ACTA CHEMICA SCANDINAVICA 27 (1973) 1487–1495

Chlorophylls

III. Keto-Enol Tautomerism of Chlorophylls *a* and *b*. The Nature of Chlorophylls *a'* and *b'*

PAAVO H. HYNNINEN

Department of Biochemistry, University of Helsinki, SF-00170 Helsinki 17, Finland

Chlorophylls *a'* and *b'*, formed in pyridine solution, were separated from the principal chlorophyll zones by means of chromatography on sugar columns. Small amounts of pheophytinlike pigments, as well as various allomerization compounds, were also observed among the transformation products of the chlorophylls.

Chlorophyll *a'* was highly soluble in petroleum ether, differed distinctly from chlorophyll *a* in terms of its visible absorption spectrum and exhibited a strong tendency to form pheophytin *a*. Spectroscopically, chlorophyll *b'* differed only slightly from the primary chlorophyll *b* zone. Chlorophylls *a'* and *b'*, as well as their respective pheophytins, yielded positive reactions to the Molisch phase test and were converted into pheophorbides *a* and *b* upon treatment with 30 % hydrochloric acid.

Chlorophylls *a'* and *b'* were characterized as hydrogen chelates of the enol forms of chlorophylls *a* and *b*, respectively. In view of the evidence presented in the present article, the possibility that chlorophylls *a'* and *b'* are C-10 epimers of the chlorophylls appears unlikely. The spectroscopic differences between chlorophylls *a* and *a'* were attributed to the electron-withdrawing effect of the conjugated ring V in the enol form of chlorophyll. The fact that the visible absorption spectrum of chlorophyll *b'* differed only slightly from that of chlorophyll *b* was interpreted as being due to the electron-withdrawing effect of the formyl group, which tends to counteract the effect of ring V in the enol.

The transformation of chlorophylls *a* and *b* into, respectively, chlorophylls *a'* and *b'*, first observed by Strain and Manning,[1] has confounded chlorophyll researchers for approximately 30 years. Since chlorophylls *a* and *a'* had been found to be interconvertible as well as spectroscopically very similar and since chlorophylls *b* and *b'* had also been discovered to behave in like manner, the transformation between these paired compounds was assumed to be an isomeric reaction. Strain [2] presented the hypothesis that chlorophylls *a'* and *b'* are C-10 epimers of the ordinary chlorophylls. Katz and co-workers [3] inter-

preted their results, obtained by nuclear magnetic resonance (NMR), in favour of this hypothesis.

In previous publications [4,5] originating from the present laboratory, convincing evidence was forwarded in support of the concept that enolization is the initial step in the reaction sequence of the allomerization leading to the 10-alkoxy-lactone derivatives. Due to the fact that formation of the intermediate (enol) occurred readily in pyridine and that "isomerization" to chlorophylls *a'* and *b'* had also been reported to take place in pyridine or propanol,[1,3] it was thought that the *a'* and *b'* "isomers" could be identical with the chelated enol forms of the chlorophylls. The experimental results given in the present article strongly support this viewpoint.

EXPERIMENTAL

Preparation of chlorophylls a' and b'. Five milligrams of chlorophyll *a* (3.0 mg of chlorophyll *b*), isolated by the method previously described,[4] were dissolved in 5.0 ml of reagent grade pyridine and the solution was then allowed to stand overnight at room temperature and in the dark.

Chromatography on sugar columns. Conventional chromatographic procedures, as described by Strain and Svec,[6] were employed in separations of chlorophylls *a* and *a'* as well as *b* and *b'*. Icing sugar, containing 0.6 % calcium phosphate, was mixed with the eluent (petroleum ether, b.p. 60 – 80°C, containing 0.5 % propanol) to form a slurry, which was then poured into a glass column having an inside diameter of 3.0 cm and a height of 50 cm. The sugar particles were allowed to settle overnight. After standing, the sugar layer utilized in the separation of chlorophylls *a* and *a'* had a height of 26 cm, while that employed for separating chlorophylls *b* and *b'* was 22.5 cm.

