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The Cytochrome Subunit of the Photosynthetic Reaction Center from *Rhodopseudomonas viridis* Is a Lipoprotein†

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ABSTRACT: Using automated procedures for Edman degradation and for the identification of the derived phenylthiohydantoin–amino acids of the cytochrome subunit in the photosynthetic reaction center from the purple bacterium *Rhodopseudomonas viridis*, the phenylthiohydantoin derivative of the first amino acid could not be detected. However, the N-terminus of the cytochrome subunit was not blocked, and a phenylthiohydantoin derivative could be isolated after manual Edman degradation. It contained two kinds of covalently bound fatty acids (18:OH and 18:1); the pattern of molecular species obtained by reversed-phase high-performance liquid chromatography and the specific fatty acid composition of the separated species were only consistent with two ester bonds per molecule. Mass spectroscopic analysis provided evidence that the N-terminal amino acid was a cysteine which was linked to a diglyceride via a thioether bond, as has been first described for the N-terminus of the major outer membrane lipoprotein from *Escherichia coli* [Hantke, K., & Braun, V. (1973) *Eur. J. Biochem. 34*, 284–296]. However, the cytochrome subunit lacked the acylation by a fatty acid at the N-terminal amino group. In addition, the DNA coded for a cysteine as the N-terminal amino acid and for a preceding peptide sequence characteristic for signal sequences of bacterial lipoproteins. The fatty acids seem to anchor the cytochrome subunit in the photosynthetic membrane which is an invagination of the inner bacterial membrane.

Photosynthetic reaction centers are complexes of pigments and integral membrane proteins which catalyze the primary steps in photosynthesis. Upon absorption of light, an electron is transferred across the photosynthetic membrane [for a review, see Okamura et al. (1982)]. Recently, the photosynthetic reaction center from the purple bacterium *Rhodopseudomonas viridis* was crystallized (Michel, 1982). The subsequent X-ray structure analysis allowed the calculation of an electron density map at 3-Å resolution. As a first result, the arrangement of the photosynthetic pigments was presented (Deisenhofer et al., 1984). For a reliable interpretation of the protein part, the amino acid sequences of the four protein subunits (H, M, L, and a cytochrome of the *c* type) were needed. The amino acid sequences of the H, M, and L subunits were elucidated by sequencing the DNA coding for these subunits and by chemically sequencing the isolated protein subunits and fragments therefrom (Michel et al., 1985, 1986). The amino acid sequence of the cytochrome subunit has now been completely determined (K. A. Weyer et al., unpublished results). During the protein sequence work on the cytochrome subunit, the unusual behavior of the N-terminal amino acid led us to further investigate its structure. In this paper, we provide evidence that the N-terminal amino acid is a cysteine linked to a diglyceride via a thioether bond as has been described for

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the major outer membrane lipoprotein of *Escherichia coli* (Hantke & Braun, 1973) and other bacterial lipoproteins [e.g., see Yu et al. (1986) and Pugsley et al. (1986)]. These findings establish that the cytochrome subunit of the photosynthetic reaction center from *Rps. viridis* is a lipoprotein.

Materials and Methods

Materials. Doubly distilled water was used. *N,N*-Dimethyl-*N*-allylamine and phenyl isothiocyanate were purchased from Pierce (Rockford, IL). The sources of other materials used have been described in a previous paper (Michel et al., 1985).

Isolation of the Cytochrome Subunit. Photosynthetic reaction centers from *Rps. viridis* were isolated as described (Michel et al., 1985). The cytochrome subunit was isolated by molecular sieve chromatography as described for the L and M subunits (Michel et al., 1985); the fractions containing the cytochrome subunits were pooled and rechromatographed.

Performic Acid Oxidation. The cytochrome subunit was precipitated at 4 °C by adding ethanol to a final concentration of 80%. The precipitate was washed once with 80% ethanol and 3 times with concentrated ethanol. The pellet was dried in vacuum for 15 min and dissolved (100 nmol/mL) in a mixture of 95 parts of 99% formic acid and 5 parts of 30% hydrogen peroxide (mixture 1 h preincubated at room temperature). After 2 h at 0 °C, the solution was diluted 1:10 with water and lyophilized.

