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Amino-Terminal Sequences of the L, M, and H Subunits of Reaction Centers from the Photosynthetic Bacterium *Rhodopseudomonas sphaeroides* R-26†

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ABSTRACT: We have determined the sequence of the 25–28 amino-terminal residues of the three subunits, L, M, and H, of the membrane-bound reaction center protein of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26. The sequences are as follows: L, H_2N-Ala-Leu-Leu-Ser-Phe-Glu-Arg-Lys-Tyr-Arg-Val-Pro-Gly-Gly-Thr-Leu-Val-Gly-Gly-Asn-Leu-Phe-Asp-Phe-(His)-Val-; M, H_2N-Ala-Glu-Tyr-Gln-Asn-Ile-Phe-Ser-Gln-Val-Gln-Val-Arg-Gly-Pro-Ala-Asp-Leu-Gly-Met-Thr-Glu-Asp-Val-Asn-Leu-Ala-Asn-; H, H_2N-Met-Val-Gly-Val-Thr-Ala-Phe-Gly-Asn-Phe-Asp-Leu-Ala-Ser-Leu-Ala-Ile-Tyr-Ser-Phe-Trp-Ile-Phe-Leu-Ala-X-Leu-Ile-. The H sequence, especially after the aspartyl residue at position 11, is rich in hydrophobic residues, consistent with the possibility that this section of the polypeptide chain is located within the membrane. The L sequence is hydrophilic near the amino terminus and then becomes moderately hydrophobic. The M sequence is of average polarity.

The primary electron transfer event in the purple photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26 is mediated by a membrane-bound reaction center composed of protein and a number of prosthetic groups including bacteriochlorophyll, bacteriopheophytin, ubiquinone, and iron [reviewed by Feher & Okamura (1978) and Okamura et al. (1982)]. The reaction center protein is isolated by treatment of chromatophore membranes with a nonionic detergent, *N,N*-dimethyllaurylamine oxide (LDAO).[1] It consists of three polypeptide chains, each of M_r ~30 000 and present in equimolar amounts. Two of these subunits, L and M, appear to be essential for photochemical activity; the function of the third, H, is not understood at present.

Initially, the subunits were isolated by elution from polyacrylamide gels after electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$) (Okamura et al., 1974). More recently, a two-step procedure has been developed (Feher & Okamura, 1978; Rosen, 1979) in which the H subunit is dissociated from a complex of L and M by treatment with a chaotropic reagent, $LiClO_4$. The LM complex is then incubated in $NaDodSO_4$, and the L and M subunits are separated by affinity chromatography with an adsorbent containing organomercurial groups. The L subunit contains a free sulfhydryl group and binds to the adsorbent; the M subunit is devoid of cysteine and does not bind.

The amino acid compositions of the isolated subunits are notable for their high proportion of hydrophobic amino acid residues, consistent with their location in the bacterial membrane (Steiner et al., 1974a; Okamura et al., 1982). This is particularly so for the L and M subunits; the H subunit has a lower proportion of hydrophobic residues but still exceeds the range of such residues found in most water-soluble globular proteins.

To understand the primary photochemistry in detail, knowledge of the amino acid sequence and the three-dimensional structure of the reaction center protein, as well as of its arrangement with respect to the bacterial membrane, is required. We report here the amino-terminal sequences of the

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[1] Abbreviations: LDAO, *N,N*-dimethyllaurylamine oxide; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; DEAE, diethylaminoethyl; GLC, gas–liquid chromatography; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography.

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three reaction center subunits. Preliminary reports of the L and H sequences have appeared previously (Rosen et al., 1977; Sutton et al., 1981).

Experimental Procedures

Materials. Sequencer grades of benzene, ethyl acetate, chlorobutane, phenyl isothiocyanate, heptane, Quadrol, and heptafluorobutyric acid were purchased from Pierce or Beckman. HPLC grades of methanol and propanol were purchased from Burdick and Jackson, Fisher, or Waters. PTH-amino acid standards and ninhydrin were from Pierce. All other reagents were of the highest grade available.

Purification of Reaction Centers and Isolation of Subunits. Reaction centers from *Rhodopseudomonas sphaeroides* were prepared as described previously [Appendix A of Feher & Okamura (1978)] with several minor modifications. To prevent proteolytic degradation, all buffers were made 0.001 M in EDTA. (Phenylmethanesulfonyl fluoride was not used.) The first $(NH_4)_2SO_4$ centrifugation (without LDAO) was omitted. After application of the sample to the DEAE-cellulose column, the column was washed with 0.03 M (instead of 0.06 M) NaCl. The reaction centers were concentrated in an Amicon ultrafiltration cell at 10 psi with a PM-10 membrane, instead of by adsorption and elution from DEAE-cellulose. In a typical preparation, 150-mg reaction centers were obtained from 90 g (wet weight) of chromatophores, prepared from bacteria in 30 L of culture. LM was prepared as described [Appendix B2 of Feher & Okamura (1978)], except that the final dialysis buffer was made 0.001 M in EDTA.

The *L subunit* was prepared from the LM complex by adsorption to Sepharose 4B containing *p*-(hydroxymercuri)-benzoate groups, followed by elution with cysteine. The procedure described by Feher & Okamura (1978; Appendix B3) was followed, except that the LM preparation was concentrated by ultrafiltration to $A_{800}^{1cm} \simeq 40$. Approximately 1 mg of L was bound by 1.0 mL of derivatized Sepharose.

The *M subunit* (together with ~10% contaminating L) was found in the unadsorbed fraction after LM was applied to the *p*-(hydroxymercuri)benzoate–Sepharose column. To reduce contamination with L, we treated the effluent (30 mL, containing ~30 mg of M) with dithiothreitol (1 mg/mL), incubated it at 65 °C for 30 min, dialyzed it overnight at room temperature against 1 L of deoxygenated 0.1 M Tris-HCl–0.001 M EDTA–0.1% $NaDodSO_4$, pH 8.0, and applied it to a column containing ~20 mL of *p*-(hydroxymercuri)-benzoate–Sepharose that had been equilibrated in the same buffer. The effluent contained the purified M subunit.

