The Shape of a Three-Dimensional Binding Site of Rhodopsin Based on Molecular Modeling Analyses of Isomeric and Other Visual Pigment Analogues. Bioorganic Studies of Visual Pigments. 11†

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Abstract: On the basis of results of binding interaction of retinal isomers with bovine opsin and assuming that all isomeric rhodopsin analogues retain the same "*points of anchor*" as rhodopsin, we have constructed a cavity representing in three dimensions the maximal boundary of the binding site of the visual protein rhodopsin. The positions of all carbon atoms in the most likely conformation of all the anchored isomeric chromophores obtained from computer assisted molecular modeling calculations were marked. The map resulting from summation of these atoms was able to account for the negative results for *all-trans*-retinal (too long) and 13-*cis*-retinal (atoms projecting into an area already occupied by the protein) and the postulated second point charge. It also suggested possible syn geometry for several of the dicis pigments. Minor modifications of the binding site became necessary when data of 10- and 18-substituted rhodopsin analogues were incorporated.

Through the remarkable effort of Wald, Hubbard, and co-workers, it was established that the visual pigment rhodopsin contains the 11-cis chromophore,[1] that it isomerizes to the all-trans chromophore during visual excitation,[2] and that the lipoprotein opsin is capable of accepting other retinal analogues by forming 9-*cis*-rhodopsin[1] and bovine porphyropsin (from 3-dehydroretinal).[3] Also, on the basis of binding studies with the limited number of geometric isomers available to them, they reached the conclusion that the binding site of opsin is highly specific, capable of accepting only the structurally similar 11-cis and 9-cis isomers of retinal.[1]

Stereoisomers of Retinal and Rhodopsin. A large number of new retinal isomers and analogues of varied structures have since become available.[4] Results of interaction of retinal isomers with opsin revealed the need to reexamine the high selectivity of opsin.[5] In addition to the six geometric isomers of retinal (all-trans, 13-cis, 11-cis, 9-cis, 9,13-dicis, and 11,13-dicis) available to Wald and co-workers,[1,6] the remaining ten stereoisomers of vitamin A are now known (Figure 1): eight with the sterically crowded 7-cis geometry (7-cis, 7,9-dicis, 7,13-dicis, 7,9,13-tricis[7] 7,11-dicis, 7,11,13-tricis,[8] 7,9,11-tricis, and all-cis[9]) and two with the 7-trans geometry (9,11-dicis[10] and 9,11,13-tricis[11]). However, the aldehydes of those containing the 11,13-dicis geometry were found to be relatively unstable.[1b,8,9,11]

Availability of these new isomers allowed testing of stereoselectivity of the binding site of opsin on a broader basis. The results with the new isomers accumulated through a period of nearly a decade have been reported separately[5] preceded by a reinvestigation of the 9,13-dicis system.[12] A few qualitative trends have been noted.[5] The binding site appears to be nonstereoselective, capable of accepting singly (7-cis, 9-cis, 11-cis), doubly (7,9-dicis, 7,11-dicis, 7,13-dicis, 9,11-dicis, 9,13-dicis), and triply bent (7,9,11-tricis, 7,9,13-tricis) isomers. The 11,13-dicis isomer probably also forms a stable pigment in spite of the earlier conclusion to the contrary.[1] The basis for the current suggestion is the observation that several analogues with stable 11,13-dicis forms (13-demethylretinal, 18-ethylretinal, 11,19-ethanoretinal, and 12-methylretinal)[13,14] (see Table III below) are found to give pigment analogues with unique UV/vis absorption maxima and also that 11-*cis*,13-*cis*-retinal is known to rearrange exclusively to 13-*cis*-retinal,[1b] which being a nonbinding isomer cannot account for the minor amount of pigments observed earlier.[1]

The notable exceptions are the all-trans and the 13-cis which failed to form stable pigment analogues. In fact, the trans isomer is the one ejected from the binding site following photobleaching of rhodopsin[2] and the 13-cis was suggested to be an unstable

Table I. The Critical Distance (Center of Cyclohexenyl Ring to Carbonyl Carbon) According to Matsumoto and Yoshizawa[16] and the Calculated Modified Critical Distance (Center of the Ring to α-Carbon of Lysine)

isomer	crit dist (M & Y),[a] Å	calcd crit dist,[b] Å	modified crit dist,[c] Å
all-trans	12.4	12.46	19.43
7-cis	10.1	11.32	18.57
9-cis	9.78	10.82	17.88
11-cis	10.1	11.08	17.75
13-cis	11.0	11.20	16.50
7,9-dicis	11.0	11.31	18.39; 16.04[d]
7,11-dicis	10.1	11.40	18.61; 17.11[d]
7,13-dicis	9.78	10.81	17.36; 17.74[d]
9,11-dicis	11.00	11.68	18.90; 16.30[d]
9,13-dicis	10.1	10.84	17.97; 17.08[d]
11,13-dicis	11.0	11.51	18.64; 17.20[d]
7,9,11-tricis	9.78	11.14	18.33
7,9,13-tricis	10.1	10.74	17.12
7,11,13-tricis	9.78	11.07	17.89
9,11,13-tricis	10.1	10.75	16.80
all-cis	10.1	10.93	17.63

[a] From ref 16. [b] On the basis of relaxed retinal isomers listed in Table II. [c] After appending a relaxed pentyl group to the relaxed retinyl Schiff base. Assuming anti geometry for the C=N bond unless specified otherwise. [d] For the syn geometry.

† A preliminary account was presented at the 8th International Symposium on Carotenoids, July 27–31, 1987, Boston. For Part 10 in the series, see: Liu, R. S. H.; Mirzadegan, T.; Trehan, A. *Carotenoids*; Krinsky, N. I., Mathews-Roth, M. M., Taylor, R. Eds.; Plenum Press, in print.

(1) (a) Hubbard, R.; Wald, G. *J. Gen. Physiol.* **1952–3**, *36*, 269–315. (b) Wald, G.; Brown, P. K.; Hubbard, R.; Oroshnik, W. *Proc. Natl. Acad. Sci. U.S.A.* **1955**, *41*, 438–451; **1956**, *42*, 578–580.

