Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 15, Issue of August 10, pp. 8596-8599, 1982
Printed in U.S.A.

The Site of Attachment of Retinal in Bacteriorhodopsin

THE ε- AMINO GROUP IN LYS-41 IS NOT REQUIRED FOR PROTON TRANSLOCATION*

(Received for publication, March 29, 1982)

Kuo-Sen Huang‡, Mei-June Liao, Chhitar M. Gupta§, Nancy Royal, Klaus Biemann, and H. Gobind Khorana

From the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Chymotryptic fragments C-1 (amino acids 72-248) and C-2 (amino acids 1-71) of bacteriorhodopsin have been shown previously to reassociate so as to regenerate the native bacteriorhodopsin chromophore in lipid/detergent mixtures and to form functional proton-translocating vesicles. The fragment C-2 has now been selectively methylated with formaldehyde and sodium cyanoborohydride to give the ε-dimethylamino derivatives of Lys-30, 40, and 41 in 96-99% average yield. The methylated and unmethylated C-2 fragments were identical in their ability to reassociate with fragment C-1 and retinal to regenerate the bacteriorhodopsin chromophore and to form functional proton-translocating vesicles. In contrast, dimethylation of the lysine residues of the C-1 fragment gave a derivative which did not form an active complex with unmethylated C-2. We conclude that the ε-amino group in Lys-41 is not required for Schiff's base formation with retinal at any step in the light-driven proton-translocation cycle.

Chymotryptic cleavage of BR[1] forms a large fragment, C-1 (amino acids 72-248), and a small fragment, C-2 (amino acids 1-71). The separated fragments can be recombined under appropriate conditions to generate the native BR chromophore and to form proton-translocating vesicles (1). In the accompanying report (2), the chymotryptic fragments prepared from [ε-^{15}N]lysine-labeled and unlabeled BR were reconstituted in different combinations, and the site of attachment of retinal in the recombined complexes was studied by resonance Raman spectroscopy. The results showed that the chromophore is not attached to Lys-41 in either the light-adapted (BR 570) or the bleached (M 412) states of BR. We now provide definitive evidence that Lys-41 is not involved in the formation of the BR chromophore or at any step in the proton-translocation process. The ε-amino groups in Lys-30, 40, and 41 of the C-2 fragment were methylated with formaldehyde and cyanoborohydride to give the corresponding dimethyl derivatives in 96-99% average yield. BR chromophores were regenerated at virtually identical rates and extents when C*-2 and unmethylated C-2 were combined with C-1 and retinal. Further, the vesicles prepared from these recombined fragments showed identical initial rates and extents of proton translocation. In contrast, C*-1 and unmodified C-2 fragments failed to regenerate the native chromophore or to form active vesicles.

* This work was supported by grants AI 11479 and GM 28289 from the National Institutes of Health and grant PCM 78-13713 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by National Institutes of Health Traineeship T 32CA09112.

§ Present address, Division of Pharmaceutics, Central Drug Research Institute, Chhattar Manzil Palace, Lucknow, India.

[1] The abbreviations used are: BR, bacteriorhodopsin; SDS, sodium dodecyl sulfate; GC-MS, gas chromatography-mass spectrometry; C*-2, dimethylated C-2; C*-1, dimethylated C-1; HFB, heptafluorobutyryl.

MATERIALS AND METHODS

Sodium cyanoborohydride was from Alfa, *N*-ε-methyl-L-lysine hydrochloride was from Aldrich, and *N*-ε-dimethyl-lysine hydrochloride was from Chemical Dynamics Corp. Other materials have been described previously (1).

Solutions—Buffer A, dimyristoylphosphatidylcholine (100 mg) was added to 2.25 ml of 0.1 M NaP_i (pH 7.0) and 0.025% NaN_3 buffer and was sonicated at 20 °C until the phospholipid formed a homogeneous suspension in the buffer. Sodium cholate (0.25 ml of 10%, pH 8) was then added, and the resulting solution was sonicated briefly at 20 °C and stored at 4 °C.[2] Buffer B, 10 mM NaP_i (pH 7.0) and 0.025% NaN_3.

Chymotryptic Fragments C-1 and C-2—The C-1 and C-2 fragments were prepared from BR as described (3). Concentrations of the fragments were determined using ϵ_{280} = 43,700 for C-1 and 16,200 for C-2 in SDS solution. These ε values were calculated from tryptophan and tyrosine content. The amounts of C-1 and C-2 used for the determination of residual lysine and *N*-ε-monomethyl lysine by GC-MS were determined by hydrolysis to amino acids. Concentrations obtained by these two methods agreed to within 20%.