Manual Edman Degradation and Separation of Products. Manual Edman degradation was performed according to Peterson et al. (1972) with the following modifications: One hundred nanomoles of the cytochrome subunit was precipitated from the sodium dodecyl sulfate (SDS)[1] solution as described above. For washing and SDS removal, the centrifuged pellet was successively extracted with the following (5 mL of each): ethanol/water (8:2 v/v) (once), ethanol (once), acetone/acetic acid/triethylamine/water (85:5:5:5 v/v) (Henderson et al., 1979; once), acetone (once), ethanol (twice), and 0.4 M *N,N*-dimethyl-*N*-allylamine adjusted to pH 9.5 with trifluoroacetic acid in 1-propanol/H_2O (3:2) (DMA buffer) (once). The pellet was suspended in 2 mL of DMA buffer. Phenyl isothiocyanate (400 μL) was added, and the coupling was performed for 25 min at 50 °C with occasional shaking (all reactions were done under nitrogen). After three extractions with 2 mL of benzene, the suspension was dried under a stream of nitrogen and then for 15 min in vacuum. Trifluoroacetic acid (500 μL) was added, and cleavage was performed for 12 min at 50 °C. The acid was evaporated with a stream of nitrogen, and the residue was extracted 3 times with 1.5 mL of methanol. The extracts were pooled and dried under a stream of nitrogen. Trifluoroacetic acid (20% v/v in water, 300 μL) was added, and the suspension was kept at 80 °C for 20 min. The acid was removed from the PTH derivative under a stream of nitrogen.

For separation by HPLC, the residue was dissolved in 150 μL of methanol/chloroform (9:1 v/v), and 50-μL portions were injected on a Lichrospher CH-8 column (Merck); the components were eluted with acetonitrile/water (99:1 v/v) and detected at 200 or 254 nm.

Gas Chromatography/Mass Spectrometry (GC/MS) Methods. The PTH derivative of the N-terminal amino acid was transesterified in methanol containing 1 N HCl in evacuated, sealed ampules at 80 °C for 2 h. The samples were diluted by adding 4 parts of water, and the fatty acid methyl esters were extracted 3 times with 1 volume of *n*-hexane (Merck, "zur Rückstandsanalyse"). The extracts were pooled, and the solvent was evaporated under a stream of nitrogen. Samples containing hydroxy fatty acid methyl esters were analyzed as their trimethylsilyl ethers.

Gas chromatographic analysis of the fatty acid methyl esters was done by using a 30-m DB 1701 fused silica capillary (J & W Scientific, Orange Vale, CA) and a 50-m CP Sil 88 fused silica capillary from Chrompac (Middelburg, The Netherlands). The GC/MS computer system consisted of a Carlo Erba 2101 gas chromatograph coupled with a CH7A (MAT) mass spectrometer operating in the electron-impact mode with an ionization potential of 70 eV and a Spectrosystem 200 data system (Finnigan-MAT). Ratios of fatty acids were determined by measuring the areas under GC peaks created from the total ion current with help of the data system.

For fast atom bombardement (FAB), the PTH derivative of the N-terminal amino acid was dissolved in 1 μL of trifluoroacetic acid and mixed with about 2 μL of glycerol. One microliter of the mixture was spread on a copper target. Spectra were taken on a MAT 312 mass spectrometer connected with the Spectrosystem 200 data system.

Protein Sequencing. For automated Edman degradation, the ethanol-precipitated and washed cytochrome subunit was dissolved in 95% formic acid. Sequencing was performed as previously described (Michel et al., 1985).

For studies on the yields of PTH–amino acids of the first three Edman degradation steps, the reaction center complex was precipitated from the detergent solution and extracted with ethanol by using the procedure described for the cytochrome subunit. In this experiment, sequencing was done with an Applied Biosystems gas phase 470 A sequenator.

DNA Sequencing. Isolation of genomic DNA, cloning, and DNA sequence analysis were done as previously described (Michel et al., 1986).

