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ScienceDirect
Journal of Structural Biology 159 (2007) 222–227
Journal of Structural Biology
www.elsevier.com/locate/yjsbi

Introduction of a rod pigment aromatic cluster does not improve the structural stability of the human green cone pigment

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Received 11 September 2006; received in revised form 16 January 2007; accepted 19 January 2007
Available online 30 January 2007

Abstract

In the course of our studies on the structure/function relationship of visual pigments, we have expressed the human green cone pigment in the baculovirus/insect cell expression system. Purification of the human green cone pigment, however, has so far proven to be severely hampered by the low thermal stability of this receptor in a solubilized state. In order to overcome this problem, we tested a variety of chemical compounds that have been described to improve protein stability in various applications. The presence of glycerol, sucrose, trehalose and lipids during extraction improved the thermal stability of the recombinant green cone pigment up to twofold. We also analyzed the effect of mutation of residues Met208, Cys212 and Cys273 into Phe in all combinations. These mutants were designed in an attempt to increase the thermal stability by replacing weakly interacting side chains in the green pigment with their counterparts in rhodopsin with strong aromatic stacking interaction. All mutants produced wild-type levels of functional pigment, but none showed an increase in thermal stability.

Keywords: Rhodopsin; Solubilization; Thermal stability; Opsin; Vision; Visual pigments; Receptor; Expression; Cone pigments

1. Introduction

Vision in vertebrates is mediated by two well-known types of photoreceptor cells, rods and cones. Rods are very light-sensitive and mediate dim light (scotopic) vision. Cones, on the other hand, are up to a thousandfold less sensitive but can provide color (photopic) vision (Wald, 1968).

The rod visual pigment rhodopsin has been the subject of extensive research for many years (Wald, 1968; Filipek et al., 2003). This has yielded an overwhelming amount of data on the structure and function of this pigment and provided a strong background for similar studies on other, related G protein-coupled receptor proteins (GPCRs) (Stenkamp et al., 2002; Lundstrom, 2005). The 2.8 Å structure of bovine rhodopsin (1HZX; Palczewski et al., 2000; Teller et al., 2001), in combination with the more recently published 2.2 Å resolution structure (1U19; Okada et al., 2004), furnish a template for detailed studies on the structure/function relationship of other members of the GPCR family.

In the past decade, we have successfully used the baculovirus expression system in *Spodoptera frugiperda* insect cells for the large-scale expression of rhodopsin and a wide variety of rhodopsin mutants (DeCaluwé et al., 1995; DeGrip et al., 1999; Breikers et al., 2001, 2002), followed by functional expression of several cone pigments (Vissers and DeGrip, 1996; Vissers et al., 1998). It appeared that expression level and stability *in vitro* of the cone pigments are much lower than that of wild-type rhodopsin. Since relatively large (mg) quantities of purified, functional protein are essential for structural studies, we set out to optimize the expression level and purification of the human green cone pigment.

In this report, we present the results of our efforts to improve the functional stability of the recombinant His-tagged human green cone pigment (HGH) by permutated

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doi:10.1016/j.jsb.2007.01.010

mutation of residues Met208, Cys212 and Cys273 into Phe. In addition, we explored the effect of chemical additives that have been described to improve protein stability in various applications.

2. Materials and methods

2.1. Cloning of M208F, C212F and C273F mutants

The synthetic cDNA encoding His-tagged human green sensitive cone pigment (HGH) that we constructed earlier in the pUC19 vector (Vissers and DeGrip, 1996) was used in a series of cassette mutagenesis cloning steps. Synthetic oligonucleotide duplexes with mutations M208F, C212F and C273F were introduced by replacing the DNA between two restriction sites (Fig. 1). Restriction enzymes *Bsg*I and *Nhe*I were used to introduce the M208F and C212F cassettes. The C273F cassette was introduced using enzymes *Apa*I and *Kpn*I. The successful introduction of the mutations M208F, C212F and C273F was verified using restriction enzymes *Bst*EII, *Afl*II or *Sph*I, respectively. Using subsequent combinations of mutation cassettes, all combinations of the mutations were cloned. The mutation cassettes were designed in such a way, that in the M208F mutants a *BstE*II site was removed, while with C212F an *Afl*II site was introduced and in the C273F mutants a *Sph*I restriction site was removed. Selected plasmid DNAs were sequenced by the dideoxy chain-termination method. All mutants were subcloned in the pFastBac Dual vector which is part of the Bac-to-Bac baculovirus expression system (Invitrogen). Using *Eco*RI restriction sites, the coding sequence was inserted under control of the polyhedrin promoter.

