The Complete Amino Acid Sequence of the Major Component Myoglobin of Dwarf Sperm Whale (*Kogia simus*)†

Francis E. Dwulet, Barry N. Jones, Lee D. Lehman, and Frank R. N. Gurd*

ABSTRACT: The complete amino acid sequence of the major component myoglobin from the dwarf sperm whale, *Kogia simus*, was determined by specific cleavage of the protein to obtain large peptides which are readily degraded by the automatic sequenator. Three easily separable peptides were obtained by cleaving the protein at its two methionine residues, and five peptides were obtained from the methyl acetimidated protein by cleavage with trypsin at the four arginine residues. Sequenator analysis of these fragments and the apomyoglobin provided over 80% of the covalent structure of the protein. The remainder of the primary structure was determined by further digestion of the two larger cyanogen bromide fragments with trypsin and staphylococcal protease. To reconfirm many of the substitutions found in this protein, the apomyoglobin was treated with 1,2-cyclohexanedione, and the resulting arginine protected protein was cleaved at its lysine residues with trypsin. This myoglobin differs from that of the sperm whale at 6 positions, and from the other cetacean myoglobins at about 16 positions. The appearance of a histidine residue at position 35 has no precedent in any myoglobin. The substitutions seen at positions 21, 51, and 132 are unique to date for cetacean myoglobins.

In preceding papers, the complete primary structure of the myoglobins from Amazon River dolphin (Dwulet et al., 1975), California gray whale (Bogardt et al., 1976), Atlantic bottlenosed dolphin (Jones et al., 1976), and Arctic minke whale (Lehman et al., 1977) were reported. All of these sequences of cetacean myoglobins were determined by automated Edman degradation. This paper reports the application of the peptide fragmentation and analytical procedures that were used in these papers in determining the complete amino acid sequence of the myoglobin from dwarf sperm whale, *Kogia simus*. In addition, the arginine blockage technique of Patthy and Smith (1975) was applied to this protein to assist in reconfirming many of the unique amino acid substitutions found within this protein. Completion of this sequence extends the number of complete cetacean myoglobin sequences to eight. In addition to the above-mentioned proteins, there are the myoglobins from the Black Sea (common) dolphin (Kluh and Bakardjieva, 1971), common porpoise (Bradshaw and Gurd, 1969), and sperm whale (Edmundson, 1965). This is the only myoglobin sequenced to date which shows a high degree of homology to the protein from sperm whale.

Experimental Section

Materials

The principal component of dwarf sperm whale myoglobin was isolated from frozen muscle tissue by the procedure of Hapner et al. (1968). Phosphate buffer (pH 6.4), ionic strength 0.1, was used to effect purification of the crude homogenate on C-50 CM-Sephadex. The homogeneity of the purified myoglobin was verified by cellulose acetate electrophoresis. The apomyoglobin was prepared essentially by the procedure of Teale (1959).

Methyl acetimidate hydrochloride was prepared according to the procedure of Hunter and Ludwig (1962) and 3-sulfophenyl isothiocyanate sodium salt was prepared according to the method of Dwulet and Gurd (1976). Tos-PheCH$_2$Cl[1]-treated trypsin was purchased from Worthington Biochemical Laboratories, and staphylococcal protease was obtained from Miles Laboratories Ltd. Sequenator reagents of "Sequencer" grade were obtained from Beckman Instruments. 1,2-Cyclohexanedione was a product of Aldrich Chemical Co. All other chemicals were the highest grade available.

Methods

All the techniques that were reported in the previous papers in this series for obtaining and isolating peptides were also used here (Lehman et al., 1977).

Reaction of Arginine Residues with 1,2-Cyclohexanedione. Apomyoglobin was treated with 1,2-cyclohexanedione using a slight modification of the procedure of Patthy and Smith (1975). Apomyoglobin (10 μmol) was dissolved in 10 mL of deionized 8 M urea and deoxygenated with nitrogen gas. To this solution was added 155 mg of boric acid (0.25 M) and 168 mg of 1,2-cyclohexanedione (0.15 M, 40-fold excess/arginine). When solution was complete, the pH was raised to 9.0 and the mixture allowed to react for 2 h at 38 °C. The reaction was then terminated by cooling the sample in an ice bath and with the addition of 1 mL of glacial acetic acid. The reacted protein was then dialyzed at 4 °C against 1% acetic acid to remove excess reagents. The modified protein was then lyophilized and stored at −20 °C until needed. Acid hydrolyses on the reacted protein and peptides were performed with 5.7 N HCl containing 0.2% mercaptoacetic acid.

