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A Mechanistic Model Study of Processes in the Vertebrate and Invertebrate Visual Cycles. Bioorganic Studies of Visual Pigments. 4[1]

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Abstract: Continued application of the model of a confined, tethered chromophore of rhodopsin employed earlier for the primary process of vision has led to a detailed mechanistic explanation for the thermal and photochemical processes of all intermediates in the visual cycle. In addition to the H.T.-*n* process, another volume-conserving process (B.P.-*m,n*) has been introduced for ground-state conformational changes in a confined medium. Specific molecular structures have been proposed for lumirhodopsin and metarhodopsin I and II. Explanations for the different chemical behaviors of the metarhodopsin intermediates of vertebrates and invertebrates and the primary process of retinochrome are offered.

Through bioorganic reasoning and photochemical mechanistic considerations, Liu and Asato recently proposed a new mechanism for the photoisomerization of confined, tethered polyenes.[2] They showed that the mechanism, which involves a concerted twist of two adjacent C-C bonds in a polyene system (known as H.T.-*n*: hula twist at center *n*; for details see drawings in previous publications[2-4] or Figure 2B), when applied to the primary process of vision led to the all-trans,10-s-cis structure of the chromophore of bathorhodopsin, the primary photoproduct in the visual cycle. However, the subsequent dark intermediates in the visual cycle also suffer from the same difficulty as in the case of the primary photoproduct, bathorhodopsin, in that none of their specific molecular structures (conformational as well as configurational) have been characterized. In this paper we have extended the same thought processes expounded in the earlier papers on the photoisomerization process[2-4] to account for the dark reactions in the visual cycle. In the process, an explanation for the different chemical properties of the visual pigments of vertebrates and invertebrates and the unique photochemical behavior of retinochrome in octopus emerged.

Visual Cycles (Background)

The visual pigments[5] of vertebrates (e.g., Mammalia) and invertebrates (e.g., Cephalopod and Insecta) contain the identical 11-*cis*-retinyl chromophore.[5] However, their visual cycles,[6,7] typified by the steps outlined in Figure 1, are not the same. Both cycles are initiated by light, giving a red-shifted primary product, bathorhodopsin, followed by its conversion to the dark intermediates lumirhodopsin and metarhodopsin I. However, in vertebrates, the initially formed meta-I is unstable, leading to eventual dissociation of the pigment, while the invertebrate metarhodopsin is stable under physiological conditions. There are differences in the secondary photochemical processes of the intermediates. While the bathorhodopsins (B) and lumirhodopsins (L) revert in both cases to the corresponding rhodopsin, only the invertebrate metarhodopsin reverts completely back to rhodopsin. Regeneration of the 11-cis geometry of the chromophore for the two systems is also different. For the invertebrates, since the chromophore is never detached from the protein, regeneration involves a slow spontaneous reaction of its metarhodopsin. For vertebrates, the regeneration procedure is more complex, involving not only detachment of the chromophore but also reversible diffusion to the epithelial layer in the reduced ester form.[8] These facts can be accounted for by a sequence of molecular mechanistic processes as elaborated below.

Bathorhodopsin to Lumirhodopsin

Lumirhodopsin is the first thermal intermediate from the primary photoproduct bathorhodopsin.[9] Its photochemical behavior is highly suggestive of its chromophoric structure. Many years ago it was reported[10] that irradiation of L at -65 °C regenerated rhodopsin with concomitant formation of 9-*cis*-rhodopsin as a minor product.[11] Being encapsulated by the protein, reactions of L continue to be limited by the restrictions of (1) limited space available for twisting of the polyene chromophore and (2) the chromophore being anchored at two ends through primary bonding with Lys-296 and secondary interaction of the trimethylcyclohexenyl ring with the protein hydrophobic pocket. With the same reasons outlined for the primary process,[2] it can be concluded that the photochemical behavior of lumi must also be H.T.-10 or 11.[2] Since rhodopsin is regenerated, the chromophoric structure of L must be all-trans,10-s-cis, i.e., the same as batho![2] Batho and lumi must then necessarily differ with respect to their protein environment. This is reasonable because the short period (~20 ps)[12] associated with the rapid primary photochemical process is not sufficient for conformational equilibration of the protein side chains near the relocated chromophore.[2] Therefore, bathorhodopsin must be a "medium-unrelaxed" species. Only after a few nanoseconds, when reorganization of the protein side chains plus compensatory minor conformational readjustment of the chromophore are allowed to take place, is the more stable lumirhodopsin obtained.[13] We note that a role for protein relaxation in visual processes was proposed by Hubbard et al. in 1959.[10] However, because it was invoked at a time before the detection of bathorhodopsin, understandably such changes were thought to have taken place during the lumi to the meta transition.

