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Effects of phosphorylation on the structure of the G-protein receptor rhodopsin

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Abstract

Upon activation by light, rhodopsin is subject to phosphorylation by rhodopsin kinase at serine and threonine residues in the carboxyl terminal region of the protein. A 19 amino acid peptide that corresponds to the carboxyl terminal end of rhodopsin (residues 330–348) and contains these phosphorylation sites was synthesized. The structure of this peptide was determined using two-dimensional proton NMR. The structure of this peptide was similar to that determined for this region in peptides corresponding to the carboxyl 33 and 43 amino acids of rhodopsin. The effect of phosphorylation on the structure of the carboxyl terminal domain of rhodopsin was determined by solving the solution structures of the peptide containing residues 330–348 with phosphorylation at one (residue 343), three (residues 343, 338, and 334) and seven residues (residues 334, 335, 336, 338, 340, 342, 343). These data indicate that the major structural change occurs upon phosphorylation of the first residue, and that an additional structural change occurs with seven phosphates.

Keywords: Rhodopsin; Phosphorylation; Membrane protein structure; NMR

1. Introduction

At low light levels, retinal rod cells are responsible for visual signal transduction. The initial events of visual transduction take place on the disk membranes which are densely stacked along the length of the outer segments of rod photoreceptor cells. Upon absorption of light, the photopigment, rhodopsin, goes through a series of spectrally defined intermediates. The transition of Metarhodopsin I to Metarhodopsin II allows the G-protein, transducin, to bind and to initiate the cGMP cascade which culminates in the hydrolysis of cGMP. Reduction in the concentration of cGMP leads to closure of the plasma membrane Na^+ channels and hyperpolarization of the plasma membrane.

Following initiation, the transduction cascade must be terminated. A mechanism of desensitization is phosphorylation of rhodopsin by rhodopsin kinase [1–4] followed by arrestin binding. Arrestin binds to photoactivated rhodopsin only following phosphorylation. The binding of arrestin prevents further activation of transducin by rhodopsin. As is the case

Abbreviations: NMR, nuclear magnetic resonance; rmsd, root mean square deviation; ROS, retinal rod cells

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with other G-protein receptors, rhodopsin can become phosphorylated at multiple sites after activation. This phosphorylation of rhodopsin occurs on the carboxyl terminal domain of the receptor [5]. However the precise roles which specific sites of phosphorylation may play in visual transduction are currently under investigation [6–8].

The molecular mechanism of desensitization by phosphorylation is unknown. However, two mechanisms are possible: (1) phosphorylation may change the conformation of the protein and thereby inhibit transducin binding [9]; and (2) phosphorylation will add negative charge which may inhibit transducin binding [10]. Furthermore, phosphorylation also provides a site for activation of arrestin [11], and binding of arrestin to photoactivated phosphorylated rhodopsin prevents further activation of transducin by rhodopsin.

A three dimensional structure of rhodopsin would allow the effects of phosphorylation to be visualized. Work is currently in progress on the three dimensional structure of rhodopsin. Electron diffraction has been successfully used to determine a low-resolution structure for the bundle of seven transmembrane helices of this G-protein receptor [12–14]. Using two-dimensional homonuclear NMR, we have determined the structure of the cytoplasmic surface of this receptor to moderately high resolution [15–19].

Fig. 1 shows the previously determined structure of the carboxyl terminal domain. Following absorption of light by rhodopsin, rhodopsin kinase sequentially phosphorylates serine and threonine residues in the carboxyl terminal region of rhodopsin. Therefore it is feasible to investigate the structure of a peptide which includes all of the sites which become phosphorylated under physiological conditions. In this study, we use two-dimensional homonuclear NMR to determine the effects of phosphorylation on the structure of the carboxyl terminal of rhodopsin. It is first shown that an unphosphorylated peptide consisting of the carboxyl terminal 19 residues of rho-

