Large-scale production and purification of the human green cone pigment: characterization of late photo-intermediates

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We present the first characterization of the late photo-intermediates (Meta I, Meta II and Meta III) of a vertebrate cone pigment in a lipid environment. Marked differences from the same pathway in the rod pigment were observed. The histidine-tagged human green cone pigment was functionally expressed in large-scale suspension cultures in Sf9 insect cells using recombinant baculovirus. The recombinant pigment was extensively purified in a single step by immobilized metal affinity chromatography and displays the expected spectral characteristics. The purified pigment was able to activate the rod G-protein transducin at about half the rate of the rod pigment. Following reconstitution into bovine retina lipid proteoliposomes, identi-

fication and analysis of the photo-intermediates Meta I, Meta II and Meta III was accomplished. Similar to the rod pigment, our results indicate the existence of a Meta I–Meta II equilibrium, but we find no evidence for pH dependence. Replacement of native Cl[−] by NO₃[−] in the anion-binding site of the cone pigment affected the spectral position of the pigment itself and of the Meta I intermediate, but not that of Meta II and Meta III. The decay rate of the ‘active’ intermediate Meta II did not differ for the Cl[−] and NO₃[−] state. However, in qualitative agreement with results reported before for chicken cone pigments, the rate of Meta II decay was significantly higher in the human cone pigment than in the rod pigment.

INTRODUCTION

Most of our knowledge on structural and functional properties of mammalian visual pigments stems from work on the rod pigment rhodopsin. Detailed information concerning the molecular properties of mammalian cone pigments, on the other hand, is almost entirely lacking. The genes encoding the human cone pigments were cloned several years ago [1], and recombinant pigments have been produced in mammalian cell lines [2–4] and recently in an insect cell line [5]. Functional expression so far has provided information about spectral properties like the wavelength of maximum sensitivity (λ_{max}) of wild-type and mutant pigments [6,7], the effect of anions on the position of the λ_{max} [8], the stabilizing effect of the disulphide bridge [9] and potential factors involved in proper folding [10]. While biochemical and structural analysis of (recombinant) rhodopsin provided a wealth of information on various aspects of its receptor mechanism, cone pigments have not yet reached this stage. For example, identification and analysis of cone-pigment photo-intermediates have only been performed on native non-mammalian pigments: chicken red and green [11–16] and gecko green and blue [17,18].

Histidine tagging in combination with immobilized metal affinity chromatography (IMAC) has been shown to be a powerful approach for purification of recombinant baculovirus-expressed bovine rhodopsin [19]. Although His-tagging slightly shifts the pH-dependency of the Meta I ↔ Meta II equilibrium, it affects neither the structural changes accompanying photo-activation nor the functionality of rhodopsin [19].

Functional expression of His-tagged human red and green cone pigments using baculovirus has already been accomplished [5]. This report employs a convenient single-step purification protocol based on IMAC, which yields purified His-tagged human green cone pigment (HGH) suitable for detailed analysis of molecular properties. Reconstitution of the purified cone pigment in bovine retina lipids allowed the first identification and analysis of photo-intermediates appearing in the late phase of the

pigment’s photocascade in a natural lipid environment. Our results indicate that pH-dependence and kinetics of the late transitions of the photocascade are significantly different from that of the rod pigment. In addition, we report that the bound anion influences the absorbance properties of the pigment and its Meta I photo-intermediate, but has no significant effect on the kinetics of the late photocascade transitions.

EXPERIMENTAL

Generation and purification of recombinant baculovirus and cell culture

Construction of the transfer plasmid pVL1393HGH (containing the cDNA encoding HGH under transcriptional control of the polyhedrin promoter), generation and purification of the recombinant baculovirus have been described elsewhere [5]. For large-scale production of recombinant protein a bioreactor (Applikon, Schiedam, The Netherlands) was used, in which Sf9 cells were grown in suspension cultures up to 10 l in serum-free INSECT-XPRESS medium (BioWhittaker, Walkersville, MD, U.S.A.) to a density of 3.5×10^6 cells/ml (oxygen saturation 50%). Infection was performed by the addition of a high-titre virus stock at a multiplicity of infection of 0.1, and cells were harvested at 4 days post infection.

SDS/PAGE and immunoblotting

Proteins were separated on a 12% SDS/polyacrylamide gel (BioRad, Mini Protean II) according to Laemmli [20].

Buffers used for regeneration, purification, analysis of anion sensitivity and lipid reconstitution of HGH

Buffer R (regeneration): 20 mM Pipes/140 mM NaCl/20% (v/v) glycerol/2 μ g/ml leupeptin (Boehringer), pH 6.5. Buffer A (IMAC purification): 20 mM bis-Tris propane (Sigma)/0.5 M

Abbreviations used: IMAC, immobilized metal affinity chromatography; HGH, histidine-tagged human green cone pigment; DoM, *n*-dodecyl- β -1-D-maltoside; β ME, β mercaptoethanol; RL, retina lipids; NTA, nitrilotriacetic acid; GTP- γ -S, guanosine 5'-O-3'-(thiotriphosphate).