Table I: Relative Yields of PTH Derivatives from Edman Degradation of an Ethanol-Extracted Reaction Center Complex[a]

	cytochrome subunit	M subunit	L subunit
step 1	X	A, 95% (71%)	A, 95% (71%)
step 2	F, 75% (67%)	D, 53% (35%)	L, 74% (67%)
step 3	E, 85% (67%)	Y, 74% (52%)	L, 97% (81%)

[a] The relative yields of the PTH derivatives refer to the amount of applied protein; 2.1 nmol of ethanol-extracted reaction centers was sequenced in each experiment by automated Edman degradation. The reaction centers had a purity index (OD_{280}/OD_{830}) of 2.0 and 2.1 (values in parentheses): respectively. Other amino acids with a yield higher than 6% could not be detected in the first degradation step. A PTH derivative of the N-terminal amino acid (X) of the cytochrome subunit could not be detected.

Results

Characterization of the N-Terminal Amino Acid. After the automated Edman degradation, a PTH derivative of the N-terminal amino acid from the cytochrome subunit could not be detected by using standard HPLC methods. Also, after performic acid oxidation of the protein, a PTH derivative could not be detected. Therefore, a cysteine in its unmodified form or forming a disulfide bridge could be excluded. A study of the yields of the PTH–amino acids over the first three degradation steps using an ethanol-extracted reaction center complex (see Table I) showed that for the cytochrome subunit yields of PTH–amino acids in degradation steps 2 and 3 were of the same size as that of the corresponding steps for the M and L subunits. The N-terminus of the H subunit is blocked

[1] Abbreviations: GC/MS, gas chromatography coupled to mass spectrometry; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; FAB, fast atom bombardment.

Table II: Percentages of 18:OH and 18:1 Fatty Acid Ratios in the PTH Derivative of the N-Terminal Amino Acid of the Cytochrome Subunit from the Photosynthetic Reaction Center and the Major HPLC Fractions Therefrom[a]

sample[b]	fatty acid type	
	18:OH	18:1
unseparated mixture	53	47
peak A	91	9
peak B	52	48
peak C	2	98

[a] Other fatty acids (16:0, 16:1, 17:0, 18:0, and 20:0) were present in a total amount lower than 5% of the sum of 18:OH and 18:1 fatty acids in the original unseparated mixture and in fractions A, B, and C. The amount of fatty acids in the minor HPLC peaks was insufficient for a reliable determination. [b] Peaks A, B, and C refer to Figure 1.

by *N*-formyl (Michel et al., 1985), and, therefore, it could not be degraded. This result clearly indicated that the N-terminus of the cytochrome subunit was unblocked.

Attempts to isolate an N-terminal peptide after chemical or enzymatic cleavage failed presumably due to the high hydrophobicity of the N-terminal peptides, which could not be recovered from reversed-phase HPLC colums.

Upon manual Edman degradation, a thiazolidine derivative could be extracted with methanol. After evaporation of the solvent, this derivative could not be redissolved in 20% trifluoroacetic acid. After transesterification in methanol/HCl, primarily two types of fatty acids could be detected in the PTH derivative by GC/MS ("unseparated mixture" in Table II). Using reversed-phase HPLC, we obtained three major fractions of the PTH derivative as detected by the absorption of the PTH group at 254 nm (Figure 1). Fatty acid analysis of the major HPLC fractions after transesterification with methanol showed that peak A contained mainly 18:OH fatty acids, peak B a nearly equimolar mixture of 18:1 and 18:OH fatty acids, and peak C mainly 18:1 fatty acids (Table II). The 18:OH fatty acids were a nearly equimolar mixture of 11-hydroxy- and 12-hydroxystearic acid, as shown by GC/MS of the trimethylsilyl ethers of their methyl esters. The 18:1 fatty acids were a mixture of *cis*-oleic, *cis*-vaccenic, and another not yet identified fatty acid in comparable amounts. The reversed-phase HPLC system used was unable to separate species differing only in the position of double bonds or of the hydroxyl groups.

