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SECOND DERIVATIVE SPECTROSCOPIC ASSAY OF CYTOCHROME P-450 OF YEAST CELLS. RESOLUTION OF CYTOCHROME a_3

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SUMMARY

The utility of 2nd derivative spectroscopy in the resolution of overlapping spectra of carbon monoxide-binding hemoproteins and in the measurement of cytochrome P-450 in whole yeast cells was explored. The linear relationship between the vertical peak to trough distance in the 450 nm region of the 2nd derivative spectrum and the cytochrome P-450 concentration determined from the 0th derivative spectrum was found useful for the quantification of cytochrome P-450. When the cells of Saccharomyces cerevisiae were grown under certain conditions, their 2nd derivative spectra showed double components around 450 nm and also around 420 nm. One of the components around 420 nm was assigned to cytochrome $\underline{a}_3$-CO.

INTRODUCTION

The method commonly used for the quantification of cytochrome P-450 is that of Omura and Sato (1). It is based on the measurement of a carbon monoxide (CO) difference spectrum from the reduced sample, and on the calculation of the amount of cytochrome P-450 from the height of the absorption band near 450 nm. Frequently the sample contains additional CO-binding compounds which may overlap the spectrum of cytochrome P-450 and consequently interfere with quantification. This concerns, in particular, measurements made from crude enzyme preparations or whole cells (2-5). One interfering compound is cytochrome $\underline{a}_3$ which is one of the two heme containing components of cytochrome $\underline{c}$ oxidase (E.C.1.9.3.1). Cytochrome $\underline{a}_3$ shows a deep trough at 441-445 nm and possibly a peak or shoulder at 430 nm in the CO difference spectrum of crude preparations (5-9). Thus the detection of cytochrome P-450 is difficult in

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mixtures where the concentration of cytochrome P-450 is low and the concentration of cytochrome c oxidase is high (2). Similarly, due to overlapping spectra, high concentrations of compounds absorbing near 420 nm may mask the bands of cytochrome P-450 and cytochrome c oxidase (2,7-16).

Derivative spectroscopy is a technique for obtaining derivatives of absorbance with respect to wavelength (a review:17). The value of this technique as an analytical tool is based on its ability to resolve overlapping bands in the normal (0th derivative) spectrum (18-20). In this report we show that the second-derivative spectroscopy can be utilized to quantificate cytochrome P-450 and to resolve the "hidden" peak of cytochrome a_3 in a suspension of whole yeast cells.

MATERIALS AND METHODS

Culturing of yeast.

Saccharomyces cerevisiae (NCYC 240) was maintained on slopes containing (in 1 litre of deionized water): 10 g of yeast extract, 20 g of peptone, 5 g of NaCl, 20 g of D-(+)-glucose and 15 g of bacto agar (21). A whole slope, grown for 2 days at 30°C, was used to inoculate 1 litre of prewarmed liquid medium (initial glucose concentration varied). The culture was incubated at 30°C with or without aeration. Foreign chemicals (chrysene, 3-methylcholanthrene, hexachlorophene), if used, were added at the beginning of the cultivation in a solution of 5 mg of the chemical/ml of dimethylsulfoxide. The control cultivation contained 1 ml of dimethylsulfoxide. Growth of the yeast was followed turbidimetrically at 600 nm. Samples containing about 0.6 g of wet cells were drawn at intervals. The cells were centrifuged at 2500 g for 5 min, washed with cold deionized water, and frozen at -20°C until analyzed.

Spectroscopic measurements.

Cytochrome P-450 was measured as described by Kärenlampi et al.(21). The samples were thawed and suspended in 6 ml of cold 0.1 M potassium phosphate buffer, pH 7.4. A few grains of sodium dithionite were added and the suspension was divided into two cuvettes (light path:1 cm). CO was bubbled through the sample cuvette. The recordings of the CO difference spectra were carried out at room temperature using a Cary Model 219 spectrophotometer (Varian Associates, Inc., Palo Alto, CA, U.S.A.). The height of the peak at about 450 nm was measured. The concentration of cytochrome P-450 was calculated by assuming that the absorptivity for the difference in absorption of the hemoprotein between 450 and 500 nm is 92 l $mmol^{-1}$ cm^{-1} (22). The results are expressed as nmoles of cytochrome P-450/mg of protein. The 2nd derivative spectra were recorded from the same samples with the same spectrophotometer using the derivative/log A accessory inserted between the signal output and the recorder input. The accessory differentiates the analog signal for the recorder with

respect to time (d^2A/dt^2). Following conditions were used: d^2A range ±10 A/min^2, response time (period) 1.0 sec, spectral band width 2.5 nm, and scan rates 10.0 nm/sec or 5.0 nm/sec. The reproducibility of repetitive scannings was high with both scan rates.

