Important Role of the Proline Residue in the Signal Sequence That Directs the Secretion of Human Lysozyme in *Saccharomyces cerevisiae*

Yoshio Yamamoto,* Yoshio Taniyama, and Masakazu Kikuchi

Protein Engineering Research Institute, 2-3 Furuedai 6-chome, Suita, Osaka 565, Japan

Received August 23, 1988; Revised Manuscript Received October 24, 1988

ABSTRACT: To elucidate the role of the proline residue in the engineered signal sequence that directs the secretion of human lysozyme in *Saccharomyces cerevisiae*, we have remodeled an idealized signal sequence L8 = Met-Arg-$(Leu)_8$-Pro-Leu-Ala-Ala-Leu-Gly [Yamamoto, Y., Taniyama, Y., Kikuchi, M., & Ikehara, M. (1987) *Biochem. Biophys. Res. Commun. 149*, 431–436] in the vicinity of the proline residue. By analyzing the secretory capability of 10 engineered signal sequences, we have shown the following. (1) The proline residue is important for the secretion of human lysozyme and is allowed at position −4, −5, or −6. (2) The secretory capability of the engineered signal sequences is correlated with their predicted conformations. (3) The functional signal sequences that we have investigated can be generalized as follows: Met-Arg-$(Leu)_n$-Pro-(Xaa)-Ala-Leu-Gly where n equals 6–12 and Xaa is Leu, Ala, or Leu-Ala or can be omitted.

Secretory proteins are generally synthesized with an N-terminal extension termed a signal sequence, which is cleaved off during or after protein synthesis. The role of the signal sequence has been studied intensively by genetic, biochemical, physicochemical, and theoretical means (Briggs & Gierasch, 1986), and three structurally distinct regions have been identified: a positively charged N-terminus, a central hydrophobic segment, and a cleavage site recognized by a signal peptidase (von Heijne, 1985). Although the signal sequences are composed of a variety of amino acids, and their primary structures are rarely conserved, they have a common function. This implies that it is possible to construct an idealized signal sequence with a simplified amino acid composition; such a signal sequence will be a great help for elucidating the structure–function relation, which has not been clarified. From this viewpoint, we have recently designed a series of signal sequences Ln = Met-Arg-$(Leu)_n$-Pro-Leu-Ala-Ala-Leu-Gly (n = 6–14) and investigated their capability to secrete human lysozyme in *Saccharomyces cerevisiae* (Yamamoto et al., 1987). These results have clearly indicated the following points for the structure–function relation of the hydrophobic segment. (1) The length, as well as hydrophobicity, of the hydrophobic segment is an important factor in the secretion of human lysozyme by yeast; the maximal secretion was obtained with L8 in which eight consecutive leucine residues are distributed in the hydrophobic segment. (2) The processing site is independent of the length of the hydrophobic segment.

A logical extension of this study is to reveal the structural requirement of the C-terminal region of the signal sequence. One feature of the C-terminal region of Ln is the presence of a proline residue at position −6. Proline is distinguished from other amino acids by the fact that it has no amide hydrogen and may play a crucial role in the function of the signal sequence. Moreover, a statistical analysis of signal sequences has indicated that a proline residue exists frequently at postion −4, −5, or −6 (von Heijne 1986). In the present study, we systematically remodeled L8 (Yamamoto et al., 1987) in the vicinity of the proline residue and elucidated its role in the secretion of human lysozyme by yeast. We also analyzed the conformations of the engineered signal sequences to reveal the structure–function relation.

Materials and Methods

Materials. Restriction enzymes and T4 ligase were purchased from New England Biolabs or Takara Shuzo. Enzyme reactions were carried out under the conditions recommended by the suppliers. *Micrococcus lysodeikticus* cells and authentic human lysozyme were purchased from Sigma.

Strains and Media. Yeast *Saccharomyces cerevisiae* AH22R$^-$ (*a leu2 his4 can1 pho80*) (Miyanohara et al., 1983) was used as the host strain. Modified Burkholder minimal medium (Toh-e et al., 1973) supplemented with 8% sucrose was used to culture *S. cerevisiae*.

