IS MITOCHONDRIAL CYTOCHROME *b*-566/558 A SINGLE HEMOPROTEIN OR TWO INDIVIDUAL COMPONENTS?

A magnetic circular dichroism study

Alexander M. ARUTJUNJAN+, Yuriy A. KAMENSKY, Elena MILGRÖM, Sergey SURKOV and Alexander A. KONSTANTINOV and Yuriy A. SHARONOV*

+*Institute of biological testing of chemical compounds, Kupavna; A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Lomonosov State University, Moscow B-234* and **Institute of Molecular Biology, Vavilova 32, Moscow B-312, USSR*

Received 10 July 1978

1. Introduction

Since the discovery of a low-potential, long-wavelength species of cytochrome *b* in the mitochondrial respiratory chain (*b*-566, *b*-566/558, b_T, reviewed [1,2]) many attempts have been made to ascribe various 'anomalous' features to this component of coupling site 2. The cytochrome's reduced state split α-absorption band [3,4] with an $A_{565-566\ nm}$ max** and a shoulder at $A_{558\ nm}$ has received little attention so far. That the split band arises from a single hemeprotein has been questioned [5–8]. Two individual cytochromes, *b*-566 and *b*-558, present in the respiratory chain in addition to a 'classical' cytochrome *b*-562, have been suggested to account for the split band.

Magneto-optical activity measurements have proved a useful tool in the studies of heme electronic structure and coordination geometry in hemeproteins [9,10]. We have described magnetic circular dichroism (MCD) and magneto-optical rotatory dispersion spectra of beef heart submitochondrial particles in [11]. Although well resolved A-terms attributed to reduced *c* and *b* cytochromes were observed, we could find no evidence for the presence of a long-wavelength cytochrome *b* species.

* To whom reprint requests should be addressed

** Wavelengths are given for room temperature spectra

We report here MCD difference spectra (reduced *minus* oxidized) of cytochromes *b*-562 and *b*-566/558 in the isolated complex III from beef heart. The bands at A_{566} and A_{558} are shown to belong to a single low-spin cytochrome *b*-566/558 with a highly asymmetric heme environment.

2. Methods

Complex III was isolated from beef heart mitochondria as in [12]. The preparation contained 3 nmol cytochrome c_1 and 6 nmol cytochrome *b*/mg protein, and showed 8 bands on a SDS–PAGE electrophoretogram.

Basic incubation medium contained 0.46 M sucrose, 8 mM Tris–HCl buffer (pH 7.5), potassium cholate, 0.2%; other additions are indicated in the figure legends. All solutions were bubbled with argon thoroughly before experiments.

Optical measurements were carried out in quartz 10 mm slit cells at room temperature. Absorption spectra were recorded with a Cary-118c spectrophotometer. MCD measurements were made with an instrument constructed in the Inst. Mol. Biol. USSR Acad. Sci. on the basis of a 'Jouan' dichrograph equipped with a 1.6 Tesla electromagnet.

Simulations of absorption and MCD spectra were performed using Hewlett Packard 9830A calculator.

Elsevier/North-Holland Biomedical Press

2.1. *Procedure for MCD difference spectra*

Individual spectra of cytochromes *b*-562 and *b*-566/558 in complex III can be obtained only as the difference between the absolute spectra of the preparation poised at appropriate stages of reduction. In order to obtain reliable difference spectra with a single-beam MCD instrument the following 3 step procedure was used:

1. After appropriate additions a difference absorption spectrum was scanned in a Cary-118c spectrophotometer.
2. MCD spectra of the sample and reference were recorded in succession in a dichrograph.
3. The sample and reference cells were transferred back to the Cary-118c and the difference absorption spectrum was scanned once more.

If the difference absorption spectra recorded before and after MCD measurements proved to be identical, an MCD difference spectrum was obtained by subtracting the spectrum of the reference from that of the sample.

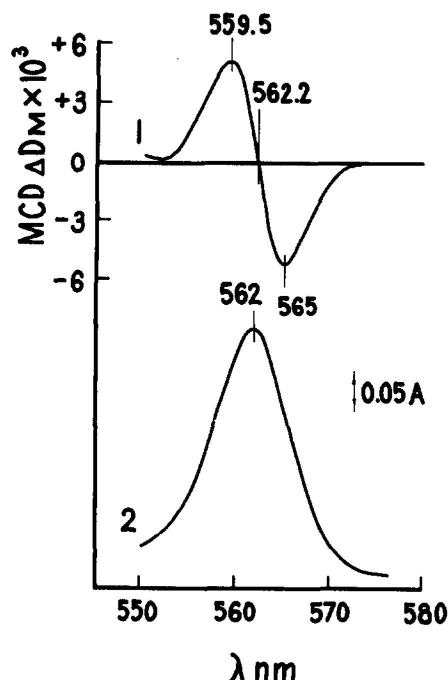

Fig.1. MCD (1) and absorption (2) spectra of cytochrome *b*-562. The sample and the reference cells contained complex III (7.5 mg protein/ml) in the basic medium supplemented with 2.5 mM ascorbate to reduce cytochrome c_1. Reduced diaminodurene, 200 μM, and 30 μM phenazine methosulfate were added to the sample.