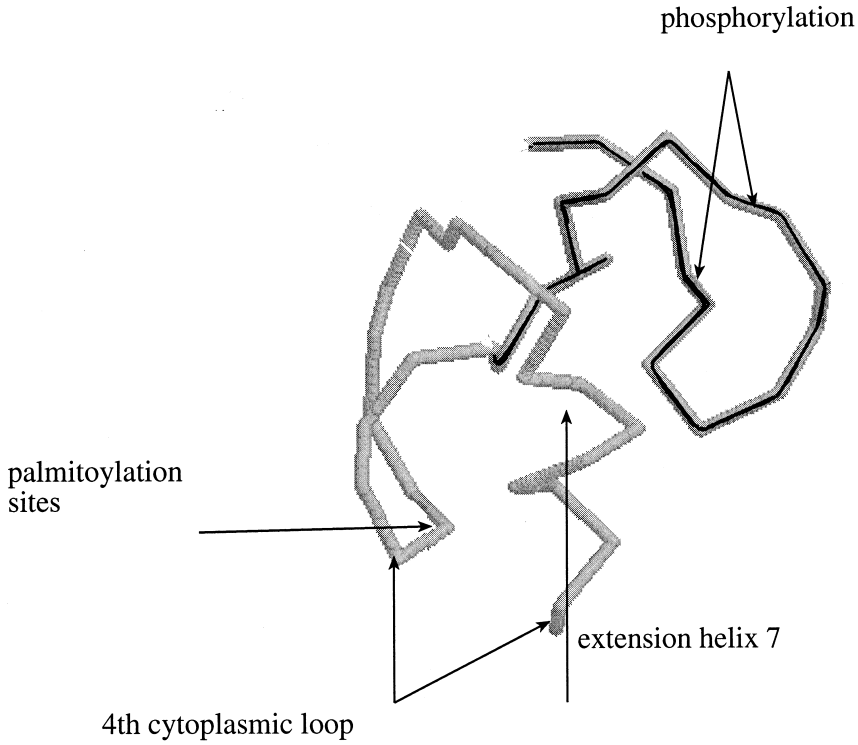


Fig. 1. Structure of the carboxyl terminal domain of bovine rhodopsin. Two-dimensional NMR was used to determine the solution structure of a 43 amino acid peptide (residues 303–348 of rhodopsin). This structure agrees well with structural determinations available on intact rhodopsin [19]. The 19 amino acid unphosphorylated peptide (330–348 in rhodopsin) whose structure was investigated in the present work is indicated in this figure by the dark line.

dopsin (330–348) retained the structural characteristics of the parent peptide (shown in Fig. 1), the 43 amino acid residue carboxyl terminal domain of rhodopsin attached to the end of transmembrane helix 7 of this receptor. Phosphorylation of the first (native) kinase site produced a conformational change in the peptide from the unphosphorylated form. Phosphorylation at the three serines caused no further conformational change from the single phosphorylation. Phosphorylation at seven sites on the peptide caused additional structural change.

2. Materials and methods

2.1. Peptide synthesis

Peptides constituting the terminal 19 amino acid residues of the carboxyl terminal domain of bovine rhodopsin were studied (residues 330–348; DDEASTTVSKTETSQVAPA). Unphosphorylated peptides were synthesized using Fmoc-Ala-Pam resin and the standard DCC/HOBt protocol. Cleavage from the resin and deprotection employed 95% trifluoroacetic acid/5% H_2O for 90 min. Phosphopeptides were made on Boc-Ala-Pam resin using Boc-*O*-(diphenylphosphono)-serine and -threonine by the DCC/HOBt protocol. Cleavage and deblocking were performed in a single step using catalytic hydrogenolysis in the presence of palladium and platinum with anhydrous trifluoroacetic acid as solvent (Arendt, 1996, no. 2869). The peptides were purified by HPLC, and had the appropriate amino acid composition and mass.

2.2. NMR spectroscopy

All NMR spectra were accumulated using 3–5 mM peptide in 10 mM phosphate buffer at pH 5.9 on a Bruker AMX-600 spectrometer at 10°C. Standard pulse sequences and phase cycling were employed to record: in H_2O (10% D_2O), double quantum filtered (DQF) COSY and NOESY (400 ms mixing time) [20]. All spectra were accumulated in a phase sensitive manner using time-proportional phase incrementation for quadrature detection in F1. Chemical shifts were referenced to a trace amount of internal methanol and expressed relative to TSP.

2.3. Structure refinement

The sequence-specific assignment of the ^{1}H-NMR spectrum was carried out using standard methods. Assigned NOE cross peaks were segmented using a statistical segmentation function and characterized as strong, medium, and weak corresponding to upper bounds distance range constraints of 2.7, 3.7 and 5.0 Å, respectively. Lower bounds between nonbonded atoms were set to the sum of their van der Waals radii (approximately 1.8 Å). Pseudoatom corrections were added to interproton distance restraints where necessary [21]. Distance geometry calculations were carried out using the program DIANA [22] within the SYBYL 6.4 package (Tripos Software, St. Louis). First-generation DIANA structures, 150 in total, were optimized from 1 to step 43 with the inclusion of three REDAC cycles. Energy refinement calculations (restrained minimization/dynamics) were carried out on the best distance geometry structures using the SYBYL program implementing the Kollman all-atom force field. Statistics on structures were obtained from X-plor. These calculations were performed on a Silicon Graphics 4D/440 computer.