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(10) Hubbard, R.; Brown, P. K.; Kropf, A. *Nature (London)* **1959**, *183*, 442-446.
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(13) The earlier suggestion (ref 2) of the possible loss of the s-cis conformation at the lumi stage is incompatible with the photochemical conversion of lumi to rhodopsin.

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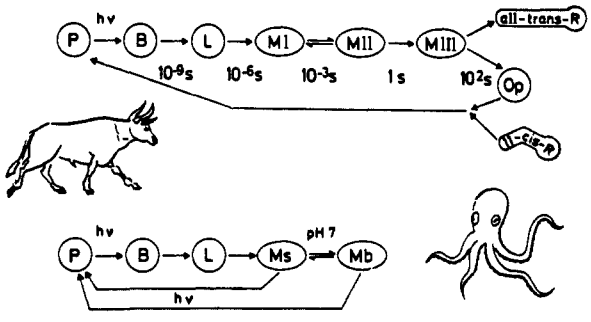

Figure 1. Visual cycles of vertebrate (bovine) and invertebrate (octopus) pigments.[6,7] Subsequent to light absorption by the rhodopsin pigment (P), spontaneous transitions to bathorhodopsin (B), lumirhodopsin (L), and metarhodopsins (M-I, -II) are shown. Metarhodopsin-III is also known as pararhodopsin, which is believed to be that of random Schiff bases. In different invertebrates, metarhodopsin may exist in the acidic and/or the basic form (Ms, Mb).[7]

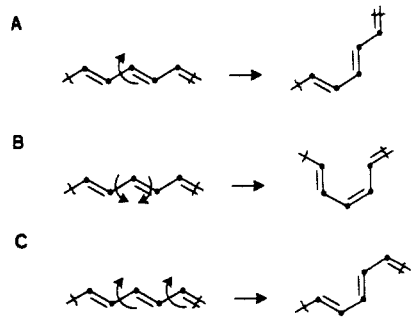

Figure 2. Three possible rotational processes in a five-carbon polyene fragment: (A) common one-bond rotational process; (B) hula-twist process (H.T.-*n*); (C) bicycle-pedaling process (B.P.-*m,n*).

The blue-shift accompanying the B (543 nm) to L (500 nm) conversion must, therefore, be a consequence of altered protein perturbation of the chromophore, which should be substantial because of the considerable lateral displacement of the polyene chain during the photochemical process.[2] This perturbation could be due to relocation of the counterion and/or the "second point charge".[14]

Lumirhodopsin to Metarhodopsins: B.P.-*m,n*

Metarhodopsin I (M-I) is still enclosed by the protein helices.[15] Therefore, reactions leading to or originating from it should continue to be controlled by protein-imposed restrictions: limited space and an anchored chromophore,[2] regardless of possible minor readjustment of the tertiary structure of the protein. Of the three possible pathways for rotational isomerization (Figure 2) in order to remove the crowded s-cis conformation, the common one-bond rotational process (A) is prohibited on both counts of protein restriction. Both remaining processes are relatively volume conserving, and they involve minor reorientation of the termini as well.[2] However, for the H.T.-*n* process (B) rotation of a formal single bond is accompanied by isomerization of a neighboring double bond,[2] therefore forbidden for a ground-state reaction. On the other hand, the bicycle-pedaling process (C) involves simultaneous rotation of two formal single bonds (a modified form of the Warshel bicycle-pedal model proposed for the primary photochemical process),[16] which is allowed as a ground-state process. For clear labeling of the reaction centers in such a process, we propose the use of the term B.P.-*m,n* (bicycle pedaling at bonds m and n).

Results of application of B.P.-*m,n* processes to the molecular model of lumirhodopsin with an immobilized α-carbon of Lys-296[17] and partially fixed cyclohexenyl ring[2,18] are shown in Figure 3. There are three successive steps of bicycle pedaling before the hindered s-cis conformation is transferred from the polyene chain to the butyl tether. The first B.P.-10,12 should be relatively fast because it involves rotation of two C–H bonds (C_{10}–H and C_{11}–H) out of the plane of the chromophore in a relatively open space in the middle of the seven trans membrane helices, which defines the binding site of opsin.[2,19] However, the second B.P.-12,14 should be more sterically hindered because it involves the rotation of a bulkier methyl group bonded to C-13 in addition to the C_{14}–H bond moving out of the plane of the chromophore. This in turn is followed by another rapid process of B.P.-14,16. Therefore, the detection of only two forms of metarhodopsin (M-I, M-II) is justifiable on kinetic grounds. The final B.P.-14,16 process, while transferring the s-cis linkage to the butyl group, causes translocation of the iminium nitrogen, eventually giving an unprotonated imine in M-II. A likely consequence is the loss of much of the polar interactions of the nearby protein, leading to the suspected disruption of its tertiary structure.[15]