3. Results and discussion

The low-spin hemeproteins are known to exhibit two optical absorption bands in the visible region. One of them, called Q_{00} or α-band, is purely electronic, and it is accompanied by a vibronic Q_v or β-band. The Q_{00} band arises from the π–π* electronic transition in the porphyrin ring from the nondegenerate ground level A_{1g} to the doubly-degenerate excited state E_u. The excited state is degenerate presumably due to the high symmetry (D_{4h}) of heme. However, rhombic distortion imposed by surrounding protein entails the symmetry being lowered to C_{2v}; consequently degeneracy is removed and the Q band splits into Q_x and Q_y components.

For a nondegenerate ground state, A- and B-type effects are possible in the MCD. The former are due to Zeeman splitting and the latter originate in mixing the ground and excited states with other excited states by the magnetic field. The A term arises only for degenerate excited states and is characterised by a dispersion curve which looks like a first derivative of the respective absorption band. The B term can arise in the case of both degenerate and nondegenerate excited state and has a shape similar to that of the absorption band. Unlike the absorption band, A and B terms can be either positive or negative. It is a crossover of the A term, but an extremum of the B term that coincides with the corresponding absorption maximum on a wavelength scale [13].

The absorption and MCD difference spectra of cytochromes *b*-562 and *b*-566/558 in the α-band region are given in fig.1,2. It is to be emphasised that, in the visible region, the difference (reduced *minus* oxidized) MCD spectra of the low-spin cytochromes are actually very close to the MCD of the reduced state.

The MCD curve of cytochrome *b*-562 (fig.1) is derivative shaped, which is characteristic of the A term. It shows a trough at A_{565}, a peak at $A_{559.5}$ and a crossover at $A_{562.2}$; it is important to note that the latter coincides with the cytochrome A_{562} max in

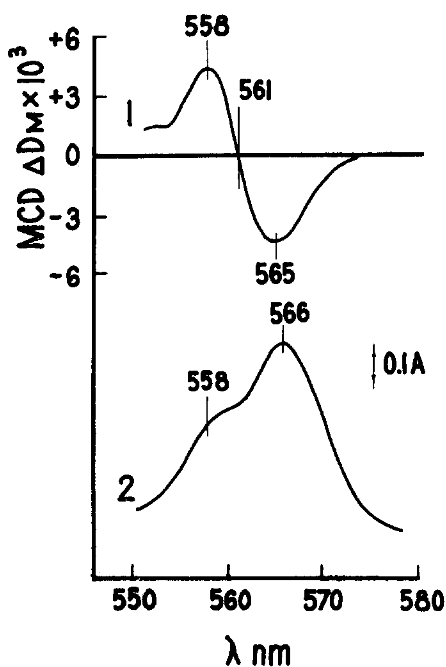

Fig.2. MCD (1) and absorption (2) spectra of cytochrome *b*-566/558. The sample and the reference cells contained complex III (7.5 mg protein/ml) in the basic medium supplemented with 2.5 mM ascorbate, 200 μM diaminodurene, 30 μM phenazine methosulfate and 2 mM NADH entailing complete reduction of cytochromes c_1 and *b*-562, and partial reduction of *b*-566/558. A few grains of solid dithionite were added to the sample.

agreement with theoretical considerations for the A term [13].

At first sight, the MCD curve of cytochrome *b*-566/558 (fig.2) also looks like the A term, with a trough at A_{565}, a peak at A_{558} and a crossover at A_{561}; this differs only slightly from the corresponding characteristics of the cytochrome *b*-562 MCD spectrum. However, the crossover point now does not coincide either with the A_{566} max or with the shoulder at A_{558} but is localized between these two wavelengths.

The A-type effects in the Q_{00} band of both cytochromes *b* are comparable in magnitude to that observed for cytochrome *c* and indicate both cytochromes *b*-562 and *b*-566/558 to be low-spin heme proteins with the heme iron coordinated to six strong ligands [11,14].

The origin of the shoulder at A_{558} in the spectrum of the low-potential mitochondrial cytochrome *b* with an A_{566} max appears to be a subject of controversy.

(i) A similarity between the spectrum of cytochrome *b*-566/558 and that of the myoglobin complex with nicotinate or with some other nitrogenous bases (the, so-called, twin-hemochromogen spectrum [15]) had been described [3] and originally both A_{566} and A_{558} bands attributed to the same cytochrome with a highly asymmetric heme environment.