The preparation of the *H subunit* was based on a method described previously [Appendix B1 of Feher & Okamura (1978)], but the sucrose gradient was omitted. Approximately 10 mg of reaction centers (A_{800}^{1cm} = 10, ~3 mL) in 0.1% LDAO–0.05 M Tris-HCl, pH 7.7, 20 °C, was layered onto a solution of 1 M $LiClO_4$ in the same buffer and centrifuged in a Spinco Ti 60 rotor at 60 000 rpm (~250000*g*) for 20 h at 4 °C. The top portion of the tube, which contained the H subunit, was dialyzed overnight at 4 °C against 0.1% LDAO–0.01 M Tris-HCl–0.001 M EDTA, pH 8.0.

The reaction centers and purified subunits were analyzed by electrophoresis in 10% polyacrylamide gels containing $NaDodSO_4$ as described by Okamura et al. (1974). The results, shown in Figure 1, demonstrated that the preparations of M and H each migrated essentially as a single component in the expected positions. A minor component was seen in the L preparation at a position corresponding to a dimer of L. Amino acid analysis showed that, compared with previous

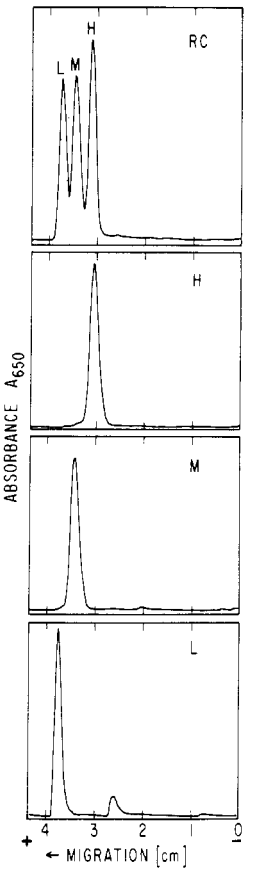

FIGURE 1: Electrophoresis of reaction centers and the isolated subunits, L, M, and H, in 10% polyacrylamide–$NaDodSO_4$ gels. The gels were stained with Coomassie Brilliant Blue and scanned in the Cary 14 spectrophotometer. The small peak seen in the bottom panel (L) is attributed to a dimer of L.

analyses (Steiner et al., 1974a; Okamura et al., 1982), the histidine content of most preparations of the H subunit was very low. All three subunits, but especially H, appear to be susceptible to destruction of histidine.

Automated Sequence Analysis. This was performed in a Beckman 890C sequencer with a dilute Quadrol program (Brauer et al., 1975) or with a modified version of this program incorporating a double cleavage procedure. Before application to the sequencer, samples were usually dialyzed against water adjusted to pH 9 with NH_4OH. Aliquots of phenylthiohydantoin (PTH)-norleucine were added to each tube in the fraction collector to serve as an internal standard. The 2-anilinothiazolinone amino acids were converted to PTH derivatives by heating at 80 °C for 10 min in 1 M HCl, as described by Ilse & Edman (1963), or with a Sequemat P6 autoconverter. In the latter case, methanol–HCl was used for conversion, and PTH-aspartic acid and PTH-glutamic acid were identified as the methyl ester derivatives. The PTH-amino acids found in the ethyl acetate phase (all except PTH-histidine and PTH-arginine) were identified by gas–liquid chromatography (GLC), high-pressure liquid chromatography (HPLC), thin-layer chromatography (TLC), and amino acid analysis after back-hydrolysis. PTH-histidine and PTH-arginine, which remain in the aqueous phase, were identified by HPLC or back-hydrolysis.

GLC was performed on a Hewlett-Packard HP5830A gas chromatograph with a 10% SP-400 column. PTH-amino acids were eluted with the following program: (1) isocratic elution at 190 °C for 5 min; (2) linear gradient from 190 to 230 °C for 8 min; (3) isocratic elution at 230 °C for 3.5 min. PTH derivatives of alanine, glycine, valine, proline, leucine, iso-

leucine, methionine, and phenylalanine were quantitated by comparing peak heights of unknowns and standards. PTH-serine (presumably as the dehydro derivative) was detected but not quantitated. The threonine derivative usually gives two peaks, a large one near PTH-proline and a smaller one near PTH-glycine; these were not quantitated. PTH-leucine and PTH-isoleucine were only slightly separated but were clearly distinguished by HPLC and back-hydrolysis. The analysis was terminated before the elution of PTH-tyrosine and PTH-tryptophan.

HPLC was performed on a Waters system liquid chromatograph consisting of two pumps (Model 6000A), an automatic injector (Model 710A), an absorbance detector (Model 440), and a system controller (Model 740). Reverse-phase chromatography was used with a Waters C_{18} μBondapak column (system I) or with a Waters RCM-C_{18} column (system II). System I [modified from Bhown et al. (1978)] consisted of 0.02 M sodium acetate (pH 5.0)–methanol (9:1 v/v) (solvent A) and methanol (solvent B). PTH-amino acids were eluted with an increasing gradient from 16% to 40% solvent B run for 20 min at a flow rate of 1.5 mL/min. System II (Sauer et al., 1981) consisted of 0.02 M sodium acetate (pH 3.0)–propanol (9:1 v/v) (solvent A) and propanol (solvent B) with the following elution steps: (1) isocratic elution (90% A, 10% B) for 4 min at 1.5 mL/min; (2) linear gradient from 10% to 32% solvent B for 4 min at 1.8 mL/min; (3) isocratic elution (68% A, 32% B) for 4 min at 1.8 mL/min. The column was then washed with a linear gradient from 32% to 52% solvent B for 4 min at 1.5 mL/min, followed by 52% solvent B for 5 min at 1.5 mL/min, and was reequilibrated to starting conditions. PTH-histidine and PTH-arginine were eluted under isocratic conditions on the C_{18} μBondapak column with 0.02 M sodium acetate (pH 4.1)–methanol (82:18 v/v) at a flow rate of 1.5 mL/min. PTH-threonine was detected, presumably as the dehydro derivative, by its absorbance at 313 nm but was not quantitated. PTH-serine (or derivatives) was detected by its absorbance either at 254 nm or at 313 nm and also was not quantitated. All other PTH-amino acids were detected by absorbance at 254 nm and were quantitated by comparing peak heights of unknowns and standards.