Plasmids. The expression plasmids were constructed as described (Yoshimura et al., 1987). The DNA encoding the signal region (Figure 1) was constructed from eight DNA oligomers that were chemically synthesized by using an automated DNA synthesizer (Model 380B, Applied Biosystems). Codons were chosen according to the codon usage in highly expressed genes in yeast (Bennetzen & Hall, 1982), as far as possible.

Measurement of Human Lysozyme Secretion. Each mutant was cultured for 5 days at 30 °C with shaking, and the time course of lysozyme secretion was monitored every day by measuring lysozyme activity as described (Yoshimura et al., 1987). A more sensitive but qualitative assay was also done on agar plates containing 0.5% *M. lysodeikticus* cells. The secretion of human lysozyme was detected as a halo formed around each colony of *S. cerevisiae*.

Western Blot Analysis. Samples from a 5-day culture were centrifuged, and the supernatant and cells were separated. The cells were washed and resuspended in physiological saline solution. The supernatant and the cell suspension were boiled for 5 min in Laemmli's sample buffer (Laemmli, 1970) and analyzed by Western blotting as described (Yamamoto et al., 1987).

Results

Replacement of the Proline Residue. First, the proline residue in L8 (Yamamoto et al., 1987) was replaced with a leucine (L8PL). We have already shown that increasing the

* To whom correspondence should be addressed.

0006-2960/89/0428-2728$01.50/0

```
L8
   M  R  L  L  L  L  L  L  L  L  P  L  A  A  L  G
 ATG AGA TTG TTG TTG CTA TTG TTG TTG TTA CCA TTG GCT GCT TTG GGT
L8PL
   M  R  L  L  L  L  L  L  L  L  L  L  A  A  L  G
                                 TTG
L8PG
   M  R  L  L  L  L  L  L  L  L  G  L  A  A  L  G
                                 GGT
L8ΔA
   M  R  L  L  L  L  L  L  L  L  P  L  A  L  G
L8LP
   M  R  L  L  L  L  L  L  L  L  L  P  A  A  L  G
                                 TTG
L10ΔLA
   M  R  L  L  L  L  L  L  L  L  L  L  P  A  L  G
                                 TTG TTG
L8PL1
   M  R  L  L  L  L  L  L  L  L  P  L  L  A  A  L  G
                                    TTG
L8PL2
   M  R  L  L  L  L  L  L  L  L  P  L  L  L  A  A  L  G
                                    TTG TTG
L6PL4
   M  R  L  L  L  L  L  L  P  L  L  L  L  L  A  A  L  G
                  CTA TTG     TTA     TTG TTG TTG CTA
L10APG
   M  R  L  L  L  L  L  L  L  L  L  L  A  P  G
                                 TTG TTG
```

FIGURE 1: Nucleotide sequence (lower strand) and corresponding amino acid sequence (upper strand) of the engineered signal sequences. For the nucleotide sequence, the upper strand alone is shown. The codons that are identical with those in L8 are omitted for simplicity. The proline residue mentioned in the text is underlined in each sequence except L8PL and L8PG, for which the residues substituted for the proline are underlined.

FIGURE 2: Time course of human lysozyme secretion with L8, L8PL, and L8PG. Each result represents an average of four observations. The standard deviation of each point is also plotted. (○) L8; (●) L8PL; (□) L8PG. The cell growth reached a stationary phase at about day 3 of culture for each mutant. The cell concentration at the stationary phase was 445, 456, and 482 Klett units for L8, L8PL, and L8PG, respectively.

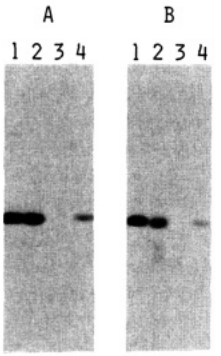

FIGURE 3: Western blot analysis of the culture medium and cell extracts of L8, L8PL, and L8PG. Samples from 5-day culture were analyzed as described under Materials and Methods. (A) Culture medium; (B) cell extracts. Lane 1, authentic human lysozyme; lane 2, L8; lane 3, L8PL; lane 4, L8PG.

hydrophobicity of the hydrophobic segment promoted the secretion of human lysozyme (Yamamoto et al., 1987). Therefore, if the proline does not play a specific role in the secretion, L8PL should be at least as functional as L8, because leucine is more hydrophobic than proline. However, the secretion of human lysozyme was very low (less than 0.1 mg/L) with L8PL (Figure 2) and was detected only slightly by the plate assay. When the proline in L8 was replaced with a glycine (L8PG), the secretion of human lysozyme was only 10% of that with L8 (Figure 2). These results clearly indicate that the proline residue plays an important role in the secretion of human lysozyme by yeast.

