Miller, E. J., Woodall, D. L., and Vail, M. S. (1973), *J. Biol. Chem. 248,* 1666.
Nagai, Y. (1973), *Mol. Cell. Biochem. 1,* 137.
Nagai, Y., Lapiere, C. M., and Gross, J. (1964), Abstracts of the Sixth International Congress of Biochemistry, Vol. 2, p 170.
Pisano, J. J., Bronzert, T. J., and Brewer, H. B. (1972), *Anal. Biochem. 45,* 43.
Sajdera, S. W., and Hascall, V. C. (1969), *J. Biol. Chem. 244,* 77.
Strawich, E., and Nimni, M. E. (1971), *Biochemistry 10,* 3905.
Trelstad, R. L. (1974), *Biochem. Biophys. Res. Commun. 57,* 717.
Wendt, P., von der Mark, K., Rexrodt, F., and Kühn, K. (1972), *Eur. J. Biochem. 30,* 169.
Woolley, D. E., Glanville, R. W., Lindberg, K. A., Bailey, A. J., and Evanson, J. M. (1973), *FEBS Lett. 34,* 267.
Woolley, D. E., Lindberg, K. A., Glanville, R. W., and Evanson, J. M. (1975), *Eur. J. Biochem. 50,* 437.
Yamada, S., and Itano, H. A. (1966), *Biochim. Biophys. Acta 130,* 538.

Photoreceptor Protein from the Purple Membrane of *Halobacterium halobium.* Molecular Weight and Retinal Binding Site†

John Bridgen* and Ian D. Walker

ABSTRACT: The apparent molecular weight of the purple membrane protein of *Halobacterium halobium* was found to be 20 000 by sodium dodecyl sulfate gel electrophoresis and by gel filtration in sodium dodecyl sulfate. However, the molecular weight value determined by gel filtration in 6 M guanidine was 28 000. To resolve this discrepancy, methods insensitive to or independent of the conformation of the protein were used to estimate the molecular weight. Analytical ultracentrifugation of the sodium dodecyl sulfate–protein complex, peptide mapping, and amino acid analysis all gave values of 25 000 ± 1000, a figure in agreement with a recent x-ray study. Borohydride reduction was used to attach the retinal cofactor covalently to a lysine residue. After digestion with thermolysin, peptide maps were prepared of the protein labeled at lysine residues with [^{14}C] succinic anhydride both before and after reduction. Comparison of the maps showed one radioactive peptide with changed mobility. This peptide was isolated and shown to have the sequence Val-Ser-Asp-Pro-Asp-Lys-Lys with only one of the two lysine residues alkylated. Solid-phase sequencing showed the succinyl group to be at position 6 and hence the retinal group to be at position 7. It was possible that a small amount of retinal was also bound to Lys-6. There was no apparent homology with the corresponding peptide of vertebrate rhodopsin. No evidence of chain heterogeneity was found by radiochemical peptide mapping and sequence analysis of peptides containing lysine residues indicating that all protein chains of purple membrane are very similar or identical.

The halophilic bacterium, *Halobacterium halobium,* exhibits optimum growth in solutions containing high concentrations of sodium chloride. On exposure to water or low salt concentration, the cells lyse and the disintegrated membranes may be separated by centrifugation into two fractions, the "red membrane" and the "purple membrane" (Stoeckenius and Kunau, 1968). The purple fraction apparently contains only one protein (Oesterhelt and Stoeckenius, 1971) which, together with membrane lipids, constitutes a system capable of carrying out the transport of protons through the membrane against a pH gradient (Oesterhelt and Stoeckenius, 1973; Oesterhelt and Hess, 1973). The purple color of the membrane has been shown to be due to the covalent attachment of 1 mol of retinal per mol of protein (Oesterhelt and Stoeckenius, 1971) and, by analogy with rhodopsin, these authors concluded that the mode of attachment was probably via a Schiff's base linkage to the ϵ-NH_2 side chain of a lysine residue. This has been confirmed in a brief report (Oesterhelt, 1971). Addition of borohydride to the native membrane normally has no effect but, after bleaching with 0.01 M cetyltrimethylammonium bromide, Oesterhelt and Stoeckenius (1971) found that borohydride treatment could be used to form a stable covalent bond between the retinal and protein. More recently, Oesterhelt and Schuhmann (1974) have briefly reported that illumination of purple membrane suspensions at 570 nm in the presence of borohydride causes simultaneous reduction of the Schiff's base linkage and bleaching of the membrane. These authors were unable to reconstitute membranes bleached under these conditions with retinal, indicating that reduction occurred at the original retinal binding site.

The molecular weight of the bacterial protein has been reported (Oesterhelt and Stoeckenius, 1971) to be 26 000 on the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, retinal content, and the finding of 1 mol of histidine per 26 000 mol of protein. Our preliminary observations indicated that the mobility of this protein on sodium dodecyl sulfate gels was incompatible with this molecular weight and we therefore decided to reinvestigate the molec-

† From the MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, England. *Received September 2, 1975.*

ular weight of this protein and to establish that all the protein chains of purple membrane were identical in sequence.