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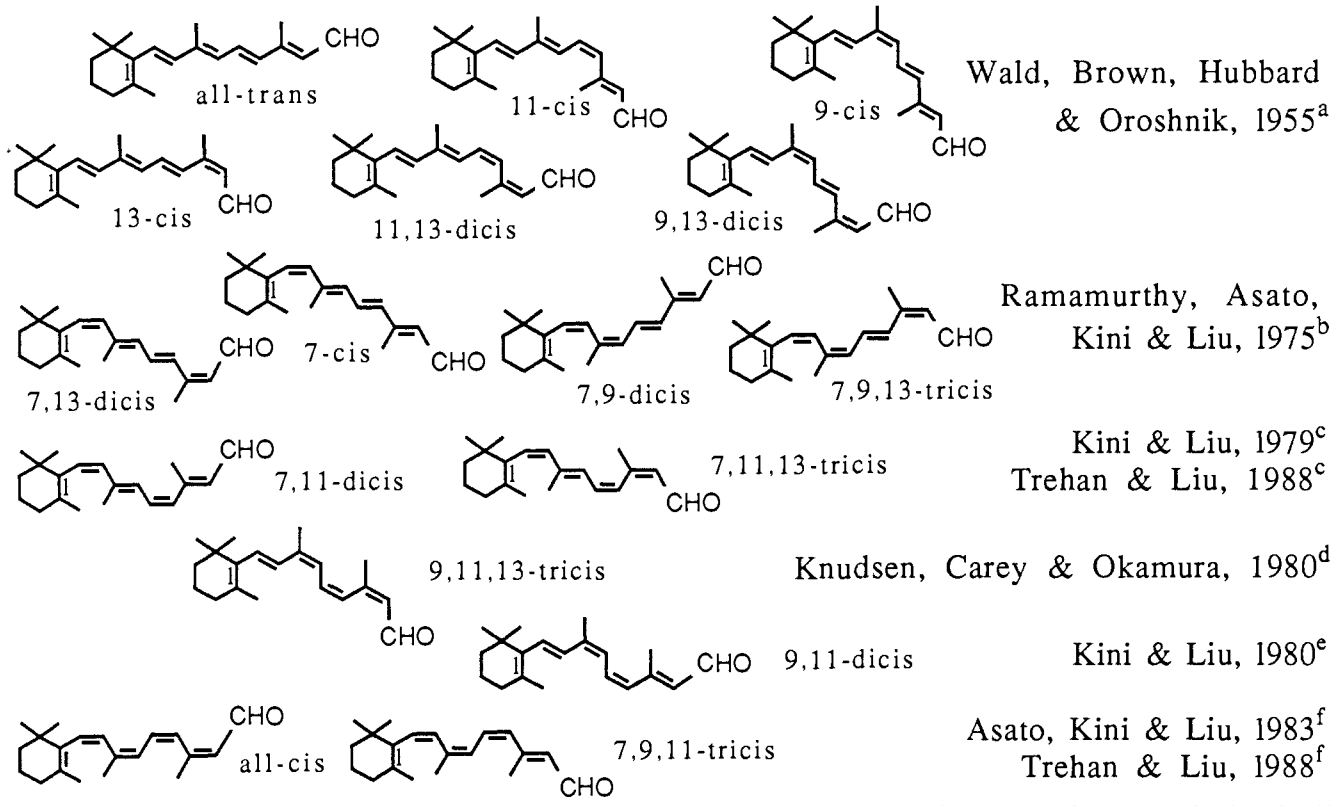

Figure 1. Sixteen possible geometric isomers of retinal arranged in chronological order of their preparation: (a) see ref 1b, 6; (b) ref 7; (c) ref 8; (d) ref 11; (e) ref 10; (f) ref 9.

photoproduct of metarhodopsin.[15] The limited data of rates of pigment formation, however, show that the binding site does exhibit an overwhelming preference for the 11-cis and the structurally similar 9-cis isomers.[5]

Early Models for the Binding Site of Rhodopsin. In 1977 Matsumoto and Yoshizawa introduced the concept of longitudinal restriction within the binding site of opsin.[16] Starting with a simple figure of fused hexagons, they categorized all 16 geometric isomers of retinal into five groups of unique distances between the carbonyl carbon and the center of the cyclohexenyl ring, known as the critical distance (see Table I). The all-trans isomer was shown to have the longest critical distance while that of 13-cis headed a group of four other isomers having the next longest critical distance. Hence they attributed the failure of all-trans and 13-cis isomers of retinal to interact with opsin to excessive length of their chromophores surpassing the longitudinal restriction within the binding site. However, in making such a postulation they neglected the positive result of the 7,9-dicis isomer,[7b] which in their scheme had the same critical distance as 13-cis. Also, it failed to account for the subsequent positive result of 9-*cis*,11-*cis*-retinal.[10] An attempt was made to modify this explanation by taking into consideration the difference in conformational properties between hindered and unhindered isomers.[17]

Table II. Calculated, MMP2(85), Energies of Retinal Isomers Relative to the All-Trans Isomer

isomer	rel energy, kcal/mole	isomer	rel energy kcal/mol
all-trans	(0)	9,11-dicis	6.8
7-cis	7.8	9,13-dicis	1.4
9-cis	0.2	11,13-dicis	7.3
11-cis	6.5	7,9,11-tricis	15.2
13-cis	0.8	7,9,13-tricis	10.0
7,9-dicis	8.8	7,11,13-tricis	15.0
7,11-dicis	13.8	9,11,13-tricis	8.1
7,13-dicis	8.7	all-cis	16.4

Recent findings of the site of attachment of the retinyl chromophore, at Lys-296,[18] and the disclosure of the Hargrave three-dimensional model for rhodopsin[19] following successful characterization of the primary structure of opsin[18] made necessary a more drastic revision of the Matsumoto–Yoshizawa model. In an earlier preliminary paper a modified model utilizing concepts derived from model construction was disclosed.[20] Now, we wish to elaborate this model using the computer-assisted molecular modeling approach. It is anticipated that such an approach will not only make the model more quantitatively meaningful, more readily adaptable to other analogues, but also better able to define the binding site in all three dimensions.