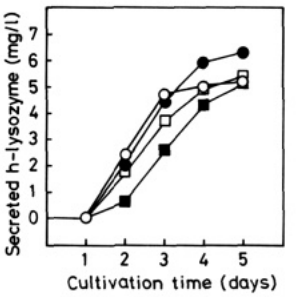

FIGURE 4: Time course of human lysozyme secretion with L8, L8ΔA, L8LP, and L10ΔLA. (○) L8; (●) L8ΔA; (□) L8LP; (■) L10ΔLA. The cell concentration at the stationary phase was 445, 438, 455, and 395 Klett units for L8, L8ΔA, L8LP, and L10ΔLA, respectively.

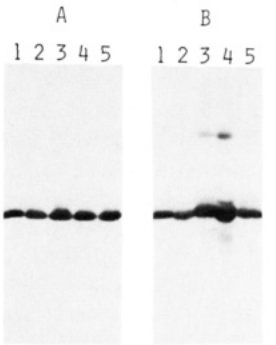

FIGURE 5: Western blot analysis of the culture medium and cell extracts of L8, L8ΔA, L8LP, and L10ΔLA. Samples from 5-day culture were analyzed as described under Materials and Methods. (A) Culture medium; (B) cell extracts. Lane 1, authentic human lysozyme; lane 2, L8; lane 3, L8ΔA; lane 4, L8LP; lane 5, L10ΔLA.

Table I: Secretion of Human Lysozyme with L8, L8PL1, L8PL2, L6PL4, and L10APG

signal sequence	plate assay	secreted lysozyme (mg/L)	growth[a] (KU)
L8	+++	5.2	445
L8PL1	±	<0.1	426
L8PL2	±	0.1	410
L6PL4	±	<0.1	436
L10APG	-	<0.1	444

[a] Cell concentration at the stationary phase is shown in Klett units (KU).

In the Western blot analysis of L8PL (Figure 3), neither mature nor precursor human lysozyme was detected either in the culture medium or in the cell extracts, indicating that the synthesis of immunoreactive protein was hindered or that proteolysis of the protein occurred. With L8PG, the secreted human lysozyme exhibited the same mobility as authentic human lysozyme, suggesting that it is correctly processed; human lysozyme extracted from the cells also comigrated with the authentic human lysozyme, and no precursor lysozyme was found. The intracellular as well as extracellular amount of human lysozyme was significantly lower with L8PG than with L8.

Position of the Proline Residue. We then constructed mutants in which the proline residue is at position −5 (L8ΔA, L8LP) or −4 (L10ΔLA) (Figure 1). As shown in Figure 4, these signal sequences were approximately as functional as L8. The Western blot analysis of these mutants (Figure 5) indi-

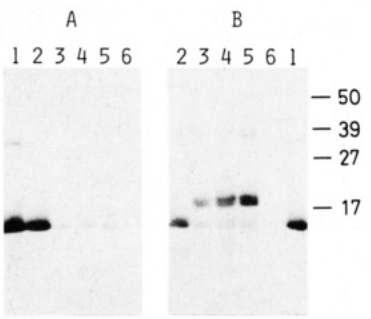

FIGURE 6: Western blot analysis of the culture medium and cell extracts of L8, L8PL1, L8PL2, L6PL4, and L10APG. Samples from 5-day culture were analyzed as described under Materials and Methods. (A) Culture medium; (B) cell extracts. Lane 1, authentic human lysozyme; lane 2, L8; lane 3, L8PL1; lane 4, L8PL2; lane 5, L6PL4; lane 6, L10APG. The numbers on the right side indicate molecular mass standards in kilodaltons.