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NaCl/20% (v/v) glycerol/0.5% (w/v) *n*-dodecyl- β -1-D-maltoside (DoM)/5 mM β mercaptoethanol (β ME)/1 mM histidine (Gibco)/2 μ g/ml leupeptin, pH 7.0. Buffer B (IMAC purification): Buffer A, with 0.25% (w/v) DoM/5 mM histidine/0.4 mg/ml bovine retina lipids (RL). These lipids were isolated from bovine retinas as described [21]. Buffer C (IMAC purification): 20 mM bis-Tris propane/140 mM NaCl/20% (v/v) glycerol/0.6% (w/v) CHAPS/5 mM β ME/50 mM histidine/0.4 mg/ml RL/2 μ g/ml leupeptin, pH 6.5. Buffer N (anion sensitivity): 20 mM Pipes/140 mM NaNO₃/20% (v/v) glycerol/0.6% (w/v) CHAPS (Sigma)/2 μ g/ml leupeptin, pH 6.5. Buffer P (reconstitution): 20 mM Pipes/130 mM NaCl/5 mM KCl/2 mM MgCl₂/2 mM CaCl₂/0.1 mM EDTA/2 μ g/ml leupeptin, pH 6.5.

Regeneration of HGH

The cone-pigment proteins were regenerated into light-sensitive pigments by the addition of the chromophore 11-*cis* retinal to the Sf9 cell-membrane fraction as described [5]. The pigment was then solubilized by the addition of CHAPS to 1% (w/v) and β ME to a final concentration of 5 mM. After 1 h (room temperature) the suspension was centrifuged (30 min, 100 000 g, 4 °C) and the supernatant used for further analysis. All manipulations of the light-sensitive pigment were done in darkness or under dim red light (Schott RG650 cut-off filter).

Purification of HGH

The CHAPS extract obtained after regeneration was purified by IMAC. The sample was diluted with an equal volume of buffer R and then solid NaCl and solid DoM were added to a final concentration of 0.5 M and 0.5% (w/v), respectively. The final concentration of CHAPS was about 0.5% (w/v). The pH was then raised to 7.0 by addition of buffer R, pH 9. The Ni²⁺-nitrilotriacetic acid resin (Ni²⁺-NTA, Qiagen, Hilden, Germany) was washed with 10 volumes of distilled water followed by 10 volumes of buffer A. The solubilized pigment was mixed with the Ni²⁺-NTA resin (250 μ l of gel/10⁹ cells) and incubated under rotation for 16 h at 4 °C. The mixture was then applied to a column and washed with 10 column volumes of buffer A, followed by 10 column volumes of a linear gradient prepared from buffers A and B in which the DoM concentration was lowered to 0.25% (w/v), and histidine and RL were raised to concentrations of 5 mM and 0.4 mg/ml, respectively. Subsequently, the column was washed with one column volume of buffer B. The bound pigment was eluted with buffer C.

Exchange of Cl⁻ for NO₃⁻

Purified HGH (0.5 nmol/ml) was applied to a Sephadex G-25 column (PD-10, Pharmacia), equilibrated with buffer N. The column was washed with buffer N and fractions were analyzed by UV-visual-wavelength spectroscopy to identify eluted HGH. To fully deplete the sample from Cl⁻, the peak fractions were pooled and subjected a second time to the same procedure.

Reconstitution of purified HGH into retina lipid proteoliposomes

The purified human green cone pigment was reconstituted into a lipid environment by the addition of bovine retina lipids in buffer P to a 200-fold molar excess over HGH. Removal of the detergents (CHAPS and a residual amount of DoM) was accomplished by addition of γ - and β -cyclodextrin (Aldrich, Milwaukee, WI, U.S.A.), respectively. The rationale behind this approach will be detailed at a later date [21a]. Solid β - and γ -

cyclodextrin were added to a final concentration of 10 mM and 20 mM, respectively, and the suspension was then layered on top of a sucrose step gradient (10% sucrose (w/v) containing 2 mM β -cyclodextrin and 4 mM γ -cyclodextrin, and 10%, 20% and 45% sucrose (w/v) in buffer P), followed by centrifugation for 16 h at 4 °C (200 000 g). The proteoliposomes containing the reconstituted green pigment were isolated from the 20–45% interface. For exchange of Cl⁻ for NO₃⁻, proteoliposomes (1–2 ml) were subjected to dialysis at 4 °C for 16 h with 3 buffer changes against buffer P (100 ml NaCl, KCl, MgCl₂ and CaCl₂ replaced by 130 mM NaNO₃) supplemented with 20% (w/v) sucrose.

Transducin activation

Activation of the G-protein transducin by HGH was assayed as described [22,23]. A reconstituted HGH sample was bleached and added to a stirred cuvette containing 100 mM NaCl, 2 mM MgCl₂, 1 mM 1,4-dithio-erythritol, 0.01% (w/v) DoM, 20 mM Hepes, pH 7.4, in a total volume of 2 ml, containing 100 nM transducin. A hypotonic extract of isotonicity washed bovine rod outer segments [24] served as the source for transducin. The final concentration of pigment was 5 nM. After 90 s, guanosine 5'-O-3'-(thiotriphosphate) (GTP- γ -S; Boehringer) was added to a final concentration of 2.5 μ M and activation of transducin was determined by means of the increase in tryptophan fluorescence using a Shimadzu RF-5301 spectrofluorometer with excitation at 295 nm (bandwidth 1.5 nm) and emission at 337 nm (bandwidth 15 nm). In view of the relatively rapid decay of HGH Meta II, the assay was performed at 11 °C.