In order to determine the position of the retinal moiety in the primary structure, and eventually in the tertiary structure, of bacteriorhodopsin, the retinal peptide has also been isolated and its amino acid sequence determined. A similar procedure has been used for the stable covalent attachment of retinal to opsin (Bownds, 1967), the protein isolated from the vertebrate retina which is responsible for the interaction with light in the first stage of the visual process, and this allows a comparison between the retinal binding peptides of rhodopsin and bacteriorhodopsin.

Experimental Section

Materials and Methods. Iodo-2-[^{14}C]acetate (33 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. 2′,2′-[^{14}C]Succinic anhydride (10 mCi/mmol) was obtained from New England Nuclear Ltd. S-[^{14}C]-Labeled carboxymethylated immunoglobulin light chain, labeled at one cysteine only, was a gift from Dr. K. Adetugbo. Thermolysin was obtained from Calbiochem, San Diego, Calif.

Bacterial Culture and Purification of Purple Membrane Protein. Cells were grown in 10-l. cultures essentially as described by Oesterhelt and Stoeckenius (1974) except that the aeration rate was reduced to 0.25 l./min. Illumination was by 8 × 75 W car headlamps placed 18 in. from the culture vessel. Typically, the culture was harvested after approximately 90-h growth. Purple membranes were prepared from these cells according to Oesterhelt and Stoeckenius (1974) with the omission of the 0.1 M NaCl washes. The final yield, measured spectrophotometrically, was approximately 30 mg/l. of culture. The membranes were separated into their protein and lipid constituents either by incubation in sodium dodecyl sulfate solutions or by extracting the lipid into acetone–NH_4OH (5:1, v/v).

Sodium dodecyl sulfate gel electrophoresis of protein samples was carried out in slabs using a Tris-glycine (pH 8.5) buffer system (Laemlli, 1970). Acrylamide concentrations of 7.5, 10, and 12.5% were used with corresponding bisacrylamide concentrations of 0.2, 0.27, and 0.33%. All of the samples were boiled in sodium dodecyl sulfate before application to the gel. After electrophoresis at 100 V for 6 h the gels were stained for 30 min with 0.2% Coomassie brilliant blue in 50% methanol containing 7% acetic acid. The gels were destained overnight in 5% methanol containing 7% acetic acid.

Estimation of Molecular Weight by Gel Chromatography. A column (110 cm × 1 cm) of Sephadex G-100 superfine was equilibrated in 50 mM Tris-HCl buffer (pH 8.5) containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. Protein samples (~20 μg of each) were mixed with Blue Dextran and ϵ-dinitrophenyllysine standards and boiled in the same buffer before application to the column. Elution volumes were measured relative to both of these standards. Delipidated protein (1 mg) was also dissolved in 6 M guanidinium hydrochloride–0.1% ammonium bicarbonate buffer containing 1% β-mercaptoethanol, and applied to a column (120 cm × 1 cm) of LKB Ultrogel AcA34 agarose–acrylamide gel. Markers were run as described above for the G-100 column.

Amino Acid Analysis. Purple membrane (1 mg), containing ~0.75 mg of protein, was suspended in 750 μl of water containing 300 nmol of norleucine. Aliquots (50 μl) were transferred to 12 hydrolysis tubes and triplicate samples were hydrolyzed in 6 N HCl under vacuum at 105 °C for 24, 48, 72, and 96 h. Amino acid analyses were performed on a Durrum D-500 analyzer. Cysteine was estimated after reduction and carboxymethylation with iodo-2-[^{14}C]acetate (Kolb et al., 1974). The results were calculated using the procedures described by Hoy et al. (1974). Tryptophan was quantitated after hydrolysis with 3 N mercaptoethanesulfonic acid (Penke et al., 1974).

Analytical Ultracentrifugation. Purple membrane (20 mg) was dissolved in 2% sodium dodecyl sulfate solution (1 ml) and then dialyzed against 1 l. of 0.1 M Tris-HCl (pH 8.0) containing 0.1% sodium dodecyl sulfate (six changes). Total dialysis time was 21 days. The resulting solution was used for measurement of partial specific volume and for the ultracentrifugation experiments.

The partial specific volume of the protein was calculated from the density increment due to the protein in solutions at osmotic equilibrium. The densities of the solution and solvent were measured with an Anton Paar Digital Densimeter DMA-02D (Anton Paar, Graz, Austria) according to Kratky et al. (1973). Protein concentrations were calculated after amino acid anaylsis of suitable aliquots.

The molecular weight was determined by sedimentation equilibrium using an MSE Analytical Ultracentrifuge Mark 2, fitted with a photoelectric scanner. Centrifugation was performed at 16 030 rpm and at 20 °C. Establishment of equilibrium was confirmed by identity of distribution after a further 24-h centrifugation. Concentrations were determined by establishing a baseline after overspeeding to sediment the protein. Molecular weights were calculated from the slopes of plots of log c vs. r^2 at various protein concentrations and extrapolated to zero concentration.