Irradiation of meta-I at −65 °C was reported[10] not to give rhodopsin.[20] This chemical behavior, different from that of L,[10] is in agreement with the presence of a new chromophore structure in M-I. Meta-II is known to give a new pigment (photo-M) absorbing at 465 nm, which is stable at temperatures below −5 °C.[21] By the process of elimination, the 13-cis geometry was assigned.[21] The change in regioselectivity of the photochemistry from isomerization at the central bonds of the chromophore in batho and lumi to the more terminal 13,14 bond in meta-II is in agreement with the current knowledge of the effect of the medium on the direction of photoisomerization of the *all-trans*-retinyl chromophore.[17] For a free molecule, i.e., one without any protein restrictions, the major product is indeed expected to be the 13-cis isomer.[2,22]

Invertebrate Visual Pigments: H.T.-12

The principal difference between the vertebrate and the invertebrate metarhodopsin photocycles is probably the inability of the latter to undergo the volume-demanding B.P.-12,14 process, which can be viewed as a reflection of possible steric congestion in this region of the invertebrate binding site. In contrast, for the energetic photochemical process, the minor protein reorganization from batho to lumi and the facile lumi to meta process should not be very sensitive to the local protein structure; thus, the reactions between the early intermediates are unaffected in all cases. Indeed experimentally it is known that in invertebrates while meta-I can exist in either the acidic or the basic form[23] (C, Figure 3), there is no equivalent protein-enclosed meta-II. Recently, the protein sequence of the fruit fly (*Drosophila*) visual pigment has been identified.[24] Its rhodopsin contains more polar amino acid residues in the portion of the protein comprising the binding site

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(20) At room temperature, the photochemistry of meta-I is quite complex: Baker, B. N.; Williams, T. P. *Vision Res.* **1971**, *11*, 449–458.
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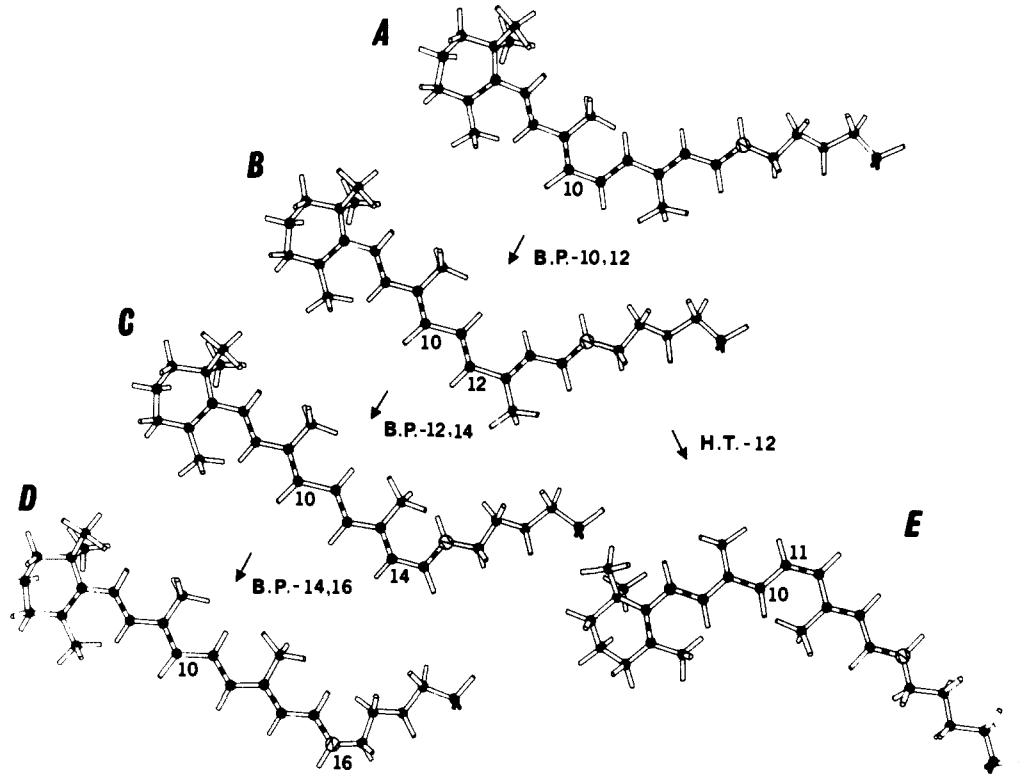

