Complete Amino Acid Sequence of the Myoglobin from the Atlantic Bottlenosed Dolphin, *Tursiops truncatus*†

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ABSTRACT: The complete amino acid sequence of the major component myoglobin from the Atlantic bottlenosed dolphin, *Tursiops truncatus*, was determined by specific cleavage of the protein to obtain large peptides that are readily degraded by the automatic sequencer. Three easily separable peptides were obtained by cleaving the protein with cyanogen bromide at the 2 methionine residues and 4 peptides were obtained by cleaving the methyl acetimidated protein with trypsin at the 3 arginine residues. By subjecting 4 of these peptides and the apomyoglobin to automatic Edman degradation, over 80% of the covalent structure of the protein was obtained. The remainder of the primary structure was determined by further digestion of the central cyanogen bromide peptide with trypsin and staphylococcal protease. This myoglobin differs from that of the sperm whale, *Physter catodon*, at 15 positions, from that of the California gray whale, *Eschrichtius gibbosus*, at 14 positions, from that of the common porpoise, *Phocoena phocoena*, at 6 positions, and from the myoglobin of the Black Sea dolphin, *Delphinus delphis* and the Amazon River dolphin, *Inia goeffrensis*, at 5 and 7 positions, respectively. All substitutions observed in this sequence fit easily into the tertiary structure of sperm whale myoglobin.

In preceding papers, the complete amino acid sequence of myoglobin from the Amazon River dolphin (Dwulet et al., 1975) and the California gray whale (Bogardt et al., 1976) were reported. Both of these sequences of Cetacean myoglobins were determined by automatic 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 the Atlantic bottlenosed dolphin, *Tursiops truncatus*. Completion of this sequence extends the number of complete Cetacean myoglobin sequences to 6, including the above mentioned Amazon River dolphin and California gray whale, the Black Sea dolphin (Kluh and Bakardjieva, 1971), common porpoise (Bradshaw and Gurd, 1969), and sperm whale (Edmundson, 1965).

Experimental Section

Materials

The principal component of Atlantic bottlenosed dolphin myoglobin was isolated from frozen muscle tissue by the procedure of Hapner et al. (1968). Phosphate buffer, pH 6.6, $\mu = 0.1$, was used to effect purification of the crude homogenate on CM-50 Sephadex. The homogeneity of the purified myoglobin was tested by vertical polyacrylamide gel electrophoresis at pH 9.2 and 5.2. The preparation of the apomyoglobin followed essentially that of Teale (1959).

Methyl acetimidate hydrochloride was prepared according to the method of Hunter and Ludwig (1962). Preparation of 3-SPITC[1] was by the procedure of Dwulet and Gurd (1976). TPCK[1]-treated trypsin was purchased from Worthington. Staphylococcal protease was obtained from Miles Laboratories Ltd. Carboxypeptidase C was purchased from Rohm and Haas, Darmstadt, Germany. Sequencer reagents of "Sequenal" grade were obtained from Beckman Instruments. All other chemicals were reagent grade.

Methods

Amino Acid Analysis. Peptides were hydrolyzed with constant-boiling HCl in evacuated tubes for 24 h at 110 °C unless otherwise specified. Amino acids were analyzed on a Model 121 Beckman amino acid analyzer by the method of Spackman et al. (1958). Tryptophan was determined by the method of Liu and Chang (1971). Chromatographic peaks were integrated by a Texas Instruments 980A minicomputer system (Bogardt, 1976).

Automated Edman Degradations. Automatic phenyl isothiocyanate degradations of peptides were performed on a Beckman Model 890C sequencer. The sequencing techniques and the methods for the identification of phenylthiohydantoin amino acids used in this sequence determination are identical to those previously reported by Dwulet et al. (1975).

Carboxypeptidase C Time Course Hydrolysis. Peptides (200–300 nmol) were digested with carboxypeptidase C as previously described (Bogardt et al., 1976).

Peptide Nomenclature. The cyanogen bromide fragments are designated by the symbol CB, the peptides isolated following tryptic cleavage at the arginine residues in the methyl acetimidated protein are given the symbol MT, and the tryptic and staphylococcal protease peptides obtained by digestion of the middle cyanogen bromide peptide, CB2, are labeled TCB2 and PCB2, respectively. All peptides are numbered from the amino terminus to the carboxyl terminus of the completed sequence.