The original unseparated mixture contained two major types of fatty acid residues (18:OH and 18:1), which were present in nearly equimolar amounts. A molecule (M) which is linked by a definite number of covalent bonds to two different types (A and B) of fatty acids should give rise to a definite number of compounds. A molecule linked with two bonds to two different types of fatty acid residues should produce four different compounds (MAA, MAB, MBA, and MBB). However, since MBA and MAB must be expected to possess very similar physical properties, only three separable "species" would be observed. Two compounds (MAA and MBB) will only contain fatty acids of the same type, and one species (MAB and MBA) will contain both types of fatty acids. If the two types of fatty acids are present in equimolar amounts, the ratio of the species containing only members of the same type (MAA or MBB) to the species containing both types of fatty acids (MAB plus MBA) should be 1:2, assuming a statistical distribution of the fatty acid residues.

By measuring the areas of peaks A, B, and C from several preparations, ratios from 21:47:32 to 30:46:24 were obtained, just approximating the 1:2:1 ratio. It was concluded that the PTH derivative of the N-terminal amino acid had two ester bonds to fatty acid residues.

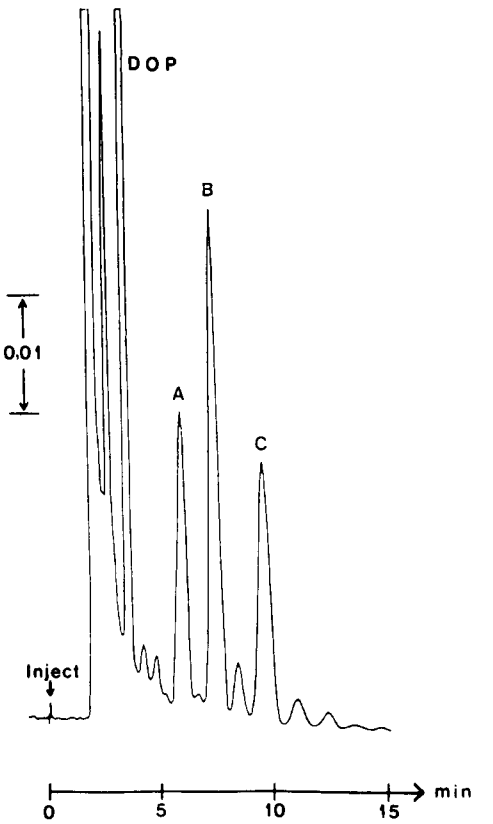

FIGURE 1: Elution profile for the PTH derivatives of the N-terminal amino acid from the *Rps. viridis* reaction center cytochrome subunit (reversed-phase HPLC column, monitored at 254 nm). When monitored at 200 nm (not shown), the elution profile was the same. For experimental conditions, see Materials and Methods. An aliquot corresponding to about 33 nmol of degraded cytochrome subunit was injected in 50 μL of 90% methanol and 10% chloroform. The dioctyl phthalate peak as verified by electron-impact mass spectroscopy is marked by "DOP".

Electron-impact mass spectra using the direct insertion technique taken from the material of peaks A, B, and C showed a fragment at m/z 135 which is typical for PTH–amino acids. A fragment at m/z 204 could principally indicate PTH derivatives of cysteine and S-modified derivatives therefrom, glutamic acid, aspartic acid, and serine (Hagenmaier et al., 1970). Assuming that there were also two fatty acid residues in the PTH derivative of the N-terminal amino acid, a modified cysteine was a good candidate for it, since such a modification had been described earlier (Hantke & Braun, 1973). N-Terminal cysteine modified by a diglyceride and acylated with a fatty acid residue was shown to be the N-terminal amino acid of the major outer membrane lipoprotein of *E. coli*. Additional bacterial lipoproteins have been identified, but in no case has a native lipoprotein with a free N-terminal amino group been described.