cated that secreted human lysozyme was correctly processed (confirmed by N-terminal sequence analysis); most of the intracellular human lysozyme was also found to be processed. We also investigated the signal sequences in which the proline residue is at position −7 (L8PL1), −8 (L8PL2), −10 (L6PL4), or −2 (L10APG) (Figure 1). As shown in Table I, these signal sequences were not functional or only slightly functional. With L10APG, no human lysozyme was detected either in the culture medium or in the cell extracts (Figure 6). However, in the cell extracts of L8PL1, L8PL2, and L6PL4, significant amounts of immunoreactive proteins, which were larger than processed lysozyme, were detected as doublets, together with slight amount of processed lysozyme (Figure 6). These proteins existed in the insoluble fraction of the cells (not shown), indicating that they were cell associated or aggregated. We suppose that these bands correspond to a precursor lysozyme with an uncleaved signal sequence and its partially degraded (or modified) derivative. However, the molecular weights of these proteins estimated from the mobility on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] were 18.6K and 17.9K, which were larger than the theoretical value, 16.5K. To test the possibility that these proteins were glycosylated, they were extracted from the insoluble fraction of the cells by Laemmli's sample buffer (Laemmli, 1970), precipitated with trichloroacetic acid, washed with acetone, and treated with α-mannosidase or endoglycosidase H. This treatment did not cause any significant change in the mobility of these proteins on SDS–PAGE (not shown). Although the possibility of other modifications cannot be denied at present, we assume that these proteins were actually precursor lysozymes and that the anomalous behavior on SDS–PAGE was caused by some property (e.g., conformation, hydrophobicity) of the signal sequences. The reason why these proteins were detected as doublets is still unknown, but partial degradation seems the most probable explanation.

These results have clearly demonstrated that the proline residue is allowed at position −4, −5, or −6 but not at other positions. This is consistent with the statistical analysis (von Heijne, 1986) showing that a proline residue is frequently found at position −4, −5, or −6.

Northern Blot Analysis. With L8PL and L10APG, no immunoreactive protein was detected either in the culture medium or in the cell extracts (Figures 3 and 6). To examine whether this was caused at the transcriptional level, we performed a Northern blot analysis. As shown in Figure 7, the amount of mRNA encoding human lysozyme did not differ significantly among L8, L8PL, and L10APG. This indicates that lysozyme synthesis was inhibited at the translational step or that synthesized lysozyme was degraded.

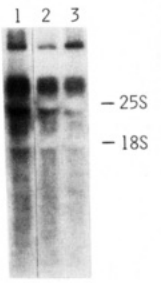

FIGURE 7: Northern blot analysis of L8, L8PL, and L10APG. Each mutant was cultured for 20–30 h, and the cells were harvested in a log phase when the cell concentration reached 200 Klett units. The total RNA was prepared by the phenol–chloroform method (Jensen et al., 1983) and analyzed by Northern blotting as described (Thomas, 1980). The *Taq*I–*Xho*I fragment of the synthetic human lysozyme gene (Yoshimura et al., 1987) was used as a probe. In each lane, 10 μg of the total RNA was applied. Lane 1, L8; lane 2, L8PL; lane 3, L10APG. The positions of yeast rRNA (25 S and 18 S) are indicated.

Discussion

It has been suggested that the conformation of the signal sequence is one of the important factors for its function (Briggs & Gierasch, 1984, 1986; Emr & Silhavy, 1983). However, definitive conclusions have not been obtained, partly because previous studies were not systematic enough. Our systematic analysis of the simplified signal sequences has made it possible to present a universal conformational model that can account for the function of the signal sequences. First, we speculated on the conformation of L8, one of the most potent signal sequences that we have engineered. It is highly probable that the hydrophobic segment of L8, which is composed of eight consecutive leucine residues, adopts an α-helical conformation in the membrane, as leucine has high potential for α-helix formation (Arfmann et al., 1977; Chou & Fasman, 1974). This is in accordance with genetic (Emr & Silhavy, 1983), physicochemical (Briggs & Gierasch, 1984; Briggs et al., 1986), and theoretical (Pincus & Klausner, 1982) analyses concerning the conformation of signal sequences. This α-helix is supposed to be interrupted by the proline residue at position −6, as proline is known to be a strong helix breaker (Chou & Fasman, 1974). It seems hard for the C-terminal segment (Pro- - -Gly) of the signal sequence to adopt an α-helical conformation, because two helix breakers (Pro, Gly) in a six-residue segment will interfere with helix nucleation (Chou & Fasman, 1974). Although it is not verified whether the method of Chou and Fasman (1974) is applicable to peptides in the membrane, it has been successfully applied to signal sequences to elucidate the structure–function relation [e.g., see Emr and Silhavy (1983)]. Considering the (−3, −1) rule for the specificity of the signal peptidase (von Heijne, 1983, 1986), the most reasonable structure of this segment would be an extended one. We now assume that this conformation, which consists of an α-helix and an extended segment, is the "active conformation" of the signal sequence that is essential for its function (Figure 8). The active conformation of L8 can span the length of about 30–35 Å, which is comparable to the thickness of the hydrophobic core of the membrane (Tanford, 1978).