Cyanogen Bromide Cleavage. The apomyoglobin was

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[1] Abbreviations used are: 3-SPITC, 3-sulfophenyl isothiocyanate, sodium salt; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; NMR, nuclear magnetic resonance.

TABLE I: Amino Acid Composition of *Tursiops truncatus* Myoglobin.

Amino Acid	No. of Residues From Acid Hydrolysates[a]	No. of Residues From the Sequence
Asp	12.0	12
Thr	5.2	5
Ser	5.1	5
Glu	16.2	16
Pro	4.0	4
Gly	13.2	13
Ala	17.2	17
Val	5.9	6
Met	1.9	2
Ile	8.0	8
Leu	18.9	19
Tyr	1.9	2
Phe	7.0	7
Lys	19.9	20
His	11.9	12
Arg	3.0	3
Trp[b]	2.0	2

[a] Samples were hydrolyzed for 24, 48, and 72 h, and duplicate analyses were performed on each hydrolysate. The results obtained for each residue were averaged except for serine, threonine, and isoleucine. The values for threonine and serine were obtained by extrapolation to zero time. The value for isoleucine was obtained by extrapolation to 96 h. The amino acid residues were calculated on the basis of 153 amino acids in the protein. [b] Tryptophan was determined by the method of Liu and Chang (1971).

cleaved with cyanogen bromide according to the method of Dwulet et al. (1975).

Tryptic Digestion of the Methyl Acetimidated Apomyoglobin. The methyl acetimidation of the myoglobin was accomplished by the method of Reynolds (1968) as modified by Garner and Gurd (1975). Tryptic digestion of the methyl acetimidated apomyoglobin was performed according to the method of Dwulet et al. (1975).

Tryptic Digestion of CB2 (56–131). Tryptic digestion of the cyanogen bromide fragment, CB2 (56–131), was accomplished as previously described by Dwulet et al. (1975).

Staphylococcal Protease Digestion of CB2 (56–131). Cleavage at the glutamyl residues of CB2 (56–131) was achieved by digestion with staphylococcal protease as previously described by Bogardt et al. (1976).

Results

Amino Acid Composition. The amino acid composition of the principal component of Atlantic bottlenosed dolphin myoglobin was obtained from 24-, 48-, and 72-h hydrolysates of the apomyoglobin. The results are summarized in Table I.

Cyanogen Bromide Fragments. The peptides obtained from the cyanogen bromide digestion of the apomyoglobin were purified on a column of Bio-Gel P-30.[2] The purification procedure was essentially that of Marshall et al. (1974) and the resulting peptides were subjected to amino acid analysis.[3]

Cleavage at Arginine Residues. Methyl acetimidated apomyoglobin was cleaved by trypsin at its arginine residues and initially purified by gel filtration on a column of Bio-Gel P-10.[2] Peptides MT3 and MT4 did not separate on the P-10 column but were isolated by gel filtration on a Bio-Gel P-6 column.[2] The amino acid compositions of these peptides were in good agreement with expected values.[3]

Tryptic Peptides of CB2. The tryptic peptides of CB2 were purified as previously described (Dwulet et al., 1975)[2] and their amino acid compositions were determined.[3]

Protease Peptides of CB2. Partial purification of the peptides resulting from the digestion of CB2 (56–131) with staphylococcal protease was achieved on a column of Bio-Gel P-4.[2] Three major fractions were collected. The first fraction to elute from the column contained the protease and partially cleaved CB2, and the last fraction to elute from the column contained peptide PCB2-4 (106–109) in pure form. The middle fraction was further purified by ion-exchange chromatography on Cellex P (Bio-Rad) using a linear gradient of pyridine acetate (pH 2.0–4.5).[2] All peptides were obtained pure. The only uncharacteristic cleavage found in the digest occurred at a Ser–His bond at positions 92 and 93. Since cleavage at serine residues by staphylococcal protease has not been previously reported, peptide PCB2-3a (86–92) was subjected to automatic Edman degradation and to carboxypeptidase C time-course hydrolysis. These procedures established the sequence and position of the peptide. The peptide containing residues 93–105 was not isolated in this digest but has been isolated from the staphylococcal protease digestion of other myoglobins containing a Ser–His bond at positions 92 and 93.[4,5,6] The positions and compositions of the staphylococcal protease peptides are reported in Table II.