The Shape of the Binding Site. First, the minimized structures of 16 possible geometric isomers of retinal were calculated with Allinger's MMP2(85) program.[21] Whenever possible the nuclear

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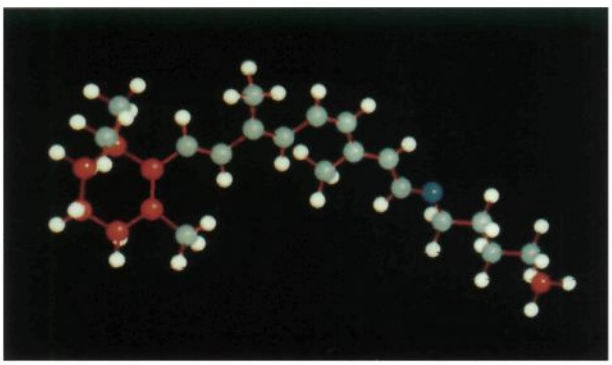

Figure 2. Computer-generated relaxed 11-cis chromophore. The minimized 11-*cis*-retinal structure was converted to the butyl Schiff base by replacing the oxygen atom with a nitrogen atom followed by appending to the nitrogen a relaxed pentyl group. The fifth carbon atom corresponds to that of the α-carbon in Lys-296, the site of attachment of the retinylidene chromophore. The two anchor points (in red) are the α-carbon atom and the center of the cyclohexenyl ring. The ring is allowed to pivot or rock about the latter in order to mimic the nonbonding hydrophobic interaction around this region.

coordinates from crystal structures (i.e., all-trans,[22] 11-cis,[23] 13-cis,[24] and 9-cis[25]) were used for calculation of the minimized structures. Other isomers were constructed from the crystal structure of 11-*cis*-retinal followed by rotation of the appropriate bond(s) by the CHEM-X program, which were then minimized with the MMP2(85) program. Results of such calculations are summarized in Table II, where energies relative to the all-trans isomer are listed. Molecular structural parameters (bond lengths and dihedral angles) comparing, where appropriate, to crystal structural data are listed separately in the supplementary material section. The calculated energies are indeed those expected, i.e., the hindered 11-cis and 7-cis isomers are much higher in energy than the unhindered 9-cis and 13-cis while the latter are only slightly higher than all-trans, thus in agreement with the observation that only the unhindered isomers are present in an equilibrated mixture of retinal isomers.[26]

Then, a structure for the 11-*cis*-retinyl chromophore was constructed. The minimized structure of retinal was converted to its butyl Schiff base by replacing the carbonyl oxygen with a nitrogen atom (parameters for sp^2 N atoms are not sufficiently complete in the MMP2 program for direct calculation of the Schiff base). The 12-S-trans conformer was used as concluded from resonance Raman studies.[27] A "relaxed" *n*-pentyl group was then appended to the N atom (Figure 2) while assuming the established anti geometry.[28] The last atom of the pentyl group corresponds to the α-carbon in Lys-296 to which the retinyl chromophore is attached. This relaxed structure is assumed to be that of the chromophore for rhodopsin. Being embedded within an α-helix (helix-7 in Hargrave's model), its position should be rigidly fixed. The presence of a hydrophobic pocket recognizing the trimethylcyclohexenyl ring was suggested by inhibition studies.[29] Hence, the longitudinal restriction within the binding site should instead be that between the α-carbon and the center of the trimethylcyclohexenyl ring.

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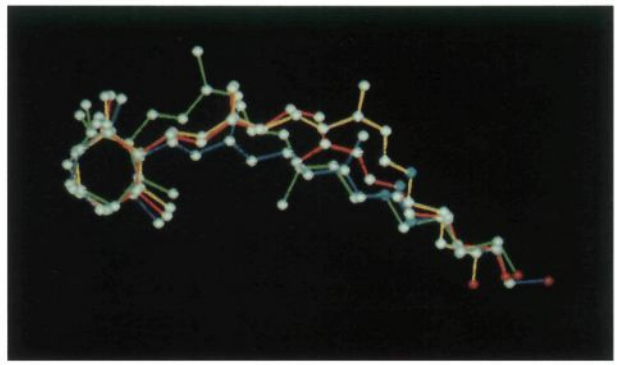

Figure 3. Relaxed structures of all-trans (blue), 13-cis (yellow), and 9-cis (green) chromophores superimposed with that of 11-cis (red) at the center of the cyclohexenyl ring. The different lengths of the tethered isomeric chromophores are reflected by the non-overlapping α-atoms (in red).

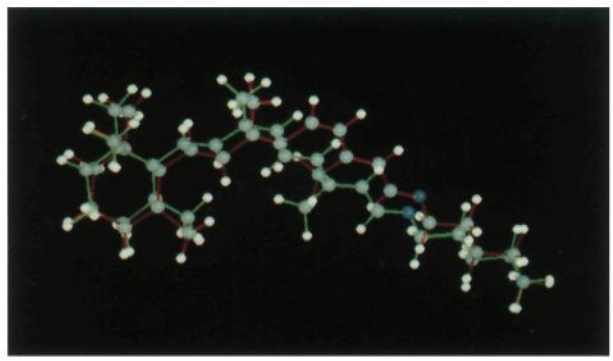

Figure 4. The 9-cis chromophore (green) with a slightly twisted butyl side chain making its points of anchor overlapping with those of the 11-cis (red).