When the proline residue in L8 is replaced with a leucine (L8PL), the α-helix of the hydrophobic segment is predicted to extend to the C-terminus of the signal sequence (Figure 8).

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

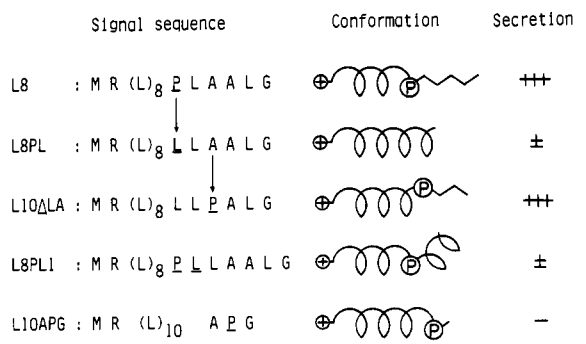

FIGURE 8: Schematic representation of the predicted conformations of the engineered signal sequences. The spiral and the zig-zag represent an α-helix and an extended segment, respectively. P denotes the proline residue.

This may affect the interaction of the signal sequence with some "translocator" [e.g., see Singer et al. (1987)] in the membrane and lead to the inhibition of the secretion.

Glycine is also known to be a helix breaker. Therefore, it seems reasonable to suppose that L8PG is nearly as potent as L8. However, L8PG was only 10% as effective as L8 (Figure 2). This apparent contradiction can be explained as follows. Although glycine is a helix breaker, it can be involved, in contrast with proline, in the middle of an α-helix, as it has an amide hydrogen. Furthermore, the glycine residue in L8PG, is surrounded by the residues that have high helix-forming potential (i.e., Leu and Ala). Thus, it is supposed that this glycine cannot break the α-helix of the hydrophobic segment as efficiently as can the proline in L8 and that the population of the active conformation will decrease significantly, leading to the low secretion of human lysozyme.

L10ΔLA can be derived from L8PL by substituting a proline for the alanine at position −4. This signal sequence was approximately as functional as L8 (Figure 4). The restoration of the secretion may be explained by supposing that L10ΔLA has acquired the ability to adopt the active conformation by the introduction of the proline (Figure 8). L8ΔA and L8LP are also allowed to assume the active conformation.

When one or more leucines are inserted into the C-terminus of the proline (L8PL1, L8PL2, and L6PL4), the helix-forming potential of the C-terminal segment (Pro- - -Gly) of the signal sequence becomes high enough to form an α-helix (Chou & Fasman, 1974). These nonfunctional signal sequences are predicted to adopt a conformation with two helices connected by a proline (Figure 8). In L10APG, which is also nonfunctional, the proline residue is adjacent to the C-terminal residue; this would also hinder formation of the active conformation (Figure 8).

The active conformation described above is essentially identical with that proposed by von Heijne (1983), which was derived by comparing various signal sequences. This agreement implies that the active conformation is not specific for our system but valid for various natural signal sequences. Although a proline residue is often found at position −4, −5, or −6 in natural signal sequences (von Heijne, 1986), it is true that some have no proline residue. In this case, however, other amino acids such as glycine and serine may be substituted for proline to adopt the active conformation. In fact, glycine is frequently present at position −4 or −5, and serine at position −5 (von Heijne, 1986).

In the above discussion, we have implicitly assumed that the active conformation is essential in the translocation step. However, there is another possibility that its role resides in the targeting step, i.e., in the interaction with the signal recognition particle (Walter & Blobel, 1980) or its related molecules. Experiments in vitro would reveal which step requires the active conformation.