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

Sequenator Results. The complete primary structure of Atlantic bottlenosed dolphin myoglobin is shown in Figure 1. The sequence strategy used to obtain the primary structure is outlined diagrammatically in Figure 2. In this diagram, sequenator analysis A represents the first 37 amino terminal residues obtained by automatic Edman degradation of the intact apomyoglobin. The amino terminal residues of sequenator analysis B of peptide MT2 (32–118) had a 6-residue overlap with the intact protein analysis and extended the sequence 29 residues to position 66. Sequenator analysis C of peptide CB2 (56–131) overlapped analysis B and extended the sequence 24 cycles to position 90. Analysis D yielded the entire sequence of peptide PCB2-3 (86–105) which overlapped with analysis C starting at position 86 and extended the sequence to position 105. Peptide TCB2-6 (103–118) (sequenator analysis E) overlapped analysis D with the only tyrosine residue in the central cyanogen bromide fragment and extended the sequence to position 118, which is also the only arginine residue of CB2. Analysis F of PCB2-5a (110–131) overlapped analysis E starting at position 110 and extended the sequence to position 121, overlapping arginine 118. Analysis G of peptide MT3 (119–139) overlapped peptide PCB2-5a and extended the sequence from residue 119 past methionine 131 to sequence position 135. The final sequenator analysis H of peptide CB3 (132–153) overlapped analysis G starting at position 132 and extended to the carboxyl terminus of the protein at position 153.

Because of the low repetitive yield of sequenator analyses D and F, despite clear cut results with little carryover, the

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

[3] The amino acid analyses of all peptides obtained by specific cleavage of the apomyoglobin and cyanogen bromide fragment CB2 (56–131) were found to be in good agreement with the expected values and the results can be found in supplementary material as described below.

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

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

[6] F. E. Dwulet, work in progress.

TABLE II: Amino Acid Composition[a] of Staphylococcal Protease Peptides from CB2.

Amino Acid	PCB2-1	PCB2-2	PCB2-3	PCB2-3a	PCB2-4	PCB2-5	PCB2-5a	PCB2-6
Asp		2.9 (3)					1.0 (1)	1.0 (1)
Thr		2.1 (2)	1.2 (1)					
Ser	1.0 (1)		0.8 (1)	0.7 (1)	1.0 (1)	0.9 (1)	0.8 (1)	
Glu	0.9 (1)	1.4 (1)	2.0 (2)	1.0 (1)	1.1 (1)	1.2 (1)	2.2 (2)	1.2 (1)
Pro			1.7 (2)	1.0 (1)		1.2 (1)	1.2 (1)	
Gly		2.8 (3)					2.1 (2)	1.9 (2)
Ala	1.2 (1)	3.3 (3)	2.4 (2)	1.0 (1)		2.1 (2)	5.0 (5)	3.0 (3)
Val		0.9 (1)				0.9 (1)	0.9 (1)	
Ile		1.2 (1)	1.6 (2)		0.9 (1)	0.5 (2)[b]	0.5 (2)[b]	
Leu		3.7 (4)	3.4 (3)	2.0 (2)		0.8 (1)	1.1 (1)	
Tyr			0.7 (1)					
Phe					1.0(1)		0.9 (1)	0.9 (1)
Lys	0.9 (1)	4.7 (5)	4.0 (4)	1.0 (1)				
His		3.1 (3)	2.3 (2)			2.8 (3)	2.8 (3)	
Arg						1.1 (1)	1.1 (1)	
Hse							0.4 (1)[c]	0.4 (1)[c]
Total Residue	4	26	20	7	4	13	22	9
Yield	99%	79%	48%	45%	57%	64%	27%	73%
Position	56-59	60-85	86-105	86-92	106-109	110-122	110-131	123-131
Pool	PCB2-III	PCB2-VII	PCB2-VI	PCB2-II	PCB2-C	PCB2-V	PCB2-IV	PCB2-I

[a] The numbers in parentheses refer to the number of residues per molecule of peptide determined from the completed sequence. [b] Low isoleucine value was due to incomplete hydrolysis of an Ile–Ile bond in the peptide. [c] Homoserine was calculated as homoserine lactone.