Tryptic Digestion of Arginine Reacted Apomyoglobin. The 1,2-cyclohexanedione-treated apomyoglobin (5 μmol) was dissolved in 5 mL of deoxygenated 0.1 M boric acid buffer. When solution was complete, and with the pH between 4 and 5, a total of 1.8 mg of trypsin (2% w/w) was added. The pH was then raised with base to 8.1. At a pH of about 6.5 some of

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47401. *Received September 28, 1976.* This is the 79th paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper, see Lehman et al. (1977). This work was supported by United States Public Health Service Research Grant HL-05556. F.E.D. and L.D.L. were supported by United States Public Health Service Grant T01 GM 1046-14.

[1] Abbreviation used: Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

TABLE I: Amino Acid Composition of *Kogia simus* Myoglobin.

Amino Acid	From Acid Hydrolysates[a]	From the Sequence
Asp	7.1	7
Thr	4.0	4
Ser	7.0	7
Glu	18.9	19
Pro	4.2	4
Gly	10.7	10
Ala	18.6	18
Val	6.9	7
Met	2.2	2
Ile	9.5	10
Leu	18.5	18
Tyr	3.0	3
Phe	5.8	6
Lys	18.3	19
His	13.0	13
Arg	4.0	4
Trp[b]	1.8	2

[a] Acid hydrolyses were performed on ferrimyoglobin for 24, 48, and 72 h at 110 °C with 5.7 N HCl and the values were averaged. The amino acid residues were calculated on the basis of 153 amino acids in the protein. The values of threonine and serine were obtained by extrapolation to zero time. The values of valine, isoleucine, and leucine were the maximum values (72 h). [b] Tryptophan was determined by the method of Liu and Chang (1971).

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                5                  10
1   Val Leu Ser Glu Gly Glu Trp Gln Leu Val Leu His Val Trp Ala
16  Lys Val Glu Ala Asp Ile Ala Gly His Gly Gln Asp Ile Leu Ile
31  Arg Leu Phe Lys His His Pro Glu Thr Leu Glu Lys Phe Asp Arg
46  Phe Lys His Leu Lys Ser Glu Ala Glu Met Lys Ala Ser Glu Asp
61  Leu Lys Lys His Gly Val Thr Val Leu Thr Ala Leu Gly Ala Ile
76  Leu Lys Lys Lys Gly His His Glu Ala Glu Leu Lys Pro Leu Ala
91  Gln Ser His Ala Thr Lys His Lys Ile Pro Ile Lys Tyr Leu Glu
106 Phe Ile Ser Glu Ala Ile Ile His Val Leu His Ser Arg His Pro
121 Ala Asp Phe Gly Ala Asp Ala Gln Gly Ala Met Ser Lys Ala Leu
136 Glu Leu Phe Arg Lys Asp Ile Ala Ala Lys Tyr Lys Glu Leu Gly
151 Tyr Gln Gly
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FIGURE 1: The amino acid sequence of dwarf sperm whale myoglobin. The three letter amino acid code has been used for simplicity and the hyphens between the amino acid residues have been omitted for clarity.

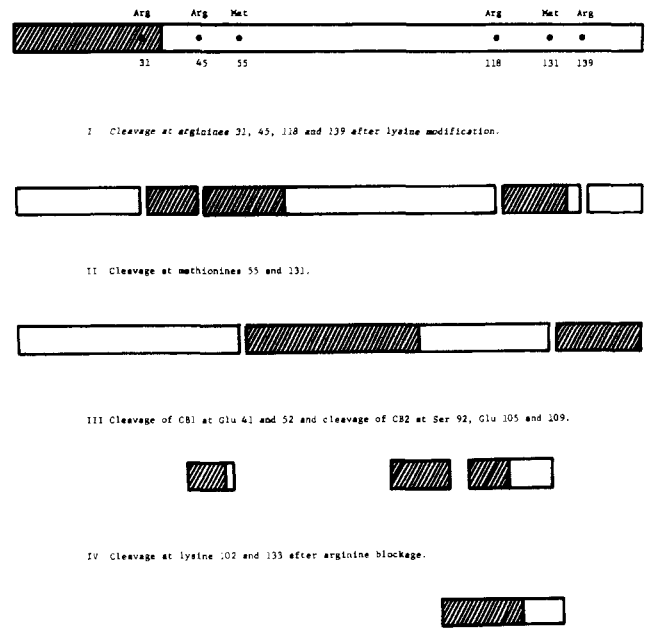

FIGURE 2: Diagrammatic summary of fragments generated from dwarf sperm whale myoglobin for sequenator analysis. The top bar represents the whole protein and the residues that are important to its fragmentation. A hatched segment in each horizontal bar indicates the segment of sequence determined by sequenator analysis on that fragment.