Succinylation. A sample of lipid-free purple membrane protein (150 mg) was suspended in water (7.5 ml) and solid guanidinium hydrochloride added gradually until the protein material had dissolved. The pH of the solution was adjusted to 9.0 by addition of 1 M NaOH. 2,2′-[^{14}C]Succinic anhydride (250 μCi) was dissolved in 200 μl of 1,4-dioxane and added slowly to the protein solution maintaining the pH between 8.5 and 9.5 by the addition of 4 M NaOH. Unlabeled solid succinic anhydride (100 mg) was then slowly added to the solution. The succinylated protein was dialyzed against 1% acetic acid (v/v) at room temperature for 3 h to deacylate threonine and serine residues and then against 0.5% (v/v) triethylamine. The clear solution was freeze-dried.

Isolation and Characterization of Peptides Containing [^{14}C]Succinyllysine Residues. Succinylated purple membrane protein (10 mg) was digested with thermolysin (0.5 mg) in 0.2 M *N*-ethylmorpholine acetate (pH 8.3) containing 5 mM $CaCl_2$, for 8 h at 45 °C. A precipitate which appeared during digestion was removed by centrifugation and the supernatant material subjected to paper electrophoresis at pH 3.5. Radioactive peptides, detected by autoradiography, were purified by paper electrophoresis at pH 6.5 followed by descending chromatography in 1-butanol–acetic acid–water–pyridine (30:6:24:30). Known aliquots (~2%) were withdrawn and counted for radioactivity in Bray's solution (Bray, 1960).

Amino Acid analysis of peptide samples (1–10 nmol) was performed with a Durrum D-500 amino acid analyzer.

Amino acid sequences were determined by the dansyl-Edman procedure of Gray (1967a,b) and peptide mobilities at pH 6.5 were measured relative to aspartic acid (= 1.00).

Reduction of the Schiff's Base Linkage. The purple

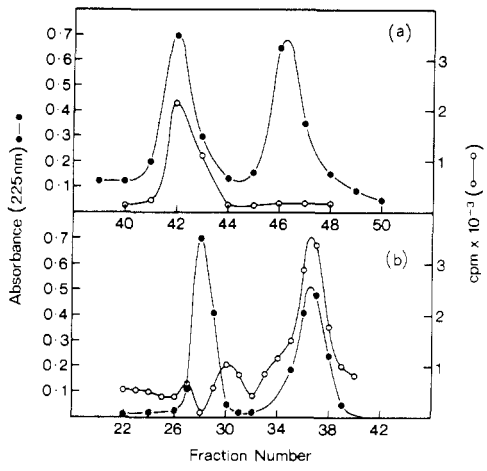

FIGURE 1: Gel filtration of purple membrane protein and *S*-[^{14}C]Ig light chain in dissociating media: (a) Sephadex G-100 in a sodium dodecyl sulfate containing buffer (see Materials and Methods); (b) Ultrogel AcA34 in a buffer containing 6 M guanidine (see Materials and Methods); flow rate was 4 ml/h and fraction volume was 1 ml. The molecular weight of the *S*-^{14}C-labeled carboxymethylated immunoglobulin light chain was 23 500 (Svasti and Milstein, 1972).

membrane was suspended in 50 mM sodium phosphate (pH 8.0) at a concentration such that A_{560} = 1.0. For fingerprint experiments, 5 mg of membrane was used whereas 25 mg of membrane was used for preparative peptide work. Suspensions were illuminated with a mercury arc lamp (Wild, Heerbrugg, Switzerland) fitted with ultraviolet (uv) and infrared (ir) filters and solid $NaBH_4$ was added with stirring until the absorption of the suspension at 560 nm was less than 0.1. In one preparative experiment reduction was carried out by additon of 25 mCi of NaB^3H_4 (Radiochemical Centre, Amersham, U.K., specific activity 274 mCi/mmol) followed by a large excess of the unlabeled compound.

Isolation and Characterization of Retinal-Binding Peptide. The reduced membrane suspension was freeze-dried and then washed with water to remove salt. Isolation of reduced bacteriorhodopsin, succinylation with [^{14}C]succinic anhydride, and digestion with thermolysin were as described above for the nonreduced form except that for preparative work the specific activity of the [^{14}C]succinic anhydride was doubled. When NaB^3H_4 was used for reduction, unlabeled succinic anhydride was added.

The amino acid sequence of peptide F was determined by the solid-phase procedure of Laursen (1971) using an Anachem SPA-1200 sequencer (Anachem Ltd., Luton, U.K.). Reagents for coupling the peptide and for sequencing were from Pierce Chemical Co., Rockford, Ill.

The peptide (20 nmol containing 100 000 cpm) was dried down twice from 200 μl of 1% triethylamine to remove ammonia and was then treated with *tert*-butyloxycarbonyl azide to reversibly block the α-amino group (Laursen, 1971).

After removal of excess reagents in vacuo, the side chain and C-terminal carboxyl groups were activated with *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide. Attachment to triethylenetetramine–polystyrene synthesized according to Horn and Laursen (1973) was performed in dimethylformamide using a procedure (Previero et al., 1973) whereby only the C-terminal carboxyl group attached to the resin.

Coupling yields were estimated by removal of 5 mg of resin, washing twice with 0.2 ml of trifluoroacetic acid to remove unbound peptide, and then counting the dried resin for radioactivity.

The sequencing program was commenced at the cleavage step to remove the butyloxycarbonyl blocking group from the N terminus of the peptide. Fractions were dried under N_2, redissolved in 100 μl of methanol, transferred to scintillation vials, and counted for radioactivity in Bray's solution (Bray, 1960).