Protein.

Protein was measured by the method of Stewart (23). Bovine serum albumin was used as a standard.

Chemicals.

Bovine serum albumin was purchased from Sigma Chemical Co., St.Louis, Mo, U.S.A.. Chrysene was obtained from Merck-Schuchardt (München, Germany), 3-methylcholanthrene from Koch-Light Laboratories (Colnbrook, England) and hexachlorophene from Yliopiston Apteekki (Helsinki, Finland).

RESULTS AND DISCUSSION

Quantification of cytochrome P-450

The carbon monoxide difference absorption spectrum of reduced yeast (*Saccharomyces cerevisiae*) cells grown on 5% glucose medium without aeration, and its 2nd derivative spectrum are presented in Fig.1. Theoretically, the inflection points of a symmetrical absorption band (0th derivative spectrum) should appear as maxima and the absorption maximum should become a minimum in the 2nd derivative spectrum. The minima in our 2nd derivative spectra were shifted to blue as compared to the absorption maxima in the corresponding normal spectra. This was partly due to the electronic differentiation process and partly to the working conditions. The most important factor in determining the wavelength shift was the scan rate. When the scan rate increased from 5 to 10 nm/sec, the shift increased from 8 to 13 nm (cf.19). The amplitude of the derivative signal also increased when the scan rate increased. Higher resolution but lower amplitude was achieved by lowering the scan rate.

For quantitative evaluation, the vertical distance between maximum (a) and the adjoining minimum (b) in the 2nd derivative spectrum (Fig.1) was used as characteristic for cytochrome P-450. A linear correlation between distance a-b and the concentration of cytochrome P-450 calculated as presented in Ma-

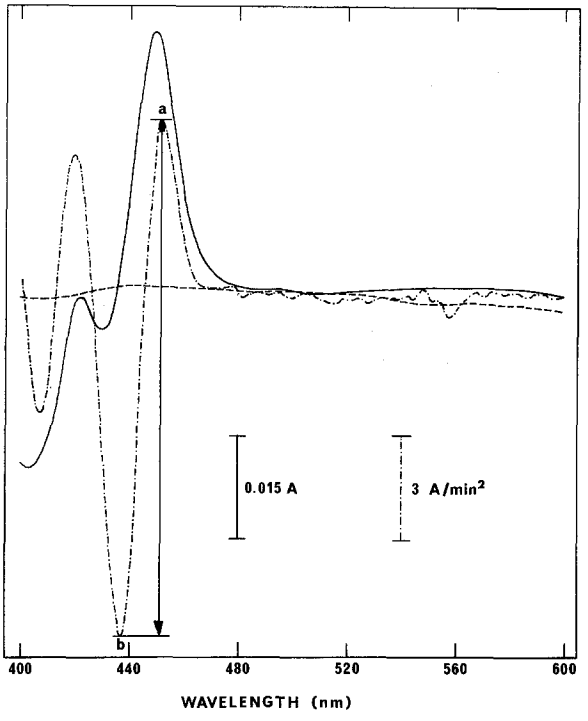

Fig. 1. CO difference absorption spectrum and its 2nd derivative from reduced yeast (Saccharomyces cerevisiae) cells. The cells were grown for 12 h 45 min in 5% glucose medium. --- baseline (reduced vs. reduced absorption spectrum); —— CO difference absorption spectrum; -·-·- CO difference 2nd derivative spectrum. Scan rate: 10 nm/sec.

terials and Methods, was obtained for both scan rates (Fig.2). The correlation coefficients were 0.954 (scan rate 10) and 0.965 (scan rate 5). The

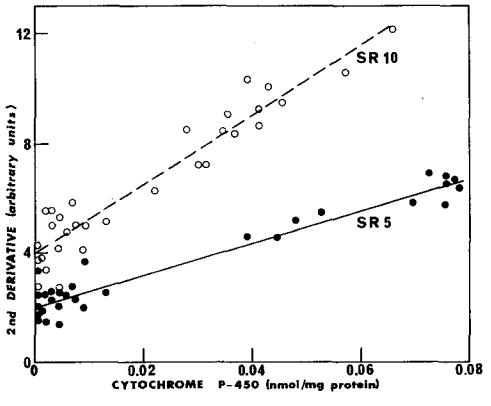

Fig. 2. Second derivative as a function of the amount of cytochrome P-450 with scan rates 10 (SR10) and 5 (SR5). The second derivative amplitudes (see Fig.1) were calculated per protein concentration (mg/ml).

following equations were found for the straight lines: $y = 127x + 4$ for scan rate 10 and $y = 59x + 2$ for scan rate 5.