the myoglobin precipitated, but by pH 7.5 all the protein had redissolved. After 3 h of reaction an additional 1.8 mg of trypsin was added and the reaction continued for a total of 5 h. The digest was terminated by lowering the pH to 6.0 and heating the sample at 90 °C for 5 min. The insoluble material which formed was removed by centrifugation and then resuspended in pH 6.0 water and again heated at 90 °C for 5 min. The insoluble peptides were isolated by centrifugation and the supernatant phases were combined and lyophilized. The pH 6.0 soluble and insoluble peptides were initially fractionated by gel filtration. Those pools found to contain blocked arginine residues had the guanidine groups regenerated with 0.2 N hydroxylamine at pH 7.0 for 24 h at 37 °C. The excess reagents were then removed from the peptide pools by gel filtration, and the peptides were finally purified by ion-exchange chromatography.

Results

Amino Acid Composition. The amino acid composition of the principal component of dwarf sperm whale myoglobin was obtained from 24, 48, and 72 h hydrolysates of the ferrimyoglobin. The results are summarized in Table I.

Peptide Isolation. All the techniques used in the previous papers in this series to isolate the needed peptides were also used here. The elution profiles and peptide compositions can be found in the supplementary material.[2]

Sequence Investigations. Only the sequence data necessary to establish the entire primary structure are reported here.

Sequenator Results. The complete primary structure of dwarf sperm whale myoglobin is presented in Figure 1. The strategy used to establish the structure is outlined diagrammatically in Figure 2. In this diagram the solid bar represents the entire myoglobin sequence and the marked residues are those amino acids important in fragmenting the protein. The hatched sections in each bar represent the amount of the structure obtained from each peptide in the sequenator.

Discussion

Comparison with other Cetacean Myoglobin Sequences. The present report is the fifth in a series of complete cetacean myoglobin sequences[3,4] determined by automated Edman degradation. The information obtained from these sequence investigations had been used to interpret proton NMR results in which the pK_a values of the individual histidine residues were assigned (Botelho, 1975), as well as in the treatment of electrostatic interactions within the myoglobin molecule (Shire et al., 1975). In addition the sequence data can be used to assist in interpreting the results of denaturation experiments using copper or hydrogen ions (Hartzell et al., 1968), oxygen binding (Rossi Fanelli and Antonini, 1958), and hemic acid dissociation

[2] Results of established procedures can be found in supplementary material as described below.

[3] L. D. Lehman, work in progress.

[4] B. N. Jones, work in progress.

Residue Number	1	4	5	8	12	13	15	21	26
Dwarf Sperm Whale	Val	Glu	Gly	Gln	His	Val	Ala	Ile	Gln
Sperm Whale	Val	Glu	Gly	Gln	His	Val	Ala	Val	Gln
Amazon River Dolphin	Gly	Asp	Gly	Gln	Asn	Ile	Gly	Leu	Gln
Gray Whale	Val	Asp	Ala	Gln	Asn	Ile	Ala	Val	Gln
Minke Whale	Val	Asp	Ala	His	Asn	Ile	Ala	Val	Gln
Common Porpoise	Gly	Glu	Gly	Gln	Asn	Val	Gly	Leu	Gln
Common Dolphin	Gly	Asp	Gly	Gln	Asn	Val	Gly	Val	Glu
Bottlenosed Dolphin	Gly	Asp	Gly	Gln	Asn	Val	Gly	Leu	Gln