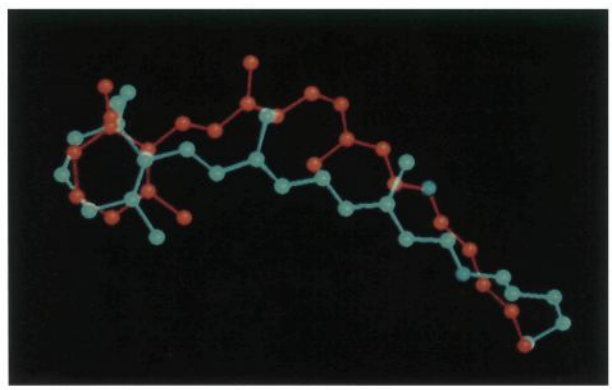

Figure 5. A forced fit of the trans isomer (blue) superimposed on 11-cis (red) resulted in a butyl side chain with substantial eclipsing interactions.

In Figure 3 are shown the relaxed structures of the binding 9-*cis*-retinylidene chromophore and the nonbinding all-trans and 13-cis superimposed to that of 11-cis at the cyclohexenyl ring. It is immediately obvious that even for the modified critical distance the all-trans chromophore is much longer than those of other isomers. In Table I the calculated distances for all 16 isomers are listed. Thus, in agreement with Matsumoto and Yoshizawa[16] we conclude that the all-trans isomer is incompatible with the longitudinal restriction within the binding site of opsin.

During binding interaction of isomeric retinals, the butyl side chain is assumed to serve the function of a flexible tether accommodating isomers of slightly different length. Thus to construct the 9-cis chromophore within the binding site, the relaxed conformation of the 9-cis isomer was converted to one with a slightly twisted butyl side chain so as to retain the same critical

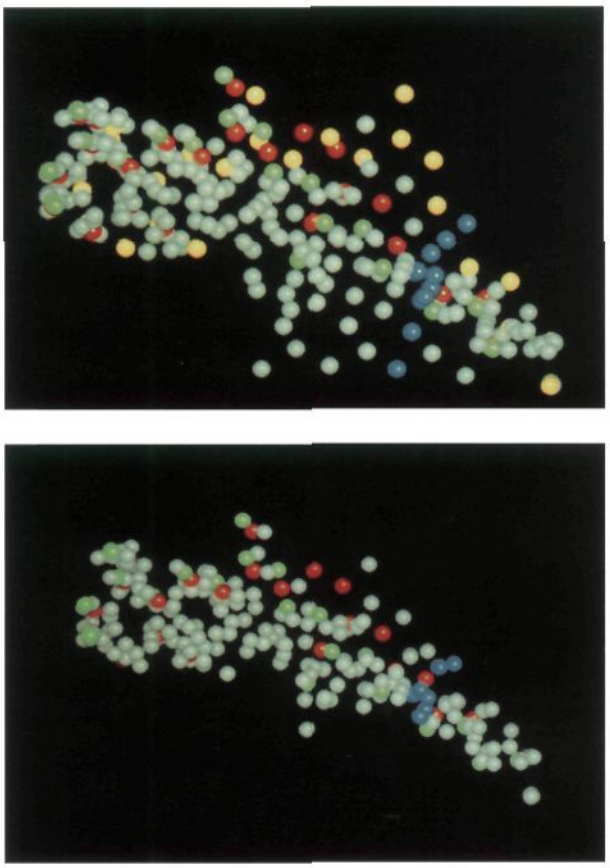

Figure 6. (a, top) Projected positions of all carbon (grey) and nitrogen (blue) atoms of binding isomeric chromophores (from Table I), all with the anti geometry. Those of 11-cis and 9-cis are highlighted in red and green, respectively, and 13-cis in yellow. (b, bottom) same as above but those of 7,11-dicis, 7,13-dicis, and 9,13-dicis are in the syn geometry and minus 13-cis.

distance as that of the 11-cis chromophore (Figure 4). By the same approach the all-trans isomer led to a structure with a severely distorted side chain (Figure 5). Similar exercises were performed on all binding isomers of retinylidene chromophores by first assuming the anti geometry established for rhodopsin and 9-*cis*-rhodopsin.[28]

All these calculated structures were then superimposed at the two points of anchor. Projected positions of the carbon atoms of these analogues were marked with solid circles. Since these atoms are within the binding site, summation of this set of atoms (Figure 6a) should then define the shape of the binding site. Figure 6a reveals considerable displacement of the imino nitrogen atoms with a range possibly too large to be readily accessible by the short tether of the aspartic acid residue which presumably serves as a common counteranion for all isomeric pigments. To alleviate this problem, we adopted the concept of possible existence of the syn geometry for the C,N bond as suggested for 13-*cis*-bacteriorhodopsin[30] and constructed the syn pigment analogues for the dicis isomers. Indeed, we found that most of the structures (7,11-dicis, 7,13-dicis, 9,13-dicis, and 11,13-dicis) are more compatible with those of the monocis while 7,9-dicis and 9,11-dicis prefer to remain in the anti form. Hence, Figure 6b was constructed in which the carbon and nitrogen atoms for several of the dicis isomers have been replaced by those of the more compatible syn isomers. The net result is a more compact, probably more realistic, binding site.

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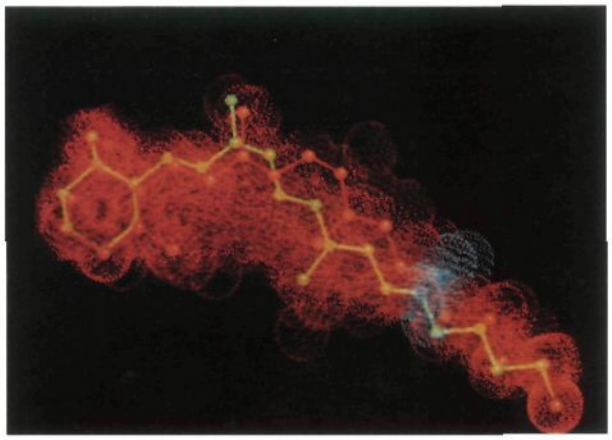

Figure 7. The shape of the binding site of rhodopsin as revealed by dot surface structure of carbon and nitrogen atoms of the binding isomeric chromophores as shown in Figure 6b.