Results

Sodium dodecyl sulfate gel electrophoresis of purple membrane protein produced a single band on gels of varying acrylamide concentrations (7.5, 10, and 12.5%) and at loadings of up to 50 μg. Measurements of mobility relative to marker proteins indicated a molecular weight of 19 500 ± 1000 for the purple membrane protein. The respective mobilities of the protein prepared separately from four different cultures were indistinguishable, indicating that the preparation was reproducible and that the apparent molecular weight of the protein product was not affected by variations between the preparations. Results in good agreement with this value were obtained after gel filtration of the protein through Sephadex G-100 equilibrated with a sodium dodecyl sulfate containing buffer. The elution volume corresponded to a molecular weight of 20 500 and complete resolution was obtained from an *S*-[^{14}C]IgG light chain (mol wt 23 500, Figure 1a).

However, after gel filtration in the presence of 6 M guanidine hydrochloride the elution order of the purple membrane protein and the light chain was reversed (Figure 1b) and comparison with marker proteins indicated an apparent molecular weight in this system of 28 000 ± 1000.

To reconcile these figures we undertook a study of the protein by analytical ultracentrifugation and chemical fingerprinting methods.

The value obtained for the partial specific volume of the protein was 0.691/g and this figure was found to be constant over a wide range of concentration values. Using this value, a molecular weight of 24 500 was calculated from the results of a sedimentation equilibrium run in buffer containing 0.1% sodium dodecyl sulfate. Since the solutions used for sedimentation and partial specific volume measurement were at osmotic equilibrium the molecular weight obtained using this apparent partial specific volume will be that of the component whose concentration was measured, i.e. the protein (Casassa and Eisenberg, 1964; Cohen and Eisenberg, 1968; Reisler and Eisenberg, 1969).

Incorporation of radioactivity into the protein from iodo-2-[^{14}C]acetate was equivalent to less than 0.01 mol/mol indicating the absence of cysteine residues. Attempts to estimate tryptophan using methanesulfonic acid were unsuccessful, possibly due to interference from either lipid or carbohydrate, but hydrolysis with mercaptoethanesulfonic acid gave reproducible results.

After subjecting the results from the amino acid analysis to the search procedures of Hoy et al. (1974) a computer plot of f, an indicator of goodness of fit to integral residue numbers, against k, a scaling factor based on an arbitrary molecular weight, is shown in Figure 2. Taking k = 1 as corresponding to a mol wt of 25 000 the effective molecular weight range scanned was from 19 750 to 31 250. A pronounced minimum is obtained at k = 1.014 and this value corresponds to a best-fit mol wt of 25 350. The amino acid composition corresponding to this molecular weight along

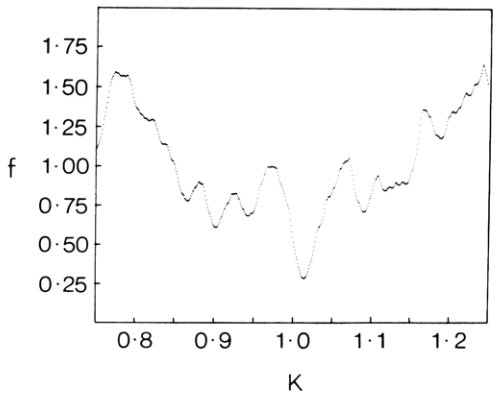

FIGURE 2: Computer plot of f, an indicator of goodness of fit to integral residue numbers, against k, a molecular weight scaling factor (Hoy et al., 1974). All amino acids except serine were included in the calculation.

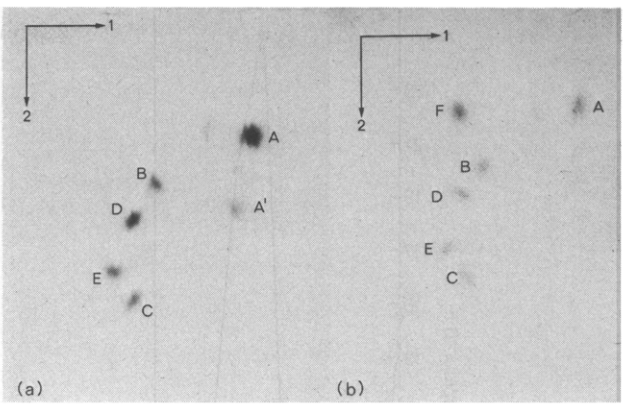

FIGURE 3: Autoradiograms of a thermolytic digest of [^{14}C]succinyl bacteriorhodopsin: dimension 1, electrophoresis at pH 6.5; dimension 2, chromatography in butanol–acetic acid–water–pyridine; (a) without prior reduction; (b) after reduction of the membrane with $NaBH_4$.