Residue Number	28	35	45	51	54	66	74	83	85
Dwarf Sperm Whale	Ile	His	Arg	Ser	Glu	Val	Ala	Glu	Glu
Sperm Whale	Ile	Ser	Arg	Thr	Glu	Val	Ala	Glu	Glu
Amazon River Dolphin	Val	Gly	Lys	Thr	Glu	Asn	Gly	Glu	Glu
Gray Whale	Ile	Gly	Lys	Thr	Glu	Asn	Gly	Glu	Glu
Minke Whale	Ile	Gly	Lys	Thr	Glu	Asn	Gly	Glu	Glu
Common Porpoise	Val	Gly	Lys	Thr	Glu	Asn	Gly	Glu	Asn
Common Dolphin	Ile	Gly	Lys	Thr	Asp	Asp	Ala	Asp	Glu
Bottlenosed Dolphin	Val	Gly	Lys	Thr	Asp	Asn	Ala	Asp	Glu

Residue Number	109	121	122	129	132	144	151	152
Dwarf Sperm Whale	Glu	Ala	Asp	Gly	Ser	Ala	Tyr	Gln
Sperm Whale	Glu	Gly	Asn	Gly	Asn	Ala	Tyr	Gln
Amazon River Dolphin	Glu	Gly	Asp	Ala	Asn	Ala	Phe	His
Gray Whale	Asp	Gly	Asp	Ala	Asn	Ala	Phe	Gln
Minke Whale	Asp	Ala	Glu	Ala	Asn	Ala	Phe	Gln
Common Porpoise	Glu	Ala	Glu	Gly	Asn	Thr	Phe	His
Common Dolphin	Glu	Ala	Gln	Gly	Asn	Ala	Phe	His
Bottlenosed Dolphin	Glu	Ala	Glu	Gly	Asn	Ala	Phe	His

FIGURE 3: Comparison of the amino acid sequences of cetacean myoglobins whose sequences have been completed to date. Only those positions in which differences occur are reported. All other positions are conserved and are the same as in the dwarf sperm whale myoglobin sequence.

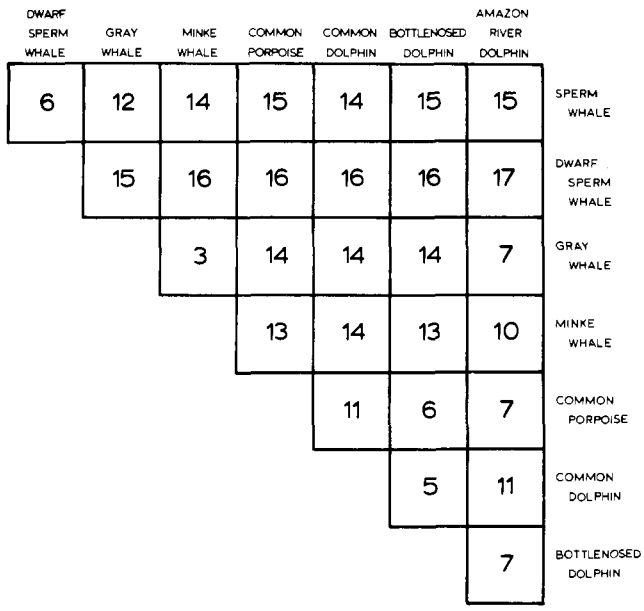

FIGURE 4: Difference matrix for cetacean myoglobins obtained by summing the number of different amino acids between pairs of proteins.

(George and Hanania, 1952). Finally, the sequence information has been used to develop a computer model of cetacean phylogenetics (Bogardt, 1977).

The sequence of dwarf sperm whale myoglobin is compared in Figure 3 with the known cetacean myoglobins, as emphasized in the difference matrix shown in Figure 4. From this it can be seen that the dwarf sperm whale myoglobin is most closely related to the myoglobin from the sperm whale. The sequence of dwarf sperm whale myoglobin will be examined here in comparison with the six differences between it and the myoglobin sequence from sperm whale. The differences will be referred to by first giving the position number, then the residue found in dwarf sperm whale myoglobin, followed by the homologous sperm whale residue in parentheses.

21 Isoleucine (Valine). This is the first time that an isoleucine residue has been seen in this position in a cetacean myoglobin. The common residue for porpoises and dolphins is a leucine, and for whales it is a valine (see Figure 3). Isoleucine at this position has only been seen in the myoglobins from horse (Dautrevaux et al., 1969) and most of the primates (Romero-Herrera et al., 1976a).

