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New Isomers of Bovine Porphyropsin: A Nondestructive Method for Determining Chromophore Configuration during Formation of Visual Pigment Analogs

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Binding interactions of six isomers (five previously unknown) of 3-dehydroretinal (3-DHR) with bovine opsin have been investigated. Of these only 7-*cis* and 7,9-di-*cis* isomers retained the original polyene configuration, giving the corresponding isomeric porphyropsin pigment analogs. The 7,13-di-*cis*, 7,9,11-tri-*cis*, and 7,9,13-tri-*cis* isomers of 3-DHR were found to lose readily the 13-*cis* or 11-*cis* geometry, yielding instead the 7-*cis* or 7,9-di-*cis* pigments. The 7,11-di-*cis* isomer yielded a pigment, believed to be the 7,11-di-*cis*, which subsequently isomerized to the stable 7-*cis* pigment. While the general behavior of the 3-DHR isomers is similar to that of the retinals, current results on the two tri-*cis* isomers were not available for the corresponding retinal isomers. Instead of using the established chromophore extraction procedure, we demonstrated that curve resolution of the spectra obtained during binding interactions, a procedure requiring a much smaller sample, could yield equally reliable information on stereochemical properties of the chromophore. © 1996 Academic Press, Inc.

INTRODUCTION

Regeneration of the visual pigment rhodopsin by reaction of the visual protein opsin with 11-*cis*-retinal or formation of the structurally similar 9-*cis*-rhodopsin with 9-*cis*-retinal is known (*1*) to proceed rapidly with complete retention of the chromophore configuration. However, for reaction with other isomers of retinal in examining stereoselectivity of the binding site of opsin, rates of pigment formation are much slower (*2*) rendering possible loss of the polyene geometry (*3*, *4*). Thus, analytical methods have been developed to assay the chromophore configurational purity either during or after binding interaction. The currently preferred procedure is to denature the pigment analog with an organic solvent and then to extract the chromophore in the form of either retinal (*5*) (complicated by a small amount of stereomutation during the denaturation process) or retinal oximes (the presence of an excess amount of hydroxylamine minimized stereomutation) (*6*) followed by HPLC analyses. It is clear that the destructive nature of the procedure requires a large amount of material for a complete analysis of chromophore configuration during pigment formation. Therefore, the procedure is applicable neither to a more complex system nor in cases where sample sizes are limited. We now wish to report

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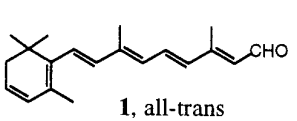
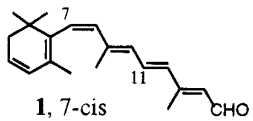
a modified procedure in studies of a new pigment analog, that allows the complete assay of chromophore configuration with a single sample used for uv–visible analysis.

This work was prompted by our recent successful effort to prepare new isomers of 3-dehydroretinal (3-DHR, vitamin A_2 aldehyde, **1**) containing the hindered 7-*cis* geometry (*7*, *8*). The compounds allow a more complete study of stereoselectivity of the visual protein in formation of isomeric porphyropsin pigments in a same manner as done for the rhodopsins (*2*). During the course of preparing such pigment analogs, we detected varying degrees of stereomutations when different isomers of 3-DHR were used. To simplify the rather tedious chromophore extraction work, we applied a more direct method of spectrum deconvolution for assaying configurational purity of 3-DHR and the pigment(s) formed.



1, all-trans



1, 7-cis

EXPERIMENTAL

New isomers of 3-dehydroretinal. The synthesis of 7-*cis*, 7,9-di-*cis*, 7,13-di-*cis* and 7,9,13-tri-*cis* isomers of 3-DHR and their characterization spectral data were reported in a preliminary paper (*7*). The detailed procedures for their preparation, along with the preparation of those isomers containing the doubly hindered 7,11-di-*cis* geometry, have appeared in a separate organic paper (*8*). For the current work, all these isomers were purified by preparative HPLC before use in binding interaction with bovine opsin. The uv–visible absorption spectra, recorded on a Perkin–Elmer-λ-19 spectrometer, of these hindered isomers are reproduced in Fig. 1.

Binding interaction of new isomers of 3-dehydroretinal with bovine opsin. Procedures for isolation of bovine opsin and formation of porphyropsin analogs were essentially the same as those reported (*9*) and modified for 9-*cis*- and 7-*cis*-porphyropsin (*10*, *11*). Briefly, bovine opsin pellet washed with cold hexane to remove retinal oximes was solubilized in 2% CHAPS detergent and stored at −85°C. Opsin concentration was determined by reaction with 11-*cis*-retinal ($\sim 2.5 \times 10^{-5}$ M), the extinction coefficient of rhodopsin being 4×10^4 mol^{-1} cm^{-1} (*12*). For isomeric bovine porphyropsin formation, a slight excess (~ 3–5×10^{-5} M) of an ethanol solution of an isomer (15 μl) of 3-DHR ($\sim$0.5 mg/ml) was added to a 0.5-ml cuvette containing 0.3 ml of the opsin solution. Pigment formation was followed by changes in uv–visible absorption in the region 300–600 nm on a Perkin–Elmer λ-19 spectrometer. Spectra were recorded at regular time intervals with selected examples shown in the Results.