Table I: Amino Acid Analysis of Purple Membrane Protein of *H. halobium*.[a]

Amino Acid	No. of Residues	Nearest Integer	Std Dev.	SEM
Asp	15.067	15	0.105	0.031
Thr	17.838	18	0.177	0.053
Ser	13.10	13	0.294	0.088
Glu	16.049	16	0.039	0.011
Pro	11.004	11	0.059	0.017
Gly	25.702	26	0.064	0.019
Ala	28.029	28	0.044	0.013
Val	20.340	20		
Met	8.039	8	0.049	0.014
Ile	13.885	14		
Leu	30.012	30		
Tyr	10.874	11	0.069	0.020
Phe	13.250	13	0.05	0.020
His	2.038	2	0.035	0.011
Lys	7.073	7		
Arg	6.662	7	0.052	0.017
Cys	0	0		
Trp	4.2	4		
Total		243		

[a] Results are the average of triplicate analyses and are corrected for norleucine recovery, incomplete hydrolysis (valine, leucine, and isoleucine), and hydrolytic destruction (serine and threonine).

Table II: Amino Acid Sequences of [^{14}C] Succinyllysine-Containing Peptides.[a]

	Peptide Sequence	Sp Radioact. (cpm × 10^3)	Mobility
A.	Val-Ser-Asp-Pro-Asp-Lys-Lys	5.50	−0.90
B.	Ala-Lys	2.64	−0.55
C.	Phe-Lys	2.87	−0.49
D.	Leu-Thr-Lys	2.47	−0.49
E.	Val-Lys-Gly	2.89	−0.43

[a] Specific radioactivities are expressed as counts per minute per nanomole of peptide. Mobilities were measured relative to aspartic acid (= −1.00) after electrophoresis at pH 6.5.

with the standard deviation and standard error of the mean for each amino acid are shown in Table I.

Isolation of Lysine-Containing Peptides. The soluble fraction from the thermolytic digest was found to contain 85% of the total radioactivity incorporated into the protein. Five radioactive peptides were obtained from this fraction, and these were shown to be pure by dansylation and amino acid analysis. The specific radioactivities (counts per minute per nanomole of lysine) of all five peptides, one of which contained two lysine residues, were identical within the limits of experimental error (Table II) and the sequence around each lysine residue was found to be unique. Furthermore, the specific radioactivity of the ^{14}C-labeled succinylated purple membrane protein (2.88×10^3 cpm/nmol of lysine) was not significantly different from that of the purified peptides, indicating that all lysine residues present had been uniformly labeled. The total amount of radioactivity in each of the lysine-containing peptides indicated that they were all present in equimolar amounts.

The precipitate contained 15% (approximately one-seventh) of the total radioactivity and contained a radioactive peptide which resisted further digestion by thermolysin, pepsin, or trypsin. Attempts to purify this peptide were unsuccessful due to its insolubility.

The peptide fingerprint of ^{14}C-labeled succinylated bacteriorhodopsin shows five major and one minor spot (Figure 3a). After elution and amino acid analysis it was found that peptide A′ was a minor cleavage peptide corresponding to peptide A but with an additional phenylalanine. Addition of the total radioactivity of peptides A and A′ showed almost exactly twice as many total counts as in any of the other four peptides and this correlates well with the two residues of lysine found in both A and A′. Since the α-amino N terminus of the protein is blocked (J. Bridgen, unpublished results) no peptide with an α-succinylamino group was recovered.

After reduction of the membrane with borohydride, the corresponding peptide map (Figure 3b) shows peptide A′ to be absent, peptide A to be of much lower intensity, and that a new peptide, F, has appeared. The amino acid compositions and mobilities of peptides A, B, C, D, and E were identical with the previous experiment. Peptide F was very similar in composition to peptide A. The specific radioactivities of these peptides are shown in Table III. The mobility of peptide F was −0.48.

The total radioactivity in the precipitate which formed after digestion was approximately equivalent to the radioactivity in spots B, C, D, or E, which is again in good agreement with the presence in the precipitate of one [^{14}C]succinyllysine-containing peptide.

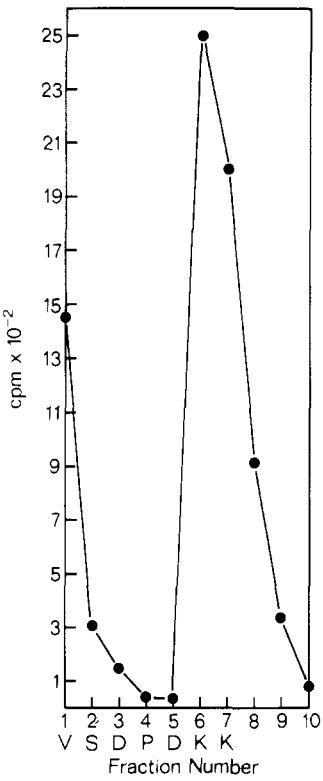

FIGURE 4: Distribution of radioactivity after automatic solid-phase sequencing of the [^{14}C]succinyl peptide F. The letters V S D P D K K refer to the amino acid sequence of the peptide.

From the preparative experiment, peptide F was obtained in a final yield of approximately 20 nmol. This material appeared to be radiochemically pure in that it was well resolved after chromatography from all of the other [^{14}C]succinyllysine-containing peptides. However, amino acid analysis indicated that there was contamination with other nonradioactive peptide material. The total number of counts recovered was 100 000. After coupling to the resin 9500 counts were attached, indicating a coupling yield of 10% which corresponds to approximately 2 nmol of peptide bound to the resin. The distribution of radioactivity in the fractions obtained from the solid-phase sequencer is shown in Figure 4. A tailing peak is seen in fraction 1 followed by a much larger peak at fraction 6 which also shows a considerable degree of overlap into subsequent fractions. This indicates the bulk of the radioactivity to be at position 6 with the retinal attached to Lys-7.