For all other known myoglobins, positions 21 and 28 appear to be examples of Fitch's "covarions" (Fitch and Markowitz, 1970). Covarions are concomitantly variable codons represented by a limited set of amino acids within a protein structure. In the majority of known myoglobin sequences, an increase in the side chain volume of residue 21 from a valine to a leucine or isoleucine is accompanied by a decrease in the side chain volume at position 28 from an isoleucine to a valine. These residues are not in direct contact with each other within the tertiary structure of the sperm whale protein (Watson, 1969). Residue 21 in the sperm whale myoglobin lies in a crevice and residue 28 is found to be inaccessible to solvent. Position 28, furthermore, is a residue involved with one of the internal cavities described by Lee and Richards (1971). The two isoleucine residues occupying both positions 21 and 28 in the present sequence represent the only time in over 40 myoglobin sequences that this amino acid has been seen at both of these positions.

35 Histidine (Serine). This is the first occurrence of a histidine residue at this position for any myoglobin reported to date. The serine residue for the sperm whale myoglobin is also a unique amino acid substitution occurring in none of the other myoglobins. This is the only position in the two proteins in which the amino acid substitution would require a two base change in the DNA codon. The loss of the serine at this position in the dwarf sperm whale myoglobin results in the loss of the hydrogen bond between the hydroxyl group of the serine residue and the peptide backbone (Watson, 1969). The loss of this hydrogen bond or its substitution by some other structural arrangement does not appear to reduce the stability of this protein to denaturing conditions in solution in relation to the sperm whale myoglobin.[5,6]

51 Serine (Threonine). Among the cetacean myoglobins sequenced to date, the dwarf sperm whale is the only example with a serine at this position. However, serine is a common residue for the seal (Bradshaw and Gurd, 1969) and sea lion (Vigna et al., 1974) and the land carnivores (Tetaert et al., 1974; Dumur et al., 1976; Romero-Herrera et al., 1976b).

[5] R. Avila, work in progress.
[6] S. Friend, work in progress.

121 Alanine (Glycine). Alanine is the more common cetacean residue, but glycine is found in many of these proteins as can be seen in Figure 3. From these and other sequences it appears that there is no evolutionary preference for either residue.

122 Aspartic Acid (Aspargine). The aspartic acid is the most common residue seen at this position. The asparagine is rarer and is only seen in sperm whale and some of the land herbivores (Dautrevaux et al., 1969; Han et al., 1970, 1972; and Vötsch and Anderer, 1975).

132 Serine (Asparagine). Up to this time all cetacean myoglobins have had an asparagine residue at this position. Serine has been limited to the myoglobins from the land herbivores (Han et al., 1970, 1972; Vötsch and Anderer, 1975) and some of the prosimians (Romero-Herrera and Lehmann, 1973; Romero-Herrera et al., 1976a-c). All substitutions characteristic of the dwarf sperm whale myoglobin appear compatible with the three-dimensional sperm whale myoglobin structure (Watson, 1969).

Having discussed the differences between sperm whale and dwarf sperm whale myoglobins, it is useful to point out the similarities between these two proteins. These two myoglobins differ from all others in having the following amino acid residues in common: histidine 12, arginine 45, valine 66, and tyrosine 151. Presumably, the events which caused these amino acid changes occurred in the evolution of the ancestor of these animals, but after divergence from the other cetaceans. These changes may have some functional or adaptive significance because these two proteins have a better stability to denaturation by acid, temperature, and copper ions than do the other cetacean myoglobins.[5,6]

Blockage of Arginine Residues. The use of the 1,2-cyclohexanedione to render the arginine residues resistant to tryptic hydrolysis was very important for the reconfirmation of the entire protein structure and for isolating all the arginine overlap peptides. This procedure was especially useful here because many of the staphylococcal protease peptides from fragments CB1 and CB2 were insoluble under the normal conditions used for ion-exchange chromatography. Thus a number of segments of the protein structure were based only on results from a sequenator run on a single peptide. This condition was acceptable but not desirable. The problem was overcome readily by limiting the tryptic cleavage to the lysine residues. Although some of the peptides obtained from this fragmentation procedure were also insoluble, procedures were developed to isolate and purify all of them.[2]

An interesting observation found in this work was the incomplete reaction of arginine residue number 31 in this protein. The other three arginine residues were found to be completely converted, but residue 31 was found to be about 20% unreacted with the 1,2-cyclohexanedione. This observation is at odds with the results for sperm whale myoglobin obtained by Atassi and Thomas (1969) and Varnes (1973). In both of these cases it was reported that arginine number 45 was resistant to reaction with diketones. However, since the reaction conditions and the protein were different for these experiments, a more detailed study of the chemistry of these reactions with myoglobins will have to be made to resolve the differences.