3. Results and discussion

Phosphorylation plays a central role in the regulation of protein activity. In this study, the effect of phosphorylation on the structure of a peptide which corresponds to the region of rhodopsin phosphorylated by rhodopsin kinase was investigated. Previous work has suggested that the conformation of a structured peptide isolated from a larger protein will reflect the structure of the region of the protein to which it corresponds [23]. The polypeptide comprising the carboxyl terminal 43 amino acids of rhodopsin has a structure that agrees well with all the available evidence on the structure of that portion of intact rhodopsin. This peptide structure begins at its amino terminal with an α-helix that is an extension of transmembrane helix 7. From the top of the helix, the structure returns to the membrane surface at Cys-322 and Cys-323, the sites that are palmitoylated in native rhodopsin. This portion of the structure forms the fourth cytoplasmic loop. Further towards the carboxyl terminal a short anti-parallel

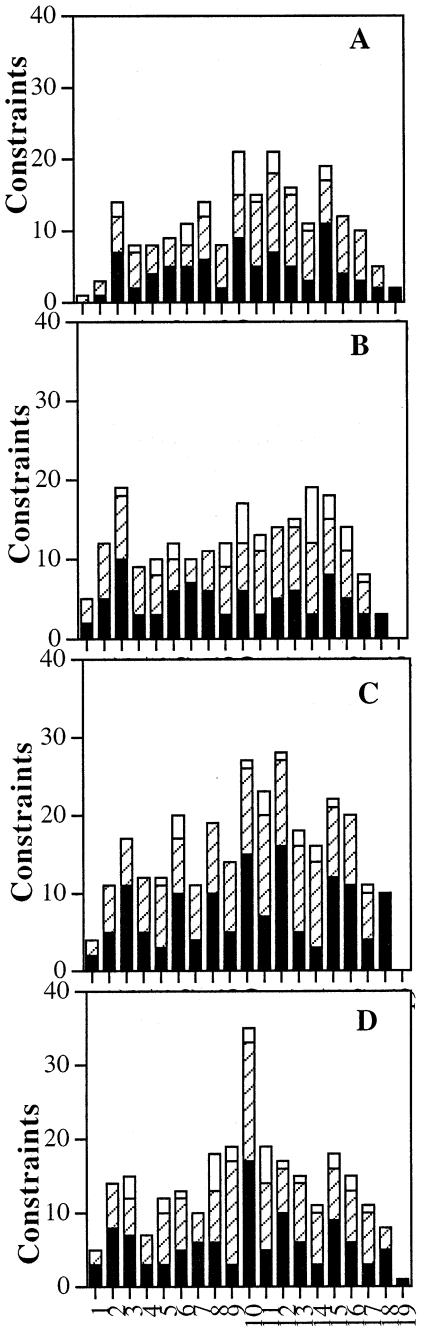


Fig. 2. Distribution of the NOE-derived constraints, as a function of amino acid in the primary sequence of non-phosphorylated peptide and the phosphorylated peptides which contain the 19 carboxyl terminal amino acid residues of bovine rhodopsin, 330–348: filled bars, intraresidue; cross-hatched bars, sequential; open bars, long range. (A) Non-phosphorylated peptide. (B) One residue phosphorylated (Ser-343 in rhodopsin). (C) Three residues phosphorylated (Ser-343, Ser-348 and Ser-334). (D) Seven residues phosphorylated (334, 335, 336, 338, 340, 342, 343).

β-sheet is formed. The principal phosphorylation sites, Thr-336, Ser-338 and Ser-343 are in or near this β-sheet. Therefore, this region is an important structural domain with respect to the effects of phosphorylation. This anti-parallel β-sheet is also conserved in the structure of the carboxyl terminal 33 amino acid peptide as previously reported [15].

