Biochimica et Biophysica Acta, 376 (1975) 561–572

BBA 46880

CHLOROPLAST CYTOCHROME b_6
MOLECULAR COMPOSITION AS A LIPOPROTEIN

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(Received August 12th, 1974)

SUMMARY

Disc electrophoretically homogeneous spinach-chloroplast cytochrome b_6 was found to be a lipoprotein whose redox potential was essentially unchanged during isolation. These results further support the hypothesis of Triton X-100/4 M urea, pH 8, as a useful extracting medium for membrane lipoproteins.

Cytochrome b_6 was found to have a heme equivalent dry weight of 1 mol of heme per 60000 g. Of this, 20000 g was lipid-extractable. The molecular weight was 60000 with a partial specific volume of 0.84 ml/g. The protein portion of the molecule (40000) consisted of 1 polypeptide chain of 20000 daltons, 1 of 9600 daltons and 2 of 6600 daltons. A simple lipid composition (relative to the original membrane) was found consisting of 7 mol of chlorophyll *a* and 6 mol of cardiolipin per mol of cytochrome; these two lipids thus account for about 75–80 % of the lipid content. An unidentified minor neutral lipid and minor polar lipid were also detected. At pH 7.0 in the presence of 0.5 % Triton X-100, E'_0 was −0.080 V, and in the absence of Triton X-100, E'_0 was −0.120 V. At pH 8 in 0.5 % Triton X-100, E'_0 was −0.084 V, thus indicating that the redox potential is independent of pH in the region 7–8. The redox reaction proceeded via a one-electron-transfer.

INTRODUCTION

A fundamental question in membrane biology is the role of lipids (if any) in the structure and function of membrane-bound proteins. The ability to extract membrane proteins into solution and to purify them without loss of lipids originally tightly-bound to or within each of the proteins would be of great value. If accomplished, the original lipid composition could be analyzed and its role in structure and function of an individual lipoprotein could be studied. The lack of a suitable solubilizing medium able to extract intact lipoproteins from membranes has so far hindered progress in this area.

A medium of 2 % Triton X-100/4 M urea (pH 8) has been successfully used for extraction of membrane proteins in the purification procedures of spinach chloroplast cytochromes b_{559} [1, 2], b_6 [3] and f [4]. The lipid content of pure

cytochrome b_{559} (about 56 % by wt) had a simple lipid composition consisting of only 4 of the 20 or more lipids present in chloroplast membranes [5]. The lipids consisted of chlorophyll *a*, β-carotene plus two as yet unidentified polar lipids. Consequently, it was proposed that 2 % Triton X-100/4 M urea (pH 8) might be of general use for extracting membrane lipoproteins. The molecular composition of pure cytochrome b_6 was accordingly examined both because of its value to photosynthesis and as a test of the above hypothesis.

We have previously reported some of the molecular properties of electrophoretically-homogeneous cytochrome b_6 [3]. This paper reports on the molecular weight, partial specific volume, polypeptide composition, lipid composition and redox potential of cytochrome b_6.

METHODS

Disc-electrophoretically homogeneous cytochrome b_6, prepared as previously described [3], was used for all experiments. Heme content was assayed as the reduced pyridine hemochromogen by the method of Appleby [6].

The procedure for dry weight analysis was essentially that of Goodrich and Reithel [7]. The instrument used was a Cahn Model RG electrobalance (Ventron Instruments Corp., Paramount, Calif.) housed in a vacuum bottle (obtained from the same source) and modified as follows: a valve was fitted onto a hangdown tube for purposes of evacuating the bottle and a glass water-jacket was constructed around the hangdown tube containing the sample. Paraffin oil at 65 °C was circulated through this water-jacket.

The deuterium oxide method of Edelstein and Schachman [8] was used for the simultaneous determination of molecular weight and partial specific volume in a Beckman Model E Analytical Ultracentrifuge. Two speeds were used for each solvent: 0.05 M Tris·HCl buffer, pH 8.0, 0.5 % Triton X-100, 32 100 and 28 200 rev./min; 0.05 M Tris·HCl buffer, pH 8.0, 0.5 % Triton X-100, 50 % ^2H_2O, 34 200 and 30 300 rev./min. Measurement of the Rayleigh interference fringes was performed with a Nikon Model 6 Shadowgraph Comparator (Nippon Kogahu K. K., Tokyo, Japan), fitted with a Micrometer Calibrator (L.S. Stamett Co., Athol, Mass.).

