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Amino acid sequence of bacteriorhodopsin

(purple membrane/hydrophobic peptides/high-pressure liquid chromatography/gas chromatographic mass spectrometry/Edman degradation)

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ABSTRACT **The complete primary structure of the purple membrane protein bacteriorhodopsin, which contains 248 amino acid residues, has been determined. Methods used for separation of the hydrophobic fragments included gel permeation and reverse-phase high-pressure liquid chromatography in organic solvents. The amino acid sequence was determined by a combination of automatic Edman degradation and mass spectrometric methods. The total sequence was derived by ordering of the CNBr fragments on the basis of methionine-containing peptides identified by gas chromatographic mass spectrometry and by analysis of *N*-bromosuccinimide fragments containing overlaps between CNBr fragments. The present sequence differs from that recently reported by Ovchinnikov and coworkers with respect to an additional tryptophan (position 138) and several amino acid assignments.**

The purple membrane of a number of extremely halophilic bacteria—e.g., *Halobacterium halobium*—functions as a light-driven proton pump (1, 2). It contains a single protein, bacteriorhodopsin (M_r 26,000) with one molecule of retinaldehyde covalently bound to a lysine residue (3, 4). Bacteriorhodopsin forms a continuum of seven α helices, each of which spans the membrane and is largely embedded in it (5). Knowledge of the primary structure of bacteriorhodopsin is a prerequisite to studies of the mechanism of the proton pump and of the biogenesis of this interesting protein. In a recent paper, we reported (6) on the sequence of the first 102 amino acids and of 39 amino acids from the COOH terminus of this membrane protein. The present paper reports on its complete amino acid sequence (Fig. 1). Although no experimental details have appeared, the complete sequence has also been deduced by Ovchinnikov and coworkers (7, 8), and partial sequences have been reported by other laboratories (3, 9, 10). The present sequence differs from that reported by Ovchinnikov and coworkers with respect to an additional tryptophan (position 138) and amino acid assignments at positions 105, 111, 117, 146, and 206. Of the total 248 amino acids present in bacteriorhodopsin, 70% are hydrophobic, and there is significant clustering of the hydrophobic as well as of the hydrophilic amino acids.

MATERIALS AND METHODS

Materials. *N*-Bromosuccinimide (NBS), fluorescamine, and isothiocyanatophenylthiocarbamoylaminopropyl (IPTAP) glass were purchased from Pierce, and trypsin treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone, clostripain, and elastase were from Worthington Biochemicals. [^{14}C]Succinic anhydride was from New England Nuclear. Tetraethyltetraamino (TETA) and aminoethylaminopropyl (AEAP) glass were gifts from Mark Horn of Sequemat, Watertown, MA. Other materials were as described previously.

Preparation of Chymotryptic and CNBr Fragments. Purple membrane was isolated from *H. halobium* and apomembrane was prepared as described (6). Digestion of the apomembrane with chymotrypsin gave two fragments, C-1 and C-2, which were collected by centrifugation and separated on a Sephadex LH-60 column with formic acid/ethanol as described (6). Cyanogen bromide cleavage of C-1 and separation of CNBr fragments 6, 7, 9, and 10 has also been described (6). In some experiments, bacteriorhodopsin obtained after delipidation of the purple membrane (6) was directly subjected to fragmentation by CNBr. In this case the fragment CNBr-10 was replaced by CNBr-10a, which contained the additional NH_2-terminal amino acid sequence Val-Pro-Phe.

NBS Cleavage of C-1. The fragment (1.5 μmol) in 200 μl of 88% (vol/vol) formic acid was mixed with 8.0 ml of 8 M urea in 0.3 M acetic acid (pH 3.5), and to it was added a solution of NBS (133.5 mg; 100-fold excess over tryptophan) in 200 μl of the above urea/acetic acid solvent. After incubation at room temperature for 30 min, the fragments were separated by gel permeation chromatography and high-pressure liquid chromatography (HPLC) (6). For analysis, aliquots of column effluents were first derivatized with fluorescamine and then subjected to polyacrylamide gel electrophoresis.