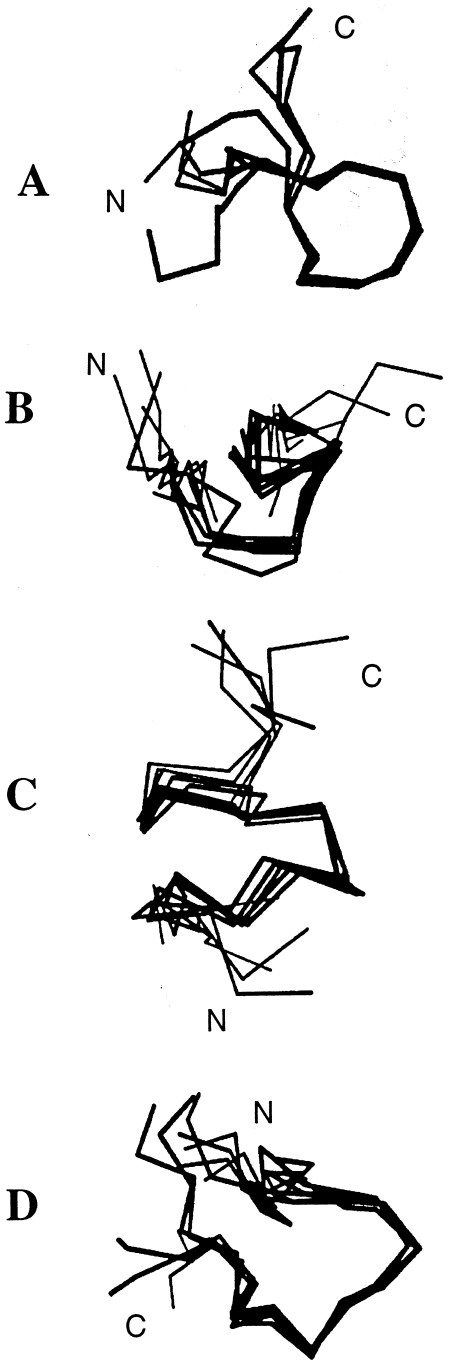
To investigate the effects of phosphorylation on the structure of rhodopsin, a peptide consisting of the carboxyl terminal 19 amino acid residues (residues 330–348) was synthesized. The length of the peptide was chosen with the following considerations. The peptide must be long enough to contain the phosphorylation sites and must also be sufficiently long to retain a well-defined structure which reflects the structure of the intact protein. However, with increasing length, synthetic difficulties mount, especially for phosphorylated species. This chosen peptide includes all the residues that form the carboxyl terminal β-sheet as well as the phosphorylation sites.

The solution structure of the 330–348 peptide was determined by standard homonuclear two-dimensional NMR protocols, as described in Section 2. From the NOESY map, 240 constraints were obtained: 141 intra-residue, 65 sequential and 34 long range. Fig. 2A shows the number of constraints per residue. Fig. 3A shows part of the family of structures obtained from the DIANA calculations and after energy minimization, and Fig. 4A shows the rmsd of the backbone as a function of residue for that family.

The fidelity of the structural elements in this peptide to longer peptides was examined by comparing the structure of the 19 mer to that of peptides consisting of the 43 and 33 carboxyl terminal amino acids of rhodopsin. The average rmsd values of the backbone atoms for a superposition of peptide structures are typically used as a measure of the similarity between peptide structures derived from a data set. In the following analysis, the independently determined structures of the various peptides were superimposed upon the 43 mer and the average rmsd of the backbone atoms determined. The values determined by superposition of independent peptides can be compared to the average rmsd determined within the structural family of a single peptide.

Superposition of residues 15–29 (330–344) of the

33 mer on the corresponding residues of the 43 mer is characterized by a rmsd of the backbone of 2.1 Å. The average rmsd of the backbone atoms for a superposition of residues 5–15 of the 19 mer on the corresponding residues of the 43 mer is 2.2 Å. The sequence of 5–15 on the 19 mer (334–344) was chosen for this analysis to avoid the disordered ends of the smaller peptide, yet still contain the β-sheet and the turn in the structure, as well as the major phosphorylation sites. A similar comparison between the 19 mer and the 33 mer gives a rmsd of the backbone of 2.4 Å. These rmsd values can be compared to those obtained for the family of peptide structures and presented in Fig. 4. This shows that the rmsd among the three peptides is only slightly higher than the rmsd obtained (typically 1.5–2) for the family of structures of a single peptide. Thus the structure of the 19 mer is similar to the 33 mer and the 43 mer in the region of interest. The similarities in structure show that major structural features of the longer peptides are retained in the shorter peptide. This is consistent with the established importance of short-range interactions as determining factors of protein structure.



