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Effect of carboxyl mutations on functional properties of bovine rhodopsin.

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Abstract

Bovine rod rhodopsin and membrane-carboxyl group mutants are expressed using the recombinant baculovirus expression system. Biosynthesis of wild-type and the mutant D83N is normal. The mutations E122L and E134D/R affect glycosylation and translocation. After regeneration, purification and reconstitution in retina lipids a wild-type photosensitive pigment with spectral and photolytic properties identical to native bovine rod rhodopsin is generated. Only the mutations D83N and E122L affect the spectral properties and then only slightly. All mutations induce a shift in the Meta I ↔ Meta II equilibrium towards Meta I (E134D/R) or Meta II (D83N, E122L). FT-IR analysis shows that the mutation E134D/R does not significantly affect the carboxyl-vibration region but, in particular in the case of E134R, affects secondary structural changes upon Meta II formation. E122L also has an effect on secondary structural changes and in addition eliminates a negative band at 1728 cm^{-1}. The mutation D83N removes a pair of negative/positive bands from the carboxyl-vibration region, indicating that Asp83 stays protonated upon formation of Meta II but undergoes a change in hydrogen bonding.

Keywords: Bovine rhodopsin; Mutagenesis; Recombinant baculovirus; Biosynthesis; Photolysis; FT-IR spectroscopy

1. Introduction

Rhodopsin, the visual pigment of the rod photoreceptor cell, is a complex integral membrane protein containing seven transmembrane helices, which harbor the chromophore, 11-*cis*-retinal, covalently linked to Lys296 via a protonated Schiff base [1]. The biosynthesis of opsin, the apoprotein, requires intermittent membrane translocation and several post-translational modifications like glycosylation [2], palmitoylation [3] and disulfide bridge formation [4,5], in order to fulfil all its structural and functional properties. Absorbance of visible light near 500 nm triggers isomerization of the chromophore to all-*trans*. The resulting fast conformational changes (Rho → Batho → Lumi → Meta I → Meta II) lead to signal site exposure, G protein (transducin) binding, and subsequent desensitization by the specific rhodopsin kinase and a 45-kDa protein (S-antigen or arrestin). Metarhodopsin II then slowly decays indirectly via metarhodopsin III or directly into the

Abbreviations: FT-IR, Fourier transform infrared; Rho, rhodopsin; Batho, bathorhodopsin; Lumi, Lumirhodopsin; Meta I, metarhodopsin I; Meta II, metarhodopsin II; Meta III, metarhodopsin III; MOI, multiplicity of infection; wt AcNPV, *Autographa californica* nuclear polyhedrosis virus; WT, wild-type (rhod)opsin; dpi, days post infection

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apoprotein opsin and the chromophore is released (for reviews see [6,7]).

The functional and structural properties of rhodopsin can be explored by in vitro expression and (site directed) mutagenesis combined with FT-IR difference spectroscopy (e.g. [8]). For this purpose we have chosen the recombinant baculovirus expression system for the expression of wild-type and mutant bovine rhodopsin in insect cells [9–12]. The high sensitivity of FT-IR difference spectroscopy provides detailed structural information of changes occurring in each step of the photoactivation cascade (for review see [13]).

Rhodopsin contains several highly conserved membrane-located carboxyl group bearing residues (Asp83, Glu122 and Glu134). Since the presence of such polar acidic amino acid side chains in the membrane environment in combination with their strong conservation suggests a specific contribution to folding and/or function of rhodopsin, we have addressed this matter using site-directed mutagenesis. It appears that mutations at the different positions affect biosynthesis and functional properties in a complex way.

2. Materials and methods

2.1. Production and purification of wild-type and mutant bovine rhodopsin

All manipulations involving rhodopsin were performed in dim red light (Schott-Jena, RG 645 cut-off filter). Site-directed mutagenesis, cloning, isolation and propagation of recombinant baculovirus, and insect cell culture were performed as described [9–12,14]. The *Spodoptera frugiperda* cell line IPLB-Sf9 was maintained at 27° C in TNM-FH medium plus 10% fetal calf serum (FCS), 50 μg/ml streptomycin and 50 units/ml penicillin. Large-scale production was achieved in suspension culture (100–1000 ml), stirred at 200 rpm with overlay aeration and with addition of Pluronic-F68 (Sigma) to a final concentration of 0.3%. Viral infections (MOI $\approx$ 10) were performed in one third of the final volume during 1–2 h and then supplemented with medium without FCS to a final density of $2 \cdot 10^6$ cells/ml. Cells were harvested at 3 dpi. Regeneration with 11-*cis*-retinal in total cellular membrane preparations, purification over ConA-Sepharose and reconstitution into retina lipids were performed as described [8,12].