Preparation of membrane films of HGH proteoliposomes on cellulose-acetate cover slips

Proteoliposomes isolated from the sucrose gradient (1 ml containing approximately 8 nmol of pigment) were diluted in 1 mM Pipes/2 mM KCl, pH 6.5 (25 ml), and centrifuged for 16 h (100 000 g, 4 °C). The pellet was resuspended in 500 μ l of 2 mM KCl and 100 μ l was used to produce membrane films (7 mm diameter) on cellulose-acetate cover slips using isopotential spin drying as described [25]. For films in the nitrate state, all chloride salts in these solutions were replaced by the corresponding nitrate salts.

UV-visual-wavelength spectroscopy and analysis of photocascade

All spectra were recorded on a Perkin-Elmer Lambda 15 spectrophotometer and analyzed using SpectraCalc software (Galactic Industries Corp, Salem, NH, U.S.A.). For the determination of difference spectra hydroxylamine was added to a final concentration of 20 mM and spectra were recorded before and after illumination (3 min exposure to a 75 W light-bulb equipped with a Schott KG1 filter). The pigment was sufficiently stable in the presence of 20 mM hydroxylamine in buffer C to allow accurate recording of difference spectra (no significant change over 15 min at 4 °C).

Photocascade intermediates were analyzed in proteoliposomes either in suspension (Meta II and Meta III formation and decay) or in a dehydrated membrane film on cellulose-acetate cover slips (Meta I formation and decay).

Analysis in suspension

Proteoliposomes isolated from the sucrose gradient were diluted with buffer P (pH 6.5). An aliquot (approximately 0.5 nmol in

400 μ l) was used for spectral analysis at 4, 10 or 20 °C. The temperature of the solution was controlled by a thermostat and measured in the optical cell in the spectrophotometer. Photolysis was induced by illumination for 5–10 s with the above-mentioned light source equipped with a Schott 530 nm cut-off filter and spectra were recorded at regular time intervals (every 50 or 90 s). For analysis at higher pH, Pipes in buffer P was replaced with Mops (pH 7.0 and 7.5) or by bis-Tris propane (pH 8.2). To demonstrate that the photoproduct at 380 nm represents Meta II and not free retinal, aliquots were rapidly acidified to protonate and stabilize the unprotonated Schiff-base linking retinal to Lys296 in Meta II. Hereto, 100 μ l of the proteoliposome suspension were diluted out under rapid mixing into 400 μ l of 1 % SDS in 100 mM sodium citrate buffer, pH 2.5. Under these conditions, the unilluminated pigment was sufficiently rapidly denatured during acidification that 90 ± 5 % conversion into a protonated Schiff-base absorbing at 440 nm could be established.

Analysis on cellulose–acetate cover slips

The cover slip containing the membrane film was inserted into a cuvette in the spectrophotometer and photolysis was induced by illumination for 10 s at 20 °C with the light source equipped with a Schott 530 nm cut-off filter. Spectra were recorded every 5 min.

RESULTS

Large-scale production and purification of HGH

During development of a suitable purification protocol it became evident that relatively large losses had to be reckoned with due to the low thermal stability of the pigment in detergent solution, in particular in small-scale purification attempts. Hence, we

turned to expression of HGH in recombinant baculovirus-infected Sf9 cells in large-scale suspension cultures (up to 10 l). After regeneration with the chromophore 11-*cis* retinal and subsequent solubilization in 1 % (w/v) CHAPS, spectroscopical analysis gave a $\lambda_{\max}$ of 527 ± 2 nm (Figure 1A). Expression levels were determined from these difference spectra as well, using a molar absorbance of $40\,000 \text{ M} \cdot \text{cm}^{-1}$ [5] and see below). Yields of photopigment up to 18 pmol/ 10^6 cells were obtained. Production per l of cell culture (typically 4×10^9 cells) varied from 45–70 nmol (2–3 mg) of pigment.

For the purification of HGH we initially followed the procedure developed for His-tagged bovine rhodopsin [19]. Unfortunately, this protocol did not prove applicable to the human cone pigment in various stages of the purification process, and had to be substantially modified. First of all, 1 % (w/v) DoM was not used for solubilization, since this detergent gave significantly (20–40 %) lower yields of photopigment compared with 1 % (w/v) CHAPS. In agreement with results reported for chicken cone pigments [26], higher detergent concentrations were less suitable because of lower pigment stability. However, the binding of CHAPS-solubilized HGH to the Ni^{2+} resin varied strongly (10–60 %) and was therefore unreliable. Eventually, a combination of CHAPS and DoM (both 0.5 % [w/v]) gave a sufficient and reproducible level of binding (70–75 %). Other modifications of the original protocol were mainly applied because of the low thermal stability of the cone pigment in detergent solution. Since lipids have been shown to stabilize solubilized chicken cone pigments [26], we investigated the effect of adding bovine RL at various stages in the purification protocol. The addition of RL to 0.2 mg/ml prior to loading of the sample on the column completely inhibited binding of the pigment. Therefore, RL were added to washing and elution buffers after the pigment was bound to the Ni^{2+} –NTA resin. In initial purification attempts we used 20 mM imidazole for washing and 200 mM imidazole for elution of the bound HGH, which was the standard procedure adopted for His-tagged rhodopsin. However, HGH was too unstable under these conditions (> 50 % loss in absorbance in 20 h at 4 °C). Consequently, imidazole was replaced by histidine in binding, washing and elution buffers. Optimal concentrations turned out to be 1, 5 and 50 mM, respectively. Typical purification results are presented in Figure 2.