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Supplementary Material Available

Experimental material including elution profiles, peptide compositions, and repetitive yield plots (37 pages). Ordering information is given on any current masthead page.

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Isolation and Characterization of an Organic Solvent Soluble Polypeptide Component from Photoreceptor Complexes of *Rhodospirillum rubrum*†

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ABSTRACT: An organic solvent soluble polypeptide has been isolated from photoreceptor complexes and chromatophores of *Rhodospirillum rubrum*. After extraction of the protein from lyophilized samples with 1:1 chloroform–methanol, it was purified by column chromatography. Its isoelectric point determined by isoelectric focusing was 7.10. When analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the purified polypeptide ran as a single band of an apparent molecular weight of 12 000. However, according to amino acid analysis, the minimal molecular weight based on one histidine residue per polypeptide is 19 000. The polypeptide contains no cysteine and no tyrosine. Amino acid analyses indicated that three methionines were present per histidine residue and cyanogen bromide cleavage gave four smaller peptides which were isolated by two-dimensional electrophoresis and chromatography. Spectroscopic analysis indicated the presence of three tryptophan residues per histidine and *N*-bromosuccinamide cleavage also gave four smaller peptides which could be isolated by two-dimensional electrophoresis and chromatography. The C-terminal amino acid was shown to be glycine by two methods, while the N-terminal amino acid appears to be blocked. The organic solvent soluble polypeptide accounts for approximately 50% of the chromatophore protein and seems to bind the antenna bacteriochlorophyll and carotenoid molecules. Using this procedure, organic solvent soluble polypeptides were isolated from several photosynthetic bacteria and were found to have substantially different amino acid contents.

The light harvesting unit in photosynthetic bacteria such as *Rhodospirillum rubrum* is contained in, or on, the membrane structure proliferating from the cell membrane of the bacterium. Cell disruptive procedures such as sonication have been used to convert the membrane into small vesicles called chromatophores, and these vesicles can be converted into even smaller particles by various detergent treatments. Some of these preparations contain the antenna pigments or "bulk bacteriochlorophyll" (Loach et al., 1963, 1970a,b; Hall et al., 1973), while others contain only the phototrap pigments (Clayton and Wang, 1971; Feher, 1971; Clayton and Haselkorn, 1972; Noel et al., 1972). The former are called photoreceptor complexes, while the latter are called reaction centers.

Photoreceptor complexes (Hall et al., 1973) appear to contain the polypeptides found in reaction centers, plus one or more smaller polypeptides, depending on the species of bacterium. The antenna bacteriochlorophyll and the carotenoids also remain in photoreceptor complexes. We have found that extraction of lyophilized photoreceptor complexes of *R. rubrum* with organic solvents will totally dissolve the low-molecular-weight polypeptide, phospholipids, carotenoids, and bacteriochlorophyll, while the remaining polypeptides are insoluble. This same low-molecular-weight polypeptide has been isolated from chromatophores by a similar procedure, and relatively large amounts of it have been purified. The work described here is concerned with the isolation of this organic solvent soluble polypeptide (OSSP[1]) from *Rhodospirillum rubrum*, as well as initial chemical and physical characterization. A preliminary report of these results has been given (Tonn et al., 1974).

Materials and Methods

Bacterial Growth. Rhodospirillum rubrum, strain 1.1.1, was propagated anaerobically in modified Hutner's growth medium (Cohen-Bazire et al., 1957) at 25–30 °C. The bacteria were grown in a light box illuminated by 500 ft-c (1×10^4 erg cm^{-2} s^{-1}) fluorescent lights.

^{14}C Bacterial Cultures. Protein contents of samples were determined by radiolabeling the bacteria with [^{14}C]phenylalanine (UL, spec act. 350 to 500 μCi/mM, International Chemical and Nuclear Corp.). [^{14}C]Phenylalanine was added to modified Hunter's growth medium at concentrations varying

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[1] Abbreviations used are: OSSP, organic solvent soluble polypeptide; $NaDodSO_4$, sodium dodecyl sulfate; $NaDodSO_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.