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                5                  10                  15
 1  Gly Leu Ser Asp Gly Glu Trp Gln Leu Val Leu Asn Val Trp Gly
16  Lys Val Glu Ala Asp Leu Ala Gly His Gly Gln Asp Val Leu Ile
31  Arg Leu Phe Lys Gly His Pro Glu Thr Leu Glu Lys Phe Asp Lys
46  Phe Lys His Leu Lys Thr Glu Ala Asp Met Lys Ala Ser Glu Asp
61  Leu Lys Lys His Gly Asn Thr Val Leu Thr Ala Leu Gly Ala Ile
76  Leu Lys Lys Lys Gly His His Asp 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 Glu Phe Gly Ala Asp Ala Gln Gly Ala Met Asn Lys Ala Leu
136 Glu Leu Phe Arg Lys Asp Ile Ala Ala Lys Tyr Lys Glu Leu Gly
151 Phe His Gly
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FIGURE 1: The amino acid sequence of Atlantic bottlenosed dolphin. The hyphens between the amino acid residues have been omitted for clarity.

carboxyl terminal sequences of peptides PCB2-3 (86–105) and PCB2-5 (110–122) were determined by time-course digestion with carboxypeptidase C.[2] This procedure reconfirmed the amino acid sequence around tyrosine 103 in peptide PCB2-3 and around arginine 118 in peptide PCB2-5.

In all sequenator runs the yields for the phenylthiohydantoins were similar to those previously discussed (Dwulet et al., 1975; Bogardt et al., 1976).

Discussion

The present report is the third in a series[4,5,6] of complete Cetacean myoglobin sequences determined by automated Edman degradation. The information derived from these sequence investigations has been used to interpret proton NMR results in which pK_a values of individual histidine residues were assigned (Botelho, 1975), as well as in the treatment of electrostatic interactions within the myoglobin molecule (Shire et al., 1975). The sequence data have also been used to develop a computer model of Cetacean phylogenetics (Bogardt, 1976).

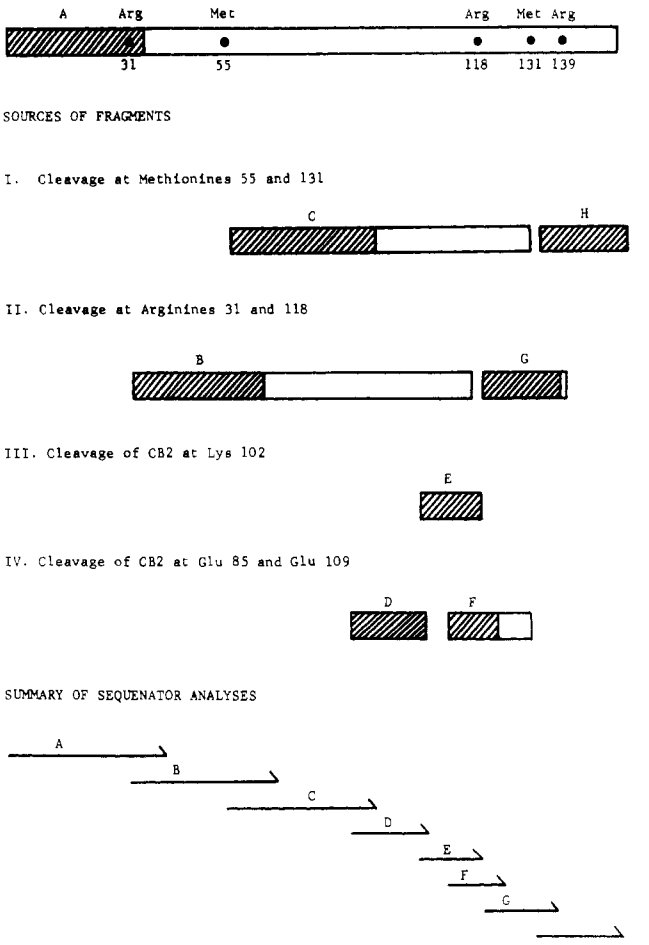

FIGURE 2: Diagrammatic summary of fragments generated from the Atlantic bottlenosed dolphin myoglobin for sequenator analysis. The top bar represents the whole myoglobin and the residues that are important for its fragmentation. The capital letters A–H identify the sequenator analyses in the order in which they are described in the text. A hatched section in each horizontal bar indicates the segment of the sequence determined by that analysis. A summary of overlaps is shown in the lower portion by the labeled arrows.