(ii) An opposing viewpoint, considered as 'currently favoured' [16], assigns the shoulder at A_{558} to a separate cytochrome *b* species or, equivalently, to a separate conformational state of cytochrome *b*-566.

(iii) The shoulder at A_{558} could be a low-frequency vibronic component at ~254 cm^{-1} of the Q_{00} band at A_{566}.

Absorption spectra per se are not sufficient to discriminate between the above three possibilities. The twin-hemochromogen problem can however be solved considering both the MCD and absorption spectra [17]. Below we are concerned with such an analysis using spectra simulations.

The absorption spectrum of cytochrome *b*-566/558 in the Q_{00} region can be decomposed into two individual bands with A_{566} max and A_{558} max at an intensity ratio of 2:1, respectively, each having a half-width of 10 nm (fig.3A) and fitting satisfactorily to the Lorentzian shape. The parameters obtained for these individual bands (wavelength positions, half-widths and intensity ratio) were used to simulate corresponding MCD spectra, and these latter were compared with the experimental MCD. The analysis of the three above possibilities is given below:

(i) If the two absorption bands were arising from the two different cytochromes *b*-566 and *b*-558 with similar midpoint potentials, or from the two states of one cytochrome, each of the two bands should give a positive A term in the MCD spectrum, with a crossover at a wavelength of the respective A_{max}. The results of the simulation are given in fig.3B, where 2 and 3 are theoretical curves for two A terms and 4 is their sum. The latter may be compared to the experimental spectrum 1. Obviously, a nonsense fit is obtained.

(ii) Let the shoulder at A_{558} be due to a mild vibronic transition at ~254 cm^{-1}. In this case it is also the

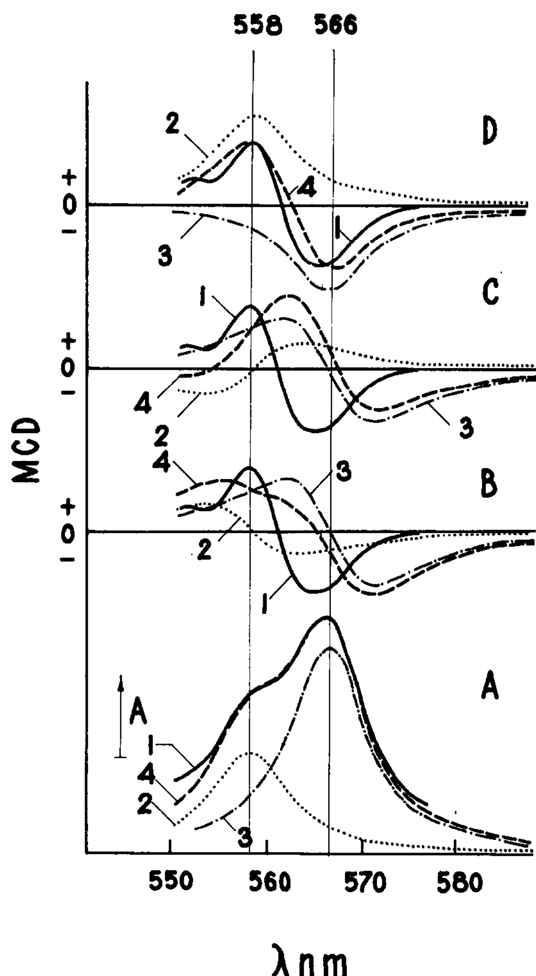

Fig.3. Simulations of the absorption (A) and MCD (B,C,D) spectra of cytochrome b-566/558. (A) 1, experimental spectrum; 2, 3, theoretical Lorentzian shape curves for A_{558} and A_{566} bands, respectively; 4, the sum of 2 and 3. (B) 1, experimental spectrum; 2, 3, theoretical curves for two positive A terms; 4, the sum of 2 and 3. (C) 1, experimental spectrum; 2 and 3, theoretical curves for two A terms of the opposite sign (positive for the A_{566} band and negative for the A_{558} band); 4, the sum of 2 and 3. (D) 1, experimental spectrum; 2 and 3, theoretical curves for two B terms of the opposite sign; 4, the sum of 2 and 3.

A term in the MCD spectrum that should be expected for this band. However, in variance with (i), this A term can be either positive or negative, depending on the symmetry of the vibration. The case of the positive A term for the A_{558} band has already been considered in fig.3B. The results of simulation assuming the negative A term are given in fig.3C. Evidently, the possibility of the low-energy vibronic transition being responsible for the A_{558} band is unlikely.