Figure 3. Molecular processes of the dark intermediates in the visual cycle as revealed by an anchored, tethered molecular model. Lumirhodopsin (A; 10-s-cis) is anchored at the α-carbon (taped to the surface) and the trimethylcyclohexenyl ring (passed through a fixed cylinder).[3,4] The B.P.-10,12 process produces the 12-s-cis structure B that is believed to be that of metarhodopsin-I. The slow B.P.-12,14 process is followed by a rapid B.P.-14,16 process. Thus, B effectively "skips" the 14-s-cis structure C and proceeds to the 16-s-cis structure. The latter, after the loss of the iminium proton and the disruption of the tertiary protein structure, is metarhodopsin II. Application of the H.T.-12 process to structure B regenerates rhodopsin (E).

than those of the vertebrates.[19,25] This structural difference could be the origin of the compactness of the binding site of the invertebrates.

Irradiation of the restricted 12-s-cis,all-trans chromophore (B) should again result in a H.T.-*n* process.[2] The location of the s-cis linkage limits the centers of reaction to carbon-12 or -13. However, H.T.-13 would require relocation of a bulkier methyl group. Hence, H.T.-12 is expected to be competitively favored, giving the observed[7] 11-cis,12-s-trans structure (Figure 3E), the stable form of rhodopsin.

The conclusions derived from deductive reasonings presented above and earlier[2] allow us to describe the visual cycle for vertebrates (Figure 2) in more structural details as shown in Figure 4.

It is interesting to note that the invertebrate visual cycle exhibits features remarkably similar to that of bacteriorhodopsin (BR).[5] Recently, a similar mechanistic proposal was also introduced for the latter.[3,4] A major difference is the absence of the proton-pumping phenomenon in the invertebrate visual process, which could be due to a lack of involvement of its charged centers in the B.P.-10,12 process as opposed to the B.P.-14,16 process in BR.[3]

Retinochrome: H.T.-12

Retinochrome is a minor retinal–protein complex common in the Cephalopod. It has the unique photochemical property of being able to convert the all-trans chromophore to the hindered 11-cis geometry;[26] thus, photochemically it is similar to the invertebrate metarhodopsin. Interestingly, its chromophore is known to be capable of exchanging with any excess unbound retinal. Therefore, effectively, retinochrome can function as a photocatalyst to convert *all-trans*-retinal to its hindered 11-cis isomer. This has been demonstrated by Hara and Hara during irradiation of retinal (pure all-trans or mixed with unhindered 9-cis and 13-cis isomers) in the presence of catalytic amounts of retinochrome with orange light.[27]

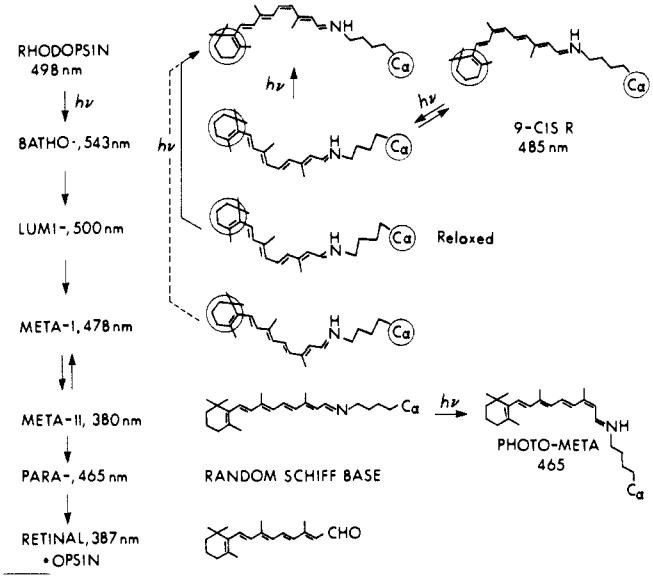

Figure 4. Chromophoric structures of the intermediates in the vertebrate visual cycle. For an invertebrate system, the process is terminated at metarhodopsin I (Figure 1). The arrows near the structures show the directions of secondary photochemical reactions. The chromophore is anchored at the two ends as indicated by the two circumscribing circles. At the meta-II stage and those after, such protein restrictions are no longer present.

On the basis of the above discussion we speculate that this photocatalytic property is a result of the presence of the 12-s-cis,all-trans conformation in the chromophore of retinochrome.