Anion sensitivity

The position of the absorbance band of the human red and green cone pigment is anion-dependent [8]. This phenomenon is known as the anion or hypsochromic effect [27], also found in other cone pigments belonging to this group [8,28]. The term hypsochromic effect is used because binding of most anions other than Cl^- results in a spectral shift to shorter wavelengths (blue-shift). This anion sensitivity was confirmed for the purified green pigment by comparing the effect of Cl^- and NO_3^- ions, which were shown to have a considerable effect on the $\lambda_{\max}$ of the pigment [8]. Exchange of Cl^- for NO_3^- indeed resulted in a 19 nm blue-shift of the absorption peak to 508 nm (Figure 1B). Upon addition of solid NaCl (final Cl^- concentration 0.5 M) the main absorbance band shifts back to its normal position (not shown). Taken together, these results indicate that the purified green cone pigment displays normal spectral characteristics and that the C-terminally located histidine tag has no significant effect on its spectral properties.

Previously, we reported that the molar absorbance coefficient of the human green cone pigment at its $\lambda_{\max}$ is very similar to that of bovine rhodopsin ($40\,000 \text{ M} \cdot \text{cm}^{-1}$) [5]. This was based on comparative analysis of difference spectra. We have now con-

Figure 1 Spectral properties of HGH, expressed using recombinant baculovirus

(A) Difference spectrum; the spectrum after illumination was subtracted from that before illumination. (B) Demonstration of the anion sensitivity of the green pigment. Substitution of Cl^- ions (curve 1, $\lambda_{\max} = 527$ nm) by NO_3^- ions (curve 2, $\lambda_{\max} = 508$ nm) results in a blue-shift of 19 nm.

Figure 2 Purification and reconstitution of HGH

(A) Typical UV–visual-wavelength absorption spectrum of HGH obtained after IMAC purification. (B) SDS/polyacrylamide gel showing protein pattern (silver stain) of crude CHAPS extract of infected insect cells (lane 1) and of purified and reconstituted pigment (lane 2), and immunoblot of purified HGH, probed with CERN956 (red/green pigment-specific [5], lane 3). Reaction with CERN9416 (anti-His-tag) gives a pattern identical to that in lane 3. Molecular mass markers are indicated on the left.

firmed this with the purified pigment. Upon conversion of the cone pigment main absorbance band ($\lambda_{\max} = 527$ nm) to all-*trans*-retinaloxime ($\lambda_{\max} = 365$ nm) by illumination in the presence of 20 mM hydroxylamine, the observed ratio A_{365}/A_{527} was 1.25 ± 0.03 . Under identical conditions a similar value was found for bovine rhodopsin (1.26 ± 0.03).

Transducin activation

Measurement of the fluorescence increase of activated rod transducin was used to assess whether the reconstituted cone pigment could act as a functional photoreceptor. In Figure 3 reconstituted HGH is compared with native bovine rhodopsin and indeed appears to be capable of activating transducin. The initial rate of transducin activation of HGH was obtained from the slope of the fluorescence measurement in the first 4 min after addition of GTP- γ -S and was $50 \pm 5\%$ of that of bovine rhodopsin. Although the transducin activation rate of HGH is lower than that of bovine rhodopsin, this analysis unequivocally demonstrates that upon illumination of HGH, an active intermediate (Meta II) is generated, which is able to interact with transducin. More detailed investigation of the photo-intermediates of HGH was accomplished by spectroscopical analysis of the photocascade.

Figure 3 Activation of rod transducin by HGH or rhodopsin as determined by increase in tryptophan fluorescence

The upper curve shows activation of transducin by native bovine rhodopsin. Identical curves are obtained with recombinant His-tagged rhodopsin (not shown). The two middle curves show two independent measurements of reconstituted HGH. Both pigments were present at 5 nM. The transducin concentration was 100 nM. The bottom curve represents a control where no pigment was added. GTP- γ -S (2.5 μ M) was added after 90 s.

Photolysis

The next objective was to study the pigment's photocascade in a native-like environment. Hereto, the purified pigment was reconstituted into proteoliposomes with bovine retina lipids. Figure 2b demonstrates that after reconstitution full-length HGH is present in the proteoliposomes. After photolysis in proteoliposomes the pigment can be regenerated with 11-*cis* retinal in good yield (85–90%). This also demonstrates the functionality of the HGH protein in this lipid environment.