Species	Residue Number 1	4	5	12	13	15	21	26	28	35	45	54
A. B. Dolphin	Gly	Asp	Gly	Asn	Val	Gly	Leu	Gln	Val	Gly	Lys	Asp
A. R. Dolphin	Gly	Asp	Gly	Asn	Ile	Gly	Leu	Gln	Val	Gly	Lys	Glu
Common Porpoise	Gly	Glu	Gly	Asn	Val	Gly	Leu	Gln	Val	Gly	Lys	Glu
B. S. Dolphin	Gly	Asp	Gly	Asn	Val	Gly	Val	Glu	Ile	Gly	Lys	Asp
Gray Whale	Val	Asp	Ala	Asn	Ile	Ala	Val	Gln	Ile	Gly	Lys	Glu
Sperm Whale	Val	Glu	Gly	His	Val	Ala	Val	Gln	Ile	Ser	Arg	Glu

Species	Residue Number 66	74	83	85	109	121	122	129	144	151	152
A. B. Dolphin	Asn	Ala	Asp	Glu	Glu	Ala	Glu	Gly	Ala	Phe	His
A. R. Dolphin	Asn	Gly	Glu	Glu	Glu	Gly	Asp	Ala	Ala	Phe	His
Common Porpoise	Asn	Gly	Glu	Asn	Glu	Ala	Glu	Gly	Thr	Phe	His
B. S. Dolphin	Asp	Ala	Asp	Glu	Glu	Ala	Gln	Gly	Ala	Phe	His
Gray Whale	Asn	Gly	Glu	Glu	Asp	Gly	Asp	Ala	Ala	Phe	Gln
Sperm Whale	Val	Ala	Glu	Glu	Glu	Gly	Asn	Gly	Ala	Tyr	Gln

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. A. B. dolphin is Atlantic bottlenosed dolphin, A. R. dolphin is Amazon River dolphin, and B. S. dolphin is Black Sea dolphin.

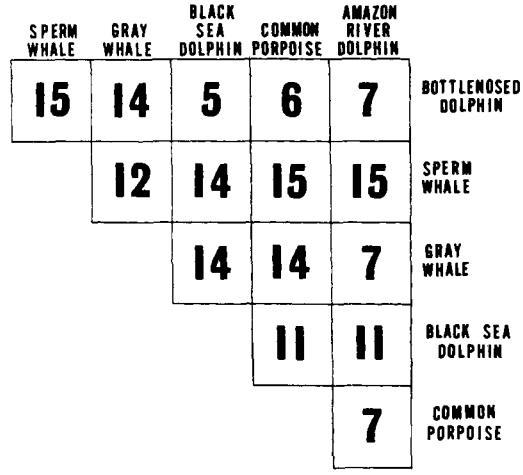

FIGURE 4: Difference matrix for Cetacean myoglobins obtained by summing the number of different amino acids between pairs of proteins.

The sequence of Atlantic bottlenosed dolphin myoglobin is compared in Figure 3 with the known Cetacean myoglobins, those of Amazon River dolphin, common porpoise, Black Sea dolphin, California gray whale, and sperm whale. As emphasized in the difference matrix shown in Figure 4, the Atlantic bottlenosed dolphin myoglobin has the closest similarity in sequence to that of the Black Sea dolphin, but is also quite similar to the myoglobin of the Amazon River dolphin and the common porpoise from the North Atlantic Ocean. The sequence of bottlenosed dolphin myoglobin will be examined here in comparison to the six differences between it and the myoglobin sequence of the common porpoise. The differences between the two sequences will be referred to by first giving the position number, then the residue found in bottlenosed dolphin myoglobin, followed by the homologous common porpoise residue in parentheses.

4 Aspartic Acid (*Glutamic Acid*). Aspartic acid is a common residue at this position and is found in the majority of known myoglobins.

54 Aspartic Acid (*Glutamic Acid*). The common residue found at position 54 is glutamic acid. Aspartic acid at this position has been found previously only in the myoglobins of the true seals (Bradshaw and Gurd, 1969; Nauman, 1973), the Black Sea dolphin (Kluh and Bakardjieva, 1971), the chicken (Deconinck et al., 1975), and the sea hare (Tentori et al., 1973).

74 Alanine (*Glycine*). Glycine is the common amino acid found at position 74. Alanine has been previously reported at this position only in the myoglobins of the sperm whale (Edmundson, 1965) and the Black Sea dolphin.