Two-dimensional thin-layer chromatography on polyamide sheets was carried out as described by Summers et al. (1973). The PTH derivatives of leucine and isoleucine were not distinguished by this method, as they comigrated, along with the internal standard PTH-norleucine. These derivatives were distinguished by HPLC and back-hydrolysis.

Back-hydrolysis of PTH-amino acids was carried out in 47–51% HI at 150 °C for 6 to 7 h in sealed, evacuated tubes (Smithies et al., 1971). Hydrolyzed samples were dried first on a water aspirator for 24–48 h and then in a vacuum centrifuge with a high-vacuum pump.

At all cycles, two or more methods for identifying the PTH-amino acids were used. For the three sequencer runs summarized in Tables I–III, the data were analyzed as follows. The amount of each quantifiable residue was determined at every cycle and corrected for recovery of the internal standard. For each identification method, the resulting values were plotted, as shown for the HPLC data in Figure 2 and in supplementary Figures 1 and 2 (see paragraph at end of paper regarding supplementary material). From these different sets of graphs, supplemented by qualitative results for residues that were not quantitated and, in some cases, by TLC identifications, the significant new residue at each cycle was determined. The net yield of this residue was obtained by correcting for background (usually the amount at the preceding cycle) and

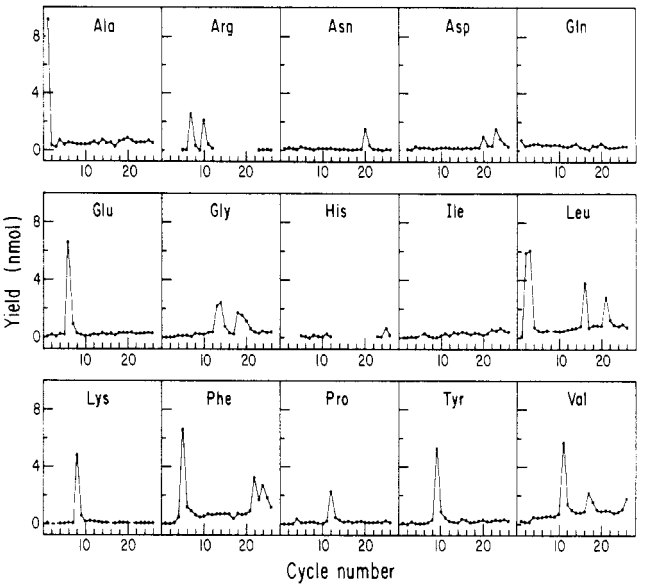

FIGURE 2: Results of HPLC determinations for the analysis of the L subunit summarized in Table I. Yields are plotted for each PTH-amino acid at each cycle. PTH-serine and PTH-threonine were not quantitated. PTH-valine coeluted with PTH-methionine; GLC showed that only PTH-valine was present. The yields of PTH-tryptophan were less than 0.1 nmol at all cycles and are not shown. Half-cystine was not derivatized and was not susceptible to detection. When no residue was found in the ethyl acetate phase at a particular cycle, the aqueous phase of that cycle and the preceding and succeeding two cycles were examined for PTH-histidine and PTH-arginine. At some cycles, it was not possible to quantitate background levels of certain residues because of interference from neighboring peaks; in these cases, the point is omitted and a dashed line is used to connect the preceding and succeeding points.

is shown in the appropriate position in Tables I–III. When the results of the different identification methods were not conclusive, data from other runs were used to determine the significant residue (e.g., M sequence, cycle 28, and H sequence, cycle 18, as discussed under Results). Repetitive yields were calculated, from the HPLC data, by linear regression analysis of the logarithms of the yields of the "stable" PTH-amino acids, i.e., those usually recovered in high yield (alanine, isoleucine, leucine, phenylalanine, tyrosine, and valine). Initial yields were obtained by extrapolation of the linear regression plots to cycle 0. The amount of sample applied to the sequencer was determined from the amino acid analysis by assuming molecular weights of 28 000 for the L subunit, 32 000 for the M subunit, and 30 000 for the H subunit. [These molecular weights are estimates based on data from amino acid analysis (Rosen et al., 1980; Okamura et al., 1982) and $NaDodSO_4$–polyacrylamide gel electrophoresis at different gel concentrations (B. Gårdlund and L. A. Steiner, unpublished results).] For other runs, quantitation was not as detailed, but the yield of the major residue(s) identified at each cycle was determined.

Amino Acid Analysis. Protein samples were hydrolyzed at 110 °C for 24 h in evacuated 10 × 70 mm ignition tubes containing 0.1 mL of constant-boiling HCl. Amino acid analyses were performed on a Dionex D-500 amino acid analyzer.