Cleavage with Clostripain. 4-Sulfophenylisothiocyanate (SPITC) derivatives of CNBr-10 and CNBr-10a, were prepared and purifed by chromatography on Sephadex LH-20 (6). These fragments were also derivatized with succinic anhydride to give water-soluble products. The derivatized fragments (100–200 nmol) were dissolved in 2.0 ml of 20 mM triethylamine, and 2.0 ml of 200 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.6) containing 2 mM dithiothreitol and 2 mM $CaCl_2$ was added. Clostripain was added at an enzyme-to-substrate ratio of 1:100 and the mixture was incubated at 37°C for 18 hr. The digest was then evaporated to dryness, the residue was dissolved in 88% formic acid, and the solution was chromatographed on Sephadex LH-20 (6). The material emerging at the void volume was used for sequence determination.

Attachment of CNBr Fragments to Glass Supports. (*i*) CNBr-11 to IPTAP glass: The fragment (50 nmol) was lactonized and allowed to react with ethylenediamine (6). Excess reagent was removed under reduced pressure and the residue was dissolved in 200 μl of dimethylformamide. To the solution

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Abbreviations: GCMS, gas chromatographic mass spectrometry; HPLC, high-pressure liquid chromatography; NBS, *N*-bromosuccinimide; SPITC, 4-sulfophenylisothiocyanate; IPTAP, TETA, and AEAP glass, isothiocyanatophenylthiocarbamoylaminopropyl, tetraethyltetraamino, and aminoethylaminopropyl glass, respectively.

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                  5                  10                  15                  20                  25
<Glu Ala Gln Ile Thr Gly Arg Pro Glu Trp Ile Trp Leu Ala Leu Gly Thr Ala Leu Met Gly Leu Gly Thr Leu
                 30                  35                  40                  45                  50
Tyr Phe Leu Val Lys Gly Met Gly Val Ser Asp Pro Asp Ala Lys Lys Phe Tyr Ala Ile Thr Thr Leu Val Pro
                 55                  60                  65                  70                  75
Ala Ile Ala Phe Thr Met Tyr Leu Ser Met Leu Leu Gly Tyr Gly Leu Thr Met Val Pro Phe Gly Gly Glu Gln
                 80                  85                  90                  95                 100
Asn Pro Ile Tyr Trp Ala Arg Tyr Ala Asp Trp Leu Phe Thr Thr Pro Leu Leu Leu Leu Asp Leu Ala Leu Leu
                105                 110                 115                 120                 125
Val Asp Ala Asp Gln Gly Thr Ile Leu Ala Leu Val Gly Ala Asx Gly Ile Met Ile Gly Thr Gly Leu Val Gly
                130                 135                 140                 145                 150
Ala Leu Thr Lys Val Tyr Ser Tyr Arg Phe Val Trp Trp Ala Ile Ser Thr Ala Ala Met Leu Tyr Ile Leu Tyr
                155                 160                 165                 170                 175
Val Leu Phe Phe Gly Phe Thr Ser Lys Ala Glx Ser Met Arg Pro Glu Val Ala Ser Thr Phe Lys Val Leu Arg
                180                 185                 190                 195                 200
Asn Val Thr Val Val Leu Trp Ser Ala Tyr Pro Val Val Trp Leu Ile Gly Ser Glu Gly Ala Gly Ile Val Pro
                205                 210                 215                 220                 225
Leu Asn Ile Glu Thr Leu Leu Phe Met Val Leu Asp Val Ser Ala Lys Val Gly Phe Gly Leu Ile Leu Leu Arg
                230                 235                 240                 245
Ser Arg Ala Ile Phe Gly Glu Ala Glu Ala Pro Glu Pro Ser Ala Gly Asp Gly Ala Ala Ala Thr Ser
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FIG. 1. Complete amino acid sequence of bacteriorhodopsin.

was added 50 mg of IPTAP glass, and the mixture was shaken at 45°C for 18 hr (11). The IPTAP glass was washed three times with 1 ml of dimethylformamide and finally with 1 ml of 88% formic acid before the start of sequencer analysis. (*ii*) CNBr-10a to TETA and AEAP glass: The fragment CNBr-10a was derivatized with [^{14}C]succinic anhydride and then treated with trifluoroacetic acid to form the homoserine lactone derivative. The latter was coupled to TETA and AEAP glass by following the published procedures (12, 13). Coupling of the peptide to the glass was monitored by measuring the radioactivity remaining in solution and in successive washes of the glass with methanol, ether, and trifluoroacetic acid.

The glass-coupled 3-carboxypropionylated CNBr-10a was cleaved with trypsin in 50 mM ammonium bicarbonate buffer (pH 7.8) at 37°C for 4 hr using an enzyme-to-substrate ratio of 1:100. The sequence of the amino acids in the resulting 36 amino acid long peptide (83–118) remaining on glass was determined by the solid phase sequencer program of Horn and Bonner (14).