83 Aspartic Acid (*Glutamic Acid*). This position in the myoglobin sequence is usually occupied by a glutamic acid residue. An aspartic acid substitution has been found only in the myoglobins from the Black Sea dolphin and the California sea lion (Vigna et al., 1974).

85 Glutamic Acid (*Asparagine*). Glutamic acid is a common residue at position 85, and the substitution constitutes the only charge change between the two proteins under comparison.

144 Alanine (*Threonine*). The majority of the known myoglobin sequences have alanine at this position. The threonine substitution is found only in the common porpoise myoglobin.

All of the above changes are considered conservative. The few positions that do vary between the bottlenosed dolphin and the common porpoise show nothing new in terms of residue substitutions among the myoglobins. An examination of the bottlenosed dolphin replacements in terms of the three-dimensional structure of sperm whale myoglobin leads to the following conclusions: the changes are generally on the surface of the molecule; all heme contact region residues are conserved; for the changes that do occur, there is no obvious involvement of their side chains in any associations with the other parts of the structure. All changes then are compatible with the sperm whale myoglobin three-dimensional structure (Watson, 1969; Lee and Richards, 1971) and no significant change in the backbone conformation of the bottlenosed dolphin myoglobin is expected to occur.

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Supplementary Material Available

Experimental material including amino acid compositions, elution profiles, sequencer repetitive yield plots, and carboxypeptidase C time course hydrolysis plots (20 pages). Ordering information is given on any current masthead page.

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Molecular Topology of the Photosynthetic Light-Harvesting Pigment Complex, Peridinin–Chlorophyll a–Protein, from Marine Dinoflagellates†

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ABSTRACT: The photosynthetic light-harvesting complex, peridinin–chlorophyll a–protein, was isolated from several marine dinoflagellates including *Glenodinium sp.* by Sephadex and ion-exchange chromatography. The carotenoid (peridinin)–chlorophyll a ratio in the complex is estimated to be 4:1. The fluorescence excitation spectrum of the complex indicates that energy absorbed by the carotenoid is transferred to the chlorophyll a molecule with 100% efficiency. Fluorescence lifetime measurements indicate that the energy transfer is much faster than fluorescence emission from chlorophyll a. The four peridinin molecules within the complex appear to form two allowed exciton bands which split the main absorption band of the carotenoid into two circular dichroic bands (with negative ellipticity band at 538 nm and positive band at 463 nm in the case of peridinin–chlorophyl a–protein complex from *Glenodinium sp.*). The fluorescence polarization of chlorophyll a in the complex at 200 K is about 0.1 in both circular dichroic excitation bands of the carotenoid chromophore. From these circular dichroic and fluorescence polarization data, a possible molecular arrangement of the four peridinin and chlorophyll molecules has been deduced for the complex. The structure of the complex deduced is also consistent with the magnitude of the exciton spliting (ca. >3000 cm^{-1}) at the intermolecular distance in the dimer pair of peridinin (ca. 12 Å). This structural feature accounts for the efficient light-harvesting process of dinoflagellates as the exciton interaction lengthens the lifetime of peridinin (radiative) and the complex topology increases the energy transfer probability. The complex is, therefore, a useful molecular model for elucidating the mechanism and efficiency of solar energy conversion in vivo as well as in vitro.

The marine dinoflagellates *Glenodinium sp.* and *Gonyaulax polyedra* contain a peridinin–chlorophyll a–protein complex (PCP)[1] which apparently acts as a photosynthetic light-harvesting accessory pigment (Prézelin and Haxo, 1976). The pigment complex (mol wt 35 500 and 34 500 for *Glenodinium sp.* and *Gonyaulax polyedra* PCP's, respectively) contains four carotenoids and one chlorophyll a molecule per protein. The dinoflagellate *Amphidinium carterae* (Plymouth 450) also contains PCP (mol wt 39 200), but its apoprotein (mol wt 31 800) is associated with nine peridinin and two chlorophyll a molecules (Haxo et al., 1976). These complexes show an efficient energy transfer from peridinin to chlorophyll a, as measured by fluorescence excitation spectroscopy (Haxo et al., 1972, 1976; Prézelin and Haxo, 1974, 1976; Prézelin, 1975).

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[1] Abbreviations used are: PCP, peridinin–chlorophyll a–protein complex; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; CD, circular dichroism; ORD, optical rotatory dispersion.