Results

Amino-Terminal Sequence of the L Subunit. The results of an automated Edman degradation of L, for 26 cycles, are summarized in Table I. Detailed HPLC data for this experiment are shown in Figure 2. The yield of PTH-glycine at cycle 19 (Table I and Figure 2) was lower than expected

Table I: Automated Edman Degradation of L[a]

cycle no.	GLC	HPLC[b]	TLC
1	Ala (9.1)	Ala (9.2)	Ala
2	Leu/Ile (7.5)[c]	Leu (5.9)	–[d]
3	Leu/Ile (7.1)	Leu (6.0)	–
4	Ser[e]	Ser[e]	–
5	Phe (5.8)	Phe (6.1)	Phe
6	Glu (6.1)[f]	Glu (6.4)[f]	–
7	–	Arg (2.4)	–
8	–	Lys (4.8)	Lys
9	–	Tyr (5.1)	Tyr
10	–	Arg (2.1)	–
11	Val (3.4)	Met/Val (4.9)[g]	Val
12	Thr/Pro (2.2)[h]	Pro (2.1)	–
13	Gly (2.1)	Gly (1.8)	Gly
14	Gly (3.3)	Gly (2.0)	Gly
15	Thr/Pro	Thr[h]	–
16	Leu/Ile (3.2)	Leu (3.0)	–
17	Val (2.5)	Met/Val (1.3)	Val
18	Gly (1.8)	Gly (1.5)	Gly
19	Gly (1.1)	Gly (1.3)	Gly
20	–	Asn (2.1)[i]	–
21	Leu/Ile (2.4)	Leu (1.9)	–
22	Phe (2.2)	Phe (2.3)	Phe
23	Asp (1.4)[f]	Asp (1.1)[f]	–
24	Phe (1.3)	Phe (1.8)	Phe
25	–	His (0.4)	–
26	Val (0.5)	Met/Val (0.8)	–

[a] The amount of sample was 12.5 nmol. Conversion to PTH-amino acids was performed in an autoconverter with methanol-HCl. The initial yield was 66%; the repetitive yield was 93.9%. Values in parentheses are yields in nanomoles of PTH-amino acids, corrected for background. [b] HPLC was performed with solvent system I (see Experimental Procedures). [c] PTH-leucine and PTH-isoleucine were not clearly separated by GLC. [d] A dash indicates that no significant new PTH-amino acid was found at this cycle by this identification method. [e] PTH-serine was not quantitated. [f] PTH-aspartic acid and PTH-glutamic acid were identified as the methyl ester derivatives. [g] PTH-methionine and PTH-valine were not distinguished by HPLC. [h] PTH-threonine and PTH-proline were not distinguished by GLC. If PTH-proline was identified by another method, it was quantitated; PTH-threonine was not quantitated. [i] PTH-asparagine was recovered as a mixture of PTH-asparagine (64%) and PTH-aspartic acid methyl ester (36%). The yield given is the sum of the ester and amide forms.

for the second residue in a pair of identical residues (compare cycles 18 and 19 with cycles 13 and 14). Further, the amount of PTH-glycine at cycle 20 (the "overlap") was high (Figure 2). However, in two previous analyses (see next paragraph), the yield at cycle 19 equaled or exceeded that at cycle 18, and the amount of PTH-glycine at cycle 20 was considerably lower. The yield of PTH-valine at cycle 17 was low, as measured by HPLC, but was in the expected range as measued by GLC (Table I). The yield of PTH-histidine at cycle 25 (0.4 nmol) was lower than that of any of the other assigned residues; however, the background level of PTH-histidine was even lower (Figure 2). Since PTH-histidine was found only in a single experiment and was in low yield, the assignment is regarded as provisional.

These results confirm and extend the sequence reported previously (Rosen et al., 1977). In these earlier studies, no identification was made at position 4. At this cycle, alanine was found after back-hydrolysis, which would be consistent with the presence of PTH-serine, but PTH-serine was not clearly identified by GLC or TLC. (HPLC was not available at that time.) At cycle 25, no PTH-amino acid was found in the ethyl acetate phase and the remaining sample, which was back-hydrolyzed, was lost. PTH-valine was found at position 26 but was not included in the reported sequence because of the gap at position 25.

Table II: Automated Edman Degradation of M[a]

cycle no.	GLC	HPLC[b]	BH[c]
1	Ala (13.2)	Ala (18.4)	Ala (9.1)
2	–[d]	Glu (10.6)	Glu (7.4)
3	–	Tyr (14.9)	Tyr (6.2)
4	–	Gln (6.7)[e]	Glu (7.2)[f]
5	–	Asn (11.8)[g]	Asp (5.6)[f]
6	Leu/Ile (8.3)[h]	Ile (11.9)	Ile (3.5)[i]
7	Phe (11.4)	Phe (11.8)	Phe (4.5)
8	Ser[j]	Ser[j]	Ala (4.2)[k]
9	–	Gln (3.1)	Glu (6.8)
10	Val (6.5)	Val (9.3)	Val (2.5)
11	–	Gln (5.1)	Glu (6.2)
12	Val (4.8)	Val (7.4)	Val (1.9)
13	–	–	Arg (2.5)
14	Gly (1.5)	Gly (4.4)	Gly (3.6)
15	Thr/Pro (1.8)[l]	Pro (3.8)	Pro (2.5)
16	Ala (2.8)	Ala (5.1)	–
17	–	Asp (2.9)	Asp (2.6)
18	Leu/Ile (2.4)	Leu (2.6)	–
19	Gly (1.0)	Gly (1.0)	Gly (0.7)
20	Met (3.0)	Met (2.4)	–
21	Thr/Pro	Thr[l]	α-Abu (1.0)[m]
22	–	Glu (1.8)[n]	Glu (2.6)
23	–	Asp (2.0)	–
24	Val (2.1)	Val (2.5)	Val (0.5)
25	–	Asn (1.6)	Asp (3.0)
26	Leu/Ile (0.9)	Leu (1.2)	–
27	Ala (1.8)	Ala (1.8)	Ala (1.1)
28	–	Asn (1.1)	–