Polacrylamide Gel Electrophoresis. Aliquots of the peptides were dried, residues were suspended in 20 μl of 0.2 M sodium borate buffer at pH 9.0, and 20 μl of fluorescamine solution (1 mg/ml of acetone) was added. The samples were subjected to electrophoresis on a 12% acrylamide gel (15) and the bands were visualized under ultraviolet light.

Gas Chromatographic Mass Spectrometry (GCMS) Sequencing. Polypeptides were subjected to partial acid hydrolysis or enzymatic digestion, the resulting mixtures were converted to polyamino alcohols, and the alcohols were identified by GCMS as described (6).

RESULTS

Purification of CNBr and NBS Fragments of Chymotryptic Fragment C-1. Chymotryptic cleavage of apomembrane yields two fragments. Of these, the shorter fragment (C-2) was sequenced earlier (6). Amino acid analysis of the larger segment (C-1) showed that it contained four methionines. Further, GCMS analysis of a partial acid hydrolysate of C-1 identified a number of methionine-containing di- to tetrapeptides consistent with four methionines. Therefore, CNBr cleavage of fragment C-1 should yield five fragments, and NH_2-terminal analysis of the digest supported this conclusion. However, separation by HPLC showed only four identifiable fragments, designated CNBr-6, CNBr-7, CNBr-9, and CNBr-10 in figure 2 of ref. 6. The peak then designated CNBr-8 had a composition identical with that of CNBr-7, the reason for the two peaks being unclear. The fragment missing in the above separation was extremely hydrophobic (see below), and either it was irreversibly absorbed to the HPLC column or it eluted slowly, possibly as mixed aggregates with other fragments. Fragments CNBr-7, CNBr-9, and CNBr-10 were further purified by gel permeation with Sephadex LH-60 (6).

Application of the total CNBr digest of C-1 on a Sephadex LH-60 column (2.5 × 80 cm) gave the pattern shown in Fig. 2*A*. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the material in each of the peaks (Fig. 2*B*) showed that pool a (void volume) contained mainly a peptide travelling faster than the other CNBr fragments (CNBr-7, CNBr-9, and CNBr-10). From its composition, this peptide proved to be CNBr-11, the fifth and smallest CNBr fragment. Its purification in monomeric form was achieved as follows: Pool a was dissolved in trifluoroacetic acid (0.5 ml) at 0°C, the solution was diluted with 7.0 ml of ethanol, and the final volume was adjusted to 10.0 ml with 88% formic acid. This was immediately applied to the same LH-60 column. Polyacrylamide gel analysis (Fig. 2*C*) showed that the different fragments now eluted essentially according to their size. CNBr-11 thus prepared was used in sequence work.

Sequence of CNBr-7. The sequence deduced and shown in Fig. 3 differs from that reported by Ovchinnikov and coworkers (7), there being two contiguous tryptophan residues (positions 137 and 138) instead of one (7). The molar extinction of CNBr-7 at 280 nm was consistent with two tryptophan residues. Sequencer analysis confirmed this conclusion and placed the second tryptophan at position 138.

Sequence of CNBr-9. A sequencer run on CNBr-9 gave unambiguous results as far as Leu-181. Sequencer analysis of the COOH-terminal NBS fragment of C-1 that started at Leu-190 gave unequivocal assignments up to position 219. The experiment also provided an overlap of CNBr-9 and CNBr-6. The only outstanding question pertained to position 182. Amino acid composition of CNBr-9 showed two tryptophan residues. From the above sequencer runs and the GCMS data, the only unassigned position is 182, and this must be occupied by the second tryptophan.

