IV. Discussion

Enzymatic biotinylation assures that all molecules will be immobilized in a uniform, bioactive orientation. Making use of enzymatic biotinylation, we introduced a biotin-tag to the N-terminus of rhodopsin. The chief advantage of this approach is that, unlike chemical reagents, enzymatic biotinylation assures that all molecules will be immobilized in a uniform, bioactive orientation. It was concluded that glycosylation at Asn-15 was required for full signal transduction activity, but apparently not for correct biosynthesis or folding (Kaushal et al., 1994), this is one reason why we preferred enzymatic biotinylation rather than chemical reaction for the subsequence functional study.

The minimum size for the C-terminal fragment of biotin carboxyl carrier protein (BCCP) that can be biotinylated as a fusion protein is about 84 residues (Li and Cronan, 1992). Compared to this, the 13 residue peptides described by Schatz (1993) which can be used as a biotinylation tag seem to bring a definite advantage for recombinant fusion protein applications at least when the size of the tag is critical. Other parameters important for the general utility of biotinylation sequences include their efficiency as biotin ligase substrates, compatibility with secretion, and possible susceptibility to proteolysis. The biotinylated lysine of BCCP is located in a highly conserved hairpin-turn (Athappilly and Hendrickson, 1995), and the cloned acceptor proteins from various bacterial as well as eucaryotic sources act as substrates for *E. coli* biotin ligase (Cronan, 1990). However, the consensus sequence of the 13 amino acid peptides differs considerably from the natural acceptors, indicating that different constraints and perhaps a different mechanism of recognition apply to the small acceptor peptides (Schatz, 1993). Despite this, the peptides have been shown to be quantitatively biotinylated in vivo, both as N- and C-terminal fusions, provided that biotin ligase is coexpressed (Tsao et al., 1996). The use of the 13 amino acid biotin acceptor peptide as fusion proteins for rhodopsin has not been attempted before.

The biotinylation reaction was optimized with recombinant MBP-Bio, showing that Bicine buffer, pH around 8.3 and a high concentration of substrate are critical factors for

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enhancing the biotinylation reaction. In addition, removal of the GST moiety of BirA before biotinylation improves the yield of biotinylation.

After optimization of the biotinylation reaction, three fusion mutants of rhodopsin were produced: Rho-Bio1, Rho-Bio2 and Rho-Bio3, which were expressed at levels between 60% and 90%, probably depending on the particular fusion site for the consensus sequence. Under the same conditions, the yield of biotinylation was different. Rho-Bio2, where the acceptor peptide was fused to the N-terminus and a glycosylation site close to the N-terminus was deleted, showed the highest degree of biotinylation (~ 90%). In the Rho-Bio3 control mutant, the same biotinylation sequence was introduced close to the C-terminus. The biotinylation sequence was not fused directly to the C-terminus because an unmodified free C-terminus is necessary for the purification of rhodopsin with the 1D4 antibody-column (Figure16). This mutant showed the lowest degree of biotinylation (67%). This argues for a different accessibility of the biotinylation sequence for BirA in these three mutants and a more unfavorable conformation of the biotin sequence in case of Rho-Bio3. Mutational analyses showed that the regions of biotin domains are important for recognition by BPL, the alteration of structurally important residues in the biotin domain results in proteins that are poorly biotinylated (Chapman-Smith, et al 1999).

We applied biotin ligase as a GST-BirA fusion protein and performed site-specific biotinylation of recombinant rhodopsins containing a 13 amino acids acceptor peptide. To facilitate the purification of the biotin ligase, GST fusion technology with the birA gene cloned into the GST fusion vector pGEX-2T was used (O´Callaghan et al, 1999). The fusion of glutathione S-transferase to the amino terminus of BirA was considered a minor risk, especially because there was an option to remove the fusion partner by thrombin cleavage at a site in the joining linker (Figure 9). On the basis of genetic data as well as sequence comparisons, Buoncristiani et al. (1986) suggested that the N-terminal part of the BirA protein contains a helix-turn-helix motif responsible for DNA binding, while the central part contains the catalytic activities. In the crystal structure of BirA (Wilson et al., 1992), there is only a loose connection between the N-terminal domain (residues 1-60) and

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the central domain (residues 68-269), suggesting that the fusion of GST to the amino-terminus of BirA would not affect the catalytic activity.