[a] The amount of sample was 25 nmol. The initial yield was 83%; the repetitive yield was 91.2%. Values in parentheses are yields in nanomoles of PTH-amino acids (GLC and HPLC) or amino acids (BH), corrected for background. [b] HPLC was performed with solvent system II (see Experimental Procedures). [c] Back-hydrolysis. Aqueous phase and ethyl acetate phase samples were back-hydrolyzed separately. Yields are not corrected for efficiency of conversion into free amino acids. [d] A dash indicates that no significant new residue was found at this cycle by this identification method. [e] PTH-glutamine was recovered as a mixture of PTH-glutamine and PTH-glutamic acid. The extent of deamidation was 38–69%. The yields given are the sums of the amide and acid forms. [f] After hydrolysis, PTH-glutamine and PTH-asparagine were recovered as glutamic acid and aspartic acid, respectively. [g] PTH-asparagine was recovered as a mixture of PTH-asparagine and PTH-aspartic acid. The extent of deamidation was 0–16%. The yields given are the sums of the amide and acid forms. [h] PTH-leucine and PTH-isoleucine were not clearly separated by GLC. [i] After hydrolysis, PTH-isoleucine was recovered as a mixture of isoleucine and alloisoleucine. The yield indicated is the sum of the two forms. [j] PTH-serine was not quantitated. [k] After hydrolysis, PTH-serine was recovered as alanine but distinguished from PTH-alanine by other identification methods. [l] PTH-proline and PTH-threonine were not distinguished by GLC. If PTH-proline was identified by another method, it was quantitated. PTH-threonine was not quantitated. [m] After hydrolysis, PTH-threonine was recovered as α-aminobutyric acid. [n] At this cycle, PTH-glutamic acid appeared as two peaks on HPLC, one slightly earlier and one slightly later than the expected position. The reason for this anomaly is unclear. In another analysis of the M subunit, PTH-glutamic acid was eluted in the expected position.

Amino-Terminal Sequence of the M Subunit. Two automated Edman degradations were carried out with the M subunit. The results of one of these experiments (28 cycles) are summarized in Table II. (Detailed HPLC data are available as supplementary material.) At cycle 28, both PTH-asparagine and PTH-glycine were elevated above background level. In the second experiment, however, PTH-asparagine was the only residue raised significantly above background at this cycle. The combined yield of PTH-glutamine and PTH-glutamic acid at cycle 9 was low, as measured by HPLC (Table II; compare cycle 9 with cycles 4 and 11), and the extent of deamidation was 69%; in the second

Table III: Automated Edman Degradation of H[a]

cycle no.	GLC	HPLC[b]	BH[c]
1	Met[d]	Met (2.3)	–[e]
2	Val (6.7)	Val (4.8)	–
3	–[f]	Gly (3.9)	Gly (6.0)
4	Val (6.3)	Val (4.6)	Val (0.3)
5	Thr/Pro[g]	Thr[g]	α-Abu (1.2)[h]
6	Ala (4.0)	Ala (5.1)	Ala (2.7)
7	Phe (3.2)	Phe (4.1)	Phe (1.9)
8	–	Gly (3.4)	Gly (2.0)
9	–	Asn (2.1)[i]	Asp (1.1)[j]
10	Phe (1.8)	Phe (3.3)	Phe (1.6)
11	–	Asp (1.6)	Asp (1.0)
12	Leu/Ile (2.7)[k]	Leu (2.5)	Leu (1.5)
13	Ala (3.6)	Ala (3.8)	Ala (2.2)
14	Ser[l]	Ser[l]	Ala (1.8)[m]
15	Leu/Ile (3.1)	Leu (2.7)	Leu (1.6)
16	Ala (2.2)	Ala (2.9)	Ala (2.4)
17	Leu/Ile (3.0)	Ile (1.6)	Ile (0.4)[n]
18	–	Tyr (1.7)	Tyr (0.7)
19	Ser	Ser	Ala (1.2)
20	Phe (1.5)	Phe (1.9)	Phe (0.9)
21	–	Trp (1.4)	Gly (0.3)[o]
22	Leu/Ile (2.1)	Ile (1.9)	Ile (0.5)
23	Phe (1.5)	Phe (1.1)	Phe (0.5)
24	Leu/Ile (1.7)	Leu (1.6)	Leu (0.8)
25	Ala (0.8)	Ala (1.1)	–
26	–	–	–
27	Leu/Ile (0.9)	Leu (1.1)	Leu (0.9)
28	Leu/Ile (1.1)	Ile (0.9)	Ile (0.3)

[a] The amount of sample was 9.6 nmol. The initial yield was 68%; the repetitive yield was 93.8%. Values in parentheses are yields in nanomoles of PTH-amino acids (GLC and HPLC) or amino acids (BH), corrected for background. [b] HPLC was performed with solvent system II (see Experimental Procedures). [c] Back-hydrolysis. Yields are not corrected for efficiency of conversion into free amino acids. [d] PTH-methionine was not quantitated by GLC. [e] A dash indicates that no significant new residue was found at this cycle by this identification method. [f] PTH-glycine was not detected by GLC, presumably due to adsorption by the column. [g] PTH-threonine and PTH-proline were not distinguished by GLC. If PTH-proline was identified by another method, it was quantitated. PTH-threonine was not quantitated. [h] After hydrolysis, PTH-threonine was recovered as α-aminobutyric acid. [i] PTH-asparagine was recovered as a mixture of PTH-asparagine (85%) and PTH-aspartic acid (15%). The yield given is the sum of the amide and acid forms. [j] After hydrolysis, PTH-asparagine was recovered as aspartic acid. [k] PTH-leucine and PTH-isoleucine were not clearly distinguished by GLC. [l] PTH-serine was not quantitated. [m] After hydrolysis, PTH-serine was recovered as alanine. [n] After hydrolysis, PTH-isoleucine was recovered as a mixture of isoleucine and alloisoleucine. The yield indicated is the sum of the two forms. [o] After hydrolysis in HI, PTH-tryptophan is usually recovered as a mixture of glycine and alanine (Smithies et al., 1971). An increase in glycine was clear at this cycle. The level of alanine at cycle 21 was approximately the same as that at cycle 20, perhaps because of overlap from the alanine at cycle 19 (derived from PTH-serine) admixed with alanine derived from PTH-tryptophan; the amount of alanine decreased significantly at cycle 22.

experiment, there was a much higher yield of PTH-glutamine at this cycle, with less deamidation. Sequence analysis of whole reaction centers also clearly showed PTH-glutamine at this cycle. The arginyl residue at position 13 is based on a single identification by back-hydrolysis; however, the yield was relatively high and the absence of any PTH derivative in the organic phase supports the identification of an aqueous phase derivative at this position. Another analysis (25 cycles), carried out with an impure preparation of M (containing ~10% L), was consistent with the proposed sequence.