Because the 19 mer is structurally similar to the longer peptides, the effect of phosphorylation on the structure of the 19 mer is likely relevant to structural effects of phosphorylation on the larger species. Although it is possible under in vitro conditions to phosphorylate all serine and threonine residues in the carboxyl terminal sequence of rhodopsin [24], under physiological conditions only a small number of these residues become phosphorylated [8]. One of the first residues to become phosphorylated is Ser-343 [2,25,26]. Therefore the monophosphopeptide in which Ser-343 was phosphorylated was synthesized as an appropriate model to investigate the structural effect of such a modification. Phosphopeptides with additional phosphorylation corresponding to the three residues Ser-343, Ser-338 and Ser-334, and corresponding to the seven residues including all the serines and threonines in this segment of the protein (residues 334, 335, 336, 338, 340, 342, 343) were then

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Fig. 3. Overlay of the six best structures for the non-phosphorylated peptide and the phosphorylated peptides which contain the 19 carboxyl terminal amino acid residues of bovine rhodopsin. These structures were determined by NMR in aqueous solution, using methods describe in the text. These are energy minimized DIANA structures overlaid to show the extent of agreement among each family of structures. (A) Non-phosphorylated peptide. (B) One residue phosphorylated (Ser-343 in rhodopsin). (C) Three residues phosphorylated (Ser-343, Ser-348 and Ser-334). (D) Seven residues phosphorylated (334, 335, 336, 338, 340, 342, 343).

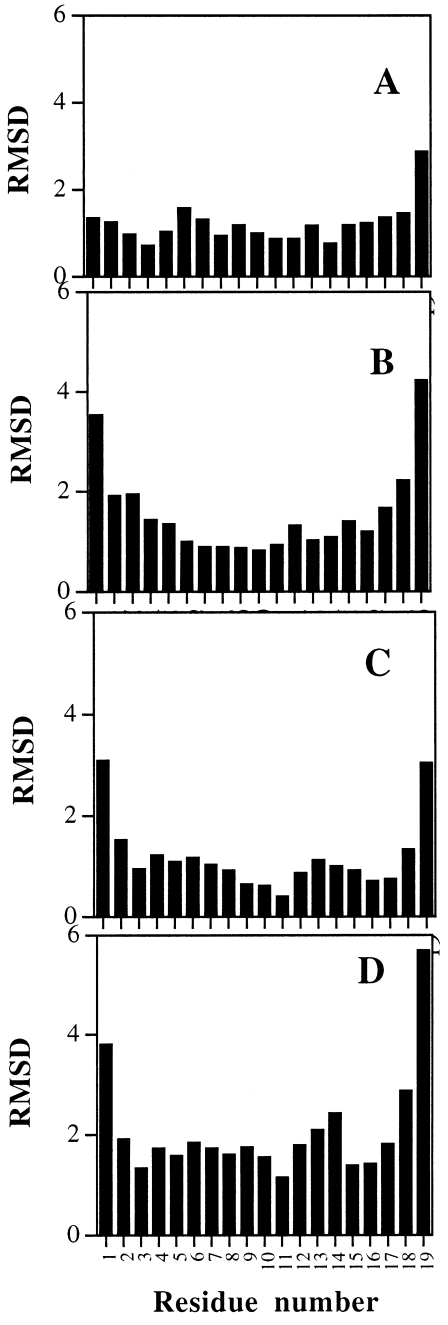

Fig. 4. Root mean square deviations (RMSD) for the families of structures in Fig. 3. Statistics graphed by amino acid position for all of the backbone atoms in the structure. Statistics were obtained from X-PLOR. (A) Non-phosphorylated peptide. (B) One residue phosphorylated (Ser-343 in rhodopsin). (C) Three residues phosphorylated (Ser-343, Ser-348 and Ser-334. (D) Seven residues phosphorylated (334, 335, 336, 338, 340, 342, 343).
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investigated to determine the structural effect of more extensive phosphorylation. The structures of three phosphorylated derivatives of the 19 mer were determined as described above for the 19 mer. Figs. 2–4 show these results.

