BBA 46279

EXTRACTION AND PURIFICATION OF ^1H, ^2H, AND ISOTOPE HYBRID ALGAL CYTOCHROME, FERREDOXIN AND FLAVOPROTEIN

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(Received October 11th, 1971)

SUMMARY

Cytochrome *c*, ferredoxin, and phytoflavin, three low molecular weight, thermally stable proteins, have been isolated from the thermophilic alga *Synechococcus lividus* grown in both ^1H_2O and ^2H_2O. The isolation, purification, and physical properties of these three proteins are given in detail and their utility for magnetic resonance studies is described.

INTRODUCTION

In recent years, problems concerning protein structure and function have come under increasing study by the techniques of nuclear magnetic resonance[1–4]. However, the extreme complexity of protein spectra has been a major difficulty in such studies. It has been shown that the ability to grow algae and other microorganisms in 99.7 atom percent ^2H_2O (ref. 5) provides one with a tool for the simplification of proton magnetic resonance (PMR) spectra of proteins[6–10]. Fully deuterated proteins present no PMR spectrum and with these proteins one can consider the re-insertion of protons at selected sites to form what has been termed "isotope hybrid" proteins[11]. We describe here the purification and characterization of three new algal proteins, a cytochrome, a ferredoxin, and a flavoproprotein, that give promise of being of great utility in magnetic resonance studies. They are small, stable, and biologically interesting molecules that lend themselves to the techniques of isotope hybridization. Some examples of magnetic resonance studies with these proteins in fully deuterated and isotope hybrid form are also presented.

EXPERIMENTAL

The thermophilic blue-green alga *Synechococcus lividus* was cultured in ^1H_2O and ^2H_2O nutrient media according to the procedures of DABOLL *et al.*[12] and TAECKER *et al.*[13]. To 800 g (wet wt.) of frozen cells (or 200 g of freeze–dried cells) are added 1.5 l of 0.01 M sodium phosphate buffer (^1H_2O), pH 6.9*, and the resuspended cells are allowed to stand for 3 h at 5° with occasional stirring. Freeze–dried ^2H_2O-grown cells are easily extracted by this procedure, but wet frozen ^2H_2O- and ^1H_2O-grown cells

* We shall refer to sodium phosphate buffer of pH 6.9 simply as "buffer".

should be sonicated for a few minutes (Branson, Model J17V, power setting 60) before being allowed to stand for 3 h. The crude extract is then centrifuged twice, 30 min each time, at 15000 × *g*, to completely sediment cells and debris. The packed cells should be reextracted. The pooled crude extracts are brought to 50 % saturation with solid $(NH_4)_2SO_4$ (Mann, Enzyme grade) added slowly with stirring. After standing 1 h, this mix is centrifuged for 2 h at 15000 × *g*. The supernatant solution should be golden brown or slightly green because of residual phycocyanin. If the supernatant is heavily colored it may be brought to 52.5 % saturation and centrifuged again. The supernatant is then brought to 90 % saturation, allowed to stand with stirring overnight, and centrifuged. Also, the precipitate from 50 % saturation can be taken up in 0.01 M buffer and reworked for the precipitate at 90 % saturation, but the added yield from this step is often marginal. All "90 %" precipitates are suspended in a minimum volume of buffer and dialyzed against water to remove all $(NH_4)_2SO_4$.