Amino-Terminal Sequence of the H Subunit. Four automated Edman degradations were carried out with the H subunit. The results of one of these experiments (28 cycles)

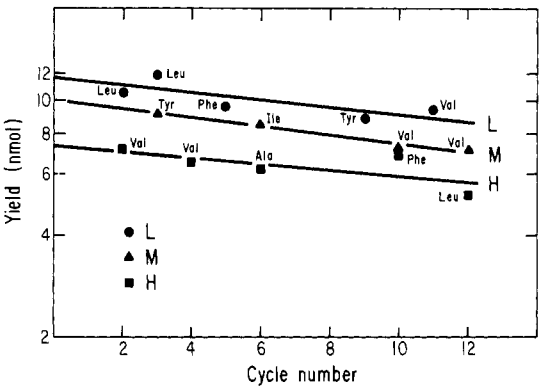

FIGURE 3: Automated Edman degradation of whole reaction centers (18.3 nmol). A blank cycle (phenyl isothiocyanate omitted) was run before the first full cycle. HPLC was performed with solvent system I (see Experimental Procedures). Only the yields of stable PTH-amino acids (those recovered consistently in high yield), corrected for background, are plotted. Residues are assigned to each subunit on the basis of the sequences proposed for the individual subunits (Figure 4). Yields for PTH-alanine at cycle 1 could not be assigned since both L and M subunits contribute to this derivative; similarly at cycle 7, PTH-phenylalanine is derived from the M and H subunits. The repetitive yields, calculated from the recovery of residues corresponding to each of the subunits, are as follows: L, 97.6%; M, 97.0%; H, 98.2%.

are summarized in Table III. (Detailed HPLC data are available as supplementary material.) No residue could be identified at cycle 26, but PTH-leucine and PTH-isoleucine were found at cycles 27 and 28, respectively. (The half-cystinyl residues of the H subunit were not modified, so these residues were not susceptible to detection; moreover, this preparation of the H subunit was almost devoid of histidine, which would, therefore, not have been identified.) In the earlier preliminary report of this sequence (Sutton et al., 1981), which was based on three of these experiments, PTH-methionine at position 1 was identified only provisionally and no residue was reported at position 18. (In addition to PTH-tyrosine, a peak corresponding to PTH-serine was found by GLC at position 18 in one of the experiments.) However, the fourth experiment confirmed the proposed sequence, including the presence of methionine at position 1 and tyrosine at position 18.

Amino-Terminal Sequence of Whole Reaction Centers and the LM Complex. Reaction centers were subjected to 12 cycles of automated Edman degradation. The mixture of residues found at each cycle was consistent with that expected from the sequences of the three individual subunits. The logarithms of the yields of the stable PTH-amino acids were plotted, and a straight line was fitted to those values that were presumably derived from each subunit. As can be seen in Figure 3, three distinct but approximately parallel lines were obtained, indicating that the initial yields for each of the subunits were different, although the repetitive yields were similar. The initial yield was highest for L (63%) and lowest for H (40%). In a parallel experiment, the reaction centers were incubated in 0.1% $NaDodSO_4$ at 56 °C for 1 h and overnight at 4 °C and then loaded directly into the sequencer cup. Again, the initial yield was highest for the sequence corresponding to the L subunit (76%) and lowest for that corresponding to the H subunit (45%). The yields in two other sequence analyses of whole reaction centers were somewhat lower, but in both cases, the yield for L was greater than that for the other two subunits.

A preparation of the LM unit was also subjected to sequence analysis (12 cycles). The initial yield of the L subunit was 34% and that of the M subunit was 15%. The data obtained from the analyses of whole reaction centers and the LM

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                5          10         15         20         25
L:  H2N-ALA-LEU-LEU-SER-PHE-GLU-ARG-LYS-TYR-ARG-VAL-PRO-GLY-GLY-THR-LEU-VAL-GLY-GLY-ASN-LEU-PHE-ASP-PHE(HIS)VAL-

M:  H2N-ALA-GLU-TYR-GLN-ASN-ILE-PHE-SER-GLN-VAL-GLN-VAL-ARG-GLY-PRO-ALA-ASP-LEU-GLY-MET-THR-GLU-ASP-VAL-ASN-LEU-ALA-ASN-

H:  H2N-MET-VAL-GLY-VAL-THR-ALA-PHE-GLY-ASN-PHE-ASP-LEU-ALA-SER-LEU-ALA-ILE-TYR-SER-PHE-TRP-ILE-PHE-LEU-ALA- X -LEU-ILE-
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FIGURE 4: Amino-terminal sequences of the three reaction center subunits. The histidyl residue in subunit L (position 25) is shown in parentheses since PTH-histidine was identified by only one method in only one experiment and was in low yield.

complex (for "unstable" as well as stable PTH-amino acids) were consistent with the proposed sequences of the individual subunits.

Discussion

The amino-terminal sequences for the three reaction center subunits are summarized in Figure 4. At almost every position, the residue shown was determined by at least two separate identification procedures and/or in at least two independent experiments. In our earlier preliminary report of the amino-terminal sequence of the L subunit (Rosen et al., 1977), we noted that sequences for the M and H polypeptides were obtained only in low yield. In the present study, the initial yields for the amino-terminal sequences of M and H, although somewhat variable, have usually been in the range found with L or other proteins (~40–80%). Moreover, we have observed that when intact reaction centers are subjected to automated Edman degradation, the yields for the sequences of each of the three subunits are also usually within this range, although that for L has in each case been greater than that for M or H (e.g., see Figure 3). In our earlier studies (L. A. Steiner and J. E. Walker, unpublished results), the analysis of intact reaction centers (up to 12 cycles) resulted in a single major sequence, which was consistent with that of the isolated L subunit. Smaller amounts of one or two additional residues were also found at most cycles, and these generally corresponded to the low-yield residues obtained with the isolated M and H subunits. However, without independent sequences for M and H, the minor residues found in the mixed sequence could not be assigned with confidence to either of these polypeptides.