Reductive Methylation of the Fragments—The procedure of Jentoft and Dearborn (4) was modified as follows. Fragment C-2 (7.0 mg, 0.9 μmol) was dissolved in 10% SDS (0.7 ml) and diluted with 6.3 ml of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (100 mM, pH 7.5). Sodium cyanoborohydride (150 μmol) and formaldehyde (15 μmol, 37% in water) were added and the mixture was stirred at 25 °C for 18 h. Additional amounts of 37% formaldehyde (50 μmol) and sodium cyanoborohydride (9.5 mg, 150 μmol) were then added and the stirring continued for another 6 h. The reaction mixture was dialyzed against 1% SDS containing 0.025% NaN_3 for 48 h and the resulting solution was lyophilized. The residue was dissolved in 2 ml of formic acid/ethanol (3:7), and the solution was chromatographed on a Sephadex LH-60 column (1.5 × 110 cm) in formic acid/ethanol (3:7) to remove SDS. Fractions containing the protein fragment were pooled and evaporated to dryness. The residue was stored at −20 °C under ethanol.

Reductive methylation of fragment C-1 was carried out by the same procedure, using fragment C-1 (3.6 mg) in 3 ml of 1% SDS and 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5) (1).

Gas Chromatography and Mass Spectrometry—Total hydrolyzates (6 N HCl, 110 °C, 24 h) of the chymotryptic fragments were converted to the HFB-isobutyl esters by the method of Pearce (5). Gas chromatographic analyses were carried out on a Varian model 3700 chromatograph with a 30-m fused silica column coated with SE-30. A Varian model 3700 GC coupled to a Finnigan MAT 212 mass spectrometer was used to analyze the standards by GC-MS for purity and authenticity.

Gas Chromatographic Analysis of Methylated Protein Fragments—The chromatographic behavior of the HFB-isobutyl esters of the protein amino acids has been described (5, 6). Derivatives of lysine and tyrosine are similarly retained on SE-30 but are completely resolved under our conditions in which lysine elutes at R_t = 27.4 min

[2] Buffer A becomes cloudy after storage at 4 °C for 2 days but becomes clear again after warming at room temperature.

TABLE I

GC analysis of C-1 and C*-2 fragments*

Bacteriorhodopsin fragment	Amount of hydrolyzate analyzed	Lysine	Mono-Me-Lys
	nmol	%	%
C*-2	20	1-2	~2
C*-2	61	<1[a]	1-2
C*-1	54	<1[a]	1-2
C*-1	12.4	1-2	~2

[a] Signal less than that recorded after co-injection of 1% authentic lysine derivative.

and tyrosine at R_t = 27.8. The HFB-isobutyl ester of monomethyl lysine elutes as a single well resolved peak at R_t = 28.4 just after tyrosine. Because it is still a base, the derivative of dimethyl lysine elutes as a very broad, flat peak ranging from approximately 24.5 to 27.0 min and lies under the peaks of phenylalanine and glutamic acid. Therefore, methylated chymotryptic fragments could not be analyzed directly for the appearance of dimethyl lysine. Instead, samples were monitored for the disappearance of lysine using the method of standard addition. Hydrolyzates of approximately 0.10 nmol of C*-1 (4 Lys) or 0.13 nmol of C*-2 (3 Lys) were injected per GC experiment. Samples were co-injected with authentic lysine (derivatized to the α,ε-diHFB-isobutyl ester) corresponding to 5, 2, and 1% of the expected lysine content (0.4 nmol) calculated for unmodified material. The amount of monomethyl derivative present was calculated by comparison to the lysine peak because the response of the two derivatives is very similar.

Reassociation of the Fragments C-1 and C-2 (or C-2) and Retinal to Regenerate the BR Chromophore*—Solutions of the fragment C-1 (0.09 ml, 10 nmol) and C-2 (or C*-2) (0.13 ml, 10 nmol) in 0.2% SDS in Buffer B were mixed and all-*trans*-retinal (4.0 μg, 1 mg/ml in ethanol) was added. The solution was immediately added to 0.2 ml of Buffer A and kept in the dark overnight at 23 °C. For light adaptation, the reconstituted complexes were illuminated for 5 min with intense light from a 500-watt projector lamp equipped with a 530 nm cutoff filter. For dark adaptation, they were stored at 4 °C in the dark overnight.

Reconstitution of C-1 and C-2 (or C-2) into Phospholipid Vesicles*—Reconstitution of the fragments into soybean phospholipid vesicles and assay of the proton-pumping activity were as described (1) except that pH 7.0 buffer was used.