2.2. Immunofluorescence assay

Sf9 cells were grown on cover slips up to a density of $1 \cdot 10^6$ cells/ml and subsequently infected with recombinant virus. Monoclonal antibodies R2-12N [15] (final dilution 1:200 in culture medium) or 1D4 [16] (final dilution 1:500 in culture medium) were added at 1 dpi to the culture medium. At 2 dpi the cells were fixed (1 min at RT in ethanol/aceton 1:1 v/v) and immunoreactivity was detected by reaction with fluorescein conjugated goat anti-mouse immunoglobulin G (IgG) (DAKO A|S, Glostrup, Denmark, dilution 1:50).

2.3. Thermolysin digestion

Sf9 cells were infected with recombinant virus and collected at 3 dpi by centrifugation (1000 $\times g$, 5 min, RT). Subsequently cells were gently resuspended in thermolysin digestion buffer (10 mM Tris-acetate pH 7.2; 150 mM NaCl; 4 mM $CaCl_2$; 10% thermolysin (w/w on a protein base)), and incubated for 5 min at RT. The reaction was stopped by addition of an EDTA-solution (0.5 M; pH 8) to a final concentration of 0.2 M. Protein extracts were then prepared and analyzed by SDS-PAGE on a 12.5% gel followed by immunoblotting as described [11].

2.4. Photolysis

Proteoliposomes were resuspended in buffer (20 mM PIPES, 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA; pH 6.5). Samples of 0.5–1 nmol pigment/ml were scanned (spectrum 1). The suspension was then illuminated for 15 s (Schott Jena, YG 430 cut-off filter) and subsequently spectra were taken at 3-min intervals (spectra 2–8). Then hydroxylamine was added to a final concentration of 50 mM (spectrum 9) followed by a final bleach (spectrum 10). The amount of metarhodopsin I remaining after illumination (spectrum 2) was calculated from $(A_{480(2)} - A_{650(2)}) - (A_{480(9)} - A_{650(9)})/(A_{500(1)} - A_{650(1)}) - (A_{500(9)} - A_{650(9)}) \times 100\%$.

2.5. FT-IR difference spectroscopy

Fourier transform infrared spectra of the Rho → Meta II transition were recorded as described [8]. Samples of 0.3–0.9 nmol pigment in proteoliposomes were prepared on AgCl windows as described [8]. Difference spectra were computed by subtracting a block of spectra taken before illumination from a block of spectra after illumination [8].

3. Results

3.1. Biosynthesis

In vitro expression of bovine rhodopsin in insect cells using recombinant baculovirus has already been described in great detail. The posttranslational processing of wild-type opsin (translocation, glycosylation, palmitoylation, targeting) are all performed correctly in this system [12]. However, some carboxyl mutations appear to impair this processing. Immunoblot analysis of the mutants Asp83 → Asn (D83N), Glu122 → Leu (E122L), Glu134 → Asp (E134D) and Glu134 → Arg (E134R) is presented in

