MAGNETO-OPTICAL ROTATION SPECTRA OF METHEMOGLOBIN IN THE PRESENCE OF ALLOSTERIC EFFECTORS

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Received 13 February 1973

1. Introduction

In earlier experiments it was shown that in the presence of allosteric effectors not only the half-saturation value of the oxygen binding curve of hemoglobin is shifted to higher partial pressures but also the high spin form in the high–low-spin equilibrium of methemoglobin (Met-Hb) is increased [1, 2]. As revealed by electron spin resonance measurements, allosteric effectors are able to change the ligand field of the iron by means of a conformational change of the protein structure in which a weakening of the bonding between iron and proximal histidine is involved [2, 3]. The conformational changes induced by the allosteric effectors have only little influence upon the absorption bands at 545 nm and 575 nm that are characteristic of the low spin form [3]. By means of the magneto-optical rotation (MOR) method, however, we can show that the Q-bands at 545 nm and 575 nm considerably decrease in the presence of allosteric effectors, indicating an increase of the high spin form. The mentioned Q-bands preponderant are assigned to π–π^*-transitions polarized in the xy-plane of the porphyrine [4] and in the MOR spectrum they have a negative A-term character [5]. Because of the intensive change of these bands in the MOR spectrum alteration of the electronic structure of the porphyrine system by means of allosteric effectors is indicated.

2. Materials and methods

Met-Hb was prepared from freshly drawn venous blood by treatment with $NaNO_2$, hemolyzed and the stroma removed by centrifugation. In order to remove phosphates, about 200 ml of the hemolysate was concentrated to 40 ml by ultracentrifugation (Diaflo membrane, PM-30) under O_2 supply at 4°, diluted to 400 ml by adding a solution of 130 mM KCl and 20 mM NaCl pH 7.2, and subsequently re-concentrated by ultrafiltration under O_2. This procedure was repeated six times. The concentration of total phosphate in the supernatant on precipitation with trichloroacetic acid using the ascorbic acid method [6] was determined to be < 0.02 M of phosphate per mole of hemoglobin. The solutions containing about 3 mM of Met-Hb were stored in liquid N_2 until use.

The concentration of Met-Hb was determined as Met-Hb cyanide using $\epsilon_{540\,nm} = 44.0\ cm^{-1}\ mM^{-1}$.

The calcium salt of inositol hexaphosphate (IHP) (Koch Light Laboratories Ltd., Colnbrook) and the cyclohexylammonium salt of 2,3-diphospho-D-glycerol acid (2,3-DPG) (Calbiochem) were transferred into free acid by incubation with Dowex 50 W-X 8 and subsequently adjusted by NaOH to pH 7.0. The concentration was determined by analysing total and inorganic phosphate using the ascorbic acid method [6]. The Di-Na salt of ATP (Boehringer and Söhne, Mannheim) was adjusted to pH 7.0 by addition of KOH. Concentration was determined by absorption measurement at 258 nm in 0.6 M $HClO_4$ using a $\epsilon_{mM} = 14.7\ cm^{-1}\ M^{-1}$. The solutions of allosteric anions were stored at −20°.

A 150 mM Tris-acetic acid buffer was used as the incubation medium for all experiments. The different pH values were adjusted by varying the mixture ratios. The experiments were carried out at a mean room temp. of 21 ± 1.5°.

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MOR measurements were performed with the spectral polarimeter, Spectropol I-B model 60 000 B (FICA), with a magnet of 0.63 Tesla. The layer thickness was 3 mm for all experiments. In the spectral region from 600 nm to 450 nm the Hb solutions were measured at a concentration of 1×10^{-3} M per heme; in the spectral region from 450 nm to 350 nm, at a concentration of 5×10^{-5} M per heme. All MOR data are given in units of the molar rotation, according to the following equation:

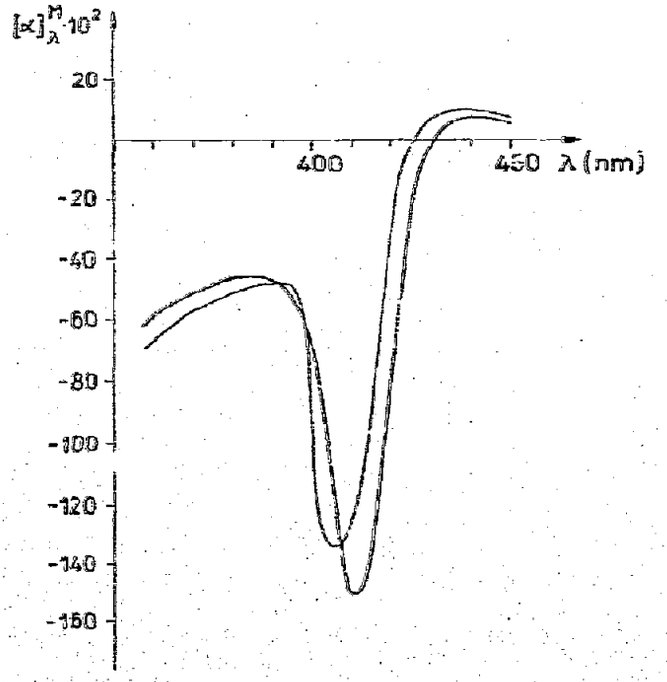
$$[\alpha]_\lambda^M = \frac{\alpha}{\ell \cdot c \cdot H}$$

where α is the angle of rotation, ℓ = layer thickness in dm, c = concentration in M, and H = the magnetic field in guass.

3. Results

The MOR spectrum of Met-Hb, in the spectral region from 350 nm to 600 nm, is characterized by the three Q-bands at 494 nm, 540 nm and 575 nm as well by the B-band at 420 nm [7]. Fig. 1 shows the influence of IHP on the MOR-spectrum of Met-Hb. With increasing concentration of IHP the magnitude of all MOR bands decreases; this being most pronounced at the band at 575 nm. The same effect, though to a lower extent, is performed by ATP and 2,3-DPG (table 1).

In order to determine the largest difference in the magnitude of the MOR-bands at 545 nm and 575 nm between the high and low spin form, the pure high spin and low spin form of Met-Hb was measured (fig. 2). This was performed by measuring Met-Hb at different pH (pH $\leq$ 6.5, high spin form; pH $\geq$ 9.0, low spin form) thus avoiding complicating effects of ligands because these produce specific MOR-spectra [8]. From fig. 2 it may be seen the largest difference in the magnitude of MOR-spectra occurs at the band at 575 nm, corresponding to the changes observed in dependence on allosteric effectors. Plotting the MOR-magnitude versus pH, the half-transition-point has been evaluated, corresponding satisfactorily with the value determined by spectral photometric titrations.