Further support for our preliminary structure came from the FAB mass spectra obtained with the material from peak C. This material contained only 18:1 fatty acids and was the purest fraction (Table II). The calculated relative molecular mass for a PTH–cysteine modified by a diglyceride containing two 18:1 fatty acids is 840 ($C_{49}H_{80}N_2O_5S_2$). This value coincided with the obtained relative mass of 841 for the quasi-molecular ion $(M + H)^+$ of this component[2] (Figure 2). Some diagnostically important ions were also present in the spectra which had similarities to the FAB mass spectra of phospholipids (Fenwick et al., 1983; Ohashi, 1984). The ion at m/z 577 can be due to the loss of an acyl chain by α-

[2] In the FAB technique, ionization usually occurs via protonation.

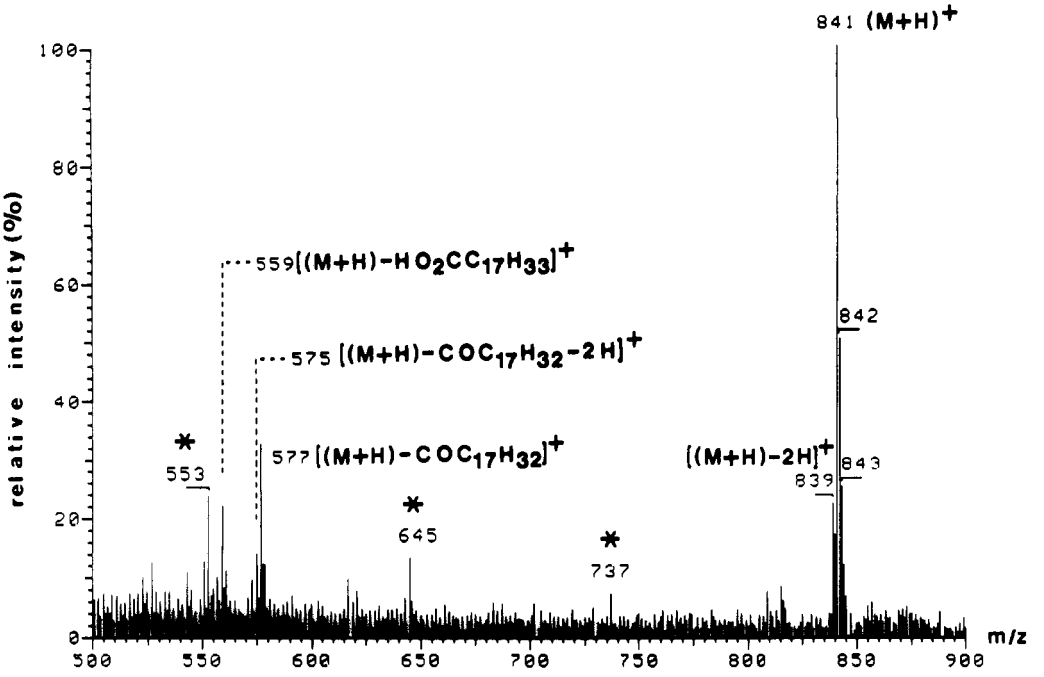

FIGURE 2: FAB mass spectrum (Xe, 8 kV) of peak C from Figure 1 (containing 18:1 fatty acids). Peaks belonging to the glycerol matrix are marked by asterisks.