Discussion

Determination of the apparent molecular weight of purple membrane protein by gel electrophoresis or gel filtration in the presence of sodium dodecyl sulfate resulted in values of about 20 000, significantly lower than the values obtained by other techniques. In order to establish the minimum molecular weight of this protein, the number of lysine residues per chain was determined by acylation with [^{14}C]succinic anhydride of known specific activity followed by isolation of the radioactive peptides. Six unique lysine residues were identified by this method in purified thermolytic peptides. The presence of these peptides in equimolar amounts indicates that all the protein chains are very similar, if not identical. The presence of an additional unique lysine-containing peptide in the precipitate produced by digestion was indicated by measurement of radioactivity. If the radioactivity in the precipitate was due solely to undigested or partially digested peptides containing sequences previously identified, redigestion with thermolysin should have solubilized at least some of the material. This was not observed and is further evidence for the presence of seven lysine residues per chain. Assuming seven lysine residues per chain, the amino acid composition of purple membrane protein allows a *minimum* mol wt of 25 100 to be calculated, indicating that the values from gel filtration and gel electrophoresis in sodium dodecyl sulfate buffer are anomalously low.

Table III: Specific Radioactivities (Counts per Minute per Nanomole of Peptide) of Radioactive Peptides from a Thermolysin Digest of Reduced ^{14}C-Labeled Succinylated Bacteriorhodopsin.

Peptide	Sp. Act.	Peptide	Sp. Act.
A	695	D	360
B	440	E	420
C	418	F	510

The measurement of the molecular weights of other membrane proteins by sodium dodecyl sulfate gel electrophoresis has previously been shown (Simons and Kääriäinen, 1970; Spatz and Strittmatter, 1971, 1973; Kyte, 1972; Rubin and Tzagoloff, 1973) to produce anomalously low values, and this also appears to be true for purple membrane protein. One possible explanation for this behavior is that hydrophobic proteins bind greater than normal amounts of sodium dodecyl sulfate. However, determination of the molecular weight of the purple membrane protein–sodium dodecyl sulfate complex by gel filtration also produced an anomalously low value, a result difficult to explain by higher sodium dodecyl sulfate binding alone. Nevertheless, the results of both the gel electrophoresis and gel filtration experiments may be reconciled if the conformation of this protein–sodium dodecyl sulfate complex was more compact than the normal extended rod proposed by Reynolds and Tanford (1970).

There is one additional piece of evidence which indicates the molecular weight to be the higher of the two values that we have found. Since the protein:lipid ratio in the intact membrane has been shown to be 3:1 (Stoeckenius and Kunau, 1968) a protein mol wt of 20 000 would give a total molecular weight for protein plus lipid of 26 630. A recent x-ray analysis (Henderson, 1975) has shown that the membrane components are packed in a P3 hexagonal lattice containing one molecule per asymmetric unit. Thus, there would be three molecules per unit cell with a total mol wt of 79 890. Assuming a membrane density of 1.18 (Stoeckenius and Kunau, 1968) the volume of the unit cell would be 67 700 cm^3/molecule. The volume of one unit cell may then be calculated as 112 830 $Å^3$ which, given the lattice dimensions of Henderson (1975), produces a membrane thickness of only 33.4 Å. A similar calculation performed using the higher mol wt of 25 000 produces a membrane thickness of 42 Å which is in much better agreement with the x-ray data.

The mol wt of 25 100 calculated from the number of lysine residues per chain represents a minimum value since not all of the lysine residues may have been labeled. However, this figure is in excellent agreement with those obtained after amino acid analysis—25 380—and analytical ultracentrifugation—24 500. The latter methods should

also be insensitive to any conformational differences which may exist between hydrophobic proteins and globular water-soluble proteins in detergent or guanidine solutions.

The molecular weight values determined by ultracentrifugation and amino acid analysis agree closely with the value of Oesterhelt and Stoeckenius (1971) determined by sodium dodecyl sulfate gel electrophoresis. In the present study, sodium dodecyl sulfate gel electrophoresis was found to be an unsatisfactory technique for determining the molecular weight of purple membrane protein and the reason for the discrepancy between our sodium dodecyl sulfate gel values and those of Oesterhelt and Stoeckenius (1971) is not clear. It is unlikely that the protein preparation of the present work differed significantly (due to strain-specific differences or preparative artefacts) from that used in earlier work since the only major difference found between the two amino acid compositions was the presence of two residues of histidine per mol instead of one.

It is clearly important to determine protein molecular weight values by at least two independent techniques, especially when dealing with intrinsic membrane proteins whose sodium dodecyl sulfate complexes may differ in charge or conformation from those of the water-soluble proteins used as molecular weight markers.

Since the position of radioactive peptides on a peptide map and their amino acid sequences are known, blocking of one lysine by covalent attachment of retinal was expected to show a difference either in the peptide map or in the amount of radioactivity in the insoluble material.