The size of polypeptide chains was determined by the method of Weber and Osborn [9]. For chains of less than 10 000 daltons the procedure was modified as follows: the acrylamide concentration was 20 % and the methylenebisacrylamide concentration relative to acrylamide concentration was double that used in the normal Weber and Osborn procedure (i.e. 20 g acrylamide and 1.08 g methylenebisacrylamide per 100 ml). These modifications were required to obtain a linear calibration graph for standard polypeptide chains between 1 000 and 12 000 daltons (see also Results). For the small polypeptide chains of cytochrome b_6 a 10 % gel was run, and the protein at the front (R_F values 0.85 and 0.90) was cut out as a single slice and eluted with the resolving gel medium and then rerun on the 20 % gel. Total mass ratios of the resolved polypeptide chains were determined both by dye-scanning and by direct biuret-protein analysis. In either method sodium dodecylsulfate-denatured b_6 was first run on a 10 % sodium dodecylsulfate gel; the protein at the front was cut out and the protein in the rest of the gel was fixed by placing it in 45 % methanol/9 % acetic acid or 40 % trichloroacetic acid. After the protein was

fixed, it was stained with 1 % amido black (in 7 % acetic acid) and then thoroughly destained. The gel was scanned spectrophotometrically at 625 nm for absorbance by amido black. A Gilford Model 2400 Spectrophotometer equipped with a gel scanner was used (Gilford Instruments, Inc., Oberlin, Ohio). The 2 smaller polypeptides migrating with the front and previously cut out were eluted from their section of the 10 % gel and rerun on a 20 % gel. The protein was fixed, stained and scanned as above. The area under each of the three polypeptide-dye peaks gave a relative index of the amount of polypeptide present [10]. The second method also employed sodium dodecylsulfate gel electrophoresis, but without subsequent staining, and the amount of protein in each band was determined by eluting each band and analyzing for protein by the microbiuret method of Singh [11] which was based on the biuret method of Gornall et al. [12]. (An alkaline control absorption using cytochrome b_6 was subtracted during this determination.) Both procedures for estimating the mass of resolved polypeptides gave essentially the same results.

Lipid extraction was performed by the Bligh and Dyer [13] procedure on samples of cytochrome b_6 previously depleted of Triton X-100 by thorough dialysis and shown to be reaggregated [3]. Thin-layer plates were prepared and dried according to Kates [14]. The adsorbant used, silica gel H (plain, no binder, Brinkmann Instruments, Inc.) was spread to a thickness of 0.5 mm. A Desaga applicator was employed (Brinkmann Instruments, Inc., stainless steel adjustable model). Lipid samples were spotted on silica gel plates under a N_2 atmosphere in a nitrogen box. Spots were routinely located by spraying the plates lightly with 40 % H_2SO_4 (v/v) and heating on a hot plate at about 200 °C for 10–20 min. Phospholipids were detected by the phosphorous stain of Vaskovsky and Kostetsky [15]. Glycolipids were detected by the α-naphthol stain for sugars [16]. The standards plant monogalactosyl diglyceride, bovine cardiolipin and phosphatidic acid (DL-dipalmitoyl phosphatidic acid) were obtained from Applied Science Laboratories Inc., State College, Pa. Squalene, squalane and the fully saturated straight chain hydrocarbons (C_{16}, C_{24}, C_{32}) were obtained from Eastman Kodak Co., Rochester, N.Y.

Total lipid dry weight was performed on the Cahn electrobalance described earlier in this section. The Bligh and Dyer [13] extraction procedure was used on samples previously thoroughly dialyzed to deplete them of Triton X-100 [3]. The final lipid extract, in chloroform, was dried to 0.1 ml under N_2 and transferred to a preweighed aluminum weighing container. To ensure quantitative transfer of the sample, the original container was rinsed successively with 5 aliquots of chloroform (5 ml, 5 ml, 3 ml, 1 ml, 1 ml) each of which was similarly dried to 0.1 ml and transferred to the aluminum container. The combined solutions (about 0.6 ml) were dried at 37 °C under vacuum for 1–12 h to constant weight. A dialysis blank of the same volume as the sample and similarly transferred was dried to constant weight, and its weight was subtracted from that of the sample.

Inorganic phosphorous was determined by the method of Fiske and SubbaRow [17]. For total lipid phosphorous, the lipid extracts were hydrolyzed by the method of Kagawa and Racker [18] and phosphorous then determined.

The oxidation-reduction potentials (E'_0) were determined by the method of Cusanovich et al. [19]. Potentials were determined with an Instrumentation Laboratories Deltamatic pH/mV Electrometer Model 145. The oxygen content of the argon gas (initially 5 ppm) was reduced by passing the gas through a column of MnO

and then a solution of $Cr_2(SO_4)_3$. The medium used was 0.05 M phosphate buffer (pH 7.0) with pyocyanin (E'_0 = −0.034 V [20]) as the redox buffer.