The reason for the difference in yields between the present and previous studies is not clear. There have been some changes in sequencer instrumentation and methodology (especially a change in buffer from dimethylallylamine to dilute Quadrol). Moreover, the methods of preparation of the M and H subunits have been modified, and the isolated proteins are now more soluble and less pigmented. Possibly, the amino termini were previously partially blocked. However, we are not certain which, if either, of these procedural changes has contributed to the improved yields in the recent experiments.

The amino-terminal residue of the L and M subunits is alanine; that of H is methionine. These two amino acid residues have been found at the amino terminus of about half of the soluble proteins in *Escherichia coli* (Waller, 1963). Formylmethionine is the initiating residue in prokaryotic proteins, and the preferential occurrence of amino-terminal methionine and alanine results from the postsynthetic enzymatic removal of the formyl group and sometimes also the methionyl residue [reviewed by Lengyel & Söll (1969) and Lucas-Lenard & Lipmann (1971)]. Cleavage after methionine appears to occur relatively easily when alanine is in the second position. Whether such cleavages account for the amino-terminal residues found in the membrane-associated reaction center protein or whether these proteins are synthesized with a precursor peptide at the amino terminus, in which case the presence of amino-terminal alanine and methionine would presumably be fortuitous, is not known.

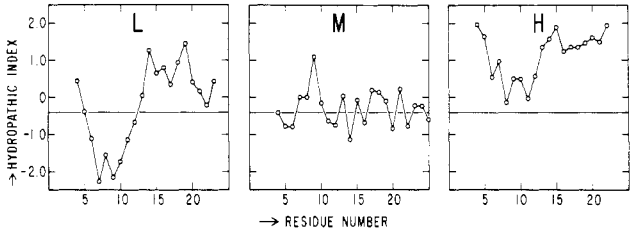

FIGURE 5: Profile of the hydropathy indices (Kyte & Doolittle, 1982) of the amino-terminal segments of the reaction center subunits. These indices were obtained by taking the average of the hydropathy values of the amino acid side chains in successive seven-residue segments and are plotted above the middle residue of each segment. We used the hydropathy values suggested by Kyte and Doolittle: Ile, 4.5; Val, 4.2; Leu, 3.7; Phe, 2.7; Cys, 2.5; Met, 1.9; Ala, 1.8; Gly, −0.4; Thr, −0.7; Trp, −0.9; Ser, −0.9; Tyr, −1.3; Pro, −1.6; His, −3.2; Glu, −3.5; Gln, −3.5; Asp,−3.5; Asn, −3.5; Lys, −3.9; Arg, −4.5. The average hydropathy of ~1300 sequenced proteins is −0.4 (Kyte & Doolittle, 1982), indicated by the horizontal line.

The amino-terminal segments of the three subunits vary considerably in their content of hydrophobic residues. As a quantitative measure of the degee of hydrophobicity, we have calculated the "hydropathic indices" according to Kyte & Doolittle (1982). This is the moving average of hydropathy values of amino acid residues within successive oligopeptide segments of predetermined length (e.g., seven residues). The hydropathy values themselves (see legend to Figure 5) are based on a variety of experimental observations such as the transfer free energy of the side chain from water into condensed vapor and the tendency of a side chain to be buried within a protein. Provided that segments of sufficient length are compared, the hydropathy profiles can be used as guides to distinguish membrane-spanning sequences from internal, hydrophobic sequences of soluble proteins (Kyte & Doolittle, 1982).

Hydropathy profiles of the amino-terminal sequences of the L, M, and H subunits are presented in Figure 5. It can be seen that the most hydrophobic sequence is that of H, especially the carboxy-terminal portion. The hydropathy of the 14-residue segment from positions 12 to 25 and the presence of leucine and isoleucine at positions 27 and 28, respectively, suggest that this portion of the chain may be buried in the membrane. The amino-terminal portion of the M subunit is of intermediate polarity throughout. The average hydropathy of this section (−0.37) is very close to that of ~1300 sequenced proteins in the data base (Kyte & Doolittle, 1982).

The L sequence begins with three hydrophobic residues and then becomes very hydrophilic. There is a negatively charged residue (glutamic acid) at position 6 and positively charged residues at positions 7 (arginine), 8 (lysine), and 10 (arginine). Secondary structure predictions, according to the method of Chou & Fasman (1978), indicate that a β turn is very likely at $Val^{11}Pro^{12}Gly^{13}Gly^{14}$. In this region, there is a marked increase in the hydrophobic character of the L sequence, and a turn could indicate a transition in the polypeptide chain from

the exterior to the interior of the membrane. However, the sequence beyond residue 15 is only moderately hydrophobic (note asparaginyl, aspartyl, and histidyl residues at positions 20, 23, and 25), and the criteria proposed by Kyte & Doolittle (1982) do not suggest that this segment of the chain is buried in the membrane.

A characteristic feature of a number of "simple" transmembrane proteins (i.e., proteins that cross the membrane once and that have their carboxy-terminal ends in the cytoplasm) is the presence of one or more lysyl or arginyl residues adjacent to the transmembrane segment, very frequently at the cytoplasmic end but sometimes also at the exterior surface (Von Heijne, 1981). Possibly, the positively charged side chains interact with negatively charged groups of the phospholipid bilayer. There is, however, no evidence that L or any of the reaction center polypeptides is a simple transmembrane protein. Bacteriorhodopsin (Henderson & Unwin, 1975; Unwin & Henderson, 1975; Engelman et al., 1980) and erythrocyte band III protein (Drickamer, 1977; Steck et al., 1978; Rao, 1979; Williams et al., 1979) have several segments that span the membrane, and this may be a characteristic feature of proteins that function in ion transport [see Engelman & Steitz (1981)].