Procedures for deconvolution of the spectra obtained during pigment formation. The absorption spectra obtained during the course of the binding interaction of bovine opsin with isomeric 3-DHR were analyzed as summation of component

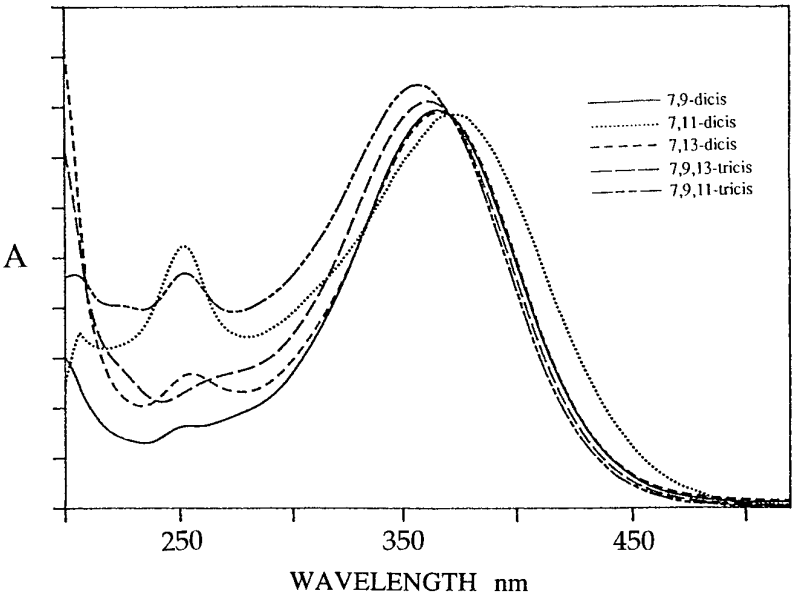


FIG. 1. Ultraviolet–visible absorption spectra of new isomers of 3-DHR recorded in hexane: 7,9-di-*cis*, 7,11-di-*cis*, 7,13-di-*cis*, 7,9,11-tri-*cis* and 7,9,13-tri-*cis*, arbitrary absorbance unit.

spectra of those species present in the reaction mixtures with the aid of the Curvefit program in the Spectra Calc software package (from Galactic Industries Corp.). The simulated spectra based on the components were then compared with the experimental curves. This iterative procedure was continued until a satisfactory match between the simulated and experimental curves was obtained.

Specifically, the procedure included the following steps: (1) The uv–visible spectra were converted to Spectra Calc file format by the file import program; (2) Curvefit calculation was selected from the Arithmatic menu; (3) number of peaks, peak types, peak widths, peak heights, and centers were entered; (4) the maximum number of fitting passes was specified (1000) to determine the relative amounts of the component peaks that best fit the experimental curve. Procedures 3 and 4 were then repeated with different initial peak parameters. The goodness of fit is judged by the small value of the calculated statistical error (χ^2) and visual inspection of closeness of match between the calculated and the experimental curves (an excellent match is usually attained with a χ^2 value less than 0.02). Results from such analyses are shown in Tables 1 and 2 (selected composition data during binding interaction of 7,9-di-*cis* and 7,11-di-*cis* 3-DHR); and spectra are shown in the figures below.

RESULTS

Three stable isomeric pigments (11-*cis*, 9-*cis*, and 7-*cis*) of porphyropsin were reported earlier (*10*, *11*). In this work, we carried out binding interactions with five new isomers containing the hindered 7-*cis* geometry: 7,9-di-*cis*, 7,13-di-*cis*, 7,11-di-

TABLE 1
Composition of Retinyl Species during Binding Interaction of 7,9-Di-*cis*-3-DHR with Bovine Opsin

Time (min)	Percentages of species present[a]			χ^2 (× 1000)
	Schiff base	7,9-Di-*cis*-CHO	7,9-Di-*cis* pigment	
3	12.8	26.4	7.1	5.5
15	19.3	16.8	11.0	3.8
21	17.6	16.1	13.0	3.3
27	15.2	16.1	15.1	6.8
33	15.9	14.5	16.1	3.3
39	14.5	15.1	16.7	7.1
45	17.4	11.9	16.8	1.9
51	16.4	12.2	17.5	2.9
57	17.3	10.8	17.8	3.7
63	16.7	10.9	18.9	4.7

[a] Determined from curvefit analysis.

cis, 7,9,11-tri-*cis* and 7,9,13-tri-*cis*. Of these we found that only the 7,9-di-*cis* retained configurational integrity throughout the reaction; the other four isomers exhibited different extent and nature of isomerization. We also reexamined the reaction of 7-*cis*-3-DHR with bovine opsin. The results are presented separately below.