(iii) Finally, the twin hemochromogen in the α-band of cytochrome b-566/558 could arise from asymmetry of the heme environment. Rhombic distortion imposed by a protein surrounding on the porphyrin ring can split a degenerate excited Q state into Q_x and Q_y components. It is important to note that the intensities of the corresponding Q_{ox} and Q_{oy} absorption bands are not necessarily equal. Mixing of the Q_x and Q_y states by the magnetic field should give rise to two B terms of opposite signs but of equal magnitudes in the MCD spectrum [18]. Figure 3D shows the simulated MCD curves for two B terms at A_{566} and A_{558} (curves 2 and 3). The sum of these two curves (4) can be seen to be in reasonable agreement with the MCD spectrum of cytochrome b-566/558 observed in experiment (curve 1). An exact fit is not to be expected since the MCD and absorption spectra measured in this study are difference spectra and therefore somewhat distorted by a contribution from the oxidized cytochromes.

Therefore, comparison of the absorption and MCD spectra proves the unusual α-absorption band of cytochrome b-566/558 to originate in the extremely strong splitting of the purely electronic Q_{oo} band into Q_{ox} and Q_{oy} bands due to rhombic distortion of the heme structure.

Interestingly, if we consider the splitting of the Q_{oo} band to be abolished, the Q_{ox} and Q_{oy} bands of cytochrome b-566/558 would converge to a single symmetrical band with a maximum at 562 nm and a half-width of 10 nm. In the MCD spectrum this band should give a positive A term with a peak at $A_{559.5}$, a trough at $A_{564.5}$ and a crossover at A_{562}. Remarkably, all these characteristics are in fair agreement (within experimental error) with those of cytochrome b-562 (fig.1). Hence, it would be tempting to speculate that both b-562 and b-566/558 are identical hemeproteins but built asymmetrically into the complex b–c_1 ensemble. One of them becoming b-566/558 suffers strong perturbation exerted by its environment, which results in rhombic distortion of the

heme structure and in the midpoint potential being shifted to the negative by some 100 mV compared to b-562 [1,2]. The strained conformation of b-566/558 would relax upon the cleavage of complex $b-c_1$, accounting for the fact that only cytochrome b, with A_{562} max, has been so far isolated from mitochondria [19].

Acknowledgements

Thanks are due to Professor M. V. Volkenstein and V. P. Skulachev for their current interest to this work and discussion.

References

[1] Wilson, D. F. and Dutton, P. L. (1971) in: Electron and Coupling Energy Transfer in Biological Systems (King, T. E. et al. eds) vol. 1, pt A, pp. 221–241, P. B., New York.
[2] Wikström, M. K. F. (1973) Biochim. Biophys. Acta 301, 155–193.
[3] Sato, N., Ohnishi, T. and Chance, B. (1971) Biochim. Biophys. Acta 275, 288–297.
[4] Davies, K. A., Hatefi, Y., Poff, K. L. and Butler, W. L. (1973) Biochim. Biophys. Acta 325, 341–356.
[5] Wikström, M. K. F. (1971) Biochim. Biophys. Acta 253, 332–345.
[6] Yu, C. A., Yu, L. and King, T. E. (1972) Biochim. Biophys. Acta 267, 300–308.
[7] Grimmelikhuijzen, C. P. J., Marres, C. A. M. and Slater, E. C. (1975) Biochim. Biophys. Acta 376, 533–548.
[8] Higuti, T., Mizuno, S. and Muraoka, S. (1975) Biochim. Biophys. Acta 396, 36–47.
[9] Sharonov, Yu. A. (1976) in: Reviews of Science and Engineering series 'Molecular Biology', (Volkenstein, M. V. ed) vol. 8, pt I, pp. 70–161, VINITI, Moscow.
[10] Vickery, L., Nozawa, T. and Sauer, K. (1976) J. Am. Chem. Soc. 98, 343–357.
[11] Arutjunjan, A. M., Konstantinov, A. A. and Sharonov, Yu. A. (1974) FEBS Lett. 46, 317–320.
[12] Rieske, J. S. (1967) Methods Enzymol. 10, 239–245.
[13] Buckingham, D. A. and Stephens, P. J. (1966) Ann. Rev. Phys. Chem. 17, 399–432.
[14] Arutjunjan, A. M. and Sharonov, Yu. A. (1973) Molek. Biol. (USSR) 7, 587–594.
[15] Keilin, J. (1966) in: Hemes and Hemoproteins (Chance, B. et al. eds) pp. 173–188, Academic Press, New York.
[16] Rieske, J. S. (1976) Biochim. Biophys. Acta 456, 195–247.
[17] Arutjunjan, A. M., Magonov, S. N. and Sharonov, Yu. A. (1978) Molek. Biol. (USSR) 11, 530–541.
[18] Sutherland, J. C. and Klein, M. P. (1972) J. Chem. Phys. 57, 76–86.
[19] Weiss, H. (1976) Biochim. Biophys. Acta 456, 291–313.