Photo-intermediates of the human green cone pigment appearing in the late photocascade were analyzed at 4, 10 and 20 °C. The suspension was illuminated for 5–10 s and spectra were recorded before and at regular time intervals after illumination (Figure 4A). The obtained results were reproducible between different batches of purified and reconstituted pigment. Upon illumination, the main absorbance band at 527 nm decreases, and an increase in absorbance can be seen in the region below 425 nm. More detailed information on spectral changes occurring upon illumination can be acquired by analysis of difference spectra. Subtraction of the dark spectrum (curve 1) from the first spectrum after illumination (curve 2) clearly shows the transition of the pigment (527 nm; negative peak) to its Meta II intermediate (375 nm; positive peak). The decay of Meta II is demonstrated by subtracting the first spectrum after illumination from those obtained subsequently. This results in a family of spectra (3–2, 4–2, etc). These spectra show a negative peak at 375 ± 3 nm and a positive peak at 440 ± 3 nm and well-defined isosbestic points. For clarity we only show the spectrum with maximal conversion (Figure 4B, spectrum 27–2). This pattern is very similar to the one documented for the human and bovine rod pigment [29,30], the chicken green cone pigment [14] and gecko cone pigments [18], and corresponds to the Meta II $\rightarrow$ Meta III conversion.

Additional evidence that the 375 nm band represents the cone Meta II intermediate and not free retinal is provided by the following observations. (i) The significant activation of rod transducin by the illuminated cone pigment can only be effected by a Meta II intermediate. (ii) Upon acidification the 375 nm band is converted into a 440 nm band, characteristic of protonation of a retinylidene Schiff base. When successive samples

Figure 4 Late photocascade of HGH

Spectral changes were recorded in suspensions of proteoliposomes of HGH at pH 6.5 at 4, 10 and 20 °C. Samples were illuminated for 10 s. (A) Spectra recorded before illumination (curve 1) and at regular time intervals after illumination (curves 2–27) showing the bleaching of the main absorbance band (centred at 527 nm) and the formation of the Meta intermediates. (B) Difference spectra. Spectrum 2–1 was obtained by subtracting the spectrum before illumination (curve 1) from the first spectrum after illumination (curve 2) and shows formation of Meta II (375 nm). The other spectrum was obtained by subtracting the first spectrum after illumination (curve 2) from that recorded 22.5 min after illumination (curve 27; 4 and 10 °C) or 1.7 min after illumination (curve 4; 20 °C), showing the conversion from Meta II (375 nm) to Meta III (440 nm). At 20 °C maximal formation of the 440 nm intermediate was already observed in curve 4. The absorbance scale for spectra 27–2 and 4–2 was expanded three-fold with respect to the spectrum 2–1.

are acidified, the 440 nm band decreases, indicative of decay of Meta II to free retinal (not shown). (iii) The activation energy of the decay of the 375 nm band is of the same order as that reported for Meta II decay in the chicken cone pigment (see below).

In the difference spectrum 27–2 of experiments performed at 4 °C a negative peak can be seen at approximately 495 nm. Under these conditions the rod pigment produces a Meta I intermediate at about 480 nm. The cone Meta I component decayed with similar kinetics as Meta II. The simplest explanation is that, as observed for the rod pigment, the Meta I and Meta II photo-intermediates are in equilibrium, and the decay of the Meta II component becomes rate-determining for the Meta I component. In rod pigments this equilibrium is strongly temperature dependent, shifting towards Meta II upon increasing temperature. Our results indicate a similar behaviour for the human green cone pigment since at 10 and 20 °C no Meta I component is observed. Because of the rapid coupled decay of Meta I and Meta II and the strongly sloping background due to the light-scattering by the proteoliposomes, reliable curve-fitting could not be accomplished on these transition spectra.

To identify the Meta I intermediate more accurately, two alternative methods can be applied. First, at a sufficiently low temperature the Meta I–Meta II transition will be blocked, which should allow a more exact identification of the Meta I intermediate. As a second approach, photolysis can be studied

Figure 5 Analysis of the late photocascade of HGH on a partially dehydrated membrane film

(A) Spectra recorded before illumination (curve 1) and 5–55 min after illumination at 5 min intervals (curves 2–12). Upon illumination the main absorbance band of the pigment decreases and a Meta I intermediate is formed, which slowly decays. (B) Difference spectrum obtained by subtracting the spectrum recorded 55 min after illumination (curve 12 in A) from the spectrum recorded 5 min after illumination (curve 2 in A), showing the $\lambda_{\max}$ of the Meta I intermediate: 490 nm (curve 1). Similar analysis of the Meta I intermediate under nitrate-saturated conditions revealed a $\lambda_{\max}$ of 463 nm (curve 2).

under dehydrated conditions. In rod pigments, the Meta I $\rightarrow$ Meta II conversion is hydration dependent [31], and in dehydrated samples the normal photocascade only proceeds to a stage which is spectrally similar to Meta I. Under such rigidified conditions this Meta I state very slowly decays to a 370 ± 10 nm product [32]. We expected a similar behaviour for the cone pigment, and since the transition temperatures for the various photointermediates are not yet known, we applied the second strategy and produced membrane films of partially dehydrated cone pigment proteoliposomes on cellulose acetate cover slips. Upon illumination (Figure 5A) the absorbance band of the pigment decreases and an increase in absorbance is observed around 490 nm, representing formation of a Meta I-like intermediate. Meta I slowly decays under these conditions to a product with a maximum absorbance at 365 ± 5 nm. From difference spectra representing this decay the $\lambda_{\max}$ of the Meta I intermediate could be determined at 490 ± 2 nm (Figure 5B, curve 1).