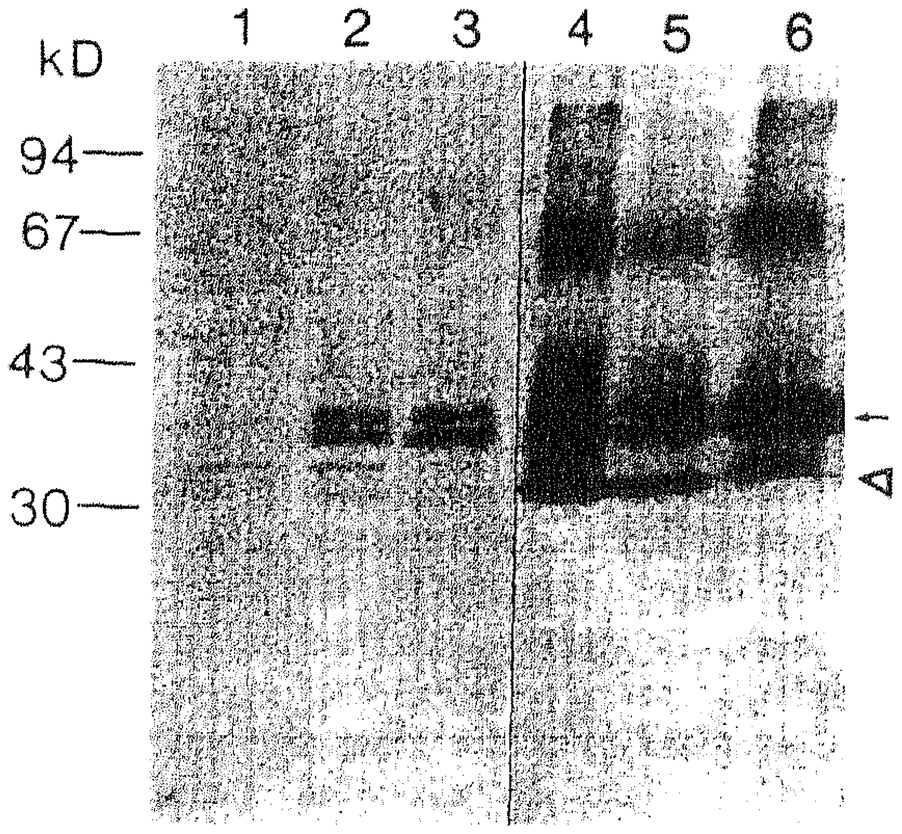


Fig. 1. Immunoblot analysis of Sf9 cells infected with wild-type or mutant recombinant opsin virus (MOI ≈ 10). Lanes: 1, wt virus (AcNPV); 2, wt opsin; 3, D83N; 4, E122L; 5, E134D; 6, E134R. Lanes represent approximately $1 \cdot 10^5$ cells. Total cell extracts (3 dpi) were separated on a 12% polyacrylamide gel and blotted onto nitrocellulose following standard procedures. Opsin was identified by incubation with antiserum CERNJS858 [31] (dilution 1:1000) followed by standard incubation with HPO-labeled antibodies and assay for HPO activity [11].

Fig. 1. Wild-type opsin was over-expressed to generate small amounts of the non-glycosylated species (arrowhead). The glycosylated species (arrow) runs at two bands, due to heterogenity in the saccharide moieties. Like wild-type opsin under non-overexpression conditions the mutant D83N only generates the 38 kDa glycosylated protein. However, the mutants E122L, E134D and E134R produce the non-glycosylated form at approximately 31 kDa, in addition to the 38 kDa glycosylated species. The products are full-length as they all react with the C-terminal-directed monoclonal antibody 1D4 (not shown) (cf. [11]). All mutant forms, including the non-glycosylated species, were palmitoylated (not shown). Immunofluorescent analysis showed that like wild-type [12], the mutant opsins produced in Sf9 cells were targeted to the plasma membrane. To examine whether correct membrane translocation of the complex opsin sequence had been achieved, intact infected Sf9 cells were probed with monoclonal antibodies directed against epitopes at the N-terminal domain (R2-12N) and the C-terminal domain (1D4). In correctly folded opsin immunoreactivity of R2-12N with the extracellularly located N-terminal domain is expected. However, in intact cells the intracellularly located C-terminal epitope should not be accessible for 1D4 and therefore no immunoreactivity should be detected. The overall results of these experiments are given in Table 1. Translocation of wild-type and the mutant D83N is performed correctly. However, the mutants E122L, E134D and

Table 1
Membrane translocation of recombinant wild-type (WT) and mutant opsin assayed by immunofluorescence assay (IFA) or thermolysin digestion and immunoblotting of intact cells

Species	IFA		Thermolysin digestion
	R2-12N	1D4	
WT	+	−	−
D83N	+	−	−
E122L	+	+	+
E134D	+	+	+
E134R	+	+	+

− = negative reaction/no digestion.
+ = positive reaction/digestion.

E134R also produce immunoreactivity of intact cells with 1D4, indicating that at least part of the protein is translocated incorrectly with the C-terminal domain extracellularly. It seems logical to assume that the non-glycosylated form is responsible for this phenomenon. We addressed this question in the following way: limited proteolysis with thermolysin cleaves (rhod)opsin only in the C-terminal domain at the intracellular site [17]. In intact cells with correctly folded protein these sites are not accessible to thermolysin digestion and the still intact protein has retained immunoreactivity for 1D4 on immunoblot. Hence, thermolysin digestion shifts the opsin band to a slightly higher mobility and abolishes reaction with 1D4. Decrease in/or absence of reactivity with 1D4 therefore indicates that part or all of the protein is translocated incorrectly and exposes the C-terminal domain at the extracellular side. The results of these experiments are given in Table 1. Wild-type opsin and the mutant D83N did not suffer significant digestion by thermolysin, again indicating correct translocation. However, the mutants E122L, E134D and E134R showed a significant decrease in reactivity with 1D4 for both the glycosylated and the non-glycosylated form. This indicates that both the non-glycosylated form as well as the glycosylated form of the protein are partially translocated incorrectly resulting in an extracellularly exposed C-terminal domain.

3.2. Functional analysis

To study the functional and structural properties of the in vitro expressed proteins, regeneration with the chromophore 11-*cis*-retinal, purification and reconstitution into proper lipids is essential [12]. A photosensitive pigment of wild-type (v-rho) and mutant opsins can be generated after regeneration (Fig. 2). The mutations D83N (492 nm) and E122L (495 nm) caused a slight blue shift of the wild-type λ_{max} (498 nm), while the mutants E134D/R have wild-type λ_{max}. However, only the glycosylated forms of the mutants E122L and E134D/R gave significant regeneration, while the non-glycosylated species did not and was not solubilized in the detergent dodecylmaltoside either (not shown). Our final purification procedure (ConA-Sepharose affinity chromatography) selects for the glycosylated species [12]. For functional analysis it is important to create a native-like environment (e.g. in detergent the pK_a of the Meta I ↔ Meta II equilibrium shows dramatic shifts from 7.3 in membrane suspension upwards to > 8.5 (dodecylmaltoside, unpublished) or down to 6.4 (digitonin [18])). This native-like environment was achieved by reconstitution in retina lipids [12]. After purification and reconstitution into retina lipids the late photointermediates of the in vitro synthesized pigments were analyzed at pH 6.5 by UV/Vis spectroscopy (Fig. 3). These photolytic transitions of wild-type v-rho are identical to native rhodopsin (inset Fig. 3A). The mutant D83N shows almost normal formation and kinetics of late photointermediates (Meta I: 480 nm; Meta II 380 nm; Meta III: 455 nm), with a slight decrease in Meta I. However, the mutant E122L produces little Meta I and a relatively large amount of Meta III. The opposite is apparent for the mutants E134D (Fig. 3D) and E134R (not shown), which produce more Meta I. These results indicate that the latter substitutions affect the Meta I ↔ Meta II equilibrium. The effect of the