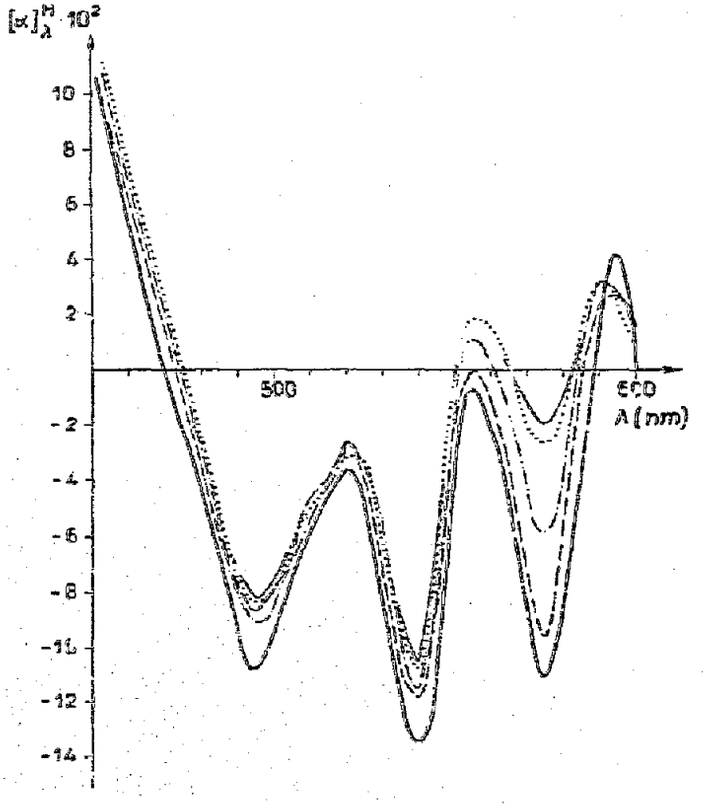


Fig. 1. MOR spectra of Met-Hb in dependence on increasing IHP concentrations: (——) control; (– – –) 0.25 M IHP/M Hb; (–·–·–) 0.5 M IHP/M Hb; (. . . .) 1.0 M IHP/M Hb; (——) 2.0 M IHP/M Hb; Met-Hb stripped; pH 7.0; Hb concentration 1×10^{-3} M per heme, in the Soret region 5×10^{-5} M per heme. (The low-spin-bands of the absorption spectrum of Met-Hb in presence of 2 M IHP decrease at increasing high-spin-bands. The differences are less than those of the MOR-spectra [3].)