Sequence of CNBr-10. This fragment formed the NH_2 ter-

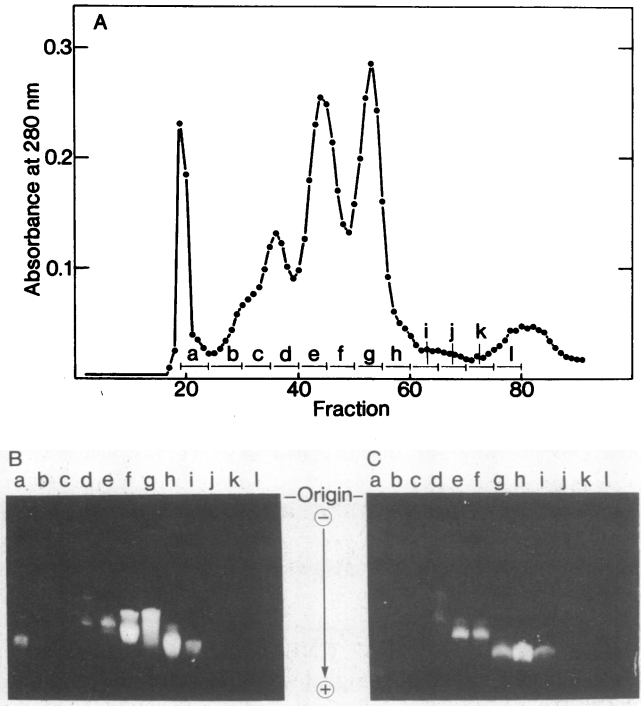

FIG. 2. (*A*) Chromatography of the CNBr fragments of C-1 on Sephadex LH-60. CNBr cleavage and chromatography were carried out as described (6). Samples were pooled as shown (a–l) and were analyzed by polyacrylamide gel electrophoresis (*B*). Pool a was dried, redissolved in 0.5 ml of anhydrous trifluoroacetic acid, and diluted with 7.0 ml of ethanol and 2.5 ml of 88% formic acid. The sample was chromatographed on Sephadex LH-60 as above and pools were subjected to polyacrylamide gel electrophoresis (*C*).

minus of C-1, and sequencer analysis of the latter gave the sequence up to Ala-103 (sequence 1 in Fig. 3). Digestion of the SPITC derivative with clostripain yielded a fragment consisting of the sequence Tyr-83 to Hse-118. Automated Edman degradation of this fragment established the sequence up to Gly-106 (sequence 2 in Fig. 3). The amino acid at position 105 was identified as glutamine by HPLC analysis of the phenylthiohydantoin. In further experiments, the fragment Tyr-83 to Hse-118 was coupled to TETA or AEAP glass and subjected to automated Edman degradation. The sequence up to Val-112 was then obtained (sequence 3 in Fig. 3). The assignment of Ile at position 117 was made on the basis of amino acid composition of CNBr-10, which showed three Ile residues for this fragment. Finally, from the amino acid composition of CNBr-10, one more amino acid, Asx, remained to be accommodated in the sequence. It could be placed only at position 115 because the sequences of the polypeptide (72–114) and the tripeptide (116–118) had been unequivocally determined. The nature of Asx, aspartic or asparagine, remains to be determined.

Sequence of CNBr-11. GCMS on acid hydrolysates of total C-1 and the unpurified aggregate (pool a of Fig. 2) gave a partial sequence for this fragment. Amino acid composition of the purified CNBr-11 and Edman degradation of the latter after attachment to IPTAP glass enabled derivation of the complete sequence.

Total Sequence of Bacteriorhodopsin. The amino acid sequences of the above four CNBr fragments are shown in Fig. 3. CNBr-10 is present at the NH_2 terminus of C-1, while CNBr-6 has been shown to be at its COOH terminus (6). GCMS analysis of a partial acid hydrolysate of C-1 yielded several methionine-containing peptides, including Leu-Phe-Met-Val (207–210) and Phe-Met-Val-Leu (208–211). The latter peptides and the sequence of the NBS fragment Leu-190 to Ser-248, described above established the overlap and order of CNBr-9 and CNBr-6. The order of (CNBr-7)-(CNBr-11) was established for these two fragments by the identification of the overlap peptide Ala-Met-Leu by GCMS.

The order (CNBr-10)-(CNBr-7) was further supported by (*i*) the amino acid composition of the NBS fragment, Leu-87 to Trp-137 and (*ii*) by the identification of the tripeptide Leu/Ile-Met-Leu/Ile by GCMS.

Thus the order of the CNBr fragments in C-1 is (CNBr-10)-(CNBr-7)-(CNBr-11)-(CNBr-9)-(CNBr-6). The resulting total sequence of bacteriorhodopsin is shown in Fig. 1.

DISCUSSION

The complete amino acid sequence of bacteriorhodopsin has been elucidated. The extreme hydrophobicity of the protein posed problems in performing fragmentation and in separating the fragments. A combination of reverse-phase HPLC and gel permeation in formic acid/ethanol mixtures proved useful for separation of the fragments, but partial aggregation of one or more fragments occurred even under these conditions. Aggregation was most striking with the fragment CNBr-11. The reason for this must be the presence of the uninterrupted sequence of 11 hydrophobic amino acids from the NH_2 terminus. Solubilization of this and other fragments was achieved by attachment of hydrophilic (3-carboxypropionyl or SPITC) groups at the termini.