RESULTS

Extent of Dimethylation of ε-Amino Groups in Lysine Residues in C-1 and C-2 Fragments—The presence of any unmethylated or monomethylated ε-amino groups in lysine residues in the chymotryptic fragments following reductive methylation was determined by GC using the method of standard addition. Total hydrolyzates of the fragments were analyzed by GC following derivatization to the HFB-isobutyl esters. Two samples of C*-1 (54 nmol and 12.4 nmol) and two samples of C*-2 (20 nmol and 61 nmol) were analyzed and the results are given in Table I. The modified fragments contained not more than 2% lysine and about the same level of the monomethyl derivative.[3] The data show that at least 96% of the total lysine in each fragment is converted to dimethyl lysine by reductive methylation.

Regeneration of Bacteriorhodopsin Chromophore by Association of Fragments C-1 and C-2 (C-2) and Retinal*—First, the ability of C-2 and C*-2 to associate with C-1 to regenerate the BR chromophore was compared. In the experiment shown in Fig. 1, C-2 and C*-2 were used at equal and constant concentrations, while the concentration of C-1 was varied. The yield of the regenerated chromophore was calculated from the concentration of C-2 or C*-2. The extent of chromophore regeneration using C*-2 was indistinguishable from that obtained with native C-2 at all molar ratios of C-1 to C-2 studied (Fig. 1). The regeneration of the chromophore was linear with the concentration of C-1 as long as the concentration of C-1 was limiting, suggesting tight association of C-1 and C-2 or C*-2 in the presence of retinal. Furthermore, in both cases, a plateau was reached at a C-1/C-2 ratio slightly higher than 1 to 1. This is probably due to the presence of a small amount of denatured C-1.

[3] Monomethyl lysine still could form an aldimine bond with retinal.

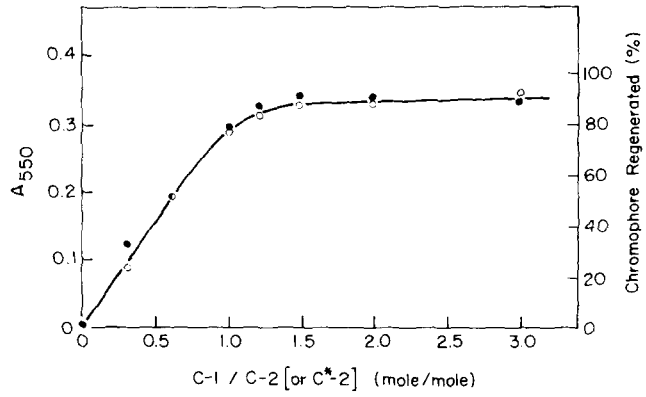

FIG. 1. **Chromophore regeneration as a function of C-1/C-2 (or C*-2) molar ratio.** For each *point* on the *curve*, C-2 (●) or C*-2 (○) (3.3 nmol) was mixed with C-1 (0–10 nmol), and the final volume was adjusted to 0.2 ml with 0.2% SDS in Buffer B. To each sample was then added retinal and Buffer A as described under "Materials and Methods." After overnight incubation, absorbance at 550 nm was recorded against a blank composed of equal volumes of Buffer A and 0.2% SDS in Buffer B. The extent of regeneration was calculated using ϵ_{550} = 47,000 for the reconstituted chromophore.[5]

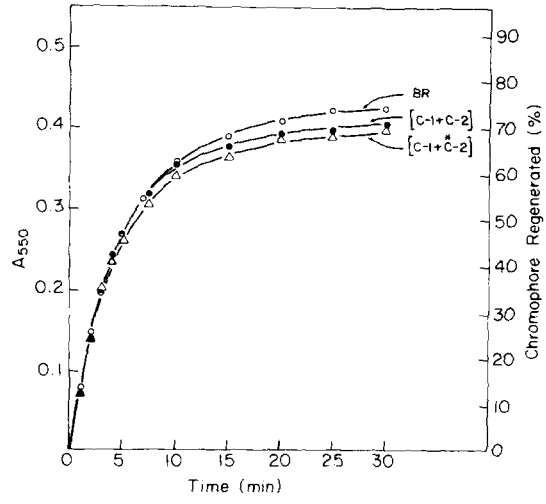

FIG. 2. **Time course of regeneration of native bacteriorhodopsin chromophore from C-1 and C-2 (●), C-1 and C*-2 (△), and denatured delipidated bacterio-opsin (○).** C-1 and C-2 or C*-2 in SDS solution at a concentration of 25 nmol of each fragment/ml (0.2 ml) was mixed with 5.4 nmol of all-*trans*-retinal (1.6 μl of 1 mg/ml in ethanol). Buffer A (0.2 ml) was then added and the solution was mixed well at time zero. Changes in absorbance at 550 nm were recorded continuously for 30 min using a Cary 15 spectrophotometer at 20 °C. Regeneration of the chromophore from delipidated bacterio-opsin was carried out under the identical conditions. The yield of regeneration for the intact protein was calculated using ϵ_{550} = 48,000.

