorophyll in sample assayed. The standard assay components were used.

servation; incubation in the absence of detergents at 58°C for 12 min resulted in no loss of activity. However, incubation at 60°C in the presence of 0.5% Nonidet P-40 or sodium dodecyl sulfate resulted in inactivation with a half-time of approximately 5 min. This is consistent with the observation (Reinman, S. and Markwell, J., unpublished data) that denaturation of the *P*-700-containing chlorophyll-protein complex A-1 [11] occurs only at temperatures greater than 55°C during 5 min of incubation in the presence of sodium dodecyl sulfate. We have also observed loss of *P*-700 activity at 35°C in the presence of 0.1% Nonidet P-40 with a half-time of approx. 2 h. However, storage overnight at 4°C in the presence of 0.1% Nonidet P-40 resulted in no detectable loss of activity. We therefore recommended that once detergent is added to a sample, it be stored at the cooler temperature until the assay is performed.

The effect on the assay of compounds commonly used in the purification of Photosystem I-enriched particles is shown in Table I. Compounds present in chloroplast isolation buffers such as EDTA, $MgSO_4$ and sucrose had no effect on the results. Detergents have been found to be particularly useful for purification [12—16]. These can be classified into three groups on the basis of their effect on the measured *P*-700 content: (i) those which caused a significant decrease in the amount of *P*-700 observed with the assay—Miranol SM, Miranol H2M and lauryldimethylamine oxide; (ii) detergents which caused no significant change in the amount of *P*-700 measured in the control (containing Nonidet P-40)—Triton X-100, Miranol M2M, Miranol S2M-SF, Depiphat 160 and digitonin; and (iii) a class of detergents which caused a significant increase in apparent *P*-700 content—Miranol HM, *N*-lauroylsarcosine, dodecyl sulfate (sodium and Tris salts) and sodium decyl sulfate. This increase, at least for sodium dodecyl sulfate, appeared to depend on the detergent's concentration. There was no obvious qualitative difference between the shape of the *P*-700 difference spectrum recorded using the standard assay with or without sodium dodecyl sulfate.

TABLE I

EFFECT OF VARIOUS COMPOUNDS AND DETERGENTS ON THE ASSAY FOR *P*-700

The sample (78 nM *P*-700) was assayed with the standard assay components present (including 0.1% Nonidet P-40) and the indicated amounts of the compounds and detergents were additionally present.

Addition	Relative *P*-700	Addition	Relative *P*-700
None	1.00	0.5% Deriphat 160	1.04
10 mM EDTA	0.96	0.5% Miranol SM	0.40
10 mM $MgSO_4$	1.07	0.5% Miranol H2M	0.85
0.2 M Sucrose	1.00	0.5% Lauryldimethylamine oxide	0.72
0.1% Nonidet P-40	0.99	0.5% Miranol HM	1.32
0.5% Nonidet P-40	1.10	0.5% *N*-Lauroylsarcosine	1.67
0.5% Triton X-100	1.10	0.5% Decyl sulfate, sodium salt	1.92
0.1% Digitonin	0.96	0.1% Dodecyl sulfate, sodium salt	1.58
0.5% Miranol M2M	1.00	0.5% Dodecyl sulfate, sodium salt	1.96
0.5% Miranol S2M-SF	1.06	0.5% Dodecyl sulfate, Tris salt	1.85

To determine if this apparent increase in the *P*-700 signal was indeed associated with Photosystem I, or was caused by some other fraction of the photosynthetic apparatus, the procedure of Shiozawa et al. [7] was used to prepare a Photosystem I-enriched fraction with a chlorophyll/*P*-700 molar ratio of 60 as determined with the standard chemical assay. This enriched fraction was treated with various amounts of sodium dodecyl sulfate (Fig. 3). The amount of apparent *P*-700 increased with increasing concentration of detergent up to approx. 0.05% and then decreased with further increases in detergent concentration.