Photolysis of the human green cone pigment was also studied at higher pH, up to 8.2. Since the Meta I $\leftrightarrow$ Meta II equilibrium in rod pigments is pH dependent, favouring Meta I at a higher pH, we investigated whether such conditions would also increase the amount of cone Meta I. However, even at 4 °C we were not able to detect an increase in the amount of cone Meta I at higher pH, in strong contrast with the bovine rod pigment (Figure 6). Apparently, in the human green cone pigment the Meta I $\leftrightarrow$ Meta II transition is not pH dependent.

Analysis of the photocascade was also performed in the nitrate state, after extensive dialysis of the reconstituted pigment to

Figure 6 pH-dependence of the Meta I ↔ Meta II equilibrium

Proteoliposomes of HGH (●) or bovine rhodopsin (▲) were illuminated for 10 s at 4 °C at the indicated pH. The %Meta I in the photoproduct was determined in difference spectra as described [36]. In HGH pH-dependence of the %Meta I could not be detected.

Table 1 Characteristics of rod and cone pigments and corresponding photo-intermediates

	Bovine rod	Human green cone	
		Cl ⁻	NO ₃ ⁻
$\lambda_{\max}$ (nm)			
Pigment	498 ± 1	527 ± 2	508 ± 2
Meta I	480 ± 2	490 ± 2	463 ± 3
Meta II	380 ± 2	375 ± 3	376 ± 3
Meta III	440 ± 2	440 ± 3	437 ± 3
Equilibrium			
Meta I–Meta II	pH-dependent temperature-sensitive	pH-independent temperature-sensitive	

substitute the (native) Cl⁻ ions in the anion-binding site [8] for NO₃⁻ ions. The general pattern was very similar to that measured for the chloride state, including appearance of the band assigned to Meta I, again only at 4 °C. With the exception of the already mentioned change in the position of the main absorbance band of the pigment, no significant changes in spectral patterns or in the $\lambda_{\max}$ of the photo-intermediates Meta II and Meta III were observed. To better characterize the Meta I intermediate, photolysis was again studied at low hydration on cellulose acetate cover slips (Figure 5B, curve 2). Surprisingly, the Meta I intermediate displayed a significant blue-shift with respect to its analogue in a chloride-saturated environment ($\lambda_{\max}$ 463 nm versus 490 nm, respectively). These results indicate that anions in the anion-binding site still affect the pigment's spectral properties up to the Meta I stage. Spectral characteristics of the human green pigment photo-intermediates are summarized and compared with rod-pigment intermediates in Table 1.

Half-life of Meta II decay

The Meta II intermediate that arises upon illumination plays a key role in the signal-transduction cascade in the photoreceptor

Table 2 Half-life of Meta II decay at various temperatures and pH 6.5

Values ± standard deviations are given, *N* = 3–6.

Temperature (°C)	<i>t</i> _{1/2} Meta II (min)	
	Human green cone	Bovine rod
4	5.3 ± 0.2	*
10	3.5 ± 0.7	30 ± 1
20	1.6 ± 0.4	10 ± 0.5
$\Delta H^\ddagger$ (kJ/mol)† at 20 °C...	51.0‡	77.6‡

* Too slow to be reliably measurable.

† Determined from Arrhenius plot. For rod pigment other available data were included.

‡ *r*², 0.997 (cone) and 0.992 (rod).

cell. It is this intermediate that interacts with the G-protein (transducin), thereby transmitting the signal from the photopigment to messenger molecules that are active further downstream in the transduction process. The kinetics of the human green cone and bovine rod Meta II decay were analyzed at several temperatures and pH 6.5 (Table 2). The decrease in absorbance measured at 380 nm in the family of spectra recorded after illumination of the reconstituted pigment was fitted with an exponential time function in order to calculate the *t*_{1/2} of the Meta II decay. This always resulted in excellent fits (*r*² ≥ 0.988). The Meta II decay of HGH in the nitrate state showed very similar kinetics to the chloride state (not shown). Overall, the cone Meta II decays considerably faster than the rod Meta II at all temperatures measured. The activation energy ($\Delta H^\ddagger$) of the cone Meta II decay is smaller, however, indicating a lower temperature dependence.

DISCUSSION

To study molecular properties of human cone pigments to a similar extent as their rod counterparts have been studied, these proteins have to be obtained in a sufficiently pure form and in a lipid environment. In the present study we demonstrate that large-scale baculovirus-mediated expression of the HGH followed by IMAC yields a purified pigment that allows detailed molecular studies. The green cone pigment could be produced in levels up to 18 pmol/10⁶ cells. In cell-culture (typically containing 4 × 10⁹ cells/l), yields varying from 45 to 70 nmol or 2–3 mg of recombinant pigment per l were obtained. This equals the amount of green pigment present in 400–600 human retinas. This illustrates the excellent properties of the baculovirus expression system for the production of heterologous membrane proteins that are present in small amounts in their native tissue.