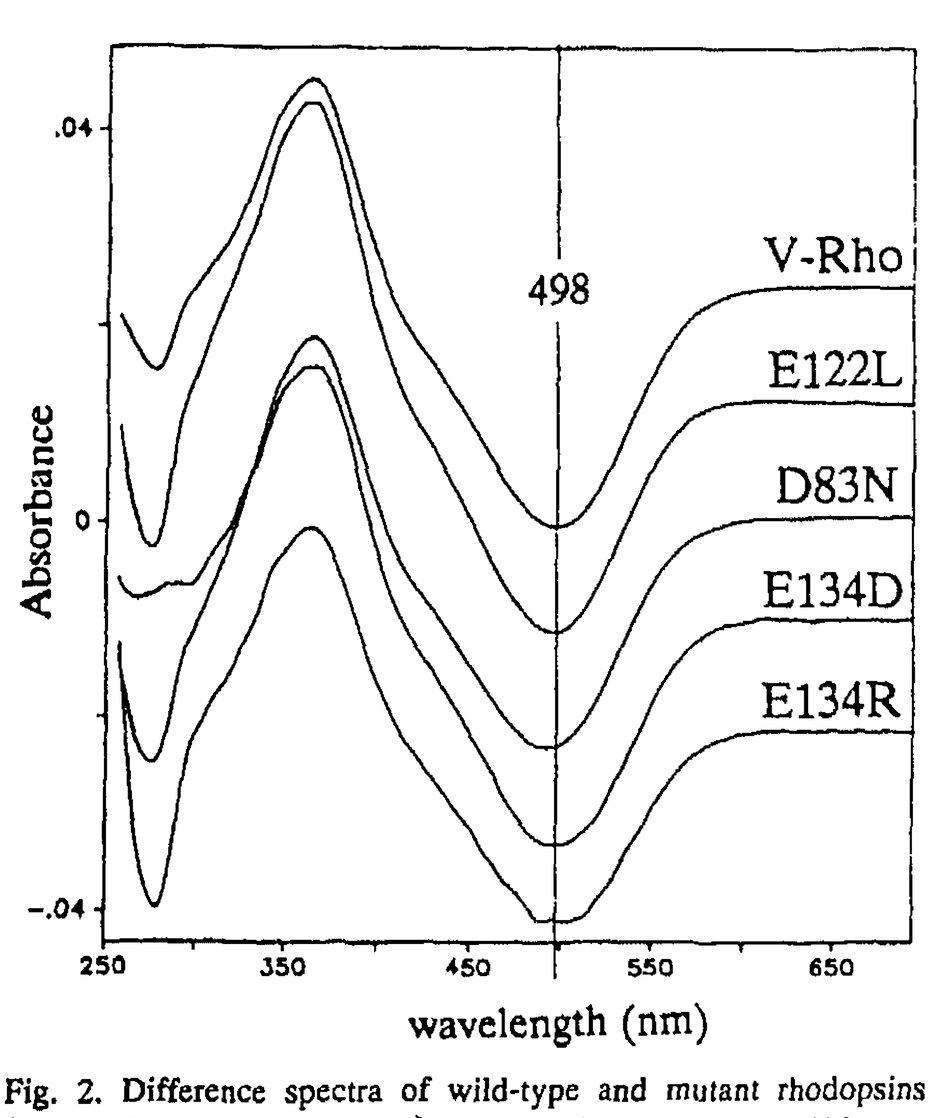


Fig. 2. Difference spectra of wild-type and mutant rhodopsins (bleached minus unbleached). λ_{max}: wild-type v-rho, 498 nm; D83N, 492 nm; E122L, 495 nm; E134D, 498 nm; E134R, 498 nm.

mutations on the Meta I ↔ Meta II equilibrium was estimated at pH 6.5 by calculating the percentage of Meta I present after illumination (Table 2). This percentage of Meta I for wild-type v-rho is slightly higher, but not significantly different from that of native bovine rhodopsin. However, in the mutants