It is evident from Figs. 1 and 2 that the 2nd derivative spectroscopy offers two advantages over normal absorption spectroscopy for the quantification of cytochrome P-450 in complex biological mixtures. Firstly, the signal amplitude can be increased to about two-fold, which implies a higher sensitivity. Secondly, the interfering effect of other absorbing compounds can be diminished or eliminated.

Further spectral analysis of yeast cells

The highly resolved 2nd derivative spectra allow one to obtain distinct signals to components possessing similar absorption properties. Thus, when the yeast was cultivated e.g. in 0.5% glucose medium (plus aeration) the normal CO difference absorption spectrum showed only one band at the 420 nm region. The intensity of the band increased in the course of the cultivation. At the early stages of growth also the 2nd derivative spectra showed only one and at the 420 nm region. However, when the culture grew older, first a shoulder and later a separate band appeared in the spectra (Fig.3:I). Commercial yeast has been reported to contain cytochrome a_3 as the only CO-binding pigment (7). A suspension of commercial yeast gave a CO difference spectrum (Fig.4) very similar to the spectrum of cytochrome c oxidase (6). The 2nd derivative spectrum showed a great similarity with the component appearing at the later growth stages of the yeast (Fig.4). Thus the component is assignable to cytochrome a_3-CO. Possible candidates for the component at the lower wavelength are cytochrome o (8,24,25), cytochrome c peroxidase (2,4,7), a hemoglobin-like pigment (24,26), catalase (27), cytochrome c (10), a degradation product of cytochrome b (8) or cytochrome o (24), nonspecific binding of excess heme (28), or structurally modified cytochrome P-450 (2,15,22,29, 30).