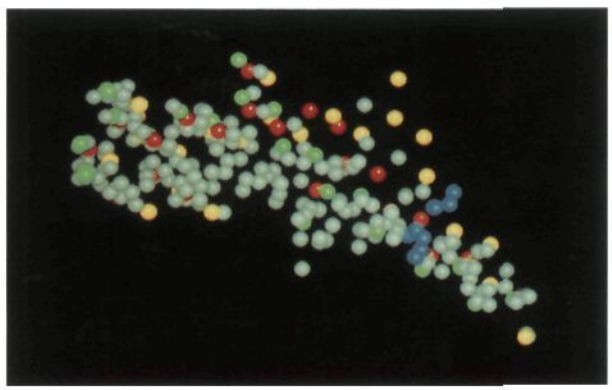

Figure 8. Atoms of the 13-cis retinylidene chromophore (in yellow) overlapping with atoms of binding isomeric rhodopsins.

The projected shape of this cavity becomes more evident when the dotted structures reflecting the van der Waal radii of the carbon and nitrogen atoms are shown instead (Figure 7). It should be noted that we do not intend to suggest that the binding site determined by this procedure reflects the presence of such a cavity within the protein matrix but rather it reflects the maximum extent that the protein side chains can give when accommodating substrates of different shape.

The negative result of the only monocis chromophore (13-cis) deserves a closer examination. It is clear from Figure 3 that longitudinal restriction cannot be the detrimental factor leading to its exclusion by the protein. When onto the binding site map one superimposes the carbon atoms of the anchored 13-cis chromophores (Figure 8) a rational explanation for the negative result emerges. It reveals that C-13, -14, -15, -20 atoms (or part of them) of the 13-cis chromophore fall into a unique region not occupied by any atom of the binding isomers. Interestingly, this region is located at a position adjacent to the positively charged iminium nitrogen with an area close in size to that of three second-row atoms (Figure 9). Hence, it appears reasonable to suggest that a carboxylate counterion protruding from the protein occupies this region, which forbids entry of any atoms from a substrate. In short, it is a forbidden zone. Indeed, the Hargrave's model of rhodopsin suggests that of the three carboxyl bearing amino acid residues among those comprising the binding site, Asp-82 is well positioned to serve as the counterion.[31] Furthermore, the proposed bidentate carboxylate ion with the negative charge shared equally by the two oxygen atoms is strategically

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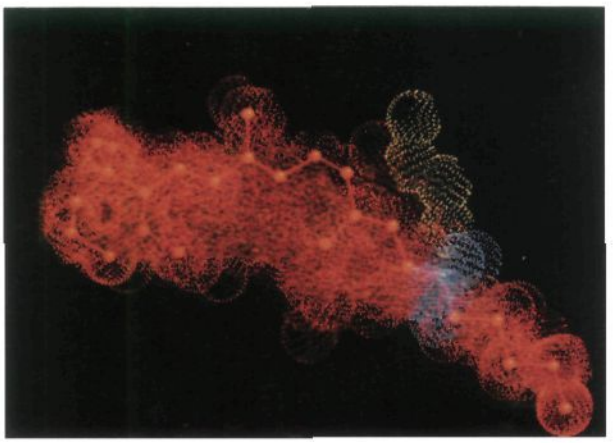

Figure 9. The area (dotted surface in yellow) occupied exclusively by C-13, -14, -15, -20 of the nonbinding 13-cis chromophore approximates that of three second-row atoms and is adjacent to N-16, the iminium ion, and C-12 of the 11-cis chromophore. The location and the size of this area suggest that it is occupied by a carboxylate ion which serves the dual functions of a counterion and a second point charge to C-12. It should orient perpendicularly to the chromophore.

located to serve the added role of a second point charge nearby C-12 as postulated earlier.[32,33]

Testing and Fine Tuning the Model with Other Analogues. The reported results of interaction of the 9-cis and 11-cis isomers of the 12-substituted retinals (12-methyl, 12-chloro, and 12-fluoro)[20] with cattle opsin (Table III) are consistent with the current model. The 12-fluororetinal isomers behaved in a manner parallel to those of retinal in that both the 11-cis and the 9-cis isomers gave high pigment yields. However, the results of the 12-methyl and 12-chloro were unexpected in that the isomer with the natural occurring 11-cis geometry gave a negligible amount of pigments while that of the unnatural 9-cis gave a moderate to high yield of pigments. The binding site map (Figure 7) provides a possible explanation to this seemingly anomalous result. Any substituent at C-12 for the 11-cis isomer would project beyond the perimeter of the binding site into the forbidden zone while the same substituent for the 9-cis isomer should remain comfortably within the binding site. This forbidden zone apparently has no effect on binding interaction of analogues with the much smaller fluorine substituent.[34]

It should be clear that the binding site defined by stereoisomers of retinal is limited by the atoms present in these isomers. It is entirely possible that for a given region the binding site could in reality be larger than that indicated in Figure 7 as suggested by results with the 10-substituted retinals[13] (data included in Table III). The high yield of pigments from the 9-cis isomer of 10-methyl- or 10-chlororetinal could only suggest that the binding site near C-10 of the 9-cis isomer should be expanded to include an added area as shown in Figure 10. Recently binding studies with isomers of 18-ethyl (5-propyl) retinal[35,4b] reveal the need to expand the perimeter of a second region. Four isomers (11-cis, 9-cis, 7-cis, 11,13-dicis) were found to form pigment analogues (Table III).[14] In the case of the 7-cis isomer, even the conformer with the most compact propyl side chain could not be fitted into