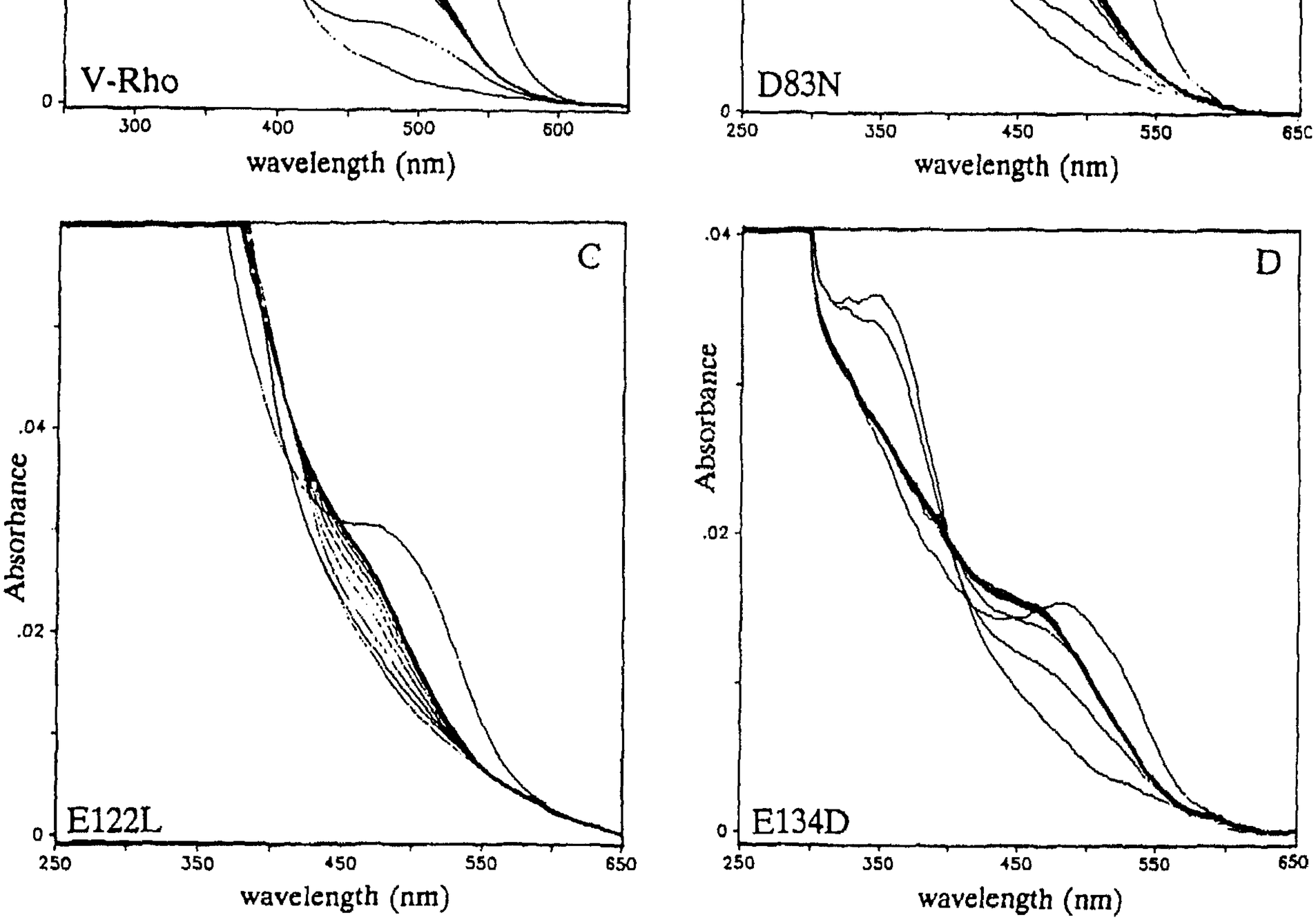


Fig. 3. Late photointermediate formation in reconstituted wild-type and mutant rhodopsins in membrane suspension at pH 6.5. (A) wild-type v-rho. The absorbance bands of rhodopsin (Rh; 498 nm), metarhodopsin I (M I; 480 nm), metarhodopsin II (M II; 380 nm), metarhodopsin III (M III; 455 nm) are indicated in the wild-type v-rho photolysis. (Inset) Photolysis of reconstituted native bovine rhodopsin under similar conditions (3 nmol rhodopsin); (B) D83N; (C) E122L; (D) E134D.

Table 2
Meta I ↔ Meta II equilibrium in recombinant wild-type (WT) and mutant rhodopsins

	% Meta I
ROS	17 ± 3 ($n = 5$)
WT	22 ± 4 ($n = 3$)
D83N	11 ± 5 ($n = 3$)
E122L	9 ($n = 1$)
E134D	59 ($n = 1$)
E134R	33 ($n = 1$)

Percentage of Meta I remaining after illumination at pH 6.5. Number of experiments between parenthesis.

D83N and E122L the equilibrium is shifted towards Meta II. Both the E134 mutants shift the Meta I–Meta II equilibrium towards Meta I.