The pyridine solution of chlorophyll, having stood overnight, was then evaporated nearly to dryness using a Thunberg tube at reduced pressure. The remaining residue was subsequently dissolved in 1.0 ml of the eluent and the resulting solution was immediately sampled into the top of the sugar column. The column was then eluted until the slower-moving principal chlorophyll zone had emerged. The absorbances of the collected fractions were measured at selected wavelengths by means of a Beckman DU spectrophotometer. The chromatographic separations were performed at a temperature of about + 4°C.

The absorption spectra of the components were determined utilizing a Cary Model 15 spectrophotometer. Measurements were performed directly upon the effluent after the components had emerged from the column as well as after they had been transferred into diethyl ether by means of a Thunberg tube at reduced pressure.

RESULTS

Fig. 1 presents the results of a typical separation of the products formed from chlorophyll *a* in pyridine. The more rapidly-moving component (B = chlorophyll *a'*) was easily separated from the principal component (C = chlorophyll *a*). The complete resolution of these components indicates the very slow equilibrium between them under the conditions prevalent during fractionation. Components B and C both yielded clearly positive reactions to the Molisch phase test, thus demonstrating that neither can be an allomerization product. The two chlorophylls differed distinctly, however, regarding their visible absorption spectra (Figs. 2 and 3; Table 1, b and c). Peak I of chlorophyll *a'* was located at a slightly longer wavelength than that of chlorophyll *a*. The primary differences, however, existed in the region of the Soret band and are clearly reflected in the values of the peak ratios as presented in Table 1. It may be

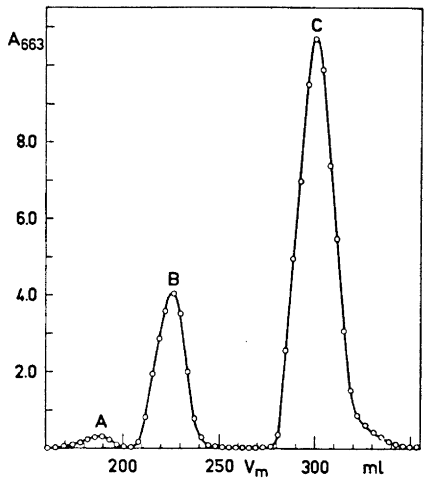

Fig. 1. Separation of chlorophylls *a* and *a'* employing chromatography on a sugar column. Experimental values (O) obtained by measuring A_{663} of the effluent fractions. *A* = pheophytin *a*, B = chlorophyll *a'*, and C = chlorophyll *a*.

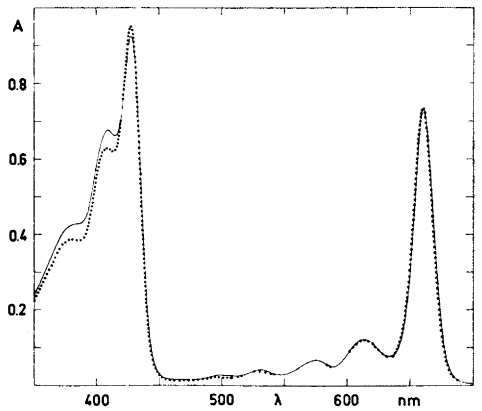

Fig. 2. Absorption spectra of chlorophylls *a'* (—) and *a* (···) in diethyl ether.