RESULTS

Heme equivalent dry weight

The heme equivalent dry weight was found to be 67 000 g (±5 %) per mol of heme. Analyses for content of Triton X-100 [21] in aliquots of dialyzed samples showed that Triton accounted, at most, for 10 % (7000 g) of the dry weight. This value probably consists of Triton bound to the cytochrome plus any soluble Triton X-100 which had not been completely removed by extended dialysis. After correction for the content of Triton X-100, the equivalent weight was found to be 60 000 g net dry weight per mol of heme. Since protein accounts for only 40 000 g per mol of heme [3], this suggests that one third of the equivalent weight (20 000 g) is contributed by non-protein material.

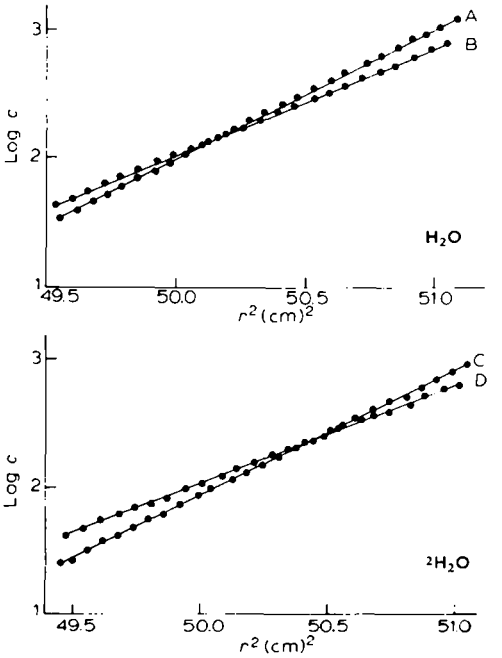


Fig. 1. Estimation of the molecular weight and partial specific volume ($\bar{v}$) of pure cytochrome b_6 by the ultracentrifugal method of Edelstein and Schachman [8]. The cytochrome was present in a medium containing 0.05 M Tris·HCl, pH 8.0, 0.5 % Triton X-100. For runs in ^2H_2O, the sample was dialysed against large volumes of a similar medium prepared with 50 % ^2H_2O and the final dialysis buffer was used as the reference medium. In H_2O, equilibrium was reached in 20–25 h and in 50 % ^2H_2O it was reached in 35–40 h. The temperature was held constant at 6 °C. The sample concentration was approximately 0.2 mg biuret protein per ml. The figure shows the straight line graphs obtained in H_2O and 50 % ^2H_2O. Cytochrome b_6 was quantitatively recovered after the runs indicating that it was stable during the course of the runs, and thus, the molecular weight and $\bar{v}$ measured were indeed those of active cytochrome b_6 and not a denatured artifact. A, 32 100 rev./min; B, 28 200 rev./min; C, 34 200 rev./min; D, 30 300 rev./min.

Molecular weight and partial specific volume

From the slopes of the lines in the log c vs r^2 plot (Fig. 1) a $\bar{v}$ value of 0.84 ml/g and a molecular weight of 67 000 were calculated. A straight line was obtained for each combination of speed and solvent. The fringes on the photographic plates could be read to the bottom of the cell, and the protein values were found to cover the entire column of the cell in all cases indicating that no heavy component had accumulated at the bottom of the cell. Since cytochrome b_6 was quantitatively recovered after each run, the properties obtained during the experiments are those of the stable molecule rather than of a denatured artifact.

The molecular weight of 67 000 is likely that of a cytochrome b_6-Triton X-100 complex. This compares with an equivalent dry weight per mol of heme of 67 000 g and a value of 60 000 g net dry weight per mol of heme after correction for Triton content. Thus the molecular weight of cytochrome b_6 is probably about 60 000. From the equivalence of the molecular weight with the equivalent dry weight per mol of heme we may also conclude that the cytochrome is not aggregated in the presence of 0.5 % Triton X-100 but dissociated non-destructively to its monomeric form (60 000 daltons). (By "monomer", we mean the smallest size of the molecule which still retains its known biological properties.)

The $\bar{v}$ value of 0.84, much higher than that expected for a molecule entirely composed of protein (0.72–0.76), gave a preliminary indication that lipid might comprise the non-protein portion of the molecule. (That Triton alone accounted for elevating the $\bar{v}$ value was unlikely since calculations showed that a Triton to protein ratio of 0.7 g/g would be required whereas experimental results indicated a maximum Triton to protein ratio of 7000/40 000 or 0.18.)