Information about the structure of rhodopsin and its conformational changes upon Meta II formation were obtained by FT-IR spectroscopy. The absolute vibrational spectra of bovine rod outer segment (ROS), and recombinant wild-type or mutant rhodopsin proteoliposomes are shown in Fig. 4. The two most intense bands at 1657 and 1543 cm^{-1} are due to the amide I and amide II modes, respectively, which are sensitive to protein secondary structure [19,20]. The band around 1739 cm^{-1} arises mainly from the carbonyl stretch of lipid ester carbonyl groups [19] and its ratio relative to the amide I band depends on the amount of lipid incorporated during reconstitution. The bandshapes of the amide bands in the spectra of ROS, wild-type, D83N and E122L is very similar, indicating that the secondary structure of these mutants is very similar to that of native rhodopsin. However, the spectra of E134D and E134R display a prominent shoulder near 1634 cm^{-1}

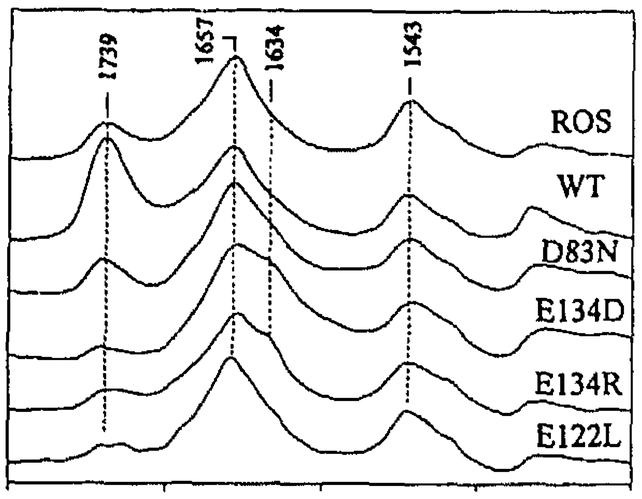
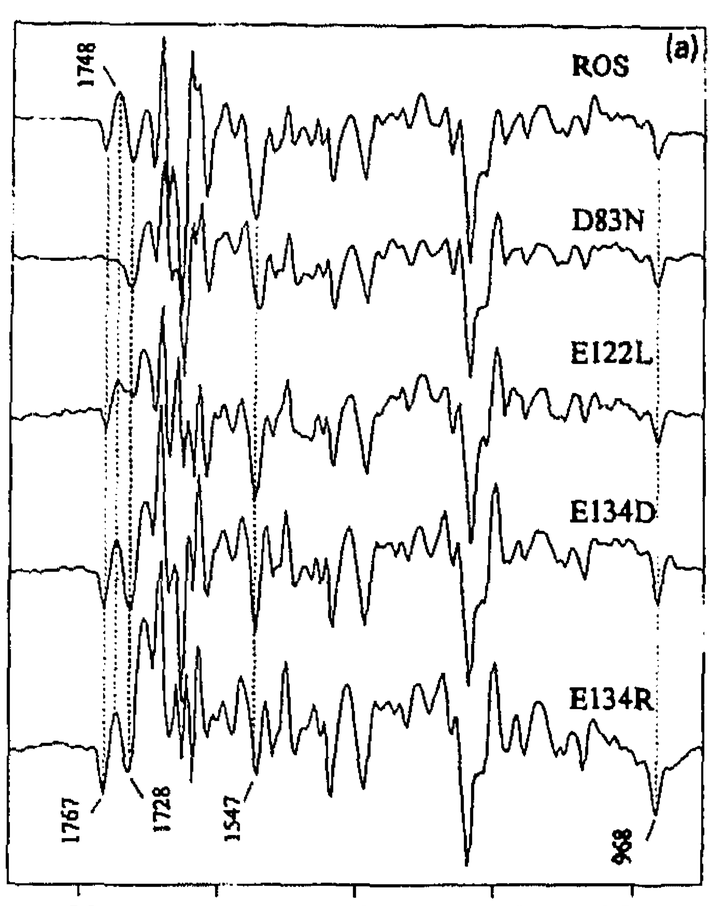


Fig. 4. Infrared absorption spectra of hydrated films of ROS and recombinant wild-type (WT) and mutant rhodopsins deposited on AgCl windows. Resolution is 2 cm^{-1}.