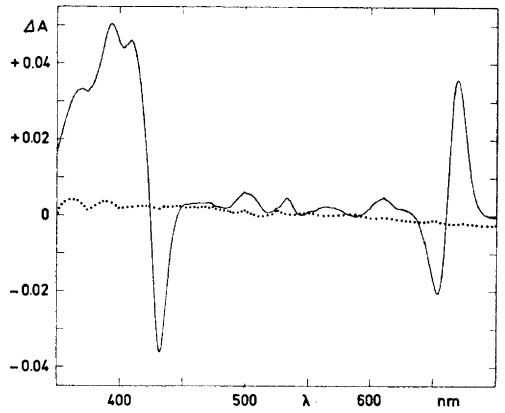

Fig. 3. Difference spectrum of chlorophylls *a'* and *a* in diethyl ether.

further observed that component C (Table 1, b) spectroscopically resembles very closely the original chlorophyll *a* (Table 1, a) utilized in the experiment. The spectroscopic differences between components B and C did not arise due to transference of the pigments from the effluent into diethyl ether, as demonstrated by the fact that similar differences had already been noticed when the spectra were recorded directly from the effluent (Table 1, d and e).

Table 1. Spectroscopic properties of the chlorophylls.

Compound	Solvent	Peak positions (nm)								Peak ratios			Halfwidths (nm)	
		I	II	III	IV	V	S	Ss1	Ss2	A_S/A_I	A_S/A_{Ss1}	A_S/A_{Ss2}	$w_{I1/2}$	$w_{S1/2}$
a. Chl. *a*[a]	Ee	660.0	614	576	530	497	428.0	409	380	1.32	1.56	2.48	17.9	39.0
b. Chl. *a* (1C)[a]	Ee	660.5	614	576	530	496	428.0	409	381	1.30	1.51	2.48	18.1	39.3
c. Chl. *a'* (1B)	Ee	661.0	614	575	532	499	428.0	409	(383)[b]	1.24	1.37	2.18	18.3	42.9
d. Chl. *a* (1C)	Pe+0.5 % PrOH	661.5	616	578	530	497	429.5	411	380	1.17	1.55	2.50	17.3	38.8
e. Chl. *a'* (1B)	Pe+0.5 % PrOH	662.5	614	576	533	502	429.0	410	(380)	1.14	1.25	(1.96)	17.8	61.0
f. Chl. *b*[a]	Ee	642.5	595				452.5	430		2.88	2.55		17.0	22.5
g. Chl. *b* (4C)	Ee	642.0	593				452.5	428		2.86	2.79		16.9	22.3
h. Chl. *b'* (4B)	Ee	642.0	592				452.5	428		2.86	2.73		17.2	21.7

[a] Number in parentheses refers to figure number and capital letter to component in that figure.
[b] Peak positions and ratios in parentheses are approximate.

S = Soret band, Ss1 = 1st satellite of Soret band, Ss2 = 2nd satellite of Soret band; Chl. = chlorophyll, Ee = diethyl ether, Pe = petroleum ether, PrOH = 1-propanol.

A small amount of yellow pigment (component A) was eluted prior to chlorophyll *a'*. This pigment exhibited a pheophytin *a*-like spectrum and yielded a positive phase test. A slight amount of pheophytin *a* was probably formed from chlorophyll *a'* in the pyridine solution, either while it was standing overnight or when it was evaporated to near dryness. This view is supported by the observation that chlorophyll *a'* is easily converted to pheophytin *a* upon standing in petroleum ether or cyclohexane, or upon washing the petroleum ether solution of the pigment with water.

Small amounts of at least three allomerization products remained in the column following the elution of component C. When the original chlorophyll *a* was fractionated under the same chromatographic conditions as the chlorophyll *a* that had been standing overnight in pyridine, no distinct chlorophyll *a'* zone separated. However, when a sugar layer of greater height (50 cm) was employed in such a fractionation, a small amount of chlorophyll *a'* was observed to separate slowly from the principal chlorophyll *a* zone. Neither allomerization products nor a pheophytin *a*-like pigment could be separated from the original chlorophyll *a* preparation.