TABLE 2
Composition of Retinyl Species during Binding Interaction of 7,11-Di-*cis*-3-DHR with Bovine Opsin

Time (min)	Percentage of species present[a]					ψ^2 (× 1000)
	Schiff base	7,11-Di-*cis*-CHO	7-*cis*-CHO	7-*cis* pigment	7,11-Di-*cis* pigment	
15	6.3	73.7	17.1	0	2.9	3.1
30	18.6	57.9	18.2	0	5.5	6.7
45	16.0	57.4	19.0	0	7.6	3.5
60	13.4	58.3	17.5	0	10.9	1.8
75	14.9	53.7	19.0	0	12.4	3.3
90	12.1	53.4	20.2	0	14.3	2.1
105	9.7	53.0	20.6	1.5	15.2	3.2
120	8.5	51.2	21.8	3.9	14.7	3.0
135	8.1	47.0	23.4	7.9	13.7	3.3
150	7.3	44.7	23.9	11.9	12.2	1.9
165	6.8	41.0	25.3	16.6	10.3	3.2
180	7.2	40.3	23.9	20.1	8.5	3.3
195	7.1	40.7	24.9	19.5	7.9	2.2
300	6.9	39.1	25.8	20.3	7.9	2.1

[a] Determined from curvefit analysis.

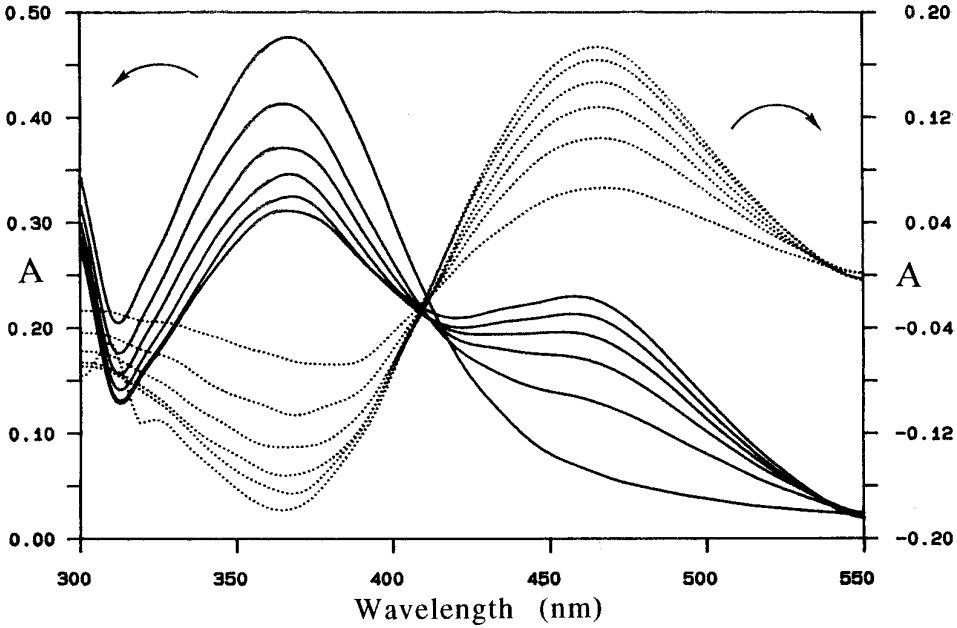


FIG. 2. Changes in uv-visible absorption spectra (solid line, scale on the left) obtained during binding interaction of 7,9-di-*cis*-3-DHR with bovine opsin recorded at 6-min intervals and interrupted after 63 min. Only selected curves are shown to minimize spectral overlap. The corresponding difference spectra (dotted line, scale on the right) were obtained by subtracting later absorption spectra from that of zero time.

Configurationally stable isomers. The progress of pigment formation of 7,9-di-*cis*-3-DHR with bovine opsin is shown in Fig. 2: the absorption spectra recorded at regular intervals and the calculated difference spectra. For the absorption spectra, a fairly sharp isosbestic point was maintained (the zero time one being more deviated from others), consistent with direct conversion of the aldehyde to the pigment. The final binding curve was first subjected to Curvefit analysis. The final simulated spectrum, virtually identical to the experimental curve, is shown in Fig. 3 along with the component curves. All of the experimental curves obtained during the course of the binding interaction were then analyzed in the same manner, using the same component peaks as those obtained above, to give similarly satisfactory results. They confirmed that only a single pigment (λ_{max} = 466 nm) was formed throughout the reaction.