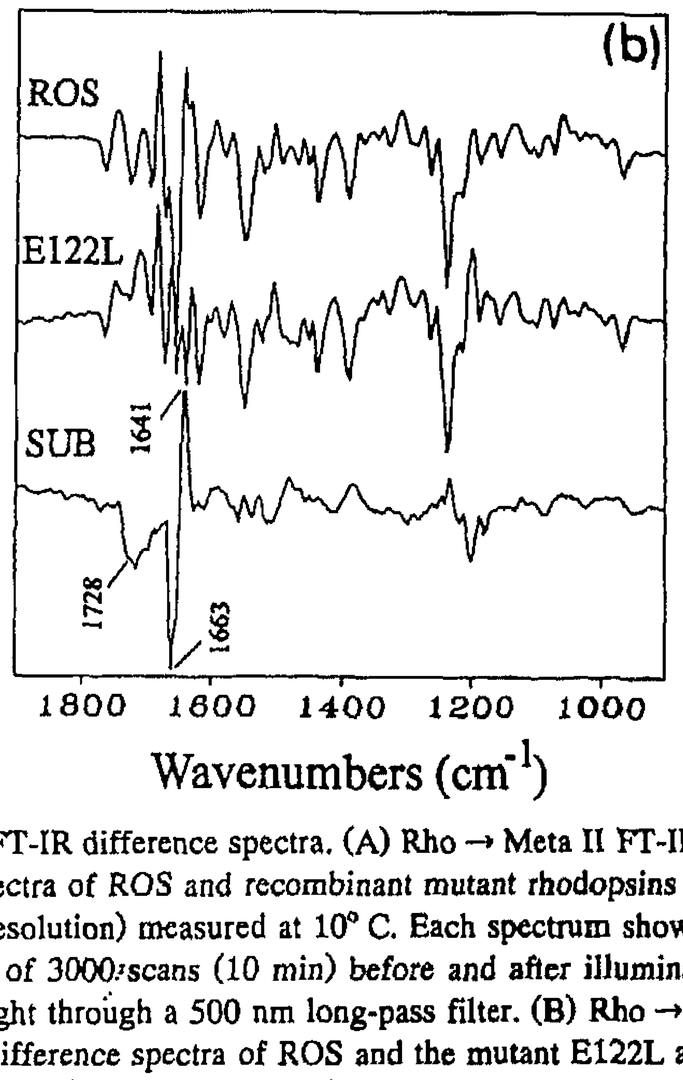


Fig. 5. FT-IR difference spectra. (A) Rho → Meta II FT-IR difference spectra of ROS and recombinant mutant rhodopsins (all at 8 cm^{-1} resolution) measured at 10° C. Each spectrum shown is the average of 3000 scans (10 min) before and after illumination by white light through a 500 nm long-pass filter. (B) Rho → Meta II FT-IR difference spectra of ROS and the mutant E122L and their substraction (SUB: ROS-E122L). The subtracted spectrum shows the missing bands at 1728, 1663 and 1641 cm^{-1} which are characteristic of the E122L mutation.

indicating the presence of at least partially misfolded protein [8].

The Rho → Meta II difference spectra of several mutants are shown in Fig. 5A. While E134D is most similar to wild-type, all the other mutants exhibit clear changes. Most dramatic is the disappearance of two bands at 1767 cm^{-1} (negative) and 1748 cm^{-1} (positive) in the D83N mutant [8,21]. These bands are also both sensitive to ^{1}H–^{2}H exchange and have been previously assigned to a protonation and/or hydrogen-bonding change of carboxyl residues during Meta I → Meta II transition [22]. The E122L mutant shows changes in the carboxyl region, with the disappearance of the negative band at 1728 cm^{-1}. This is also evident in the difference of the ROS and E122L spectra (Fig. 5B). A similar effect is also observed in detergent micelles [21], although a positive band located at 1735 cm^{-1} was reported by these authors to disappear, which we do not observe. E122L also displays drastically altered amide I region (Fig. 5B) with the disappearance of prominent bands at 1663 cm^{-1} (negative) and 1641 cm^{-1} (positive) most likely reflecting altered structural changes during Meta II formation. Finally, the mutant E134R has a relatively normal carboxyl region but shows reduced band intensities in the amide I region as well as changes in the amide II region (1547 cm^{-1} band).

4. Discussion

4.1. Wild-type

In vitro expression of bovine rhodopsin using the recombinant baculovirus expression system results in a protein with correct posttranslational processing (translocation, glycosylation, palmitoylation and targeting). After regeneration with the chromophore 11-*cis*-retinal, purification over ConA-Sepharose and reconstitution in retina lipids a pigment with identical spectral and photolytical properties to native bovine rod rhodopsin is generated.

Since high conservation of polar side chains in membrane domain suggests their involvement in essential structural or functional properties, we investigated the effect of mutation of residues D83, E122 and E134 on posttranslational processing, and spectral and photolytic properties of (rhod)opsin. Our results so far indicate mosaic effects of the various mutations on (rhod)opsin properties, i.e. each mutation affects more than one of the properties investigated but in different combinations. Hence we will discuss the various mutations separately.