Size and number of polypeptide chains

When cytochrome b_6 was incubated with sodium dodecylsulfate and run on a 10 % polyacrylamide gel, three bands with R_F values of 0.58. 0.85 and 0.90 were found. A linear calibration graph of logarithm of molecular weight vs R_F was obtained using standard proteins (bovine serum albumin 68 000, ovalbumin 43 000, pepsin 35 000, trypsin 23 300 and lysozyme 14 300). The band with R_F of 0.58 corresponds to a polypeptide molecular weight of 20 000. The R_F values of 0.85 and 0.90 suggested polypeptide chains of molecular weight less than 10 000. When the latter two bands were eluted from the 10 % gel and rerun on a 20 % polyacrylamide gel, two bands were

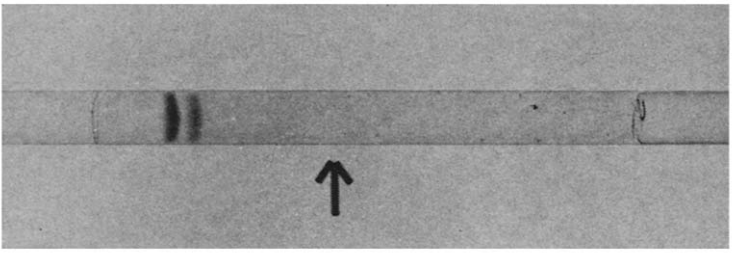


Fig. 2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of pure cytochrome b_6 on a 20 % gel with twice the "normal" amount of methylenebisacrylamide. Only the two smaller subunits (9600 and 6600) were run. The arrow indicates the position of the front before staining with Coomassie brilliant blue R.

observed in the 20 % gel (Fig. 2) with R_F values of 0.32 and 0.35. A linear calibration graph of logarithm of molecular weight vs R_F was obtained using cytochrome *c* 11 700, thrombin 9250, trypsin inhibitor 6500, ACTH 4400, secretin 3200 and bradykinin 1300. The bands with R_F 0.32 and 0.35 correspond to polypeptides of 9600 (±1000) and 6600 (±1000) daltons, respectively.

To eliminate the possibility that the small polypeptides were produced by proteolysis during incubation, aliquots of cytochrome b_6 were incubated at 100 °C for 5 min [22] immediately following the addition of sodium dodecylsulfate and mercaptoethanol. This treatment destroys any proteolytic activity. No change in the patterns was observed using this method. There was also no change in the patterns when mercaptoethanol was omitted from the incubation medium. When a b_6-sample was first delipidated by the method of Bligh and Dyer [13], the polypeptide results were identical to those obtained above.

The ratios of total mass of the subunits found either by dye-scanning or by direct biuret protein measurement were essentially the same: the ratios based on protein mass of the three polypeptides (20 000 daltons: 9600 daltons: 6600 daltons) were found to be 2 : 1 : 1.2–1.3. Since this is a ratio by mass, then for every one polypeptide of 20 000 there must be one 9600 polypeptide and two 6600 polypeptides. Adding up the weights using this ratio gives 42 800 (±10 %) as the minimum protein mass of cytochrome b_6. This value corresponds within experimental error to the heme equivalent weight of 40 000 g biuret protein per mol of heme and thus independently corroborates the polypeptide composition found. The same results were obtained with three different preparations of the cytochrome.

Lipid composition (*All lipid data presented are representative for two separate pure b_6 preparations, and, for chlorophyll, three pure b_6 preparations*)

1. Dry weight of total lipids. Cytochrome b_6 had a lipid content of 25 000 g per mol of heme and, correcting for 7000 g Triton per mol of heme, a net dry weight of 18 000 g lipid per mol of heme. The lipid content of b_6 is thus 30 (± 5) % by weight of the molecule. This percentage by weight agreed, within experimental error, with the value indicated by difference between the dry weight of b_6 (i.e. protein + non-protein) and the biuret protein content which gave a "non-protein" content of 33 %. Thus the Bligh and Dyer procedure was indeed extracting all the "non-protein" material (within ± 5 %). The content of covalently-linked lipid (or material other than protein or lipid) would thus appear to be very small, if present at all.

2. Neutral lipid analysis. Spectrophotometric analysis (400–700 nm) of the total lipid extract in acetone revealed a sharp peak at 662 nm with no peak or shoulder at 645 nm. Using the equation of Arnon [23], the chlorophyll *a* content was quantitated as 7 molecules of chlorophyll *a* per molecule of cytochrome b_6. The chlorophyll (mol. wt = 892) content was calculated as about 11 % of the dry weight of cytochrome b_6.