Table III. Visual Pigment Analogues from 10-, 12-, and 18-Substituted Retinals

retinal[a] analogue	isomer	pigment λ_{max}, nm	yield[b]	(detergent)[c]
12-fluoro[d]	all-trans	—	0	(D)
	13-cis	—	0	(D)
	11-cis	507	+++	(D)
	9-cis	493	+++	(D)
	7-cis	—	–	(D)
12-chloro[e]	11-cis	—	–	(D)
	9-cis	488	++	(D)
12-methyl[e]	11-cis	489	+	(C)
	9-cis	487	+++	(C)
	11,13-dicis	486	+	(C)
18-ethyl[f]	11-cis	475	++	(D)
	9-cis	482	+	(D)
	7-cis	470	+	(D)
	11,13-dicis	480	+	(D)
10-fluoro[g]	11-cis	489	+++	(C)
	9-cis	486	+++	(C)
	7-cis	484	+	(C)
	11,13-dicis	461	++	(C)
10-chloro[g]	11-cis	—	–	(D)
	9-cis	488	++	(D)
10-methyl[g]	11-cis	498	+	(D)
	9-cis	497	+++	(D)
	7-cis	506	+	(D)
10-ethyl[g]	11-cis	480	–	(D)
	9-cis	494	+	(D)
13-demethyl[f]	11-cis	498	++	(D)
	9-cis	483	+++	(D)
	11,13-dicis	500	++	(D)
	9,11-dicis	477	+	(D)
11,19-ethano[e]	11-cis	483	+	(D)
	11,13-dicis	471	+	(D)

[a] Extracted from ref 4b, see also other specific references below. [b] +++ ≡ >70% yield; ++, 30–70%; +, 3–30%; –, 0–3%. [c] D ≡ digitonin; C ≡ CHAPS. [d] Liu, R. S. H.; Matsumoto, H.; Asato, A. E.; Denny, M.; Schichida, Y.; Yoshizawa, T.; Dahlquist, F. *J. Am. Chem. Soc.* **1981**, *103*, 7195–7201. [e] Reference 14. [f] Reference 35. [g] Reference 13.

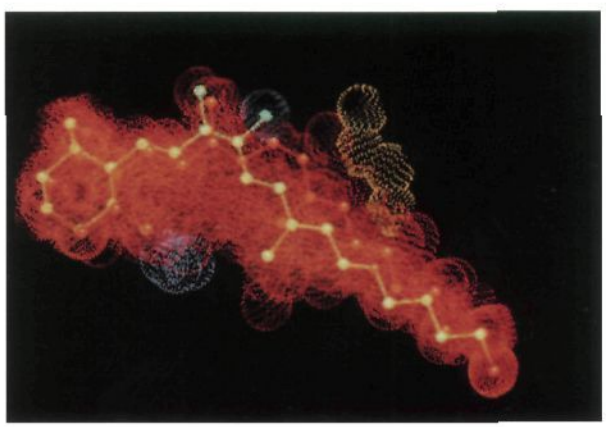

Figure 10. An expanded binding site map. Results of binding interactions with 10-substituted 9-cis chromophore and 18-substituted retinal analogues require expansion of the binding site map of Figure 7 by areas as indicated in blue.

the binding site map shown in Figure 7. Hence, the region nearby 5-CH_3 should also be enlarged at least to an extent shown in Figure 10.

The method of utilizing the substrates (relaxed keys) to map the shape of the binding site (the lock) should, in principle, lead to an answer defined in all three dimensions. In Figure 11, the binding site as sketched in Figure 7 is shown in several different perspectives in order to emphasize the overall length, breadth, and thickness of the cavity. Again, it should be pointed out that the shape of the cavity obtained in this manner only represents the maximal possible boundary ascribing the binding site.

(32) Honig, B.; Dinur, U.; Nakanishi, K.; Balogh-Nair, V.; Gawinowicz, M. A.; Arnaboldi, M.; Motto, M. G. *J. Am. Chem. Soc.* **1979**, *101*, 7084–7086.

(33) In addition to our suggestion of the dual function of the carboxylate ion (ref 20), two additional research groups independently reached the same conclusion: (a) Kakitani, H.; Kakitani, T.; Rodman, H.; Honig, B. *Photochem. Photobiol.* **1985**, *41*, 471–479. (b) Birge, R.; Murray, L. P.; Pierce, B. M.; Akitani, H.; Balogh-Nair, V.; Findsen, L. A.; Nakanishi, K. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 4117–4121.

(34) The van der Waal radius of a fluorine atom is about 20% larger than that of a hydrogen atom while a methyl group is only 10% larger than a chloro group but 80% larger than a hydrogen. See, e.g.: Pauling, L. *The Nature of the Chemical Bond*; Cornell University Press: Ithaca, 1960.

(35) Unpublished results of A. E. Asato, M. Denny, and B.-W. Zhang.