In view of the successful purification of recombinant His-tagged bovine rhodopsin by IMAC [19], the same approach was taken initially for HGH. However, due to the notoriously low thermal stability of cone pigments in detergent solution, we had to substantially modify the procedure developed for rhodopsin. Eventually, a satisfactory and reliable procedure could be developed. Optimal solubilization of the regenerated pigment required 1% CHAPS (w/v). Binding to the Ni²⁺ resin was optimal when the CHAPS concentration was lowered to 0.5% and DoM was added to 0.5% (w/v). To maximally stabilize the pigment upon elution from the column RL were added to 0.4 mg/ml. HGH processed in this way displayed the expected sensitivity of its $\lambda_{\max}$ towards anions [8]. The ratio of the protein to pigment absorbance (indicative of the purity of the pigment

sample, A_{280}/A_{527}) was typically 3.5 ± 0.5 . The lowest A_{280}/A_{527} ratio we observed was about 3. This is in the same range as the one reported for the chicken red cone pigment, which also belongs to the LW (long wavelength) class [26]. Although our preparations contain at least 80% green opsin according to densitometric analysis of Coomassie Blue-stained polyacrylamide gels we cannot exclude the presence of denatured opsin, which will increase the A_{280}/A_{527} ratio. The amino acid sequence of HGH contains 14 Trp residues and 15 Tyr residues, compared with 5 Trp and 18 Tyr for the bovine rhodopsin. Since the molar absorbance of the main band in the visible region is similar for both proteins, and the Trp residues have the most dominant influence on the molar absorbance coefficient of a protein at 280 nm [33], we estimate that the A_{280}/A_{527} ratio for the green pigment will be much higher than the value of 1.7 ± 0.1 [34], measured for the A_{280}/A_{500} ratio of rhodopsin. A crude estimation, taking the 1.7 ratio for rhodopsin and the different contents of Trp and Tyr residues into account [33] leads to a ratio of approx. 3 for HGH. Hence, our reconstituted preparations indeed contain at least 80% functional HGH.

Purified HGH was reconstituted into a lipid environment to simulate native conditions in the cone photoreceptor cell. Bovine RL are somewhat less unsaturated (60% unsaturated fatty acid) than rod outer-segment lipids (68%), and contain a lower percentage of poly-unsaturated fatty acids (42% versus 58%) [21]. Nevertheless, native and recombinant as well as recombinant His-tagged rhodopsin, all reconstituted in RL proteoliposomes, exhibit late photocascade kinetics very similar to native rhodopsin in rod outer-segment membranes [19,30]. This is irrespective of the reconstitution procedure used (detergent dilution, dialysis or cyclodextrin extraction ([19,30], W. J. DeGrip, J. VanOostrum and P. H. M. Bovee-Geurts, unpublished work). As a matter of fact, the lipid and fatty acid composition of chicken outer-segment preparations, which contain 70–80% cone outer segments (50% unsaturated, 34% poly-unsaturated fatty acyl chains) [35], is not markedly different from that of bovine RL. Hence, we considered it most proper to use bovine RL also for reconstitution of cone pigments. Reconstituted HGH was shown to activate the G-protein transducin and is therefore able to sustain phototransduction as a fully functional photoreceptor. In the transducin activation assay, native bovine rhodopsin and recombinant His-tagged rhodopsin produce identical rates (C. H. W. Klaassen, P. H. M. Bovee-Geurts, M. D. Portier and W. J. DeGrip, unpublished work). The somewhat lower activity of HGH in this assay compared to bovine rhodopsin is to be expected, both since HGH Meta II shows a much faster decay and since the rod transducin will have lower affinity for the activated cone pigment.

Reconstituted samples were also used to study the generation and decay of intermediates in the late phase of the photocascade. Upon illumination, formation of a Meta II (375 ± 2 nm) and a Meta III (440 ± 2 nm) intermediate can be observed. Meta I could still be identified at 4 °C, but no longer at 10 and 20 °C, indicating that, similar to the rod pigment, the green cone Meta I–Meta II transition is an equilibrium, which is strongly temperature-dependent, higher temperatures favouring Meta II. In contrast to the rod pigment, we could not detect a pH effect on the Meta I $\leftrightarrow$ Meta II equilibrium of the cone pigment. Since this pH effect in the rod pigment is dependent on the charge distribution [36], the difference might be due to a significantly altered charge distribution in the cone pigment. This is not inconceivable in view of the much higher pI of cone pigments relative to rod pigments [28].