The total lipid extract of cytochrome b_6 was then analyzed by thin-layer chromatography. No quinones were detected using the solvent system of 0.1 % diethyl ether in chloroform [24], and no carotenoids were found using 0.5 % diethyl ether in hexane [24]. In both systems a spot, colorless before charring, was found at the front where highly non-polar compounds such as hydrocarbons would be expected if present. When the total-lipid-extract of cytochrome b_6 and that derived

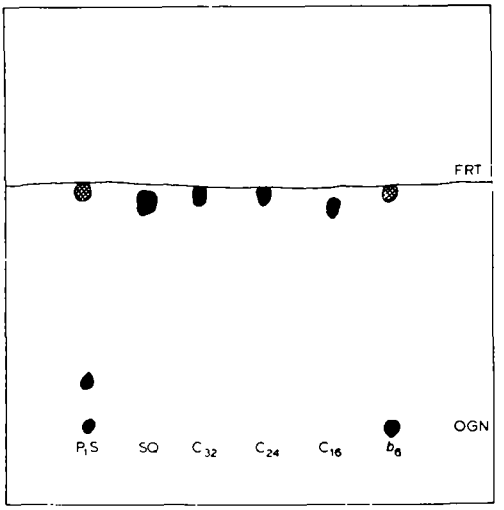


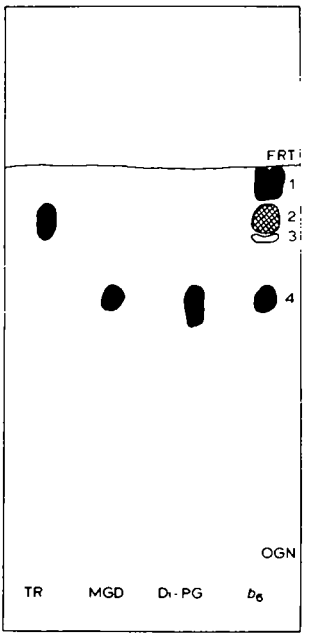
Fig. 3. Thin-layer chromatography on silica gel of the total lipid extract of cytochrome b_6 using the solvent system, heptane/benzene, (9 : 1, v/v). P_1S, chloroplast grana membranes; SQ, the C_{30} hydrocarbon squalane; C_{32}, dotriacontane; C_{24}, tetracosane; C_{16}, hexadecane; b_6, total lipid extract of pure cytochrome b_6. The spots were detected by charring with sulfuric acid. FRT and OGN designate the solvent front and origin, respectively.

from P_1S (chloroplast grana membranes as prepared by Whatley and Arnon [25]) were each chromatographed using the system, heptane/benzene (9 : 1, v/v) [26], each gave a spot at the front which was colorless before charring (Fig. 3). The saturated hydrocarbons dotriacontane (C_{32}) and tetracosane (C_{24}) spotted as standards on the thin-layer plate moved at the front; the C_{30} hydrocarbon squalane and hexadecane (C_{16}) moved slightly behind the front. (Squalene, not shown, moves with an R_F of 0.4 in this solvent system; chlorophyll, quinones, polar lipids and Triton X-100 (if present) remain at the origin; β-carotene and other carotenoids have an R_F of 0.2–0.3 and likely constitute the second spot in the P_1S column.)

It is tentatively concluded that the unknown neutral lipid of b_6 is possibly a hydrocarbon. Further work will be needed to confirm or negate this. From charring densities (estimated approximately by eye) this unknown neutral lipid accounts for only about 2–3 % (at most 5 %) of the dry weight of cytochrome b_6.

3. Polar lipids. Thin-layer chromatographic analysis on silica gel plates employed the solvent system of Nichols [27] (chloroform/methanol/water (82 : 25 : 3, v/v/v)). A control run (not shown) using a total lipid extract of P_1S (chloroplast grana membranes) gave good resolution of the polar lipids. Fig. 4 shows the pattern obtained with a total lipid extract of cytochrome b_6. Four spots were visible after charring. Spot 1 corresponds to chlorophyll and neutral lipid. Spot 2 moved with the same R_F as Triton X-100. Spot 3 was barely, but clearly, separated from the Triton spot and was a minor component (< 5 %). Spot 4 was the only major polar lipid present. It moved with the same mobility as monogalactosyl diglyceride (MGD) and diphosphatidyl glycerol (cardiolipin). The only other polar lipid which moves in this region in this solvent system is phosphatidic acid [27].