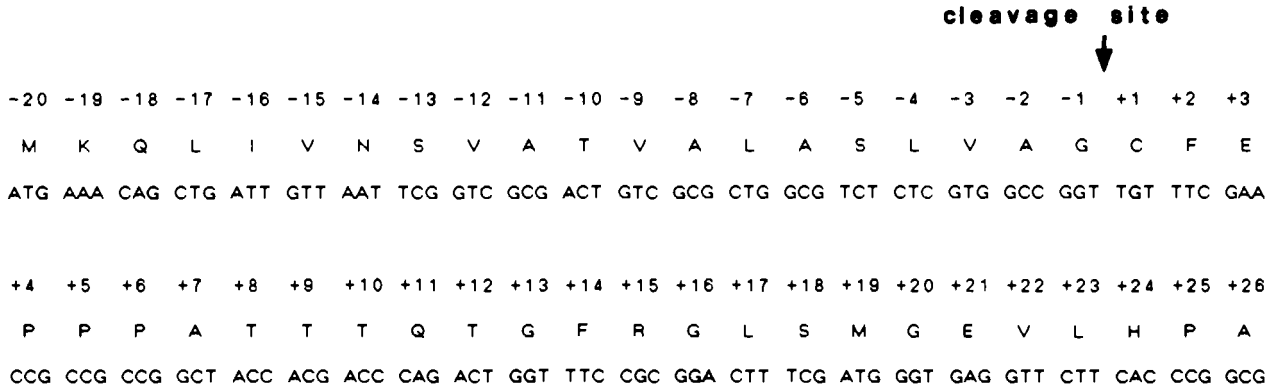

FIGURE 3: DNA sequence (and amino acid sequence derived by translation of the DNA sequence) coding for the signal peptide and the first 26 amino acids of the mature cytochrome subunit from *Rps. viridis*.

cleavage with associated hydrogen rearrangement. The ion at m/z 559 can be explained by the loss of an intact fatty acid. The presence of ions at 2 daltons below the expected m/z values $\langle[(M + H) - 2H]^+$, $[(M + H) - OCC_{17}H_{32} - 2H]^+\rangle$ is a typical feature of FAB spectra obtained by using a glycerol matrix (Fenwick et al., 1983).

Dissolving the PTH derivative in trifluoroacetic acid prior to the addition of the glycerol matrix was necessary to get a FAB mass spectrum of high intensity.

Characterization of the Signal Peptide. The DNA sequence coding for the cytochrome signal peptide and the first 77 amino acids of the mature cytochrome was obtained as described (Michel et al., 1986). The DNA indeed coded for cysteine as the N-terminal amino acid of the mature cytochrome subunit. Figure 3 presents the DNA sequence coding for the signal peptide and the first 26 amino acids of the mature cytochrome subunit.

The peptide preceding the mature N-terminus is 20 amino acids long and exhibits all features characteristic for a signal peptide [for a review, see Duffaud et al. (1985)]. It contains a positive charge in the N-terminal region, followed by a highly hydrophobic region of 15 amino acids. It contains four valines, three alanines, three leucines, and one isoleucine. The most striking characteristic for bacterial prolipoproteins is the nature of amino acids +1 to −3. This sequence is known to be the recognition site for the signal peptidase II (Lai et al., 1981; Inouye et al., 1983; Politt et al., 1986). Figure 4 compares the cytochrome subunit signal sequence and the signal sequences of 10 lipoproteins for which the signal sequences are available. Signal peptidase II cleaves the peptide bond between the cysteine [which has already been linked to the diglyceride; see Inouye et al. (1983)] and its preceding amino acid. With only one exception, the preceding amino acid is glycine. Alanine is found in position −2 nine times, including the proapocytochrome. In nine cases, a leucine is in position −3, which is substituted by valine in the proapocytochrome. Leucine at position −4 is conserved between seven prolipoproteins, including the proapocytochrome.

Discussion

Following the work of Hantke and Braun (1973) on the major outer membrane lipoprotein of *E. coli*, several proteins from eubacteria were found to possess a cysteine, modified by acylation of the amino group and condensation of a diglyceride to the side chain, as the N-terminal amino acid (see Figure 4 and references cited therein). In the Gram-negative bacteria, these lipoproteins seem to be anchored to the outer membrane with two exceptions: Ichihara et al. (1982) reported that two