A variety of methods have been used to investigate the topography of the reaction center subunits in the membranes of *Rhodopseudomonas sphaeroides* and the related organism *Rhodospirillum rubrum*. To date, information has been obtained in experiments utilizing probes such as highly specific antibodies (Steiner et al., 1974b; Reed et al., 1975; Valkirs et al., 1976; Feher & Okamura, 1976; Valkirs & Feher, 1981, 1982), radiolabeling reagents that selectively do or do not penetrate the membrane (Zürrer et al., 1977; Oelze, 1978; Francis & Richards, 1980; Odermatt et al., 1980; Bachmann et al., 1981), and proteolytic enzymes that can digest exposed portions of the polypeptides (Hall et al., 1978; Oelze, 1978; Bachmann et al., 1981). A limitation in all of these studies has been the lack of information regarding the amino acid sequence of the reaction center subunits. Thus, it has not been possible to localize residues within the sequence or to determine which portions of the polypeptides have been cleaved by proteolytic digestion. The determination of the amino-terminal sequence of each of the subunits should now facilitate the localization of these segments with respect to the bacterial membrane.

A search for homologies in sequence between the amino-terminal segments of the reaction center subunits and ~1300 other proteins was performed by R. F. Doolittle, using a computer program and data bank as described (Doolittle, 1981). No significant homologies were found. Moreover, the amino-terminal segments of the three subunits do not appear to be similar in sequence or, as judged by the criteria of Chou & Fasman (1978), in secondary structure.

Since the reaction center protein is highly hydrophobic, standard approaches to total sequence determination might be expected to work poorly. Indeed, our efforts to determine the sequence of the L subunit have been severely hindered by the propensity of this subunit and large derivative fragments to aggregate. However, a number of small peptides have been isolated (B. Gårdlund and L. A. Steiner, unpublished experiments). It seems likely that the most promising approach to obtain the complete amino acid sequence will be to isolate and sequence the DNA encoding each of the subunits. Toward this end, the partial amino acid sequence data we have obtained should be useful as a basis for constructing nucleic acid probes, which can then be used to identify the structural genes for these polypeptides. Further, the sequence of the amino-terminal sections of the subunits, as well as that of individual soluble peptides, can serve as a useful check of the results obtained by DNA sequencing [e.g., see Putney et al. (1981)].

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Supplementary Material Available

Two figures showing results of HPLC determinations for automated Edman degradations of the M and H subunits (3 pages). These data are summarized in Tables II and III, respectively. Ordering information is given on any current masthead page.

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Intramolecular Electron Transfer in *Chlorobium thiosulfatophilum* Flavocytochrome *c*†

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ABSTRACT: The electron-transfer reactions of photoproduced lumiflavin semiquinone and fully reduced lumiflavin with oxidized *Chlorobium thiosulfatophilum* flavocytochrome *c* have been studied by using laser flash photolysis. The *Chlorobium* flavocytochrome *c* contains one heme and one flavin per M_r 50000, and thus the possibility exists for intramolecular electron transfer. We find a complex kinetic pattern which is consistent with the transient formation of a spectrally perturbed protein-bound flavin semiquinone which transfers an electron intramolecularly to the heme ($k = 1 \times 10^3$–1.8×10^3 s^{-1} for the neutral semiquinone, depending upon the pH). Evidence is presented that electron transfer from exogenous lumiflavin to the heme moiety occurs through the protein-bound flavin. We have also performed redox titrations which determine the midpoint potentials of the heme and flavin prosthetic groups at various pH values and the p*K* values for the semiquinone (6.4) and fully reduced flavin (6.1). Thus, at pH 7, the semiquinone is predominantly in the anionic form at equilibrium. The reactions of *Chlorobium* flavocytochrome *c* with photoreduced lumiflavin are similar to those previously found with *Chromatium vinosum* flavocytochrome *c* [Cusanovich, M. A., & Tollin, G. (1981) *Biochemistry 19*, 3343–3347] in that a protein-bound flavin semiquinone is an intermediate in the pathway of reduction. However, the rate constants are substantially different. As a class, the flavocytochromes *c* appear to operate by analogous mechanisms involving rapid intramolecular transfer between the heme and flavin moieties.

In a recent publication (Cusanovich & Tollin, 1980) kinetic studies of electron transfer from fully reduced lumiflavin (LFH^-) and lumiflavin semiquinone radical ($LFH\cdot$) generated by laser flash photolysis to *Chromatium vinosum* cytochrome *c*-552 (flavocytochrome *c*) were reported. This cytochrome is representative of a class of low-potential flavin-containing heme proteins which have been isolated from four species of purple and green phototrophic bacteria (Bartsch, 1978). It was found that both the heme and flavin moieties of *Chr. vinosum* flavocytochrome *c* were reduced simultaneously on a millisecond time scale, with the transient formation of a protein-bound flavin anion radical. Further, studies on the redox process following photolysis of the CO–ferrocytochrome complex in which the flavin was partly oxidized established that intramolecular electron transfer from ferrous heme to oxidized flavin occurred with a first-order rate constant of greater than 1.4×10^6 s^{-1}.

Cytochrome *c*-553 from *Chlorobium thiosulfatophilum* is also a member of the low-potential flavin-containing cytochrome class discussed above. However, this protein contains one heme and one flavin per *Mr* 50000, as opposed to two hemes and one flavin for the *Chr. vinosum* flavocytochrome (Bartsch et al., 1978). In addition, the midpoint oxidation–reduction potential of the *Chlorobium* flavocytochrome is approximately 100 mV compared to 35 mV for the *Chromatium* protein (Bartsch et al., 1978; Meyer et al., 1968; Vorkink, 1972). Recent studies (Kusai & Yamanaka, 1973;

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