4.2. D83N

Substitution of Asp83 for Asn has no detectable effect on posttranslational processing of opsin. This actually is not surprising since the mutation D83N occurs 'naturally' in frog rod rhodopsins [23] and hence is not expected to perturb the delicate balance of signals in the opsin sequence which regulate translation, translocation, folding and further posttranslational processing. However, the mutation does affect spectral and functional properties. A small but significant blue shift of the maximal absorbance band (498 → 492 nm) is observed, in agreement with earlier suggestions [24]. Whether this mutation causes a similar blue shift in the frog pigment (λ_{max}: ≈ 500 nm in the A_1 form) cannot yet be ascertained since there are multiple differences in amino acid composition in the transmembrane domains between frog and bovine rhodopsin [23]. Although the kinetics of the slow photointermediates (Meta II → Meta III) are not affected in the mutant D83N, the Meta I ↔ Meta II equilibrium is slightly shifted towards Meta II, in agreement with results reported by Weitz et al. [25] obtained in digitonin solution. Quite interesting is the disappearance of the two bands at 1767 cm^{-1} (negative) and 1748 cm^{-1} (positive) in the Rho → Meta II FT-IR difference spectrum of D83N. As previously discussed [8,21], this, along with the absence of changes in the carboxylate region around 1400 cm^{-1}, indicates that Asp83 is structurally active in this transition and undergoes an increase in hydrogen bonding upon Meta II formation. Apparently, the Asn substitution is able to maintain the normal hydrogen bonding pattern and does not significantly perturb the Meta I → Meta II transition.

4.3. E122L

While E122 is absolutely conserved in rod visual pigments, cone pigments have an apolar residue at this position (L,I,M). Indeed, substitution of Glu122 for Leu much less affects the spectral properties of

bovine rhodopsin (λ_{max} 498 → 495 nm), than that reported for Gln, Asp or Ala (λ_{max} 475–480 nm) [24,26–29]. The mutation E122I also induces only a small λ_{max} shift [24]. This suggests that replacing E122 by similar polar groups like Q or D perturbs the tertiary structure and induces an appreciable blue shift in absorbance. Direct interaction of E122 with the chromophore is unlikely since then much larger effects of the mutations E122L and E122I should be expected. Hence apolar residues like Leu and Ile are good substitutions for E122 with regard to a correct structure for spectral tuning. However, they strongly affect other properties of opsin. During biosynthesis a large amount of non-glycosylated product is produced (Fig. 1) which has not been properly translocated and/or folded (Table 1). The 'normal' glycosylated product, which has been purified, shows no aberrant folding as far as can be judged from the bandshape of the amide I band in the FT-IR spectrum (Fig. 4). Apparently the mutation E122L interferes with the translocation process of opsin. Furthermore it shifts the Meta I ↔ Meta II equilibrium towards Meta II (Table 2). This is opposite to what Weitz et al. [25] found by difference spectroscopy in digitonin solution. Although we tentatively contribute this discrepancy to the fact that our data are obtained in a native-like membrane environment, this requires further investigation. The structural importance of a Glu at position 122 is also evident from changes induced by the Leu substitution in the Rho → Meta II difference spectrum. First, bands in the amide I region which most likely reflect structural changes involving the rhodopsin backbone are absent. Second, a negative band in the carboxyl region at 1728 cm^{-1} disappears. This band has been previously tentatively identified as a buried carboxyl group [22], and assigned to Glu122 due to its disappearance in E122Q [21]. However, in view of the fact that a corresponding positive carboxyl band is not observed in our spectra (Fig. 5B), it appears that this group does not undergo hydrogen bonding changes as occurs for Asp83. In addition, we also do not observe a significant change in the carboxylate region near 1400 cm^{-1} due to the E122L mutation which would be expected if Glu122 undergoes deprotonation. An alternative possibility is that this band arises from a protein vibration such as a Pro residue as suggested previously [30].

4.4. E134D / R

Neither E134 mutations interfere with spectral tuning, but severely affect glycosylation and translocation. We already postulated that this residue could be part of an important internal translocation signal [11,12]. While the non-glycosylated form again appears to become translocated incorrectly (Table 1), now also the FT-IR spectrum of the purified glycosylated species shows the presence of some misfolded protein (Fig. 4). Furthermore, both mutations shift the Meta I ↔ Meta II equilibrium towards Meta I (Table 2). This again is in contrast with results obtained in digitonin solution for the mutants E134Q, E134L, E134L/R135L and E134R/R135E [25]. FT-IR analysis also shows that secondary structural changes, as reflected in the amide I region, upon Meta II formation are altered in these mutants. This is particularly evident in the E134R mutant. In contrast, the structural changes of carboxyl groups as reflected in the carboxyl region are unaffected. This further supports our earlier conclusion that E134 is not structurally active in the Meta I → Meta II transition [8].

5. Conclusions

We have demonstrated that in vitro expression and mutagenesis of bovine rhodopsin in combination with FT-IR difference spectroscopy is a suitable method to investigate the structural changes which occur during photoactivation. It is striking that a single amino acid substitution can have mosaic effects, i.e. causes changes in very different functional properties like glycosylation, translocation, folding, Meta I ↔ Meta II equilibrium, and primary or secondary structural changes during photoactivation. Hence, when analyzing effects of mutations on specific functional properties one should always consider the potential concomitant effects on folding and structural stability.

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