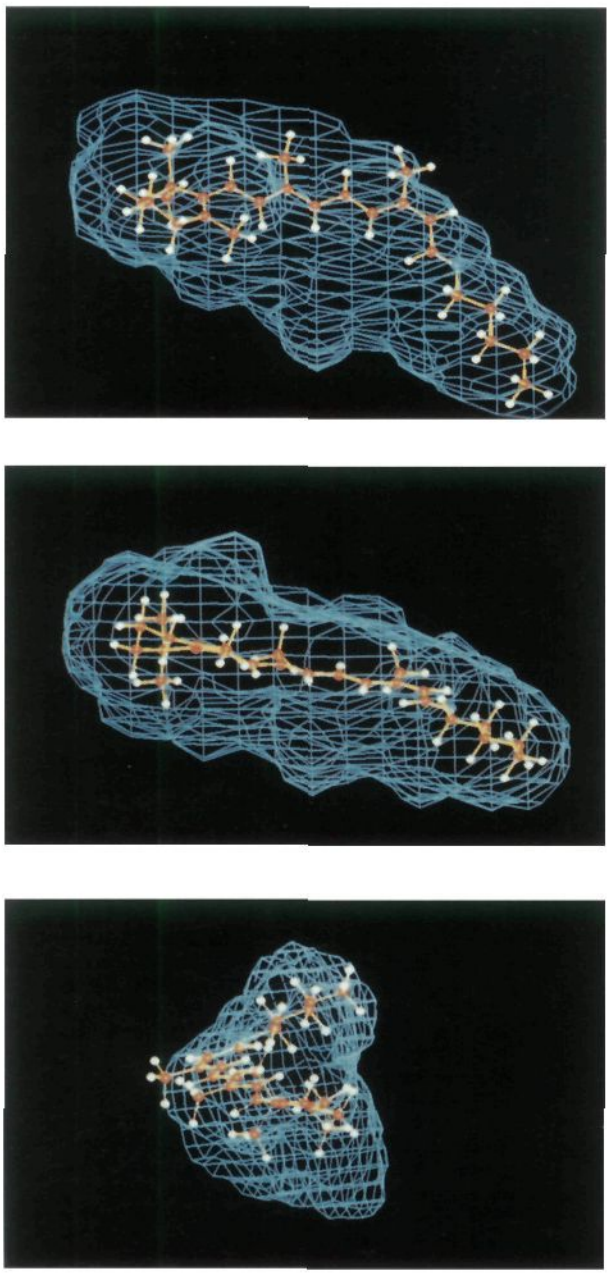

Figure 11. The volume map of the binding site of rhodopsin with the nonbinding 13-cis chromophore viewing from several different angles: (a, top) 0°, same as in Figure 10, (b, middle) after rotation of 90° around the x axis of a, (c, bottom) after rotation of 90° around the y axis of a.

In the future, it will be of interest to apply similar analysis to all other visual pigment analogues.[4a] But by inspection, it should be clear that many analogues such as those derived from the adamantylallenic retinal[36] or ring truncated retinals[37] are quite compatible with the concepts introduced and the results obtained in this paper. In some cases, e.g., the allenic system, similar calculations will have to await development of molecular mechanics parameters for such π-systems. A desirable but difficult feature to be introduced in the future is the inclusion of a procedure to differentiate among cases of analogues that exhibit differences only in a quantitative manner (e.g., in yields of pigment or rates of pigment formation). At this time, such an attempt is complicated by the absence of yield data in many reports and pigment formation carried out under varied conditions in different laboratories.

Also, it will be of interest to allow minimization through relaxation of the polyene chromophore in addition to the butyl tether. This can be approximated, as is presently being done in this laboratory, by replacing the imino bond by a C=C bond so as to overcome the lack of knowledge of iminium nitrogen parameters. While sacrificing any information related to charge interactions, such an approximation should yield meaningful information related to steric interactions. It is hoped that in the near future one will be able to deal with minor structural variations of retinal analogues including minor conformational changes as a result of structural variation. And it will be of particular interest to compare such a cavity with that generated from the protein helices and the protruding side chains. It should be noted that preliminary efforts toward the latter emphasizing orientation of protein helices and secondary protein structures have already appeared in the literature.[19,38]

Experimental Section

Procedure for Calculations. Methods. Energy calculations were performed with the MMP2(85) molecular mechanics software package for π systems implemented on a micro-VAX computer. The force-field parameters were those established without further modification.[21] The empirical energy of the system is represented in terms of bond stretching, bending, torsional and nonbonded energies, and π energies by a SCF method as documented.[21,39] The 85 update of MMP2 involved refinement of parameters, part of which led to softening of nonbonded interactions giving more realistic polyene conformation than those from the earlier version.

The Chemical Design MICROGRAF-5 Molecular Modelling package was used which included the Chem-X graphics software and the Sigmex 6234 color graphics terminal. Chem-X, an advanced graphics program, among many options, provides rigid and flexible molecular fitting or atom matching facilities which was used to fit the substrate molecules.

Procedure. The coordinates from crystal structures of all-trans,[22] 11-cis,[23] 13-cis,[24] and 9-cis[25] were input in Chem-X as the starting geometry for calculations. The remaining isomers were constructed from the crystal structure of 11-*cis*-retinal by rotating the appropriate bond(s) via Chem-X. The minimized structures were converted to the corresponding butyl Schiff base by replacing the oxygen atom with a sp^2 nitrogen atom followed by appending a relaxed pentyl group to the nitrogen. The added fifth carbon atom corresponded to that of the α-carbon in Lys-296, the site of attachment of the chromophore.

In constructing the shape of the cavity that defined the binding site of rhodopsin from structural parameters of the isomeric chromophores it was necessary to adjust the overall lengths of the chromophore of rhodopsin analogues so that the same points of anchor as that of the 11-cis isomer were involved for all isomers. This was accomplished by matching the critical distances (center of the ring to the C_α atom) of all isomeric chromophores with that of the 11-cis isomer by the following step-by-step procedure: (1) mark the position of the center of the cyclohexenyl ring of an isomeric chromophore by a dummy atom; (2) calculate the distance from the dummy atom to the C_α of Lys-296 (the last atom of the pentyl group); and (3) adjust this distance to match that of 11-cis, by rotating only the C–C bonds of the appended butyl group.

All the isomers were fitted to the 11-cis chromophore. Rigid and flexible fitting were done sequentially on each isomer. In rigid fitting the bond lengths, angles, or torsion angles remained constant. Seven atom pairs were specified for matching (the C_α and the C atoms of the ring). To mimic the situation of the more rigid site of attachment at C_α relative to the hydrophobic pocket for the cyclohexenyl ring a large weighing constant was chosen for C_α and a much smaller one (one hundredth) for each C atom of the ring. A flexible fit followed where the torsion angles of the pentyl side chain were allowed to vary in order to find the best fit. In several cases a small weighing constant was also assigned to C_β so as to maximize matching of these atoms as well. The critical distances for all fitted, binding isomeric chromophores fall within 0.7 Å of that of the 11-cis.

The spatial analysis was done by "dot surfaces" and "set map" facilities in Chem-X. The spherical dot surfaces consist of dots drawn on the surface of the spheres centered on atom positions. The van der Waals

(36) A low-yield pigment unstable in hydroxylamine: Blatchly, R. A.; Carriker, J. D.; Balogh-Nair, V.; Nakanishi, K. *J. Am. Chem. Soc.* **1980**, *102*, 2495–2497.

(37) (a) Crouch, R.; Or, Y. S. *FEBS Lett.* **1983**, *158*, 139–142. (b) Rao, V. J.; Zingoni, J. P.; Crouch, R.; Denny, M.; Liu, R. S. H. *Photochem. Photobiol.* **1985**, *41*, 171–174.