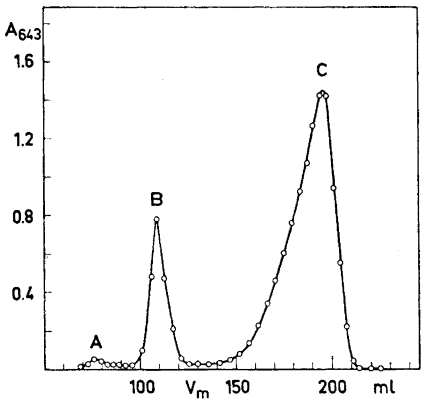

Fig. 4. Separation of chlorophylls *b* and *b'* employing chromatography on a sugar column. Experimental values (O) obtained by measuring A_{643} of the effluent fractions. A = pheophytin *b*, B = chlorophyll *b'*, and C = chlorophyll *b*.

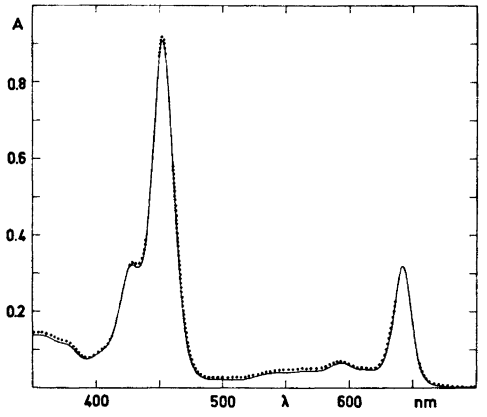

Fig. 5. Absorption spectra of chlorophylls *b'* (—) and *b* (···) in diethyl ether.

The result of a fractionation of the products formed from chlorophyll *b* in pyridine are presented in Fig. 4. The resolution of components B (chlorophyll *b'*) and C (chlorophyll *b*) was not as complete as in the chlorophyll *a* fractionation. The concentration profile reveals that slow equilibrium has probably existed between these components during fractionation. This difference, as compared to the chlorophyll *a* fractionation, is understandable, since the time required for the separation of chlorophylls *b* and *b'* was considerably longer than that needed for the separation of chlorophylls *a* and *a'*. Spectroscopically,

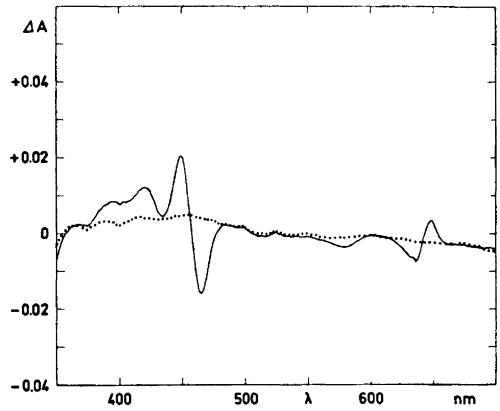

Fig. 6. Difference spectrum of chlorophylls *b'* and *b* in diethyl ether.

chlorophylls *b* and *b'* differed only slightly from each other (Figs. 5 and 6; Table 1, g and h). Chlorophyll *b'* yielded a positive phase test and was converted to normal pheophytin *b* or pheophorbide *b* upon treatment with 13 % or 30 % hydrochloric acid, respectively.

Component A consisted of a pheophytin *b*-like pigment. When components B and C were refractionated after standing overnight in pyridine, the pheophytin-containing zone was considerably smaller, thus suggesting that the original chlorophyll *b* preparation contained a slight amount of pheophytin *b*. This viewpoint is also supported by the fact that the spectra of components B and C differed slightly from that of the original chlorophyll *b* (Table 1, h, g and f, respectively).

The results described above may be satisfactorily interpreted by utilizing the reaction scheme previously presented for the chlorophylls.[4] Chlorophylls *a'* and *b'*, which are easily separated from the principal chlorophyll zones, probably represent chelated enol forms of the chlorophylls. The following evidence supports this concept and is contrary to the postulation of Strain [2] and Katz *et al.*[3] that the chlorophyll *a'* and *b'* "isomers" are merely C-10 epimers of the chlorophylls:

1. Chlorophylls *a'* and *b'* differ very significantly from chlorophylls *a* and *b* by their higher solubility in nonpolar solvents (*e.g.* petroleum ether), a phenomenon which is to be expected if it is assumed that they are chelated enol forms of the chlorophylls. Conversely, if it is assumed that chlorophylls *a'* and *b'* are merely diastereoisomers of chlorophylls *a* and *b*, then the great difference in the solubility properties of the components is not easily explained. Also, under the latter supposition, the components would probably not be so readily separable as has been experimentally observed.