The major change in the peptide maps before and after reduction of the retinal linkage was the partial disappearance of peptide A from the map of the native protein and the accompanying appearance of peptide F on the map of the reduced protein. Both peptides were of similar composition although in the case of peptide F the small amount of material (~0.5 nmol) did not allow an accurate amino acid analysis to be obtained. However, the specific radioactivity of peptide A was approximately twice that of peptide F indicating that in the latter case only one of the the two lysine residues was available for radioalkylation. The mobility of peptide F (−0.48) corresponds to a charge difference of two relative to peptide A and this would correspond with the substitution of an acidic succinyl group by a retinal group attached via a secondary amine linkage.

Since peptide A and, by analogy, peptide F were known to contain two lysine residues at the C terminus, it was necessary to determine which lysine in peptide F was alkylated and therefore which contained the retinal group.

Because of the high hydrophobicity of retinal peptides, manual methods of sequence analysis were not used since the small quantity of peptide was liable to be lost in organic solvent extractions. Using the solid-phase procedure of Laursen (1971) no such losses should be incurred although the coupling yields to resins using carbodiimide procedures are known to be somewhat variable. Hence, the large amount of radioactivity emerging at cycle 1 (Figure 4) was not surprising and presumably corresponds to noncovalently bound peptide. Most of the remaining radioactivity was found at cycle six indicating that the lysine residue at this position in most, but not necessarily all, of the chains was available to succinic anhydride and did not contain the retinal group in intact bacteriorhodopsin. However, a significant amount of radioactivity was also found at residue seven. This may be due to overlap from the previous cycle but could indicate some [^{14}C]succinyllysine at position 7, i.e. retinal at position 6. Two peptides differing only in the position at which retinal is bound (6 or 7) would be expected to copurify. Incorporation of ^{3}H into the protein after reduction with $NaB^{3}H_4$ was very low (<5%) and most of the incorporation appeared to be into the lipid. Thus, it was not easy to follow the purification of this peptide and insufficient material was purified for sequence studies.

The sequence of the peptide modified by retinal binding is Val-Ser-Asp-Pro-Asp-Lys-Lys. This sequence has also been found in a CNBr peptide derived from bacteriorhodopsin (J. Bridgen, unpublished results) whose complete sequence is Gly-Val-Ser-Asp-Pro-Asp-Lys-Lys-Phe-Tyr-Ala-Ile-Met which correlates well with the finding of a minor form of peptide A with an additional phenylalanine residue. The structure of the purple membrane (Henderson and Unwin, 1975) shows that the protein is arranged in seven α-helices, of approximately equal length, running perpendicular to the plane of the membrane. These helices account for about 75% of the total protein and are almost entirely buried in the lipid portion of the membrane. It is possible that the hydrophilic sequence Ser-Asp-Pro-Asp-Lys-Lys represents a link between two helices, particularly as proline is known to be incapable of being incorporated into an α helix. Also, the sequence following this region is entirely composed of hydrophobic residues and may well represent the start of an α helix penetrating into the membrane.

The retinal binding peptide from bovine rhodopsin has been shown (Bownds, 1967) to have the composition Phe-(Phe,Ala,Lys) which does not appear to resemble any part of the peptide isolated here. However, although the retinal binding sites do not appear homologous in primary structure there may be similarities in tertiary structure which enable homologous enzymes in the eye and in *H. halobium* to specifically recognize the retinal attachment sites in opsin and the apo forms of bacteriorhodopsin, respectively.

Acknowledgments

We wish to thank Dr. R. Henderson, Dr. P. J. G. Butler, and Dr. W. Ferdinand for advice on various aspects of this work, and Mr. K. Gooderham for technical assistance.

References

Bownds, D. (1967), *Nature (London) 216,* 1178.
Bray, G. A. (1960), *Anal Biochem. 1,* 279.
Casassa, E. F., and Eisenberg, H. (1964), *Adv. Protein Chem. 19,* 287.
Cohen, G., and Eisenberg, H. (1968), *Biopolymers 6,* 1077.
Gray, W. R. (1967a), *Methods Enzymol. 11,* 139.
Gray, W. R. (1967b), *Methods Enzymol. 11,* 469.
Henderson, R. (1975), *J. Mol. Biol. 93,* 123.
Henderson, R., and Unwin, P. N. T. (1975), *Nature (London) 257,* 28.
Horn, M. J., and Laursen, R. A. (1973), *FEBS Lett. 36,* 285.
Hoy, T. G., Ferdinand, W., and Harrison, P. M. (1974), *Int. J. Peptide Protein Res. 6,* 121.
Kolb, E., Harris, J. I., and Bridgen, J. (1974), *Biochem. J. 137,* 185.
Kratky, O., Leopole, H., and Stabinger, H. (1973), *Methods Enzymol. 27D,* 98.
Kyte, J. (1972), *J. Biol. Chem. 247,* 7642.
Laemlli, U. K. (1970), *Nature (London) 227,* 680.
Laursen, R. A. (1971), *Eur. J. Biochem. 20,* 89.
Oesterhelt, D. (1971), *Biochem. Cell Differ., Fed. Eur. Biochem. Soc. Meet., 7th, 1971,* 205 (Abstr. 517).