We repeated the same analysis for 7-*cis*-3-DHR. The resultant spectra were virtually identical to those reported earlier. However, after curve resolution we found the absorption maximum for 7-*cis* bovine porphyropsin (in CHAPS) was at 458 nm compared with 464 nm (in digitonin) reported earlier (*11*).

Configurationally unstable isomers. The difference absorption spectra obtained in binding interaction of 7,13-di-*cis*-3-DHR with bovine opsin are shown in Fig. 4. The general trend of the stacked plots is clearly different from that in Fig. 2, showing now the absence of an isosbestic point and the continued rise in absorbance in the

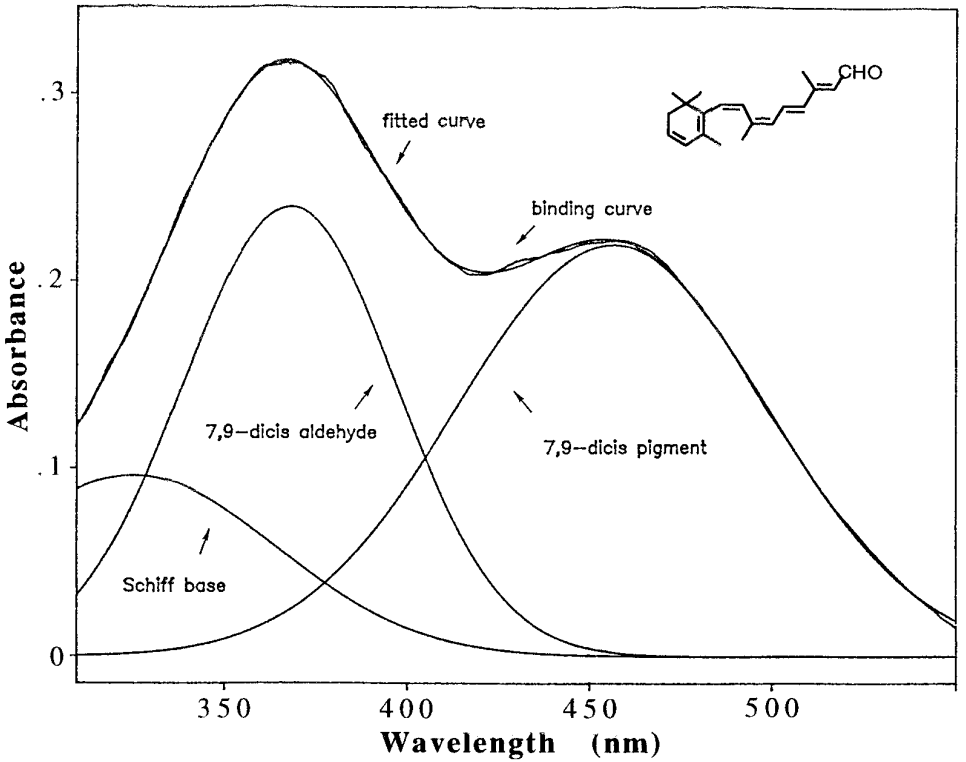


FIG. 3. Calculated curve from a summation of the curves of 7,9-di-*cis*-3-DHR, its Schiff base, and the pigment peaks superimposed with the experimental curve (final absorption curve from Fig. 2).

aldehyde region accompanied by a red shift of the same peak. These trends are consistent with prior isomerization of the 7,13-di-*cis* aldehyde to the 7-cis isomer followed by pigment formation, thus reminiscent of that of 7,13-di-*cis* retinal with opsin yielding primarily 7-cis-rhodopsin (*3*) as shown by chromophore extraction experiments. The absorption spectra of 3-DHR were therefore analyzed by assuming a similar sequence of events, i.e., isomerization of 7,13-di-*cis*- 3-DHR to the more planar and red-shifted 7-*cis*-3-DHR followed by formation of 7-*cis*-porphyropsin. The calculated curve is indistinguishable from the final binding curve (Fig. 5).

The results of the binding interaction of 7,9,13-tri-*cis*-3-DHR with bovine opsin are similar to those of the 7,13-di-*cis* isomer, thus giving a similar conclusion with respect to the loss of the 13-*cis* geometry to form the reactive 7,9-di-*cis*-3-DHR and then its 466-nm pigment. The simulated curves are shown in Fig. 6.

The reaction of the doubly hindered 7,11-di-*cis*-3-DHR with bovine opsin was then investigated. The stacked absorption spectra obtained during binding interactions showed the absence of an isosbestic point, an increase in aldehyde absorption, and the red shift of the latter. Additionally, the difference spectra showed the trend of a blue shift of the pigment peak, more obvious at the later stage of binding interactions. The latter observation suggested to us the possible involvement of