The biotin ligase activity consists of two steps: the formation of biotinyl-5'-AMP from biotin and ATP and the transfer of the activated biotin moiety to the acceptor protein (BCCP). If all acceptor proteins are already biotinylated, the biotinyl-5'-AMP remains bound to BirA and acts as the corepressor of biotin biosynthesis (Cronan, 1989). Not only is biotinyl-5'-AMP required for the binding of BirA to the bio operator but, in turn, also the presence of the DNA-binding domain is important for the binding of biotinyl-5'-AMP to the catalytic domain. In a study of a truncation mutant of BirA lacking the N-terminal DNA-binding domain, Xu and Beckett (1996) found that the affinities for biotin and biotinyl-5'-AMP were 100- and 1000-fold decreased, respectively, although the maximal catalytic rates for the formation of biotinyl-5'-AMP and the transfer of biotin to BCCP were unchanged. These results implied that the N-terminal fusion of GST to BirA could, in an unfortunate case, affect the enzymatic activity. In our experiments, deletion of the GST moiety appeared to be a critical factor for improving biotinylation yield, so we removed the GST part by taking advantage of the thrombin cleavage site. In figure 15, with soluble MPB-Bio at the same concentration and same reaction conditions, the biotinylation was 42.3% for the fusion protein (GST-BirA), compared to 96.4% biotinylation for purified BirA (with GST moiety removed).

Monomeric avidin binds biotin with a K_D of 10^{-7}, allowing reversible binding of biotinylated proteins. The Soft Link Avidin Resin was used for isolation and purification of biotinylated rhodopsin.

The enzymatically biotinylated rhodopsin was characterized by biophysical and biochemical techniques in order to assess the effect of biotinylation on the photochemistry and activity of rhodopsin. First, UV/Visible absorption spectroscopy was used to test the interaction between biotinylated rhodopsin and the chromophore (11-*cis*-retinal). Spectra of purified biotinylated rhodopsin mutants were taken in the dark and after illumination.

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The same shift of the absorption maximum was seen for all three mutants, suggesting that the photoreaction was not influenced by the biotinylation. This absorption shift was independent of whether the tag was fused to the N-terminus or inserted close to the C-terminus. To test whether biotinylation affects the capability of rhodopsin to catalyze nucleotide exchange in transducin, GTPγS uptake rates were determined with fluorescence spectroscopy. The measurements for wild type rhodopsin and the Rho-Bio mutants were almost identical. As expected, the biotinylation does not affect the activity of rhodopsin neither at the N-terminus nor C-terminus.

A biochemical pull-down assay was performed to measure interaction between biotinylated rhodopsin and transducin in the soluble state. By using SDS-PAGE for analysis of bound Gt, the same results were obtained with wild type rhodopsin and biotin-tagged rhodopsin mutants. It can be concluded that enzymatic biotinylation provides the uniform site-specific biotin tag to rhodopsin without affecting the interaction between rhodopsin and Gt.

Rhodopsin-transducin interaction is amenable to analysis by the IAsys biosensor device.
Rhodopsin has been reconstituted into planar phospholipid membranes, immobilized in a uniform, bioactive orientation onto functionalized sensor surfaces, and served as a model for the investigation of G protein-coupled receptors with surface-sensitive optical techniques. Rhodopsin activity was assessed by its coupling to the G protein, transducin, following photoexcitation. Supported lipid bilayer was formed by critical micellar dilution, resulting in an artificial membrane for rhodopsin. All reaction steps, from membrane formation to transducin binding and release, were monitored in situ using the IAsys cuvette system.

The IAsys cuvettes contained a sensor surface which was modified with biotin. Covering this surface with tetrameric NeutrAvidin, provided binding sites for immobilization of biotinylated rhodopsin. Due to the strong affinity of NeutrAvidin to biotin ($K = 10^{-15}$ M), the monolayer of neutrAvidin is homogeneous and stable, so biotinylated rhodopsin can be immobilized via the NeutrAvidin linker. The procedure for rhodopsin reconstitution in

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supported membranes in this work was designed to fulfill the following criteria: (i) to avoid loss of rhodopsin functionality by always keeping the protein in contact either with detergent or with lipids; this is a profound difference to the method of Salamon et al. (1996); (ii) relying on a defined system composed of a few, pure compounds, so the data from IAsys measurement can be readily interpreted .

According to the manufacturer, the sensitivity of the biotin cuvette is 600 arc sec per ng/mm^2 , which can be used to calculate the density of protein immobilized. The resonance angle increase due to rhodopsin binding is 345 ± 52 arc sec. Thus the density of that on the sensor surface calculated 1 rhodopsin per ~100 nm^2. The calculated density is lower as compared to the rhodopsin density in native disc membranes (1 rhodopsin/33 nm^2 (Hofmann et al 1996)).

After immobilization of rhodopsin, a supported lipid bilayer (SLB) (Sackmann, 1996) was formed by micellar dilution (Lang et al 1994). The obtained data indicate that the SLB was stable, allowing fast G protein binding to the SLB, which saturated after some minutes. The rhodopsin containing SLB showed a behavior comparable to pure disc membranes (Schleicher et al 1987).