2.2. Cell culture and virus production

Recombinant baculovirus was generated according to the Bac-to-Bac manual (Invitrogen) with some minor adjustments as described before (Bosman et al., 2003). Cells of a *S. frugiperda*-derived cell line Sf9 (ATCC CRL1711) were cultured at 27 °C in TNM-FH medium (Sigma) with 10% FCS during virus selection and virus production, and in serum-free Insect Xpress medium (BioWhittaker) for protein production. Plaque assays were carried out to obtain pure recombinant virus particles and to determine the concentrations of plaque forming units (pfu) in the virus stocks. For recombinant protein production, Sf9 cells were seeded in three 175 cm^2 cell culture flasks and infected at a multiplicity of infection (MOI) of 0.5–1. The cells were harvested at 4 days post infection (dpi) by pipetting and collected by centrifugation (5 min 1000*g*, RT).

2.3. SDS–PAGE and immunoblotting analysis of membrane proteins

Cell samples of 1–3 × 10^6 cells were lysed by adding 1 ml of ice-cold lysis buffer (Bosman et al., 2003) with 2 μg/mL leupeptin and vortexing for 1 min. Cell membranes were collected by centrifugation for 10 min at 13 000 rpm, 4 °C, and resuspended in ice-cold PBS at 1.5 × 10^7 cells/ml. Samples were analyzed using SDS–PAGE (12%) followed by Western blotting as described before (Vissers and DeGrip, 1996). Blots were probed with the antiserum CERN956, that recognizes mammalian red and green cone pigments (Vissers and DeGrip, 1996). As a second probe, peroxidase-labeled anti-rabbit antiserum (Jackson Immunoresearch Labs) was used, after which the proteins were visualized using Supersignal (Pierce).

2.4. Regeneration and extraction

After harvesting the cells at 4 dpi, the cell pellet was resuspended in buffer A (6.5 mM Pipes, 10 mM ethylene diamine tetraacetic acid (EDTA), 2 μg/mL leupeptin, 1 mM dithioerythritol (DTE), pH 6.5) and homogenized with five strokes in a Potter–Elvehjem tube. After centrifugation (20 min. 40 000*g*, 4 °C), the pellet was resuspended in buffer

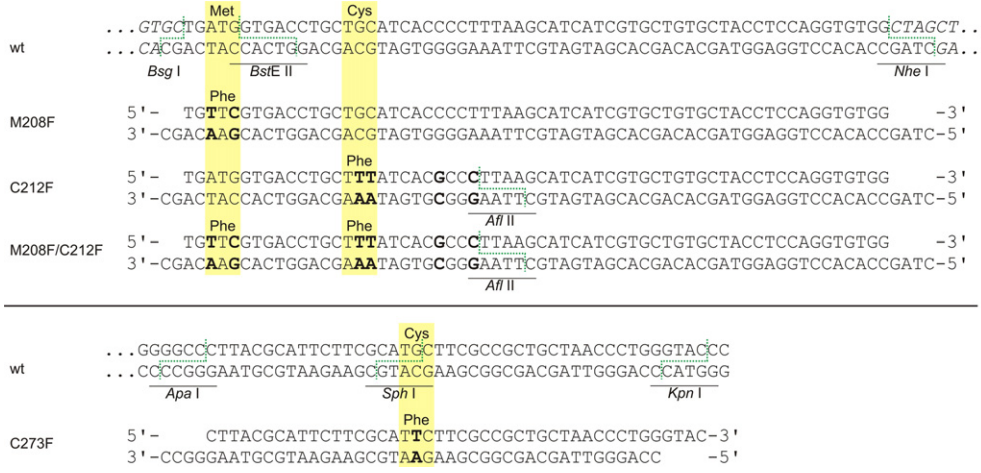

Fig. 1. Mutations in the HGH cDNA were introduced using synthetic oligonucleotide duplexes with the shown sequences. Mutated nucleotides are indicated in bold and restriction sites are underlined. The restriction sites located on the outside were used for cloning, and the inner *Bst*EII, *Afl*II and *Sph*I sites were used to distinguish between wild-type (wt) and mutated plasmids. Correct mutagenesis was always verified by dideoxysequencing.