2. The visible absorption spectrum of chlorophyll *a'* differs considerably from that of chlorophyll *a*. Again, this is to be expected upon the formation of the enol form of chlorophyll *a*. The conjugated double bond system of ring V will draw electrons from the tetrapyrrole system, thereby resulting in a

weakening of the bonds between the magnesium atom and the nitrogen atoms. If chlorophylls *a'* and *a* were indeed C-10 epimers, then no spectroscopic difference between them would be expected.

3. Chlorophyll *a'* was observed to exhibit a strong tendency to undergo conversion to pheophytin *a*, thus also indicating that electronic rearrangements occur upon the formation of chlorophyll *a'*.

4. The fact that chlorophyll *b* and *b'* are more similar spectroscopically than are chlorophylls *a* and *a'* may be interpreted upon the basis of the electron-withdrawing effect of the formyl group, which tends to counteract the effect of the conjugated ring V in the enol form.

5. The fact that the chlorophylls contain an exchangeable hydrogen at the C-10 position supports the concept of enolization. The presence of this hydrogen was demonstrated by Fischer and Goebel [7] upon the basis of Zerewitinoff's test and later confirmed by Dougerthy *et al.*[8] utilizing NMR.

6. The relationship between epimerization and enolization is a well-known phenomenon which may be found, for example, in sugar chemistry.

7. The probable intermediate in the formation of some chlorophyll derivatives (*e.g.* the 10-alkoxy-lactone derivatives and chlorin e_6-triester) is the enolate ion.[5] Assuming that the intermediate is a C-10 epimer, the lability of ring V towards oxygenation and solvolysis is then more difficult to comprehend.

8. Chlorophylls *a'* and *b'* have never been detected when multiple liquid-liquid partition methods have been employed for fractionation of the chlorophylls.[4] This is obviously not due to the lower selectivity of these methods compared to that of chromatography. A more plausible explanation is that the hydrogen bridge of the chelated enol is unstable in the highly-polar lower phases of the solvent systems utilized. Thus, if the enol form is originally present in the mixture to be fractionated or if it is formed during fractionation, it will rapidly be allomerized in the presence of alcohol and oxygen. This is in accord with the experimental results previously obtained.[4,5]

DISCUSSION

The formation of chelated enol forms of the chlorophylls is not a new concept, since it has already been proposed by Holt and Jacobs [9] in order to account for the IR absorption bands of chlorophyll *a* at 1640 cm^{-1} in the crystalline state and at 1652 cm^{-1} in carbon tetrachloride and carbon disulfide. This interpretation was supported by the IR studies of Russian investigators.[10–12]

A group of researchers [13] at the Argonne National Laboratory (USA) later demonstrated that the chlorophylls have a strong tendency to form aggregates in nonpolar solvents and that the absorption peak at 1652 cm^{-1} in the IR spectrum of chlorophyll *a* is an "aggregation peak". These results were confirmed by Ballschmiter and Katz.[14] However, the conclusions drawn by Katz and co-workers [8,13,15] regarding the keto-enol tautomerism of the chlorophylls have led to some controversy. The afore-mentioned investigators have stated that in solution, chlorophylls occur almost completely in the all-keto form. The present author cannot agree with this statement as long as the solvent is