Kinetics of Regeneration of the Chromophore—Fig. 2 shows the time course of chromophore regeneration using BR and combinations of fragments C-1 and C-2 or C-1 and C*-2. The initial rates and extents of regeneration were practically identical in all the three cases. Regeneration was complete after overnight incubation, with final extent close to 80%. The extent of renaturation with BR was only about 5% higher than that obtained with the fragments.

[5] Unpublished observation.

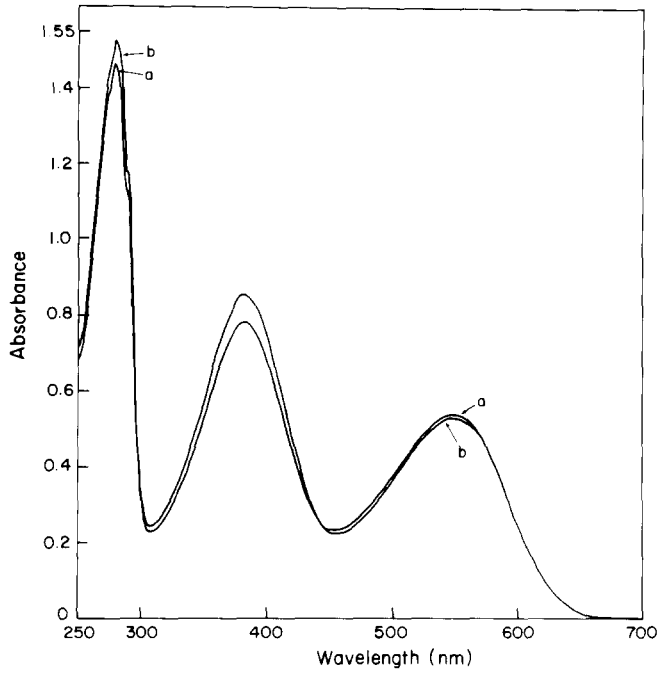

FIG. 3. **Absorption spectra of reconstituted BR complexes in dimyristoylphosphatidylcholine/SDS/cholate mixtures.** *Curve a,* chromophore was regenerated from C-1 and C-2; *curve b,* chromophore was regenerated from C-1 and C*-2 under the identical conditions.

TABLE II

Proton translocation by vesicles reconstituted from fragments C-1 and C-2 and their dimethylated derivatives

Reconstitution of fragments into vesicles was carried out as described under "Materials and Methods" using 11.2 nmol of each of C-1 and C-2 or their dimethylated derivatives. Aliquots used per assay contained 0.046–1.83 nmol of the fragments in the vesicles.

Experiment	Fragments in vesicles	Initial rate	Total H^+ pumped
		H^+/fragment complex/s	*H^+/fragment complex*
1	C-1 + C-2	0.32	17.0
2	C-1 + C*-2	0.33	17.0
3	C*-1 + C-2	0	0
4	C*-1 + C*-2	0	0

Absorption Spectrum of the Chromophores Regenerated Using Fragment C-2 or C-2*—Chromophores were regenerated using an excess of retinal and 1:1 molar ratios of C-1 to C-2 or of C-1 to C*-2. The absorption spectra of the resulting complexes are shown in Fig. 3. The strong absorbance at 380 nm is due to unbound retinal. The chromophores reconstituted using C-2 and C*-2 have both identical spectral characteristics. Thus, they both have λ_{max} = 547 nm at 20 °C in the dark, and the difference in the absorbance at this wavelength between methylated and unmethylated complexes is less than 2%. They also showed equal light-induced shifts from λ_{max} = 547 nm (dark adapted) to λ_{max} = 553 nm (light adapted).

Light-dependent Proton Translocation by Vesicles Reconstituted from C-1 and C-2 or C-2*—Vesicles were reconstituted from C-1 and C-2 or C*-2 under identical conditions. The initial rate and extent of proton translocation for the two vesicle preparations were virtually identical (Table II) and linear with fragment concentrations in the range of 0.046–1.83 nmol. Furthermore, they were equally sensitive to carbonyl cyanide *m*-chlorophenylhydrazone.

Experiments Using Methylated C-1 Fragment—The chromophore was not regenerated when dimethylated C-1 and unmodified C-2 were treated with dimyristoylphosphatidylcholine/cholate mixed micelles in the presence of all-*trans*-retinal. Furthermore, attempts to reconstitute the above fragments into soybean phospholipid vesicles showed no proton-translocating activity (Table II, Experiment 3).