To more accurately characterize the Meta I intermediate we utilized partially dehydrated membrane films of HGH proteo-

liposomes. Although the rhodopsin protein structure does not respond in an identical way to rigidification by dehydration or by lowering the temperature [37,38], the spectral properties of the Meta I-like photoproducts generated under either condition are very similar. Since dehydration blocks the normal Meta I $\rightarrow$ Meta II transition even at room temperature, it is considered an appropriate approach to characterize the spectral properties of the Meta I intermediate [38]. In the rod pigment the Meta I formed under rigidified conditions very slowly decays to a 370 ± 10 nm product [32]. Upon illumination of largely dehydrated HGH a Meta I intermediate is formed with a $\lambda_{\max}$ of 490 ± 2 nm, which slowly decays to a 365 ± 5 nm product. This behaviour very much resembles that of the rod pigment, and we therefore are confident to assign the 490 nm band to HGH Meta I.

Replacement of Cl^- anions by NO_3^- anions in the proteoliposomes blue-shifted the $\lambda_{\max}$ of HGH from 527 nm to 508 nm. The kinetics of the Meta II decay were not affected by this exchange of anions. Neither was the $\lambda_{\max}$ of the Meta II and Meta III intermediates. The Meta I intermediate, again isolated in largely dehydrated membrane films, however, was blue-shifted from 490 nm to 463 nm in the nitrate form. Apparently, anions can modify the spectral properties of photo-intermediates up to the Meta I stage. The blue-shift in Meta I (27 nm) is even larger than in the parent pigment (19 nm). This suggests that the relatively small conformational changes leading to Meta I have led to a closer contact between the chromophore and residues in the anion-binding site. Meta II intermediates from various pigments all exhibit the same $\lambda_{\max}$ (375–380 nm), indicating that the spectral tuning by the protein is no longer effective at this stage. Future studies will address the question of whether anions induce structural changes in the cone opsin or directly interact with the chromophore.

Our results demonstrate that the Meta II intermediate of HGH decays much faster than the human and bovine rod pigment Meta II under comparable conditions. A faster Meta II decay is also reported for chicken cone pigments. Chimeric pigments of gecko blue and bovine rhodopsin [39] indicate that this faster decay primarily originates in transmembrane helices I–III. Recent mutagenesis studies suggest that this effect is largely controlled by one residue in helix III [16], which is Glu in rod pigments (E122 in bovine), but Gln (chicken green), Ile (chicken red, HGH), Leu (human blue) or Met (chicken blue) in cone pigments. Mutation of this residue in chicken rhodopsin to Gln or Ile accelerated the Meta II decay by seven–ten-fold [16]. On the other hand, the substitution Glu122 $\rightarrow$ Leu that we reported previously [40] only produces a three-fold increase in the Meta II decay rate of bovine rhodopsin. Hence we consider it most likely that other factors also contribute to this characteristic difference between rod and cone pigments. The $t_{1/2}$ we measure for the HGH Meta II decay at 10 °C in RL is significantly longer than the one we calculate from Arrhenius plots for chicken red and green pigments [13,15]. These differences may arise in species differences. However, we rather consider this at least partially to be due to a micro-environment effect. The chicken pigments were all measured in detergent/lipid mixed micelles. Our results were obtained in a more native-like lipid environment. Table 3 shows that the $t_{1/2}$ of the Meta II decay of the rod pigment is significantly shorter when measured in detergent/lipid mixtures than when measured in proteoliposomes. In our opinion, photocascade analysis of visual pigments, and in particular of the less-stable cone pigments, is best performed in a membrane environment. The $\Delta H^\ddagger$ of the Meta II decay for the bovine rod and human green cone pigments we calculate (78 and 51 kJ/mol, respectively) are not very

Table 3 Comparison of the $t_{1/2}$ of Meta II decay in various rod and cone pigments

Species	Pigment	$t_{1/2}$ (s, 10 °C)	
		Mixed micelles*	Membrane†
Chicken	Rod	400	—
	Red cone	45	—
	Green cone	15	—
Ox	Rod	900	1800
Human	Green cone	—	210

* Estimated by extrapolation from Arrhenius plots [13,15].

† This study.

different from that of the chicken rod and green cone pigments (80.7 and 58.5 kJ/mol, respectively) reported by Imai et al. [15]. Apparently, the temperature dependence of the Meta II decay is less strongly affected by the micro-environment.

In general, our results support the conclusion drawn from the chicken-cone-pigment studies that the $t_{1/2}$ of the cone Meta II decay is substantially shorter than that of the rod Meta II. It has been claimed that this is related to the difference in photo-sensitivity between rods and cones [13]. It should be noted, however, that inactivation of the photoresponse in a human cone cell is accomplished within 200 ms [41]. When we extrapolate our data (Table 3) to physiological temperature, the corresponding $t_{1/2}$ of the Meta II decay is in the order of 25–35 s. Hence, this decay cannot be a rate-determining factor in that process. Other mechanisms will be primarily responsible for the inactivation of the Meta II intermediate, like phosphorylation and arrestin binding.

In conclusion, baculovirus-expressed HGH could be produced in large-scale cultures and efficiently purified using IMAC. This allowed the first analysis of biochemical properties of a mammalian cone pigment. The characteristics of the slow part of its photocascade (Meta I → Meta II → Meta III) are markedly different from that of the rod pigment, and the kinetics are not dependent on the type of anion bound. An important next step will be analysis of the conformational changes that occur in the pigment upon photo-activation. We expect to be able to perform such structural studies in the near future.

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