Specific stain tests were then used. The use of a phosphorus stain [15] re-



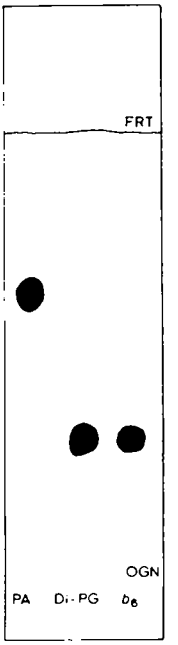


Fig. 4. Polar lipid analysis by thin-layer chromatography on silica gel of the total lipid extract of cytochrome b_6. The solvent system was chloroform/methanol/water (85 : 25 : 3, v/v/v). TR, Triton X-100; MGD, monogalactosyl diglyceride; di-PG, Diphosphatidyl glycerol (cardiolipin); b_6, total lipid extract of pure cytochrome b_6. Spot 1, chlorophyll (green before charring) and neutral lipid; spot 2, Triton X-100; spot 3, minor (<5 %) polar lipid; spot 4, major polar lipid of cytochrome b_6. Spots were detected by charring with sulfuric acid.

Fig. 5. Polar lipid analysis by thin-layer chromatography on silica gel of the total lipid extract of cytochrome b_6. The solvent system was diisobutyl ketone/acetic acid/water (45 : 25 : 5, v/v/v). PA, phosphatidic acid; Di-PG, diphosphatidyl glycerol (cardiolipin); b_6, total lipid extract of pure cytochrome b_6. Spots were detected by phosphorus staining. Upon charring with sulfuric acid an additional spot (not shown) occurred at the front. This spot was green before charring and was likely due to chlorophyll, which moves at the front in this system. (Triton X-100 remains at the origin in this system.)

vealed that the major polar lipid (spot 4) of cytochrome b_6 was phosphorus positive. As well, all the b_6 lipid spots were negative with respect to sugar (α-naphthol stain [16]) thus eliminating the possibility of monogalactosyl diglyceride.

To differentiate between cardiolipin and phosphatidic acid, thin-layer chromatography of the total-lipid extractof b_6 was performed using the solvent system, diisobutyl ketone/acetic acid/water (40 : 25 : 5, v/v/v) [28]. The results of Fig. 5 indicate that the phospholipid in cytochrome b_6 is cardiolipin rather than phosphatidic acid.

4. Phosphorus analysis of the lipids of cytochrome b_6. The digestion of the components in the lipid extract and analysis for inorganic phosphate is described in Methods. The analyses showed that there are 12 mol of phosphorus per mol of cytochrome b_6. Since there are 2 mol of phosphorus per mol of cardiolipin, it is concluded that there are 6 mol of cardiolipin per mol of cytochrome b_6. If we assume, for purposes of calculation, that the four fatty acids in cardiolipin are each 16 carbons

long, then the cardiolipin would have a molecular weight of about 1300. 6 mol of cardiolipin would then represent about 8000 g of cardiolipin per mol of cytochrome b_6 or about 13 % of the dry weight of the molecule.

Redox potential

From Fig. 6 (A and B) it can be seen that in the presence of 0.5 % Triton X-100 the E'_0 (at pH 7.0) for cytochrome b_6 is −0.080 V and −0.120 V (at pH 7.0) in the absence of Triton X-100. Using the equation

$$n = \frac{RT}{F} \cdot \frac{2.3 \; \mathrm{d} \log (\mathrm{ox})/(\mathrm{red})}{\mathrm{d}E_h},$$

a plot of log $Y/1-Y$ (i.e. log (ox)/(red)) vs E_h (where Y is the fractional oxidation and E_h the potential) gave values for n of 0.99 and 1.02 in the presence and absence of Triton X-100, respectively. Thus in both cases the oxidation-reduction of cytochrome b_6 was a one electron transfer at pH 7.0. Redox potential determinations performed at pH 8 in 0.05 M Tris·HCl buffer containing 0.5 % Triton X-100 (not shown) gave an E'_0 value (pH 8) of −0.084 V with a one-electron-transfer (n 1.01) for the reaction. Thus the E'_0 of b_6 in the pH 7–8 region is independent of pH.