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As stated above, such a geometry when confined in the cavity of the binding site should preferentially undergo the H.T.-12 process, giving the 11-cis isomer. Presently, there is no direct evidence on the existence of such a conformation in retinochrome. However, in the Diels-Alder reaction, it is common knowledge that *all-trans*-vitamin A reacts selectively in the 12-s-cis form.[28] A similar selective reaction of aporetinochrome with *all-trans*-retinal would indeed lead to the proposed structure.

Concluding Remarks

On the basis of the above discussion and that presented previously,[2] it is apparent that all chemical transformations of the chromophores of vertebrate and invertebrate visual pigments during the visual processes can be described by combinations of H.T.-*n* and B.P.-*m,n* processes. Not only has a complete rational picture linking many known but seemingly unrelated experimental results emerged but also the processes have led to proposals of molecular structures for all intermediates in the visual cycle.

We also wish to emphasize that these thoughts should be viewed as tentative proposals derived from bioorganic mechanistic reasoning assisted by the use of molecular models. It is hoped that the specifics of the conclusions will stimulate future definitive experimental or theoretical investigations in this area. Also, the current and related approaches[2-4] emphasize the role of the protein, which clearly is a factor that cannot and should not be overlooked in the design of future corroborative experiments.

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NMR Studies of Monoliganded Fe-Co Hybrid Hemoglobins: Their Quaternary Structure and Proximal Histidine Coordination

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Abstract: Tricobalt-substituted Fe-Co hybrid hemoglobins have been synthesized by cross-linking symmetric Fe-Co hybrid HbA and Co-substituted mutant HbC (β6 Glu→Lys) with bis(3,5-dibromosalicyl) fumarate. Carbon monoxy derivatives of these molecules can serve as important models of monoliganded hemoglobins, the intermediate species produced in the first step of oxygen binding to hemoglobin. Acceptance of the first ligand in an α subunit gave essentially no change in the proximal histidine coordination in the remaining deoxy subunits and a small alteration of deoxy quaternary structure. However, when the first ligand was bound to a β subunit, significant change in the proximal histidine coordination and complete elimination of one hydrogen bond in the deoxy quaternary structure occurred. Such substantial differences in the structures of the two monoliganded hemoglobins obtained by NMR allow us to postulate the possible course of oxygen binding. Also, the observation of asynchronous decreases in the intensities of so-called "T-state" markers and the absence of concomitant increases in the so-called "R-state" marker indicate the existence of more than two quaternary structures for Hb and contradict the two-state allosteric theory.

The applicability of the concept of allostery[1] to the ligand binding in hemoglobin (Hb) has been proven, to a first approximation, by a number of experimental data, particularly structural studies by X-ray crystallography[2] and nuclear magnetic resonance (NMR) spectroscopy.[3] However, detailed understanding of the control mechanism of ligand affinity in Hb may well be achieved only by analyzing the tertiary and quaternary structures and functional properties of the protein as a function of the degree of ligation. However, the physical and chemical characterizations of Hb species at intermediate states of ligation have been thus far elusive, owing to the difficulty in physically isolating such molecules.

CoHb, in which ferrous porphyrin is replaced by cobaltous porphyrin, has been shown not only to possess homotropic and heterotropic behaviors similar to those of natural FeHb but also to acquire physical and chemical properties derived from the Co(II) ion.[4] We had previously prepared symmetric Fe-Co hybrid Hbs such as $\alpha(Co)_2\beta(Fe)_2$ and $\alpha(Fe)_2\beta(Co)_2$. Under an anaerobic carbon monoxide (CO) atmosphere, only the ferrous subunits in these molecules are ligated, but the cobaltous subunits remain unligated.[4b] Thus, it was possible to isolate the diligated species $\alpha(Co)_2\beta(Fe{\cdot}CO)_2$ and $\alpha(Fe{\cdot}CO)_2\beta(Co)_2$ and to characterize them by various spectroscopic, thermodynamic, and kinetic techniques.[5] Recently, a cross-linking technique[6] was utilized to prepare asymmetric valency hybrid FeHbs.[7] We have applied this approach in the preparation of asymmetric Fe-Co hybrid Hbs containing three cobaltous porphyrins and one ferrous porphyrin. The reagent bis(3,5-dibromosalicyl) fumarate links the two β subunits (Lys-$\beta_1$82-Lys-$\beta_2$82) across the 2,3-diphosphoglycerate binding site.[6] Cross-linking between symmetric Fe-Co hybrid HbA and Co-substituted tetrameric HbC (β6 Glu→Lys) enables

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