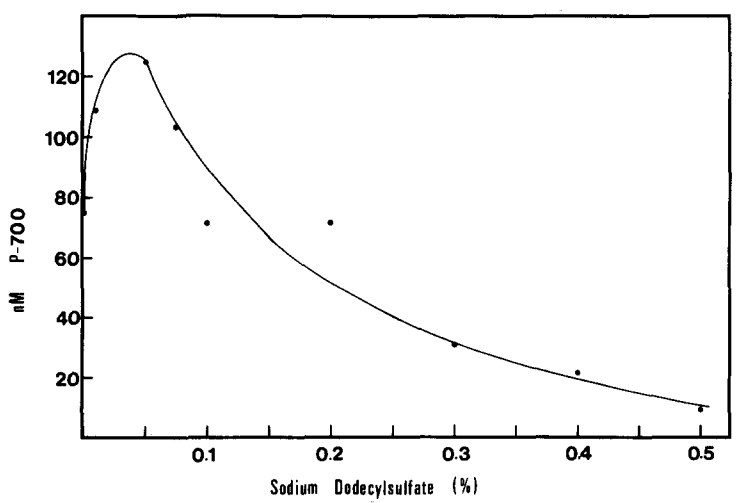

Fig. 3. Effect of various concentrations of sodium dodecyl sulfate on the apparent *P*-700 content of a fraction enriched in Photosystem I. The sample contained 75 nM *P*-700 as determined with the standard assay.

Discussion

The chemical assay for *P*-700 when performed as described in this report appears to be a valid procedure for the determination of this Photosystem I component. Quantitation of *P*-700 was linear with respect to the amount of sample added over a wide range, and the assay was sensitive enough to allow a precise determination in samples with high chlorophyll/*P*-700 ratios (e.g. 500/1). We recommend that samples be assayed at several different chlorophyll concentrations. This will establish that the amount of sample is not above the linear region of the assay (cf. Fig. 2) due to inadequate concentrations of the redox reagents and will assure experimenters of the accuracy of their determinations. We emphasize that care must be exercised to ensure that the solutions in the cuvettes have equilibrated in temperature. The decrease in interference from oxidation of chlorophyll species other than *P*-700, which in our experience has been considerable with the conventional (oxidized minus reduced) method, is a significant improvement. Using the assay described under Methods, both the sample and reference cuvettes undergo equivalent non-reversible oxidation of chlorophyll (for 90 s) and thus their resulting contribution to the ultimate difference spectrum is negligible. The widespread availability of microprocessor-linked spectrophotometers should allow broad use of this new assay procedure by researchers interested in a more accurate determination of *P*-700.

The assay was not significantly affected by the presence in the test solution of compounds which are normally used during the purification of Photosystem I (EDTA, Mg^{2+}, sucrose, Triton X-100 and digitonin). Among the other detergents tested, some had a marked effect on the results obtained, and caution should be taken in interpreting results from the chemical determination of *P*-700 concentration in the presence of such detergents.

The surprising observation of the effect of sodium dodecyl sulfate on the amount of apparent *P*-700 present in a sample requires comment, since sodium dodecyl sulfate is widely used in studies on Photosystem I and *P*-700. One possible explanation for the increased value is that the presence of sodium dodecyl sulfate is causing an approximate doubling of the extinction coefficient of *P*-700; this appears unlikely. Alternatively, the true size of the photosynthetic unit may be much smaller than the conventionally accepted value of about 400 chlorophyll/*P*-700. It seems more feasible that the detergent 'unmasks' some chlorophyll species that is not detected with this assay in the presence of several other detergents. This other species apparently has a difference (oxidized minus reduced) spectrum similar to that of *P*-700 in the 650—750 nm region, and, if it occurs in a 1 : 1 stoichiometry with *P*-700, it has approximately the same extinction coefficient. It also seems to copurify with *P*-700, indicating that it may also be a component of Photosystem I. All of these criteria are met by a chlorophyll species (A_1) thought to act as an intermediary electron acceptor in Photosystem I [17—19]. The A_1 species has a difference spectrum which closely resembles that of *P*-700 [17]. However, until an identity is established we will refer to our component as *A*-700. Our current understanding of the sequence of intermediates on the reducing side of Photosystem I is then as follows [9]: *P*-700 → A_1 → X → *P*-430(A-B). Both *P*-700 and A_1 are believed to be dimeric forms of chlorophyll. It is still not certain that A_1 is