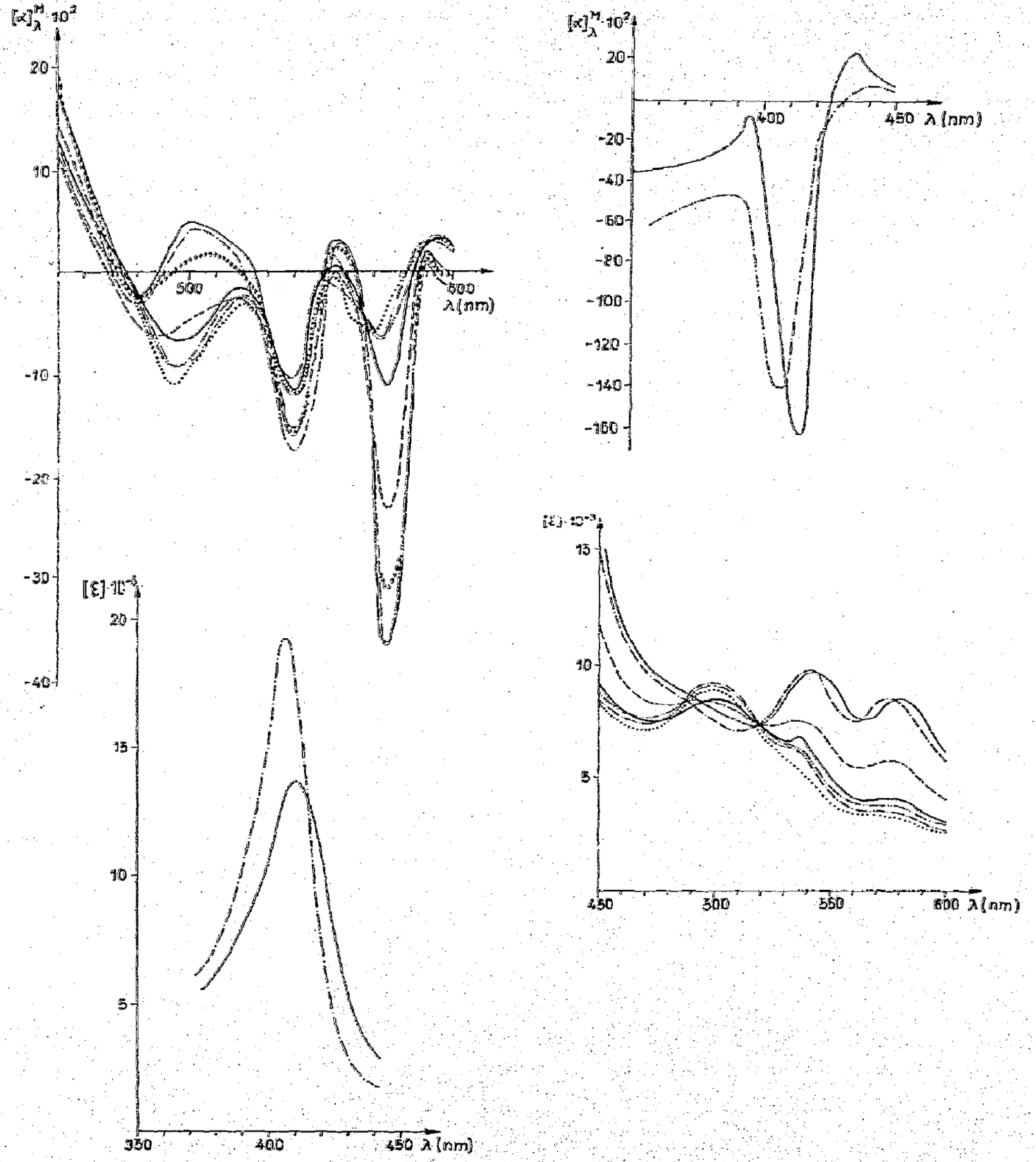


Fig. 2. MOR and absorption spectra of Met-Hb in dependence on pH; (. . . .) pH 5.5; (– . – . –) pH 6.0; (– .. – .. –) pH 6.5; (——) pH 7.4; (– – –) pH 8.0; (+ + + +) pH 8.5; (——) pH 9.0; (- - - - - - -) pH 9.5; pH adjustment by 150 mM Tris buffer; Hb concentration 1×10^{-3} M per heme, in the Soret region 5×10^{-5} M per heme.

Table 1

Magneto-optical activity of the 3 Q-bands of methemoglobin in the presence of allosteric effectors.

	$[\alpha]^{M}_{495\ nm} \times 10^2$	$[\alpha]^{M}_{540\ nm} \times 10^2$	$[\alpha]^{M}_{575\ nm} \times 10^2$
Met-Hb, pH 7, stripped			
$c = 1 \times 10^{-3}$ M per heme	−10.72	−13.50	−11.11
+ 2 M 2,3-DPG per M Hb	−10.70	−13.08	− 9.52
+ 2 M ATP per M Hb	− 9.52	−12.70	− 7.93
+ 2 M IHP per M Hb	− 8.20	−10.59	− 1.98

4. Discussion

Met-Hb is known to exist in two electronic configurations in the basic state [9]; the configuration with 5 unpaired electrons represents the high spin form, and that with 1 unpaired electron, the low spin form. In the visible absorption spectrum the low spin form is characterized by 2 bands at 540 nm and 575 nm [10]. These 2 Q-bands correspond to the degenerated transitions $a_{1u}, a_{2u} \rightarrow e_g$. In the case of the low spin form the symmetry of this 6-fold coordinated complex is D_{4h}. If the symmetry is lower (high spin form), the degenerated transitions are lower or removed. At conversion from the high spin to the low spin form the iron approaches the heme disc [11], which causes increase not only in the iron—porphyrin interaction, but also in the symmetry. In the di-cation of porphyrin there exists symmetry because of the degeneration of transition corresponding to the Q-bands.

Therefore in the MOR spectra of porphyrin bands with A-term character are observed [8]. A-term only occur when the excited transition corresponding to the optical transition is degenerated. Hence, the bands at 540 nm and 575 nm of the low spin form of Met-Hb that are typical forms of A-term indicate that the symmetry of this complex is similar to the di-cation of porphyrin. Possibly the observed reduction of MOR magnitude of the A-term at the conversion from the low spin to the high spin form is caused partially by changes of the charge-transfer transitions which are present between iron and porphyrin system [12].

Contrary to hemoproteins with reduced iron, the Fe^{3+} in Met-Hb is able to bind anions. Therefore it had to be excluded that the observed effects at Met-Hb being caused by binding of allosteric anions to the iron. The results obtained justify the assumption that the allosteric anions are bound to the protein moiety of Met-Hb. The MOR-effects can be explained by conformational changes triggered by this binding.

Because of apparative limitation in our studies we were not able to analyze the high-spin-band at 630 nm. This band is suggested to decrease at increasing low-spin-character of the investigated Met-Hb-compounds. This assumption has been shown to be correct by ligand-binding at the iron of myoglobin by Atanasow et al. [13].

The allosteric effectors decrease the magnitude of the bands with A-term character. That does not mean only a decrease in symmetry of the iron—porphyrin complex but also a decrease of the bonding strengths in this system. By means of other methods it could be observed according to these results that the strength of the ligand field of iron is decreased and the electronic structure of the ligand is changed in the presence of allosteric effectors [2, 14]. Transferring our results to oxy-Hb it may be suggested that allosteric effectors enforce a state of lower symmetry in the iron—porphyrin—ligand complex and therefore the consequence is a facilitated release of oxygen. Further investigations to prove this effect in oxy-Hb are in preparation.

Acknowledgements

The authors are indebted to M. v. Löwis for many discussions concerning the use of the MOR-spectrometer.

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