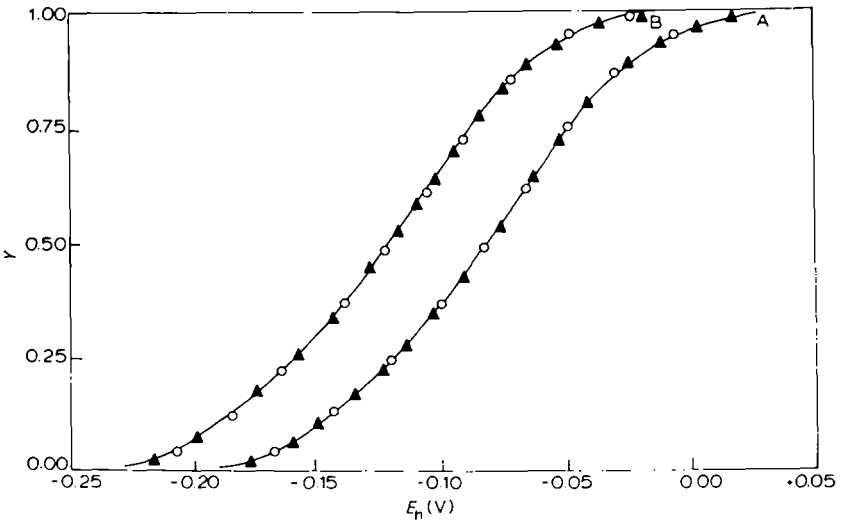


Fig. 6. Oxidation-reduction equilibrium curves of cytochrome b_6 in 0.05 M phosphate buffer, pH 7.0, in the presence and absence of Triton X-100. A, 0.5 % Triton X-100; B, no Triton X-100; ○, reductive titration with $Na_2S_2O_4$; ▲, oxidative titration with $K_3Fe(CN)_6$. Ordinate (Y) is fractional oxidation value ((b_6 oxidized)/(total b_6 present)); abscissa is potential referred to the hydrogen electrode, in volts.

DISCUSSION

Triton X-100/4 M urea (pH 8) as an extraction medium for membrane lipoproteins

The purification procedures for chloroplast cytochromes b_{559} and b_6 both employed 2 % Triton X-100/4 M urea (pH 8) as an initial extraction medium. Examination of molecular composition of disc-electrophoretically homogeneous prepara-

tions of each cytochrome has now found each to be a lipoprotein of relatively simple lipid composition (4 lipids in each) in contrast to the 20 known lipids (and additional unknowns) present in spinach chloroplast lamellae [29]. The lipid composition of cytochrome b_6 was however different from that in b_{559} (ref. 5) both in terms of total lipid present in the molecule (about 33 % for b_6 vs 56 % for b_{559}) and in the identity of individual lipids. The major unknown polar lipid of b_{559} (which was not a phospholipid) and the neutral lipid β-carotene were not present in cytochrome b_6. Chlorophyll *a* was the only lipid found common to both cytochrome preparations. As yet, we have not shown, however, that the lipids of either b_6 or b_{559} are essential for its structure or function.

Lack of chlorophyll a absorption in the b_6 spectrum

As in the case of cytochrome b_{559}, the absorbance at 662 nm by chlorophyll *a* does not appear in the absolute absorption spectrum of cytochrome b_6 in aqueous solution [3], despite the relatively large amount of chlorophyll *a* (7 mol per mol of cytochrome b_6) which can be extracted from the same b_6 preparations via organic solvents and subsequent spectrophotometric assay in acetone. This lack of chlorophyll *a* absorption in the b_6 spectrum thus poses the same enigma observed for cytochrome b_{559}.

Mol. wt, $\bar{v}$ and Triton X-100

The hydrodynamic molecular weight, determined in the presence of Triton X-100, is likely that of a monomeric cytochrome b_6 · Triton complex. The close agreement of this molecular weight (67 000) with the equivalent weight per mol of heme (60 000, after correction for a maximum content of 7000 g of Triton) indicates that most (90 %) of the hydrodynamic molecular weight is contributed by cytochrome b_6 rather than Triton. The experimental $\bar{v}$ of 0.84 (beyond the usual range of 0.72–0.76 for molecules composed entirely of protein) also can be explained by the composition of b_6 as a lipoprotein without invoking large quantities of bound Triton: assuming a partial specific volume of 0.73 for its protein content and a $\bar{v}$ of about 1.0 for its lipid content, a calculated $\bar{v}$ of 0.82 (i.e. $0.67 \cdot 0.73 \div 0.33 \cdot 1.0$) is obtained, in substantial agreement with the observed value of 0.84. The small amount of bound Triton (10 % or less by wt for b_6 and about 7 % for b_{559}) present during ultracentrifuge molecular weight determinations also is probably not in micellar form (assuming a micelle size for Triton of at least 7000). For both cytochromes b_{559} and b_6, Triton thus produced no serious errors in determination of molecular weight and $\bar{v}$ for these lipoproteins. In contrast, the assumption for a membrane protein of a $\bar{v}$ of 0.73 (i.e. an implicit assumption that the molecule contains only protein) without finding $\bar{v}$ experimentally, seriously reduces the molecular weight value, because $\bar{v}$ appears in the Svedberg equation as a $(1-\bar{v}\rho)$ term rather than as a simple factor. For cytochrome b_6, an assumed $\bar{v}$ of 0.73 (rather than 0.84) would produce a molecular weight 41 % lower (39 600 vs 67 000); for b_{559} a $\bar{v}$ of 0.73 (rather than 0.91) would give a specious molecular weight 67 % lower (39 000 vs 117 000).