(38) Findley, J. B. C. *Photobiochem. Photobiophys.* **1986**, *13*, 213–228.

(39) See e.g.: Berkert, U.; Allinger, N. L. *Molecular Mechanics*; American Chemical Society: Washington, D.C., 1982.

volume of the binding site was calculated and displayed with the "set map", and this cavity was then rotated and viewed in 3-D, with the help of the Chem-Movie facility in Chem-X.

Comments on the Method. It is well-known that molecular mechanics calculations of this type are more accurate for neutral molecules than those involving polar interactions. The success of the current study probably can be attributed to the following. First, the binding site of visual pigments is known to be hydrophobic. The only major polar interaction in these analogues is near the common imino center. Thus, any possible errors associated with polar interactions at such centers could well be present in a similar amount, thus mutually cancelled when comparing differences in energy. Second, and probably more important, the ease of binding interaction for all the analogues, a point of concern here, is probably determined primarily by steric interactions rather than any polar interaction. A meaningful observation along this line is the close homology between the unprotonated blue cone pigment and the protonated green and red cone pigments.[40] The ability of the 11-cis chromophore to occupy these binding sites is apparently unaffected by the presence or absence of such polar interactions.

(40) Nathans, J.; Thomas, D.; Hogness, D. S. *Science* **1986**, *232*, 193-202.

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Registry No. *all-trans*-Retinal, 116-31-4; 7-*cis*-retinal, 24315-14-8; 9-*cis*-retinal, 514-85-2; 11-*cis*-retinal, 564-87-4; 13-*cis*-retinal, 472-86-6; 7,9-*dicis*-retinal, 56085-53-1; 7,11-*dicis*-retinal, 67737-35-3; 7,13-*dicis*-retinal, 56085-54-2; 9,11-*dicis*-retinal, 67711-05-1; 9,13-*dicis*-retinal, 23790-80-9; 11,13-*dicis*-retinal, 564-88-5; 7,9,11-*tricis*-retinal, 67737-38-6; 7,9,13-*tricis*-retinal, 56085-55-3; 7,11,13-*tricis*-retinal, 67737-39-7; 9,11,13-*tricis*-retinal, 67737-36-4; *all-cis*-retinal, 67737-37-5.

Supplementary Material Available: Tables listing calculated bond lengths (Table IV) and calculated dihedral angles (Table V) of retinal isomers (3 pages). Ordering information is given on any current masthead page.

Amplified Mass Immunosorbent Assay with a Quartz Crystal Microbalance

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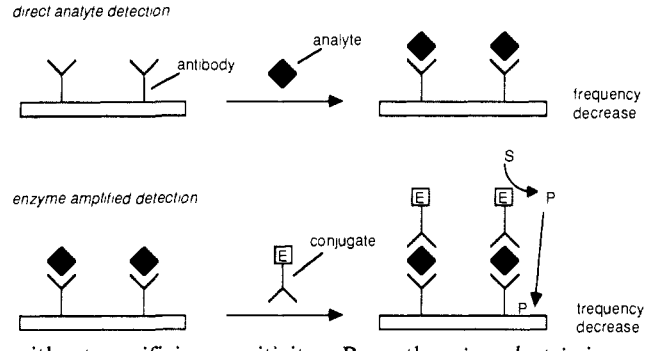
Abstract: Amplified mass immunosorbent assays (AMISA) using a quartz-crystal microbalance (QCM) are described for the detection of adenosine 5'-phosphosulfate (APS) reductase and human chorionic gonadotropin (hCG). APS reductase detection is accomplished by binding APS reductase to an anti-APS reductase antibody immobilized on the surface of the QCM, followed by addition of an anti-APS-alkaline phosphatase reductase conjugate. Subsequent exposure of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to the bound sandwich complex results in enzymatically amplified deposition of the oxidized dimer of BCIP on the QCM surface, resulting in a frequency change that corresponds to the level of APS analyte. The enzymatic amplification leads to significant enhancement of the detection limits; levels of ca. 5 ng/mL (10^{-14} M) of APS reductase can be detected whereas the direct binding of APS reductase at even more elevated concentrations cannot be measured. Assay methodology providing for a reusable biosensor is also described. In this example, hCG is detected with a horseradish peroxidase conjugate in which a sandwich complex is immobilized on a separate nylon membrane positioned in close proximity to a polyvinylferrocene (PV-Fc) film on the QCM. This enzyme catalyzes the oxidation of I^- to I_2/I_3^-, which subsequently reacts with the PV-Fc film with concomitant insertion of I_3^-, resulting in an observable decrease of the QCM frequency. Since the formation of PV-Fc$^+I_3^-$ can be reversed electrochemically to regenerate the original PV-Fc film, this configuration provides for reuse of the detection crystal.

Immunoassay methodology for the diagnostic determination of biological analytes (drugs, enzymes, metabolites, hormones, antigens, etc.) has proven valuable for clinical analyses, primarily because of the highly specific recognition between analytes and antibodies elicited for those analytes.[1-3] Although extensively used, the cost and time intensiveness of these procedures and the safety hazards of radioimmunoassay have stimulated investigations of new methods. Much attention has been given to "biosensors" in which an immunological reaction that occurs at the interface of a transducer results in output of an electrical signal. Critical features of biosensors are low cost, simplicity, and disposability, without sacrificing sensitivity. Recently, *piezoelectric* immunoassay procedures employing the quartz-crystal microbalance (QCM) and surface acoustic wave (SAW) devices have been described in attempts to address these criteria.[4] The fundamental

Scheme I



(1) *Enzyme Labelled Immunoassay of Drugs and Hormones*; Pal, S. B., Ed.; de Gruyter: New York, 1978.
(2) Engvall, E., Pesce, A. J., Eds.; *Scandinavian Journal of Immunology*; Blackwell: Oxford, 1978; Supplement No. 7, Vol. 8.
(3) Lunte, C. E.; Heineman, W. R. *Top. Curr. Chem.* **1988**, *143*, 1, and references therein.

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