Oesterhelt, D., and Hess, B. (1973), *Eur. J. Biochem. 37,* 316.
Oesterhelt, D., and Schuhmann, L. (1974), *FEBS Lett. 44,* 262.
Oesterhelt, D., and Stoeckenius, W. (1971), *Nature (London), New Biol. 233,* 149.
Oesterhelt, D., and Stoeckenius, W. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2853.
Oesterhelt, D., and Stoeckenius, W. (1974), *Methods Enzymol. 21,* 667.
Penke, B., Ferenczi, R., and Kovacs, K. (1974), *Anal. Biochem. 60,* 45.
Previero, A., Derancourt, J., Colletti-Previero, M. A., and Laursen, R. A. (1973), *FEBS Lett. 33,* 135.
Reisler, E., and Eisenberg, H. (1969), *Biochemistry 8,* 4572.
Reynolds, J. A., and Tanford, C. (1970), *J. Biol. Chem. 245,* 5161.
Rubin, M. S., and Tzagoloff, A. (1973), *J. Biol. Chem. 248,* 4269.
Simons, K., and Kääriäinen, L. (1970), *Biochem. Biophys. Res. Commun. 38,* 981.
Spatz, L., and Strittmatter, P. (1971), *Proc. Natl. Acad. Sic. U.S.A. 68,* 1042.
Spatz, L., and Strittmatter, P. (1973), *J. Biol. Chem. 248,* 793.
Stoeckenius, W., and Kunau, W. H. (1968), *J. Cell. Biol. 38,* 337.
Svasti, J., and Milstein, C. (1972), *Biochem. J. 128,* 427.
Waley, S., and Watson, J. (1953), *J. Chem. Soc.,* 475.

Isolation, Chemical, and Physical Properties of α-1-Antitrypsin†

Piero Musiani and Thomas B. Tomasi, Jr.*

ABSTRACT: A method of isolation of α-1-antitrypsin (α-1-AT) in good yield from normal human plasma is described. A key step was affinity chromatography employing an antiserum which had been depleted of α-1-AT antibodies. The final preparations were homogeneous by immunological and physicochemical criteria. The specific activity of the purified α-1-AT was 0.363 mg of active bovine trypsin inhibited per 1.0 mg of inhibitor. Polyacrylamide gel patterns at both alkaline and acid pH of highly pure preparations frequently, but not invariably, showed multiple bands. Molecular weight studies by sedimentation equilibrium ultracentrifugation in aqueous buffer and in 6 M guanidine as well as sodium dodecyl sulfate polyacrylamide gel electrophoresis suggest that α-1-AT is a single polypeptide chain having a molecular weight of 49 500. Other physical and chemical properties of the inhibitor are described. A limited N-terminal sequence (Glu-Asp-Pro-Gln-Gly-Asx-Ala-Ala) was obtained. It was found that α-1-AT easily forms polymers and higher aggregates when exposed to denaturing agents such as 8 M urea and 6 M guanidine. The results suggest that aggregation is determined by both covalent and noncovalent forces.

Human serum has long been known to inhibit the activity of several proteolytic enzymes. The most important protease inhibitor in human serum is α-1-antitrypsin (α-1-AT)[1] designated as such by Schultze et al. (1962). This protein has approximately 90% of the trypsin inhibitory capacity of human serum and it has also been shown to be active against other proteolytic enzymes including chymotrypsin, collagenase, elastase, plasmin, and thrombin and a protease from human leukocytes (Schwick et al., 1966; Gans and Tan, 1967; Kueppers and Bearn, 1966).

Interest in α-1-AT has been stimulated by studies which suggest a possible role in several human diseases. The genetically determined severe α-1-AT deficient state is associated with pulmonary emphysema in certain families (Eriksson, 1965) and with infantile cirrhosis in others (Sharp et al., 1969) while in occasional families (Glasgow et al., 1973) both organs are affected.

Although several methods have been reported for the purification of α-1-AT (Bundy and Mehl, 1959; Schultze et al., 1962; Heimburger et al., 1971; Myerowitz et al., 1972; Crawford, 1973; Liener et al., 1973) in our experience these techniques have not always yielded pure preparations of α-1-AT, particularly when analyzed immunologically with certain potent antisera to whole human serum. We report here a method for the isolation of α-1-AT which yields highly purified preparations of this protein from human plasma. Some of the physical and chemical properties of the inhibitor are described, including a limited N-terminal sequence.

Materials and Methods

Isolation of α-1-AT. Citrated plasma obtained from outdated blood was dialyzed for 24 h against 10 volumes of 0.01 M calcium chloride in 0.01 M phosphate (pH 7.4) containing 0.14 M sodium chloride. The resulting serum was then precipitated with an equal volume of saturated ammonium sulfate. The 50% saturated ammonium sulfate super-

† From the Department of Immunology, Mayo Medical School, Rochester, Minnesota 55901. *Received October 9, 1974.* This investigation was supported by Public Health Research Grant RO 1 AM 17554 from the National Institute of Arthritis and Metabolic Diseases.

[1] Abbreviations used are: α-1-AT, α-1-antitrypsin; $\bar{v}$, partial specific volume; PEC, *S*-β-(4-pyridylethyl)cysteine; TIC, trypsin inhibitory capacity; PCA, pyrrolidonecarboxylic acid.