When the secretion of human lysozyme was low, two distinct states of the intracellular lysozyme were observed. In one (L8PL1, L8PL2, L6PL4), the accumulation of precursor lysozyme was detected (Figure 6). This result seems reasonable, as it is common in prokaryotes that inhibition of secretion leads to the accumulation of precursor proteins. In the other state (L8PL, L8PG, L10APG), however, no accumulation of precursor or mature lysozyme was observed (Figure 3 and 6). We examined mRNA encoding human lysozyme by Northern blot analysis and confirmed that mRNA was produced as normal (Figure 7). One possibility is that these signal sequences rendered the precursor lysozyme sensitive to proteases, while the signal sequences L8PL1, L8PL2, and L6PL4 stabilized the precursor. As mentioned above, L8PL1, L8PL2, and L6PL4 are supposed to adopt a conformation with two helices connected by a proline, which is distinguished from the predicted conformations of the other signal sequences (Figure 8). It is therefore conceivable that this conformational difference may affect the stability of the precursor lysozyme. We are now trying to elucidate the details by pulse-label experiments.

Finally, the functional signal sequences that we have investigated can be generalized as follows:

Met-Arg-(Leu)$_n$-Pro-(Xaa)-Ala-Leu-Gly

where n equals 6–12 and Xaa is Leu, Ala, or Leu-Ala or can be omitted. Among these, L10ΔLA, where n equals 10 and Xaa is omitted, is the most simplified signal sequence that satisfies the minimal requirement for functional signal sequences: the initiation methionine, a positively charged residue, the hydrophobic segment composed of leucine residues alone, the proline residue discussed above, and C-terminal three residues, two of which (positions −1 and −3) seem to be important for the interaction with the signal peptidase (von Heijne, 1983). It will be of great interest to determine whether these signal sequences are effective for the secretion of other proteins in yeast or other organisms.

ACKNOWLEDGMENTS

We thank Dr. M. Ikehara for helpful advice and encouragement, Drs. Y. Sugino and A. Kakinuma for providing the expression vector pGLD906-1, and Drs. K. Nakahama and K. Yoshimura for valuable discussions. Thanks are due to Y. Kaisho for advice on Northern blot analysis and K. Ishimaru and C. Seko for technical assistance.

Registry No. L8, 118949-80-7; L8PL, 118949-81-8; L10ΔLA, 118949-82-9; L8PL1, 118949-83-0; L10APG, 118978-01-1; L8PG, 118949-84-1; L8ΔA, 118949-85-2; L8LP, 118949-86-3; L8PL2, 118949-87-4; L6PL4, 118949-88-5; Pro, 147-85-3; lysozyme, 9001-63-2.

REFERENCES

Arfmann, H.-A., Labitzke, R., & Wagner, K. G. (1977) *Biopolymers 16*, 1815–1826.

Bennetzen, J. L., & Hall, B. D. (1982) *J. Biol. Chem. 257*, 3026–3031.

Briggs, M. S., & Gierasch, L. M. (1984) *Biochemistry 23*, 3111–3114.

Briggs, M. S., & Gierasch, L. M. (1986) *Adv. Protein Chem. 38*, 109–180.

Briggs, M. S., Cornell, D. G., Dluhy, R. A., & Gierasch, L. M. (1986) *Science 233*, 206–208.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222–245.

Emr, S. D., & Silhavy, T. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4599–4603.

Jensen, R., Sprague, G. F., Jr., & Herskowitz, I. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80* 3035–3039.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Miyanohara, A., Toh-e, A., Nozaki, C., Hamada, F., Ohtomo, N., & Matsubara, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1–5.
Pincus, M. R., & Klausner, R. D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3413–3417.
Singer, S. J., Maher, P. A., & Yaffe, M. P. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1015–1019.
Tanford, C. (1978) *Science 200*, 1012–1018.
Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5201–5205.
Toh-e, A., Ueda, Y., Kakimoto, S., & Ohshima, Y. (1973) *J. Bacteriol. 113*, 727–738.
von Heijne, G. (1983) *Eur. J. Biochem. 133*, 17–21.
von Heijne, G. (1985) *J. Mol. Biol. 184*, 99–105.
von Heijne, G. (1986) *Nucleic Acids Res. 14*, 4683–4690.
Walter, P., & Blobel, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7112–7116.
Yamamoto, Y., Taniyama, Y., Kikuchi, M., & Ikehara, M. (1987) *Biochem. Biophys. Res. Commun. 149*, 431–436.
Yoshimura, K., Toibana, A., Kikuchi, K., Kobayashi, M., Hayakawa, T., Nakahama, K., Kikuchi, M., & Ikehara, M. (1987) *Biochem. Biophys. Res. Commun. 145*, 712–718.