After clarification by centrifuging for a few minutes at 15000 × *g*, the concentrated crude extract is applied to a Cellex D (Calbiochem) column, 5 cm × 13 cm. The column is washed with 4 column volumes of 0.001 M buffer and then the cytochrome is eluted with 0.005 M buffer. The column is then washed with 0.01 M buffer containing 0.3 M NaCl and eluted with 0.01 M buffer containing 0.5 M NaCl. Crude flavoprotein and ferredoxin fractions are collected. The crude cytochrome is dialyzed to 0.001 M buffer, applied to a Cellex D column, 1.7 cm × 15 cm, washed with 10 column volumes of 0.001 M buffer and eluted with 0.005 M buffer to give pure cytochrome. The crude flavoprotein and ferredoxin are diluted with 10–20 vol. of water and are adsorbed on DE-52 (Reeve-Angel micro-granular, pre-swollen, decoarsed and defined[14]). Columns are poured, eluted with 0.01 M buffer containing 0.5 M NaCl, and the eluates are desalted (Sephadex G-10, Pharmacia) to give protein solutions in 0.001 M buffer. These solutions are applied to hydroxylapatite (Calbiochem) columns and washed with 0.01 M buffer. Ferredoxin is eluted with 0.04 M buffer. The flavoprotein column is washed with 0.04 M buffer and flavoprotein is eluted with 0.1 M buffer. These eluates can then be adsorbed on DE-52 and eluted in a small volume. If the cytochrome is not sufficiently pure after the second Cellex D column, it may be concentrated (dilute and adsorb on DE-52), desalted (G-10, 0.001 M buffer), adsorbed on a hydroxylapatite column, washed (0.001 M buffer) and eluted (0.005 M buffer). All hydroxylapatite columns are operated at 2–4 lb pressure of nitrogen.

As an alternative to the above procedure, ferredoxin and flavoprotein can be adsorbed on DE-52 directly from the crude extract (the cytochrome is then isolated according to the procedure outlined above). The adsorbed ferredoxin and flavoprotein are concentrated and purified through a series of adsorption and elution steps using DE-52, with extensive washing of each column with 0.1 M buffer before each elution. A concentrated eluate of 3–5 ml is applied to a Sephadex G-50 column, 2.5 cm × 35 cm, equilibrated with 0.05 M Tris (pH 8) or 0.01 M sodium phosphate (pH 6.9). Ferredoxin and flavoprotein fractions are then adsorbed on DE-52, eluted, desalted and applied to hydroxylapatite columns as described above. At all elution steps of all of the above procedures, only the very dilute beginning and end fractions are discarded. Yields in the range of 0.1–0.3 mg protein per g (dry) of algae are obtained for each of the three proteins for both ^1H_2O- and ^2H_2O-grown algae. No differences were observed in the preparative behavior of the deuterated as compared to the ^1H proteins.

Amino acid analyses were performed with a Beckman 120 Amino Acid Analyzer after hydrolysis of the native or performic acid oxidized protein[15] in 6 M HCl. Tryptophan was determined after alkaline hydrolysis and also spectrophotometrically[16,17]. Ferredoxin and flavoprotein were extracted with trichloroacetic acid before performate oxidation[18]. Flavin mononucleotide (FMN), was determined according to the method of SWOBODA AND MASSEY[19] as well as by methanol extraction of thermally denatured, dried flavoprotein. Flavoprotein was dry-ashed and analyzed spectrochemically for metal content by the copper-spark method (analysis performed by E. HUFF of this Laboratory). FMN was identified chromatographically by the method of HARRIS[20] and was further distinguished from flavin adenine dinucleotide (FAD) by treatment with venom diesterase (Worthington) while monitoring fluorescence[21]. The redox potential of the cytochrome was determined according to the method of HENDERSON AND RAWLINSON[22] and the cytochrome prosthetic group was characterized spectrophotometrically[23]. Disc electrophoresis and electrofocusing (cytochrome and flavoprotein) experiments were performed by Dr. Carl Peraino of this Laboratory. Molecular weights were determined by the gel filtration method of ANDREWS[24] and by calculation from amino acid content. End groups were determined by the method of SANGER AND THOMPSON[25]. Extinction coefficients were calculated from dry weight measurements. Photochemical reduction of the flavoprotein was accomplished by ultraviolet irradiation in the presence of ethylenediaminetetraacetic acid[26,27]. The phrase "apparent pH" refers to the pH meter reading given by ^2H_2O buffers.