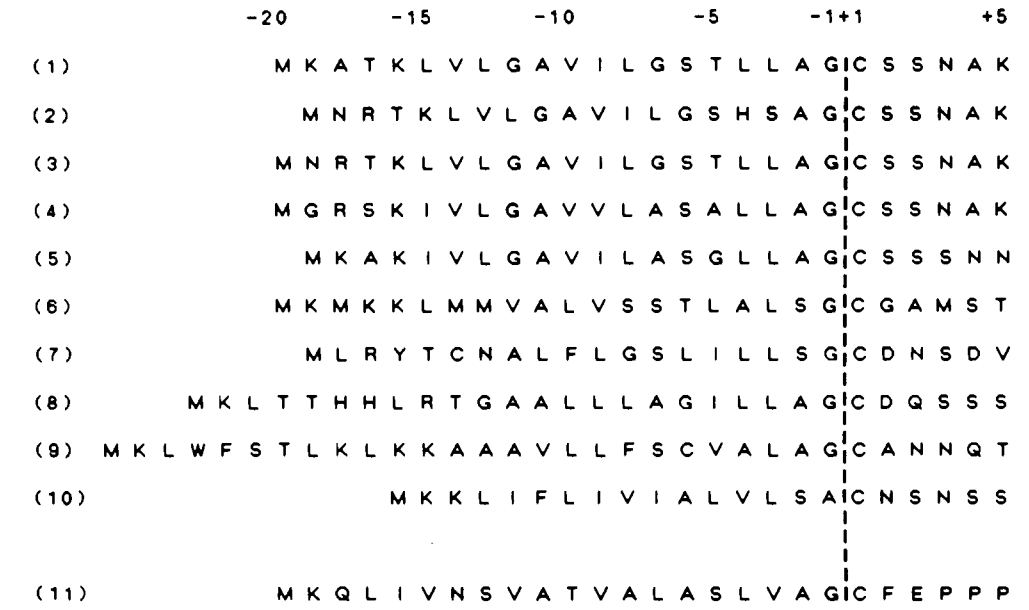

FIGURE 4: Comparison of the signal and N-terminal sequences of the cytochrome subunit from the *Rps. viridis* photosynthetic reaction center with those from other bacterial lipoproteins. (1) Major outer membrane lipoprotein (LP) *E. coli* (Nakamura & Inouye, 1979); (2) LP *Serratia marcescens* (Nakamura & Inouye, 1980); (3) LP *Erwinia amylovora* (Yamagata et al., 1983); (4) LP *Morganella morganii* (Huang et al., 1983); (5) LP *Proteus mirabilis* (Ching & Inouye, 1985); (6) TraT protein *E. coli* (Ogata et al., 1982); (7) pullulanase *Klebsiella pneumoniae* (Chapon & Raibaud, 1985); (8) lipoprotein 28 *E. coli* (Yu et al., 1986); (9) penicillinase *Bacillus licheniformis* (Neugebauer et al., 1981); (10) penicillinase *Staphylococcus aureus* (McLaughlin et al., 1981); (11) cytochrome subunit *Rps. viridis* photosynthetic reaction center. The cleavage sites, which are not always experimentally proven, are indicated by a dotted line.

minor lipoproteins of *E. coli* are localized in the cytoplasmic membrane. One of these lipoproteins is probably identical with "lipoprotein 28", which could be cloned and expressed. The function of this lipoprotein is not known (Yu et al., 1983). Since the photosynthetic membranes of the purple bacteria are invaginations of the cytoplasmic membrane, the cytochrome subunit of the photosynthetic reaction center from *Rps. viridis* is a member of this new class of lipoproteins from Gram-negative bacteria, which are anchored to the cytoplasmic membrane. However, one important difference may exist: To our knowledge, the cytochrome subunit is the only bacterial lipoprotein which has been found not to become N-acylated at its N-terminus under standard growth conditions. Possible reasons for the absence of N-acylation are the following: (i) The absence of the responsible *N*-acyltransferase in *Rps. viridis* may mean that the major outer membrane lipoprotein is also not acylated. (ii) The *N*-acyltransferase which acylates the outer membrane lipoprotein may not be able to recognize the N-terminus of the cytochrome subunit, e.g., due to a rapid association of the cytochrome subunit with the residual reaction center complex. (iii) The *N*-acyltransferase may be localized exclusively in the outer membrane of *Rps. viridis*. Therefore, the N-terminus of the cytochrome subunit would not be accessible to the *N*-acyltransferase. It is interesting to note that no report exists stating whether the two minor lipoproteins from the inner membrane of *E. coli* are N-acylated. Therefore, absence of N-acylation may be a more general phenomenon for cytoplasmic bacterial lipoproteins. (iv) The *N*-acyltransferase may be localized elsewhere in the cytoplasmic membrane but not at those sites where the cytochrome is incorporated into the photosynthetic membrane.