As in previous work (6), the combined use of automated Edman degradation, both in solution and in solid phase, and GCMS greatly facilitated the sequence analysis. Because the methods are independent, the results obtained by their combined use are more reliable than those obtained from either technique alone. This is of particular importance for those residues for which our sequence differs from the sequences of other groups (7, 9).

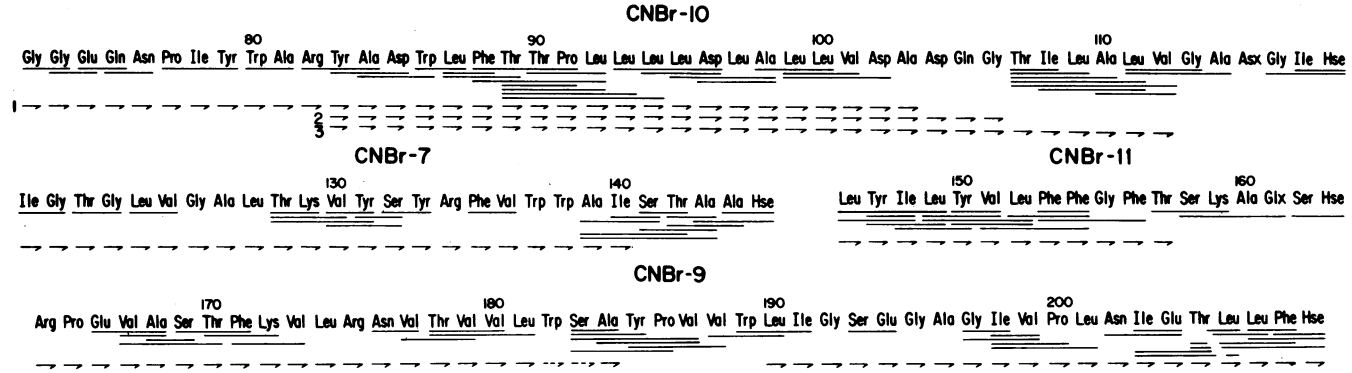

FIG. 3. Amino acid sequences of CNBr fragments. Underlinings indicate peptides identified by GCMS; the arrows indicate data obtained by automated Edman sequencing. The three sets of arrows under CNBr-10 indicate three sequencer experiments as described in text.

The sequence shown in Fig. 1 is complete except for the specification of Asx (position 115) and of Glx (position 161). Furthermore, the assignments at positions 111 and 117, Leu and Ile, respectively, need confirmation. There are several differences between the present sequence and that recently reported by Ovchinnikov *et al.* (7). First, these authors assigned the amino acids Ile and Leu at positions 111 and 117, whereas in our sequence these assignments are reversed. Second, following Trp-137, we find a second Trp (138), the total length of the protein thus being 248 residues rather than 247. Two independent automated Edman degradations performed on CNBr-7, which contains the above sequence, clearly showed a second Trp before the Ala-Ile sequence. Third, we assign Leu rather than Ser at position 146. This amino acid forms the NH_2 terminus of CNBr-11 and, as expected, a single cycle of Edman degradation on the CNBr digest of C-1 showed equimolar amounts of five residues: Gly, Leu, Ile, Arg, and Val, no Ser being detected. Further, four GCMS peptides placed a Leu between Met-145 and Tyr-147, and automated Edman degradation on purified CNBr-11 indicated Leu-146. The next discrepancy with the reported sequence (7) is at residue 206, which the Russian group has assigned as Ala. On the basis of four GCMS peptides and one sequencer experiment we place Leu at this position. Finally, we determined residue 105 to be Gln rather than Glu (sequencer analysis). The reasons for the above differences are unclear. There is the possibility of mutations in the *H. halobium* strains used. However, it should be noted that the Russian workers have commented on their failure to isolate large hydrophobic peptides in a pure state (7), and most of the differences in amino acid assignments are, in fact, in these polypeptides.