RESULTS AND DISCUSSION

Fig. 1 shows the optical absorption characteristics of cytochrome *c*, ferredoxin, and flavoprotein from *S. lividus*. The cytochrome appears to be a "*c*" type cytochrome, similar in properties to other algal cytochromes, with α bands at 552–553 nm[23,28]. Heme was not extracted by 0.1 M HCl in methyl ethyl ketone and the reduced pyridine hemochromogen had an absorption spectrum essentially identical to that of horse-heart cytochrome *c* pyridine hemochromogen. Thus, the heme group is of the meso

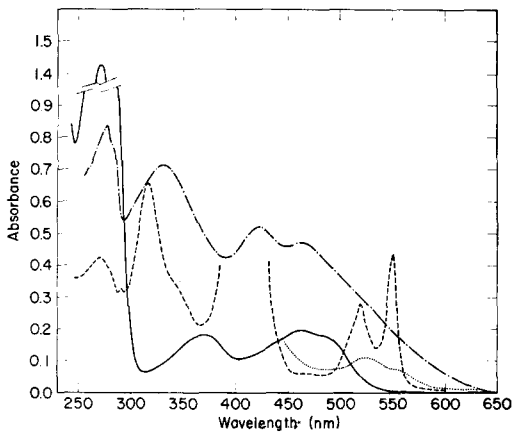

Fig. 1. Optical absorption spectra in neutral phosphate buffer of cytochrome *c*, ferredoxin, and flavoprotein from the blue-green alga *S. lividus*. ——— and ·····, reduced and oxidized cytochrome *c*, respectively; —·—, oxidized ferredoxin; ———, flavoprotein.

type and covalently attached to apoprotein. In the reduced state, this cytochrome *c* autooxidizes very slowly at neutral pH.

The optical spectrum of the ferredoxin indicates it to be a typical plant ferredoxin. This ferredoxin is essentially identical to spinach ferredoxin in terms of molecular weight, iron content, circular dichroism, and electron spin resonance spectrum (G. PALMER AND R. ANDERSON, personal communication). The PMR spectra of the contact shifted lines in the oxidized and reduced states closely match those reported by POE *et al.*[29] for spinach ferredoxin. Fig. 2 gives the diamagnetic portion of the PMR spectra of reduced and oxidized [^{1}H]ferredoxin from *S. lividus*. The pattern of lines is again very similar to that of spinach ferredoxin[29]. Details of PMR studies with ferredoxin and with cytochrome *c* and phytoflavin from *S. lividus* will be reported elsewhere, but we present spectra of the ^{1}H-labeled protein as part of the general characterization of these proteins. PMR spectra of the [^{1}H]cytochrome *c* and [^{1}H]-phytoflavin have already appeared[11].

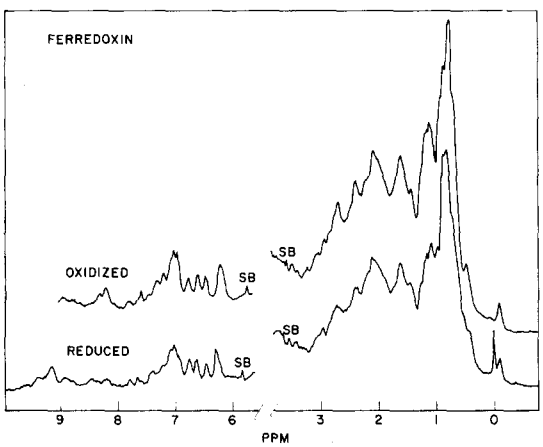

Fig. 2. Proton magnetic resonance spectra at 220 MHz of oxidized and reduced [^{1}H]ferredoxin from *S. lividus*. The spectra were obtained from 6% solutions of [^{1}H]ferredoxin in 0.05 M sodium phosphate buffer (^2H_2O), containing 0.5 M NaCl, at an apparent pH of 6.8 for the oxidized material and 7.4 for the reduced material. The ferredoxin was reduced by the anaerobic addition of $Na_2S_2O_4$ dissolved in 1.0 M dibasic potassium phosphate in ^2H_2O. Peaks labelled SB are sidebands. The small sharp line at 0 ppm of the lower trace is internal standard, trisilyltetradeuteropropionate (TPP). Spectra were recorded with a Varian spectrometer.