The structures of the unphosphorylated peptide and the three phosphopeptides were compared by superposition of the average structures of the families, as described above for the unphosphorylated 19 mer and the larger peptides. For the 19 mer with one phosphate, superposition on the 43 mer gives a rmsd of the backbone of 4.2 Å. Superposition of the 19 mer with one phosphate on the 19 mer with no phosphates shows an rmsd of 4.3 Å. Superposition of the 19 mer with three phosphates on the 43 mer shows a rmsd of 4.2 Å. Superposition of the 19 mer with three phosphates on the 19 mer with one phosphate shows a rmsd of 2.1 Å. The 19 mer with seven phosphates shows an rmsd of 3.4 Å with respect to the 43 mer, and an rmsd of 4.5 Å with respect to the 19 mer with one phosphate and an rmsd of 4.1 Å with respect to the 19 mer with three phosphates. The structural analysis here suggests that the deviations in structure between any of the phosphorylated derivatives and the unphosphorylated 43 mer are substantially greater than the deviations in structure between the unphosphorylated 19 mer and the unphosphorylated 43 mer. The latter might be taken as the 'noise level' in this structural 'experiment'. This noise level may represent the degree of agreement feasible between these kinds of solution structure determinations of small peptides and larger, better ordered peptides containing the same sequence as part of their structure. This analysis further shows that while the addition of one phosphate induces a conformational change, the addition of two more phosphates does not introduce any further conformational change. The conformational change that is induced by these lower levels of phosphorylation reflects a twisting of the β-sheet relative to the unphosphorylated peptides. Fig. 5 shows this twist. It is interesting to note that, although not likely physiological, the addition of seven phosphates causes a different conformational change, in which the β-sheet is broken (the interstrand hydrogen bonds are broken), but the turn that leads to the antiparallel strands is still observed.

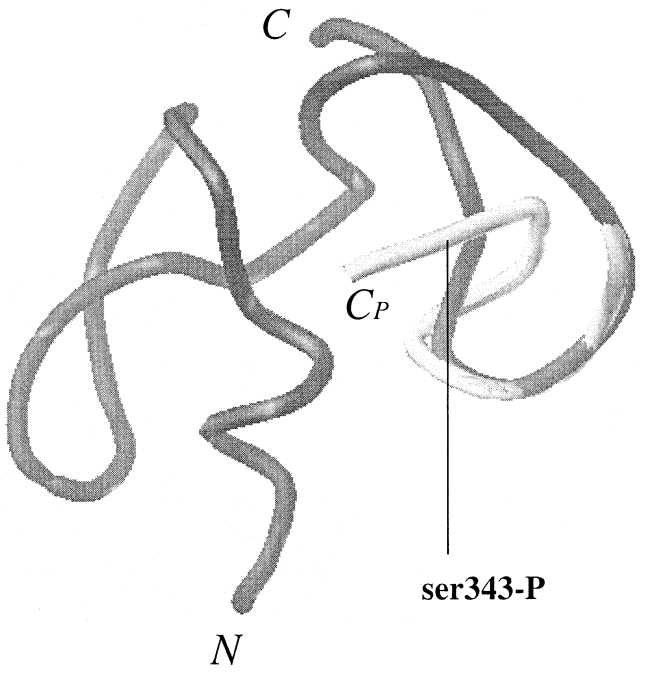

Fig. 5. Comparison of the structure of the monophosphorylated (ser343) peptide (light gray) with the unphosphorylated structure of the 43 mer (dark gray), showing the twist in the β-sheet induced by the phosphorylation. Cp is the carboxyl terminal of the monophosphorylated peptide (19 mer), and C is the carboxyl terminal of the unphosphorylated 43 amino acid peptide from the C-terminus of rhodopsin.

While it may be argued that phosphorylation may induce a conformational change on an adjacent loop, this is unlikely. The phosphorylation sites are well exposed and are oriented away from other portions of the protein. Furthermore, studies of phosphorylation effects on structure of another proteins have indicated conformational changes are localized near the residue which is phosphorylated [27]. This analysis suggests that the addition of one phosphate by rhodopsin kinase may introduce a conformation change in the carboxyl terminal detectable by transducin. Since the first phosphate appears to produce just as great a conformational change as an additional two phosphates appear to do, any further diminution of the ability of the G-protein to bind with increased phosphorylation of rhodopsin may be due to the increase in negative charge from the additional phosphates rather than to a change in conformation. In this context, it is interesting that the introduction of only a single phosphate is sufficient in vivo to facilitate the binding and activation of arrestin which initiates shut-off of the visual transduction cascade [28]. Therefore, the initial twisting of the β-sheet induced by phosphorylation at one site may be an important factor in arrestin binding.

Acknowledgements

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