DISCUSSION

Fragments C-2 and C*-2 are indistinguishable in their ability to recombine with fragment C-1 and retinal to regenerate the native BR chromophore and to catalyze the translocation of protons. At least 96% of the total lysines (Lys-30, Lys-40, and Lys-41) in C-2 were dimethylated. Although most unlikely, it could be argued that Lys-30 and Lys-40 were 100% dimethylated; in this event, a maximum of 12% of Lys-41 would have a free ϵ-amino group. Even this hypothetical fraction could not account for the regeneration of the chromophore to the extent of 80% of the theoretical maximum since the latter process requires stoichiometric interaction of the two functional fragments. Similarly, the catalytic reaction of proton translocation also cannot be accounted for by the presence of unmethylated C-2. When vesicles reconstituted from C-1 and C-2 or from C-1 and C*-2 were tested in the concentration range of 0.046–1.83 nmol, the initial rates and extents of proton pumping were linear throughout as a function of the concentration. These results rule out Lys-41 as a functional site of attachment of retinal in BR.

The present results and those in the accompanying report (2) further confirm the earlier conclusion that Lys-216 is the unique site of retinal attachment in BR (7–9). We must, therefore, conclude that the multiple retinal-binding sites observed by Ovchinnikov *et al.* (10) and Katre *et al.* (11) are artifacts of their experimental conditions, a possibility noted in the latter report. One possible explanation (8) for the assignment of Lys-41 as a binding site is that reduction of the Schiff's base in BR with $NaBH_4$ can also form a new fragment carrying the retinal moiety, and this can be mistaken for the C-2 fragment.[4] It should also be noted that the assignment of retinal to Lys-41 has never been supported by detailed amino acid sequence analysis of the appropriate protein segment. Another important point is that, in order to obviate illumination during $NaBH_4$ reduction of the Schiff's base, both Ovchinnikov *et al.* (10) and Katre *et al.* (11) used BR samples which had been precleaved with chymotrypsin (cleavage at Phe-71). It is possible that this cleavage facilitates migration of retinal between Lys-41 and Lys-216 under the alkaline conditions of $NaBH_4$ reaction. In the three-dimensional models currently proposed (11, 12) Lys-41 and Lys-216 are located on adjacent helices.

Finally, the present approach, using reconstitution of BR fragments, should offer additional opportunities for studying the effects of selective chemical modifications of specific amino acids in the individual fragments for structure-function studies of bacteriorhodopsin.

REFERENCES

1. Huang, K.-S., Bayley, H., Liao, M.-J., London, E., and Khorana, H. G., (1981) *J. Biol. Chem.* **256,** 3802-3809
2. Rothschild, K. J., Argade, P. V., Earnest, T. N., Huang, K.-S., London, E., Liao, M.-J., Bayley, H., Khorana, H. G., and Herzfeld, J. (1982) *J. Biol. Chem.* **257,** 8592-8595
3. Gerber, G. E., Anderegg, R. J., Herlihy, W. C., Gray, C. P.,

[4] We found that about 70% of the peptide bond between Gly-155 and Phe-156 of BR was cleaved after incubation of purple membrane with $NaBH_4$ in the dark at 4 °C for 1 day. This cleavage corresponds to that reported by Lemke and Oesterhelt (8).

Biemann, K., and Khorana, H. G. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 227–231
4. Jentoft, N., and Dearborn, D. G. (1979) *J. Biol. Chem.* **254,** 4359–4365
5. Pearce, R. J. (1977) *J. Chromatogr.* **136,** 113–126
6. Desgres, J., Boisson, D., and Padieu, P. (1979) *J. Chromatogr.* **162,** 133–152
7. Bayley, H., Huang, K.-S., Radhakrishnan, R., Ross, A. H., Takagaki, Y., and Khorana, H. G. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 2225–2229
8. Lemke, H.-D., and Oesterhelt, D. (1981) *FEBS Lett.* **128,** 255–260
9. Mullen, E., Johnson, A. H., and Akhtar, M. (1981) *FEBS Lett.* **130,** 187–193
10. Ovchinnikov, Y. A., Abdulaev, N. G., Tsetlin, V. I., Kiselev, A. V., and Zakis, V. I. (1980) *Bioorg. Khim.* **6,** 1427–1429
11. Katre, N. V., Wolber, P. K., Stoeckenius, W., and Stroud, R. M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4068–4072
12. Engelman, D. M., Henderson, R., McLachlan, A. D., and Wallace, B. A. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 2023–2027