The flavoprotein has an optical spectrum quite similar to the phytoflavin isolated from the blue-green alga *Anacystis nidulans*[30,31] and the flavodoxin from bacteria[32]. Anacystis cultures must be iron-deficient in order to elicit phytoflavin production[33] as phytoflavin serves in place of ferredoxin. The flavoprotein of *S. lividus* is present under our normal culture conditions, but sufficient iron may not be available, for we have noted occassional striking variations in flavoprotein yields from algae cultured in the presence of exogeneous amino acids. Preliminary assay (G. PALMER AND R. ANDERSON, personal communication) of this flavoprotein indicates no ferredoxin–NADP reductase activity, so at this point we judge this flavoprotein to be a phytoflavin.

Table I lists the amino acid contents of the three algal proteins. The flavoprotein

TABLE I

AMINO ACID COMPOSITION OF ALGAL PROTEINS

Amino acid	*Flavoprotein*		*Ferredoxin*		*Cytochrome c*	
	μmoles/mg	*Residues*	*μmoles/mg*	*Residues*	*μmoles/mg*	*Residues*
Lysine	0.203	5 (5.06)	0.412	4 (3.89)	0.578	7 (6.64)
Histidine	0.040	1 (1.00)	0.106	1 (1.00)	0.174	2 (2.00)
Arginine	0.175	4 (4.38)	0.298	3 (2.81)	0.126	1 (1.45)
Aspartic acid	0.893	22 (22.3)	1.14	11 (10.8)	1.00	12 (11.5)
Threonine	0.308	8 (7.70)	0.608	6 (5.74)	0.206	2 (2.37)
Serine	0.168	4 (4.20)	0.405	4 (3.82)	0.169	2 (1.94)
Glutamic acid	0.982	25 (24.6)	1.77	17 (16.7)	0.874	10 (10.0)
Proline	0.142	4 (3.55)	0.576	5 (5.43)	0.132	2 (1.5)
Glycine	0.746	19 (18.7)	0.679	6 (6.41)	0.817	9 (9.35)
Alanine	0.616	15 (15.4)	0.646	6 (6.09)	1.38	16 (15.9)
Half cystine	0.076*	2 (1.9)	—	5 (4.7)*	0.142	2 (1.63)
Valine	0.233	6 (5.83)	0.742	7 (7.00)	0.475	5 (5.46)
Methionine	0.042	1 (1.05)	None	—	0.292	3 (3.36)
Isoleucine	0.489	12 (12.2)	0.392	4 (3.70)	0.228	3 (2.62)
Leucine	0.463	12 (11.6)	1.08	10 (10.2)	0.437	5 (5.03)
Tyrosine	0.287	7 (7.18)	0.375	4** (3.54)	0.185	2 (2.13)
Phenylalanine	0.365	9 (9.13)	0.306	3 (2.89)	0.258	3 (2.97)
Tryptophane	0.132	3 (3.3)	None**	—	0.070***	1 (0.80)
Total residues		154		96		87

* Determined as cysteic acid.
** Also determined spectrophotometrically.
*** Alkaline hydrolysis.

contains an unusually large number of acidic amino acids and electrofocusing experiments indicated an isoelectric point in the region of pH 2 for this protein. The amino acid composition of the ferredoxin is generally similar to the composition of *Scenedesmus* ferredoxin[34] and contains no tryptophan. In the cytochrome *c*, the presence of two half-cystine residues indicates the problable presence of one heme prosthetic group per protein chain. Table II lists a number of chemical and physical properties of these molecules. The low molecular weights of these molecules makes them particularly well suited to study by nuclear magnetic resonance techniques. Four quantitative determinations of the FMN content of the flavoprotein gave 0.8 ± 0.1 mole of FMN per mole of flavoprotein. Many flavoproteins contain two or more of flavin prosthetic groups[35] but some contain only one flavin prosthetic group and a cystine residue. The flavoprotein described here appears to fall into this latter class. Two iron atoms per ferredoxin molecule is typical of plant ferredoxins. Alanine was found to be the amino end group of all three proteins. The DNP-alanine derivative from the flavoprotein was obtained in 80–85 % yield and the yields of DNP-alanine from the cytochrome and ferredoxin were also very good, but were not determined quantitatively. We judge that none of the end groups are acetylated. Alanine is a frequent end group of ferredoxin and plant cytochromes[34]. The three proteins are thermally very stable, as might be expected from the fact that the source alga, *S. lividus* is a thermophilic organism.