An antibody directed against the nine C-terminal (intracellular) amino acids of rhodopsin (denoted 1D4 (Molday et al, 1983)) was used to detect the immobilized rhodopsin in SLB. Stable binding signals were observed with Rho-Bio1 and Rho-Bio2, which were immobilized in the correct orientation with the C-termini facing away from the sensor surface. No binding signal was detected with Rho-Bio3, which was immobilized up-site down to the sensor surface, because the biotin tag was close to the C-terminus of rhodopsin. This is a further indication that rhodopsin has been reconstituted in a lipid bilayer with the desired orientation.

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Rhodopsin-Transducin Interaction on Supported Membranes Exhibits the Same Basic Reactions As Observed on Disc Membranes. As in light-scattering measurements on rod outer segments and disc membranes (Kühn, et al 1981; Schleicher et al, 1987; Bennett et al, 1985), we observed binding signals upon illumination of rhodopsin membranes in the absence of GTP, indicating the formation of the R* Gt_{empty} complex. Addition of GTP to the cuvette after illumination led to fast dissociation signals, which have been also observed in light scattering experiments on disc membranes (Kühn, et al 1981; Schleicher et al, 1987). This dissociation was reversible; eventually rebinding of Gt to membrane occurred, an effect also observed for disc membranes reconstituted with transducin (Bennett et al, 1985). These phenomenons can be observed with Rho-Bio1 and Rho-Bio2. With the same procedure however, the mutant of Rho-Bio3 gives no detectable signal either with illumination or illumination plus addition of GTP, due to its wrong orientation in the SLB.

In rod cells, R* is deactivated within seconds by phosphorylation and arrestin binding (Helmreich et al., 1996 and Hofmann et al 1995), in the case of reconstituted system (as in our case) bleached rhodopsin remains active for minutes. It slowly decays to all-*trans*-retinal and opsin, with a half-time of ~5 min, for washed membrane, the process with a half-time of 15 minutes (Farrens et al 1995) and the faster transducin rebinding is due to GTP being used up (Bennett et al, 1985).

Continuous R* activity after illumination, transducin rebinding as a function of GTP depletion, and slow R* decay have been demonstrated on washed membranes (Ernst et al 2000). The application of GTP pulses of low concentration led to transducin desorption-rebinding cycles, the amplitude of which decreased with time.

Rhodopsin immobilized via the N-terminal biotin tag decayed faster than native rhodopsin. A possible reason may be that the immobilization affected the compact structure of the N-terminal domain of rhodopsin which contributes to the firm binding of retinal (Palczewski, 2000;)

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The reconstituted rhodopsin-Gt system was remarkably stable, and repeated activation/deactivation cycles could be monitored readily. Rhodopsin was not constitutively active (as GTP addition alone did not induce transducin desorption), and could be stimulated by illumination. When rhodopsin was activated by illumination, this led to a slight increase in resonant angle in the absence of GTP, due to trapping of the R* Gt complex. This complex can be dissociated by addition of GTP. The steps following illumination and fast photoprocesses are as follows: (1) transition of rhodopsin from the MI to the active MII state (here termed R*, when correctly oriented in the supported membrane), which expresses the signalling state for Gt; (2) Diffusion of transducin toward MII; (3) binding of transducin to MII and GDP release; (4) GTP binding, dissociation of the subunits, release from MII; (5) release of the transducin subunits from the membrane.

Our observation is that with addition of GTP, the R*Gt complex is released fast, after some minutes at low level, a slow increase of the response angle can be detected due to the decay of rhodopsin (additional GTP didn't induce decreased signal). The consecutive illumination steps (20 sec each) induce repeatable desorption/rebinding signals. 11-*cis*-retinal was applied for regeneration of rhodopsin, the regenerated rhodopsin showed the same activity compared to that of the native rhodopsin. Three or even more activation /deactivation cycles were performed without losing significantly its functionality even though no fresh G protein was added during this time.

In membranes on sensor surface, the binding signals allow us to estimate that about 20-60% of the rhodopsin bound via biotin tag can be activated by a illumination (20 sec). In the procedure described, biotin tagging to the N-terminus ensures that the rhodopsin is immobilized to the sensor surface with the correct orientation, this avoid that some rhodopsin molecules assemble randomly with the cytoplasmic (transducin-binding) face toward the support, and are thus inaccessible to transducin (Heyse et al 1998). Introducing a site-specific biotin tag under mild reaction condition ensures that rhodopsin can be reconstituted with correct orientation without affecting biological activity.

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Total internal reflection spectroscopy as used in the IAsys system has been shown to be a valuable tool to study membrane protein (Hovius et al 1999), there is no loss of the reflected light intensity associated with the resonance condition (reviewed by Hall, 2001). We applied enzymatic biotinylation for functional immobilization of rhodopsin with defined orientation to the sensor surface. By using the IAsys device, we were able to follow ligand binding, G protein activation, and deactivation/ activation cycle of rhodopsin directly in real-time.

Experimental data from this approach can be used for fundamental reference to improve the accuracy of biosensor-based assays. The work presented may be useful to develop a routine technique for assaying interactions of rhodopsin mutants with other proteins of the visual cascade.

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