the primary electron acceptor in Photosystem I. Recently [18] an even faster component (10 ns) of the charge recombination than that between A_1^- and P^+-700 [6] was detected and could be interpreted as being due to the existence of an electron carrier between *P*-700 and A_1.

The absorbance changes attributed to *A*-700 could arise from either the A^--700/*A*-700 or the *A*-700/A^+-700 redox couple. To determine which case it was, we performed assays in the presence and absence of sodium dodecyl sulfate. Using the standard assay, the presence of sodium dodecyl sulfate gave the expected increase in absorbance change. However, when the assays were performed, and potassium ferricyanide was replaced with water, there was no appreciable absorbance change in either the presence or absence of sodium dodecyl sulfate (data not shown). We interpret these results to indicate that it is not the A^--700/*A*-700 couple, but rather the *A*-700/A^+-700 couple that gives rise to the observed increases in the absorption difference. Furthermore, if it were the former couple, then its absorption change would be of the opposite sign to that of the P^+-700/*P*-700 couple, probably resulting in an almost zero change in absorption around 700 nm.

One possible interpretation of the data here is that in the presence of detergents such as Triton X-100, *A*-700/A^+-700 has a midpoint potential lower than that of ascorbate and therefore would not be detected by the assay used here since sodium ascorbate would not reduce A^+-700. However, in the presence of sodium dodecyl sulfate its midpoint potential might increase, enabling it to be oxidized by ferricyanide and then reduced by sodium ascorbate. Such an altered potential for *A*-700 could explain the observation [6] that the recombination half-time between P^+-700 and A_1^- is more rapid in particles isolated with Triton X-100 (3 μs) than in those isolated with sodium dodecyl sulfate (7 to 15 μs). Sodium dodecyl sulfate is known to destroy components X and *P*-430 (A-B) [20,21] and it is not unreasonable that it could affect the environment of *A*-700 sufficiently to raise its midpoint potential. An alternate explanation is that *A*-700 is normally in an environment that is inaccessible to the redox reagents and that some detergents, including sodium dodecyl sulfate, cause changes in the components surrounding *A*-700 thereby allowing access of these reagents to *A*-700. In either case, increasing amounts of sodium dodecyl sulfate affected the loss of both the *P*-700 and *A*-700 signals in the purified Photosystem I particles. It is this phenomenon that may account for the decrease in apparent *P*-700 signal seen with several of the detergents tested. The effects may be related and differ only in the extent of denaturation caused at the concentrations used.

Regardless of the actual source of the additional absorbance in the 700 nm region, the observation that sodium dodecyl sulfate can result in an increase in the *P*-700 signal is significant: considerable discrepancies between chemical and photochemical assayed values for *P*-700 have been reported. For example, Mathis et al. [6] measured the *P*-700 content of sodium dodecyl sulfate-treated particles and found a chlorophyll/*P*-700 ratio of 70 by photochemical assay, but 40 to 50 by chemical assay. The data in this report could explain this discrepancy. A similar lowering in the chlorophyll/*P*-700 ratio upon treatment of Photosystem I particles with sodium dodecyl sulfate was seen by Bengis and Nelson [22]. These authors [22,23] used a chemical assay to establish that

P-700 is associated with a 70 000 molecular weight-equivalent apoprotein of the so-called CPI-chlorophyll-protein [1,4,5]. In view of the data in this report, it is conceivable that they may have detected *A*-700 rather than *P*-700. Thus the actual site of *P*-700 is a matter that should be carefully reexamined.

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