TABLE II

PROPERTIES OF SOME DEUTERATED ALGAL PROTEINS

For the [^{1}H]cytochrome *c*, $E'^\circ = +0.33 \pm 0.01$ V and for the [^{2}H]cytochrome *c*, $E'_0 = 0.32 \pm 0.01$ V, both in ^1H_2O at pH 7.0. Absorption maxima for the reduced cytochrome appear at 552, 520, 414, 315 and 275 nm. In the oxidized form maxima appear at 525, 408, and 358 nm. Some absorbance ratios are: Soret/α, 6.7; α/β, 1.6; Soret (ox)/Soret (red), 0.84. Flavoprotein: absorption maxima at 490 (shoulder), 465, 373, 290 (shoulder) and 273 nm. The molar extinction at 465 nm is $8.1 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. Ferredoxin: absorption maxima at 465, 423, 331 and 276 nm. Mol. wt. determination from gel filtration and amino acid analysis. Estimated error, 5%. The absorbance ratios are the same for both ^{1}H and ^{2}H proteins. At these absorbance ratios, disc electrophoresis and electrofocusing showed no impurities.

Protein	*Mol. wt.*	*Prosthetic group*	*Amino end group*	*Isoelectric point (pH)*	*Absorbance ratio*
Cytochrome *c*	9 500	Mesoheme	Alanine	4.47_{red} 5.00_{ox}	$A_{552\ nm}/A_{273\ nm}$, 1.05**
Flavoprotein	17 000	Flavin (one) Mononucleotide*	Alanine	About 2	$A_{464\ nm}/A_{273\ nm}$, 0.15
Ferredoxin	10 500	2 Fe	Alanine	—	$A_{420\ nm}/A_{273\ nm}$, 0.65

* No metals detected. With a precision of a factor of two, we found 0.01% Zn, 0.001% Mn and Mo, and 0.005% Fe.

** Reduced form.

Fig. 3 shows the changes in the spectrum of the flavoprotein upon irradiation with light in the presence of EDTA[26]. A blue semiquinone radical is formed that yields a typical electron paramagnetic resonance (EPR) signal[36]. Spectra generated by the [^{1}H]flavoprotein semiquinone radical have a linewidth of 19 gauss (low power level) are essentially featureless, and show "anomalous" saturation properties because the correlation time of the protein makes it more difficult to saturate the anisotropic part of the spectrum (the wings) as compared to the isotropic part of the spectrum (the center)[36]. In spectra of the [^{1}H]flavoprotein radical, the wings appear as unresolved shoulders and interpretation is difficult. However, EPR spectra of the [^{2}H]flavoprotein semiquinone radical exhibit resolved hyperfine splitting patterns, because of the decreased hyperfine interaction of the deuteron as compared to the proton, and these spectra lend themselves to detailed analysis of the motional pro-

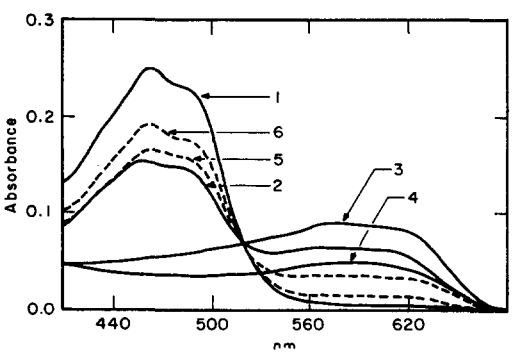

Fig. 3. Changes in optical absorption of flavoprotein upon anaerobic irradiation with ultraviolet light in the presence of EDTA at pH 7. A blue semiquinone radical is formed which is then further reduced (Curves 1–4). Upon exposure to air, the flavoprotein is re-oxidized (Curves 5, 6, 1) to give a spectrum identical to the original.

perties of the radical, both *in vitro* and *in vivo*. Details of the ESR spectral properties of [^{2}H]FMN and [^{2}H]phytoflavin were reported elsewhere[38].