Further experiments are required to discriminate between the various possibilities.

The X-ray structure analysis of the intact reaction center complex (Deisenhofer et al., 1985) together with biochemical experiments (Brunisholz et al., 1984) shows that the protein part of the cytochrome subunit is localized in the periplasmic space. The X-ray structure analysis also indicate that the N-terminus of the cytochrome subunit is located near the presumed periplasmic surface of the membrane [see Figure 1a in Deisenhofer et al. (1985)]. The fatty acids seem to anchor the cytochrome in the photosynthetic membrane. However, since there are substantial polar interactions between the cytochrome subunit and the other three protein subunits of the reaction center, the fatty acids cannot be expected to contribute substantially to the integrity of the whole reaction center. Their main function may be to retain the cytochrome subunit bound to the photosynthetic membrane prior to its association with the residual reaction center complex.

The fatty acids are not visible in the high-resolution electron density map due to disorder in the crystals. The only way to firmly prove their existence was by protein–chemical methods, as described in this paper. It is interesting to note that without the protein–chemical sequence work, the presence of these fatty acids might have escaped our attention despite the availability of a high-resolution electron density map and the DNA sequence.

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Dependence of the Activity of Beef Heart Mitochondrial Adenosinetriphosphatase on the Properties of the Catalytic Metal Ion†

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ABSTRACT: Several divalent metal ions were used as kinetic probes of the beef heart mitochondrial adenosinetriphosphatase (F_1) under a variety of conditions, and the relationship between the properties of the catalytic metal ion and the catalytic activity of the enzyme was examined. V_{max} for ATP hydrolysis was largest when metal ions characterized by intermediate values of acidity of coordinated water molecules (pK_a) and metal-nucleotide stability constants (K_{stab}) were present. As temperature increased, the peak of V_{max} vs. pK_a (or K_{stab}) shifted to lower initial values of pK_a or K_{stab}. The solvent deuterium isotope effect on V_{max} (^DV) was normal and largest when the metal ion present during F_1-catalyzed ATP hydrolysis was most acidic and the metal nucleotide stability constant was large. When an active site tyrosine on F_1 was nitrated, V_{max} was most affected when the metal ion present was least acidic and the metal nucleotide stability constant was small. The isotope effect on V/K ($^DV/K$) was normal, small, and apparently independent of the metal ion present. ADP inhibition of F_1-catalyzed ATP hydrolysis is competitive, and the K_i is independent of the metal ion present. The degree of P_i inhibition of F_1 is dependent on the metal ion present. The inhibition by P_i is competitive at low temperature and becomes noncompetitive as temperature increases. These and previous results support a mechanism whereby a water molecule coordinated to the metal ion of an enzyme-bound γ-monodentate metal-ATP complex is deprotonated to begin a series of events whereby a β,γ-bidentate metal-ATP complex is produced. Upon hydrolysis, the bond between the metal ion and the β-phosphate of ADP in the P_i-metal-ADP complex is broken before products (ADP and metal-P_i) are released.

The regulatory and catalytic mechanisms of beef heart mitochondrial ATPase (F_1)[1] have been extensively studied for many years. Several recent reviews discuss a wide variety of the regulatory and catalytic aspects of F_1 as well as the structure of this multisubunit complex (Cross, 1981; Pedersen, 1982; Amzel & Pedersen, 1983; Wang, 1983).

It has been shown that beef heart mitochondrial F_1 requires a divalent metal ion for catalytic activity and that any of several different ones will suffice. Furthermore, it has been shown that the rate of catalysis depends on the identity of the

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[1] Abbreviations: ATPase, adenosinetriphosphatase; F_1, soluble beef heart mitochondrial ATPase; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; ATPβS, adenosine 5'-*O*-(2-thiotriphosphate); DCCD, dicyclohexylcarbodiimide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; P_i, inorganic phosphate; CHES, 2-(cyclohexylamino)-ethanesulfonic acid; HPLC, high-performance liquid chromatography; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole.

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