E'_0 of pure b_6 vs membrane-bound b_6

Since no acceptable enzyme assay exists for the component, the redox potential of cytochrome b_6 offers the only functional index for assessing whether its

molecular integrity has been retained after isolation from chloroplast membranes. We will presume, as is commonly done for any redox component, that the E'_0 for the membrane-bound component is correct, despite several potentially serious hazards of electrochemical logic: other, unknown membrane components may be participants in the redox equilibrium reaction and biological membranes may either bind the added redox titrants or offer permeability barriers to them. If E'_0 values determined in situ offer little assurance of accuracy, E'_0 values of tightly-bound membrane proteins determined in vitro after purification have heretofore been even more suspect because of the possibly drastic methods employed to extract and purify these components. As examples, cytochrome *b* in beef heart mitochondria had a potential of about 0.06 V which dropped to $E'_0 = -0.34$ V, almost a 0.3 V drop, for the purified component. Of interest is that the purification procedure employed the anionic detergents deoxycholate and sodium dodecylsulfate, and the purified cytochrome *b* preparation contained negligible lipid content and was relatively insoluble [30]. A similar drop of about 0.3 V occurred when particulate *Escherichia coli* cytochrome b_1 (E'_0 of about 0.0 V) was solubilized via prolonged sonication and purified to an octomeric form ($E'_0 = -0.34$ V) [31]. To our knowledge, whenever a tightly-bound redox component has shown a change in its E'_0 value after isolation, the change has always been a drop, never an increase. E'_0 values reported for particulate cytochrome b_6 consist of -0.060 V [32], recalculated later as 0.0 V [33], -0.180 (± 0.02) V [34] and -0.100 V [35]. E'_0 for pure cytochrome b_6 reported in this article is about -0.08 V (for the soluble monomeric cytochrome in 0.5% Triton) and -0.120 V (for the soluble, aggregated cytochrome in the absence of Triton). It would appear that the E'_0 value for b_6 was essentially unchanged following extraction with Triton X-100/4 M urea (pH 8) and subsequent purification; or at least no change of the order of 0.3 V occurred. Also, Triton present during the redox determination produced no marked change in E'_0 (a slight rise in E'_0 of 0.04 V) indicating, possibly, that this nonionic detergent produced little or no change in the b_6 molecule. We have previously shown [3] that purified b_6 retains all of its known spectrophotometric properties, which are less sensitive indices of molecular integrity than its redox potential.

The extraction and purification of tightly-bound membrane proteins with retention of their original molecular properties has been a difficult art, and there is insufficient evidence at present to precisely correlate changes in molecular properties after purification with either the methods of isolation or with the molecular composition of the pure preparations. (Very few membrane proteins have in fact been purified to electrophoretic or chromatographic homogeneity, and data on total molecular composition of such pure preparations is scarce.) A working hypothesis would propose (1) that tightly-bound membrane proteins are in fact lipoproteins with lipid being essential to structure and function; (2) that isolation steps which detach lipids intrinsic to the molecule (e.g. via the use of sodium dodecylsulfate, prolonged sonication, lipases, autolysis and possibly cholate and deoxycholate) release the protein with altered catalytic properties either in a soluble form when the protein is sufficiently polar and in an insoluble form which requires strong anionic detergents for resolubilization for proteins of relatively non-polar amino acid composition; (3) that in the latter case readdition of specific or non-specific lipids may restore the original molecular (lipoprotein) properties; (4) that the extraction medium

of Triton X-100/4 M urea (pH 8) solubilizes the intact lipoprotein without alteration of molecular composition and catalytic properties.

ACKNOWLEDGEMENTS

We thank Harinder S. Garewal for advice in lipid methodology, Dr Edward Meighen for advice in ultracentrifuge measurements and Craig Southwell for expert, part-time, technical assistance. The Graduate Faculty of McGill University contributed part of the cost of the Cahn microbalance. This project was supported by operating grant MA4385 (to A.R.W.) from the Medical Research Council of Canada.

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