The proteins described here have been used in a variety of magnetic resonance experiments. Three classes of protons can be considered for PMR visualization using the techniques of isotope hybridization: (1) the "core" amide protons of peptide bonds *plus* other very slowly exchangeable protons; (2) protons associated with the prosthetic group of a protein or an enzyme; (3) protons of selected amino acids. In each of these three types of experiments, only a few of the hundreds of hydrogen atoms of the protein are observed in the proton magnetic resonance spectrometer, as the proteins are largely deuterated. Details of these types of experiments with the proteins described here have already appeared [9-11,39]. Fig. 4 illustrates an additional example of the type of results made possible with isotope hybrid proteins. The PMR spectrum of [^{2}H]cytochrome *c* ([^{1}H]phenylalanine[^{1}H]tyrosine) indicates at least two or perhaps three conformational states, an observation that would be most difficult with the [^{1}H]cytochrome *c*. With the availability of fully deuterated and isotope hybrid proteins, the problem of the assignment of PMR lines in proteins is, in principle, solved, and the full potential of magnetic resonance techniques can be brought to bear upon some of the problems of structure and function of proteins and enzymes.

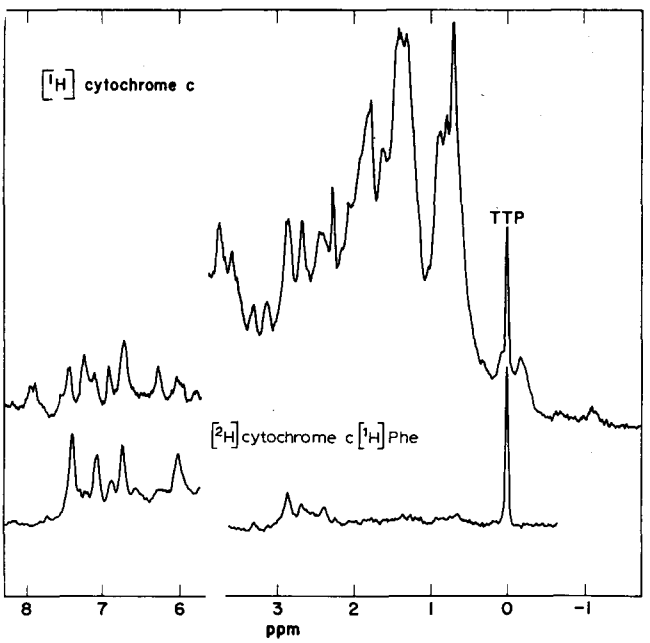

Fig. 4. Upper trace, the PMR spectrum at 220 MHz of [^{1}H]cytochrome *c* from the blue-green alga *S. lividus*, oxidized form. Lower trace, the PMR spectrum at 220 MHz of [^{2}H]cytochrome *c* ([^{1}H]phenylalanine[^{1}H]tyrosine), oxidized form. In the aromatic region, the spectrum of the isotope hybrid is interpretable in terms of three sets of three lines due to the three phenylalanine and two tyrosine residues. One major and probably two minor conformations of the phenyl groups are indicated under the conditions of this analysis (about 5% protein, 0.05 M sodium phosphate buffer, 19°, apparent pH 6.8). The incorporation of [^{1}H]phenylalanine into *S. lividus* cells growing in ^2H_2O leads also to the production of [^{1}H]tyrosine. These spectra, and those of Fig. 2, were taken by repetitive scanning techniques using an audio-sideband trigger and Fabri-tek 1074 computer.

ACKNOWLEDGEMENTS

We would like to acknowledge the assistance of David Bevan, Mark Burlingame, Solomon Rosenblatt, and Lewis Waber, undergraduate honors research students sponsored by the Central States Universities, Incorporated.

This work was performed under the auspices of the U.S. Atomic Energy Commission. One of the authors (T.T.) is a Resident Associate on leave from Beloit College, Beloit, Wisc., 1969, and R.M.A. is a Resident Associate on leave from Timothy Christian College, Palos Heights, Ill., 1968.

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