insufficiently specified. According to the experience of this laboratory, the behavior of the chlorophylls is sensitive to the properties of the solvent. In completely nonpolar solvents (aliphatic hydrocarbons, carbon tetrachloride) and in water, the chlorophylls exist primarily in an aggregated state which thus precludes keto-enol tautomerism. In electron donor solvents (diethyl ether, acetone, pyridine, dioxane, tetrahydrofuran, the lower alcohols), however, the chlorophylls occur as monomers and will therefore undergo the tautomerization. The rate of enolization and the concentration of free enol are, in this case, sensitive to the particular properties of the solvent in question. Thus, in the lower alcohols (methanol, ethanol), the alcoholysis and oxidation of ring V, along with the concomitant solvation, consume the free enol form and draw the enolization reaction forward.[5] Formation of the chelated enol is probably not possible in these solvents due to their strong hydrogen-bonding capability. In the higher alcohols (propanol, butanol), the oxidation of ring V probably competes with the formation of the chelated enol. In solvents (pyridine, tetrahydrofuran, petroleum ether containing a small amount of propanol) which are polar enough to prevent aggregation but which do not possess a strong hydrogen-bonding capability, the formation of the hydrogen chelate appears to be the principal reaction that consumes the free enol. Thus, the keto-enol tautomeric equilibrium shifts towards the enol form at the expense of the all-keto form. When the reaction that consumes the free enol proceeds more rapidly than the reaction that produces it, the concentration of the free enol may be rather low. Indeed, the argument presented by Katz and co-workers may be true only in regard to relatively few solvents (diethyl ether, acetone).

Upon the basis of the slow exchange rate observed for epi-C-10-resonance, Katz *et al.*[3] have excluded the possibility that this resonance is due to an enolic hydroxyl group. It appears, however, that the afore-mentioned authors have not taken into account the possibility of the enol existing predominantly in the chelated form under the conditions prevalent during the exchange studies. The hydrogen of a chelated enol would be expected to exchange more slowly than the hydrogen of a free enol. The hydrogen chelate ring of a chelated enol may be quite stable in nonpolar solvents, since there exists the possibility of additional resonance.[4] Therefore, the present author does not consider evidence based upon the rate of hydrogen exchange strong enough to exclude the possibility that the satellite resonance peak observed by Katz *et al.*[3] is due to an enolic hydroxyl group.

REFERENCES

1. Strain, H. H. and Manning, W. M. *J. Biol. Chem.* **146** (1942) 275.
2. Strain, H. H. *Agr. Food Chem.* **2** (1954) 1222.
3. Katz, J. J., Norman, G. D., Svec, W. A. and Strain, H. H. *J. Am. Chem. Soc.* **90** (1968) 6841.
4. Hynninen, P. H. and Ellfolk, N. *Acta Chem. Scand.* **27** (1973) 1463.
5. Hynninen, P. H. and Assandri, S. *Acta Chem. Scand.* **27** (1973) 1478.
6. Strain, H. H. and Svec, W. A. In Vernon, L. P. and Seely, G. R., Eds., *The Chlorophylls*, Academic, New York–London 1966, p. 21.
7. Fischer, H. and Goebel, S. *Ann. Chem.* **522** (1936) 168.
8. Dougherty, R. C., Strain, H. H. and Katz, J. J. *J. Am. Chem. Soc.* **87** (1965) 104.

9. Holt, A. S. and Jacobs, E. E. *Plant Physiol.* **30** (1955) 553.
10. Sidorov, A. N. and Terenin, A. M. *Opt. i Spektroskopiya* **8** (1960) 259.
11. Sidorov, A. N. *Opt. i Spektroskopiya* **3** (1962) 206, 374; **14** (1963) 834.
12. Karyakin, A. J. and Chibisov, A. K. *Opt. i Spektroskopiya* **13** (1962) 379.
13. Katz, J. J., Closs, G. L., Pennington, F. C., Thomas, M. R. and Strain, H. H. *J. Am. Chem. Soc.* **85** (1963) 3801.
14. Ballschmiter, K. and Katz, J. J. *J. Am. Chem. Soc.* **91** (1969) 2661.
15. Katz, J. J., Dougherty, R. C. and Boucher, L. J. In Vernon, L. P. and Seely, G. R., Eds., *The Chlorophylls*, Academic, New York – London 1966, p. 185.

Received November 28, 1972.