The extreme hydrophobicity of bacteriorhodopsin is evident from the amino acid composition (Table 1): over 70% hydrophobic amino acids. The hydrophobicity of specific regions may be enhanced by the clustering of hydrophobic and of hydrophilic amino acids. Thus, 80% of hydrophobic amino acids occur in clusters of four or more, the longest region containing 14 contiguous hydrophobic amino acids (143–156). The presence of tracts of hydrophilic and hydrophobic amino acids may be a general property of membrane proteins. The membrane-associated segments of a number of integral membrane proteins (17–19) seem to acquire hydrophobicity in this way (20), although the overall compositions of these proteins are not markedly hydrophobic. Thus, tracts of hydrophobic and hydrophilic amino acids could be of general importance in incorporation and stabilization of the proteins in the membrane and, consequently, in their function.

Derivation of the primary structure of bacteriorhodopsin should facilitate work on its tertiary structure and, therefore, in studies of the mechanism of the proton pumping cycle. The electron diffraction data of Unwin and Henderson (5) indicate that bacteriorhodopsin probably consists of seven helices, which traverse the membrane and are largely embedded in it. Because

Table 1. Amino acid composition of bacteriorhodopsin

Hydrophilic		Hydrophobic	
Asp 9	Gln 3	Pro 11	Ile 15
Asn 3	<Glu 1	Gly 25	Leu 36
Thr 18	Lys 7	Ala 29	Tyr 11
Ser 13	Arg 7	Val 21	Phe 13
Glu 9		Met 9	Trp 8
Total 70		178	
% 28.2		71.8	

Amino acids were classified as recommended in ref. 16.

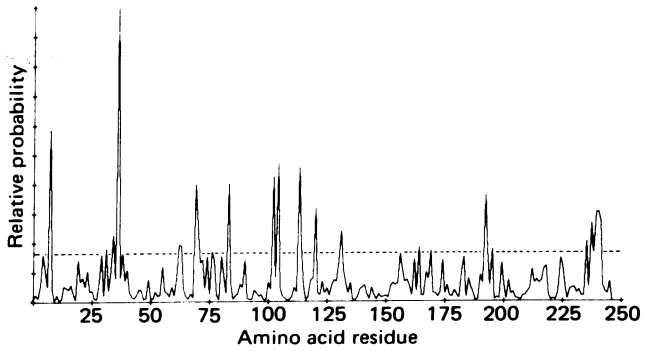

FIG. 4. Probability of β turns at different tetrapeptide sequences in bacteriorhodopsin calculated according to Chou and Fasman (21). The broken line indicates the minimum probability for prediction of a β turn.

the COOH terminus of bacteriorhodopsin is on the cytoplasmic side of the membrane (4), the NH_2 terminus must be at the exterior surface. Some inferences concerning the approximate location of the presumed six turns can be drawn from the sites of enzymatic cleavages in purple membrane sheets and from the location of clusters of hydrophilic amino acids and of proline residues. In this connection, we have performed calculations (Fig. 4), using the method of Chou and Fasman (21) for the occurrence of β turns, although this empirical method, which was originally derived from the tertiary structures of soluble proteins, may not be applicable to membrane proteins. Thus, starting from the NH_2 end, the enzyme cleavage (7–9) near this terminus indicates that several residues may extend out of the bilayer. This conclusion is supported by the presence of hydrophilic amino acids (Thr-Gly-Arg-Pro-Glu^9) and the prediction of a β turn at Arg^7···Trp^{10} (Fig. 4). Another turn seems certain near the sequence where the chymotryptic cleavage (Phe-71) (6) and the papain cleavage (Gly-72) (8) occur. The presence of this turn is also supported by the calculations in Fig. 4 (Pro^{70}···Gly^{73}). In the intervening 63 residues, the hydrophobic sequence Ser^{35}···Lys^{40} is the most probable place for an additional turn. Ovchinnikov and coworkers (7) propose a turn at residues 30–37. The β turn calculations, however, predict a turn at residues 36–39 similar to the model of Walker *et al* (9). This turn very probably is on the cytoplasmic side and therefore the retinyledene Schiff base (Lys-41) is also close to the same face of the membrane. Nothing is known about the location of the remaining turns except that a papain cleavage has been observed at residue 162 (7).

Note Added in Proof. Derivatization of the COOH terminus in CNBr-10 with [^{14}C]ethylenediamine followed by degradation with elastase yielded a radioactive fragment that contained Ile and had the composition expected for the COOH-terminal pentapeptide. This result supports the assignment of Ile at position 117. Further, carboxypeptidase Y treatment of *N*-(3-carboxypropionyl)-CNBr-10 has given evidence for the assignment of Asp at position 115.

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