Regeneration of Bovine and Octopus Opsins in Situ with Natural and Artificial Retinals†

Y. Koutalos,*,‡ T. G. Ebrey,‡ M. Tsuda,§ K. Odashima,‖ T. Lien,‖ M. H. Park,‖ N. Shimizu,‖ F. Derguini,‖ K. Nakanishi,‖ H. R. Gilson,⊥ and B. Honig⊥

Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, 524 Burrill Hall, 407 South Goodwin Avenue, Urbana, Illinois 61801, Department of Physics, Sapporo Medical College, Sapporo 060, Japan, and Departments of Chemistry and of Biochemistry and Molecular Biophysics, Columbia University, New York, New York 10027

Received August 9, 1988; Revised Manuscript Received November 21, 1988

ABSTRACT: We consider the problem of color regulation in visual pigments for both bovine rhodopsin (λ_{max} = 500 nm) and octopus rhodopsin (λ_{max} = 475 nm). Both pigments have 11-*cis*-retinal (λ_{max} = 379 nm, in ethanol) as their chromophore. These rhodopsins were bleached in their native membranes, and the opsins were regenerated with natural and artificial chromophores. Both bovine and octopus opsins were regenerated with the 9-*cis*- and 11-*cis*-retinal isomers, but the octopus opsin was additionally regenerated with the 13-cis and all-trans isomers. Titration of the octopus opsin with 11-*cis*-retinal gave an extinction coefficient for octopus rhodopsin of 27 000 ± 3000 M^{-1} cm^{-1} at 475 nm. The absorption maxima of bovine artificial pigments formed by regenerating opsin with the 11-cis dihydro series of chromophores support a color regulation model for bovine rhodopsin in which the chromophore-binding site of the protein has two negative charges: one directly hydrogen bonded to the Schiff base nitrogen and another near carbon-13. Formation of octopus artificial pigments with both all-trans and 11-cis dihydro chromophores leads to a similar model for octopus rhodopsin and metarhodopsin: there are two negative charges in the chromophore-binding site, one directly hydrogen bonded to the Schiff base nitrogen and a second near carbon-13. The interaction of this second charge with the chromophore in octopus rhodopsin is weaker than in bovine, while in metarhodopsin it is as strong as in bovine.

Rhodopsin is an integral membrane protein participating in the light-transduction process that takes place in the photoreceptor cells of higher organisms. Current models of the process propose that incoming photons excite the light-sensitive rhodopsin, inducing protein conformation changes that initiate an enzymatic cascade resulting in the electrical excitation of the photoreceptor cell (Stryer, 1986; Pugh & Cobbs, 1986; Koutalos & Ebrey, 1986; Tsuda, 1987). In this vein the photochemistry of rhodopsin is of fundamental importance for understanding the visual process.

Rhodopsin is composed of a light-insensitive protein moiety and a light-sensitive chromophore, linked to it by a protonated Schiff base. The use of retinal (vitamin A aldehyde) as the chromophore by rhodopsins is almost universal, the few exceptions using closely related retinals. Even the light-sensitive proteins of some unicellular organisms, like Halobacteria (Oesterhelt & Stoeckenius, 1971) and Chlamydomonas (Foster et al., 1984, 1988; Nakanishi et al., 1989), use retinal.

Accordingly, the widely varying enzymatic and spectral properties of rhodopsins are to be explained by differences in the protein moiety and its interaction with the chromophore. Studies substituting artificial chromophores for the natural one in order to probe the chromophore-binding site are very useful. The type of artificial chromophores used in the present study are the dihydro series, which have already been used to

† The work was supported by NIH Grants EY01323 (T.G.E.), GM36564 (K.N.), and GM30518 (B.H.) and NSF Grant DMB85-03489 (B.H.).
‡ Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign.
§ Department of Physics, Sapporo Medical College.
‖ Department of Chemistry, Columbia University.
⊥ Department of Biochemistry and Molecular Biophysics, Columbia University.

0006-2960/89/0428-2732$01.50/0