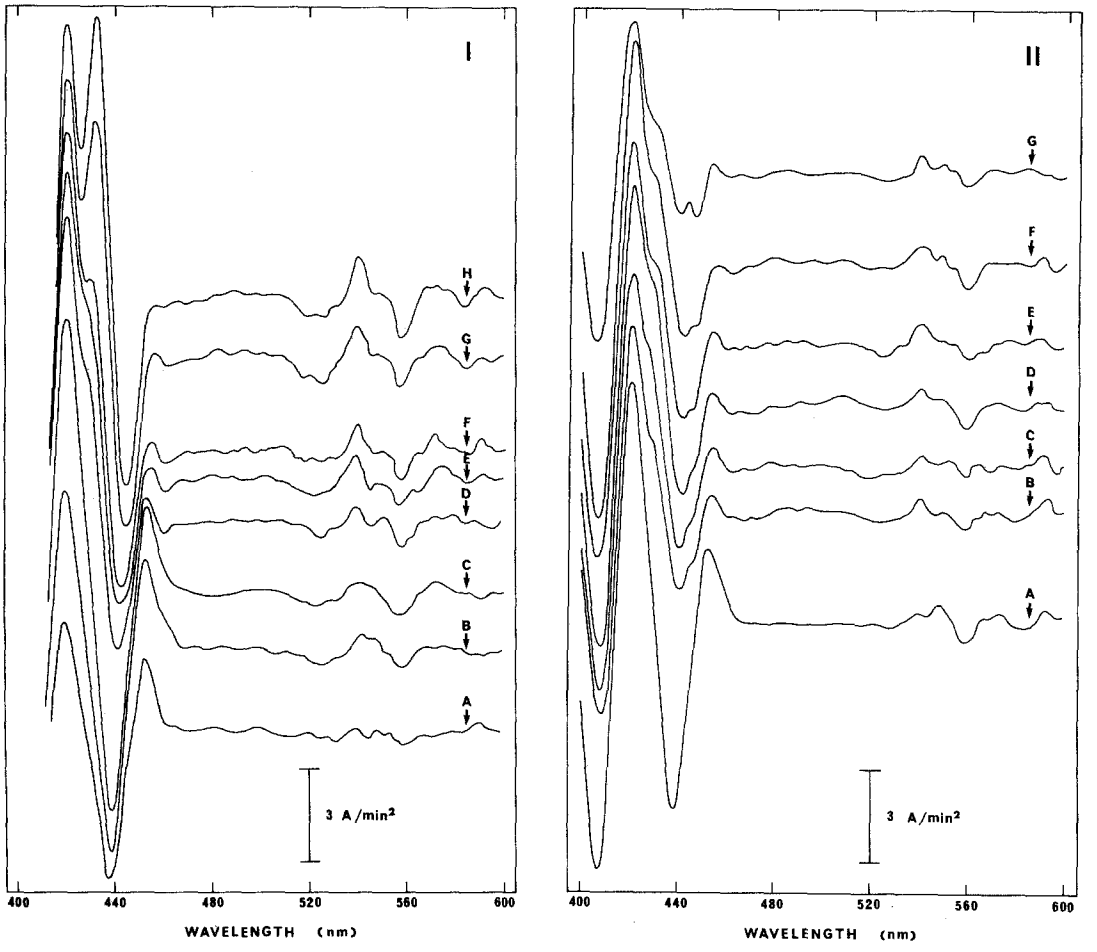

Fig. 3. CO difference 2nd derivative spectra of reduced yeast cells at different growth stages. I. Cells grown aerated in 0.5% glucose medium. Growth times: A. 10 h 15 min, B. 12 h 45 min, C. 16 h 50 min, D. 20 h 50 min, E. 24 h 15 min, F. 27 h 30 min, G. 31 h 40 min, H. 35 h 30 min. II. Cells grown aerated in 0.5% glucose medium in the presence of 5 mg of chrysene/l. Growth times: A. 16 h 50 min, B. 20 h 50 min, C. 24 h 15 min, D. 27 h 30 min, E. 31 h 40 min, F. 35 h 30 min, G. 39 h 30 min. Scan rate: 10 nm/sec.

The fact that the 2nd derivative spectroscopy is capable of resolving cytochrome a_3 from other components offers new possibilities for the quantification of this cytochrome. On calibration with purified cytochrome a_3, the method could be used to measure the formation and degradation of this cytochrome in whole yeast cells. This might be of great value for studies concerning respiratory metabolism and bioenergetics.

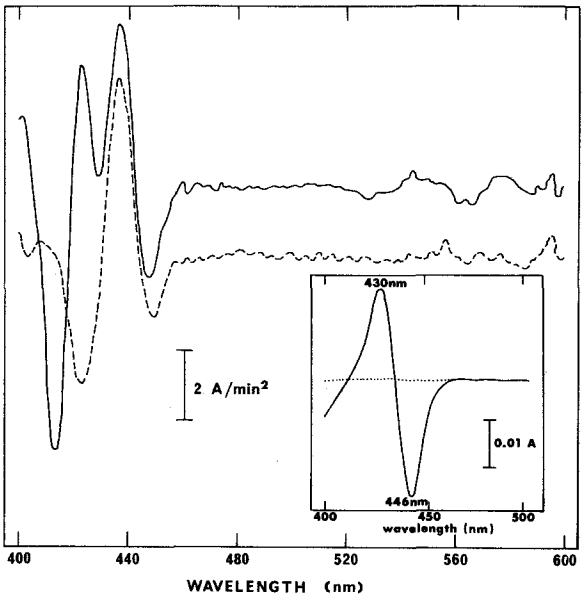

Fig. 4. Resolution of cytochrome a_3 in CO difference 2nd derivative spectra of reduced yeast cells. —Cells grown for 35 h 30 min aerated in 0.5% glucose medium. --- Commercial yeast cells (0.05 g/ml). Small figure: CO difference absorption spectrum from reduced commercial yeast cells.

Also the cytochrome P-450 band was observed to split into two bands when the 2nd derivative spectra were run (Fig.3:II). The splitting occurred at later stages of growth and was most evident when the cultivation proceeded in the presence of a foreign compound like chrysene, 3-methylcholanthrene or hexachlorophene, but was observed also in some control cultivations. The origin of the band is unknown. It may represent a new type of cytochrome P-450 or a degradation product of cytochrome P-450. However, the 2nd derivative spectrum obtained from Aroclor 1254 -induced rat liver 9000 g supernatant did not show double bands, although this PCB-mixture is known to contain compounds inducing cytochrome P-450 and compounds inducing cytochrome P-448 (31).

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REFERENCES

1. Omura, T., and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378.
2. Ishidate, K., Kawaguchi, K., Tagawa, K., and Hagihara, B. (1969) J. Biochem. (Tokyo) 65, 375-383.
3. Daniel, R. M., and Appleby, C. A. (1972) Biochim. Biophys. Acta 275, 347-354.
4. Delaissé, J. M., and Nyns, E. J. (1974) Arch. Int. Physiol. Biochem. 82, 179.
5. Ferris, J. P., MacDonald, L. H., Patrie, M. A., and Martin, M. A. (1976) Arch. Biochem. Biophys. 175, 443-452.
6. Yonetani, T. (1960) J. Biol. Chem. 235, 845-852.
7. Lindenmayer, A., and Smith, L. (1964) Biochim. Biophys. Acta 93, 445-461.
8. Cartledge, T. G., Lloyd, D., Erecińska, M., and Chance, B. (1972) Biochem. J. 130, 739-747.
9. Poole, R. K., Lloyd, D., and Chance, B. (1974) Biochem. J. 138, 201-210.
10. Appleby, C. A. (1969) Biochim. Biophys. Acta 172, 71-87.
11. Cartledge, T. G., and Lloyd, D. (1972) Biochem. J. 127, 693-703.
12. Duppel, W., Lebeault, J.-M., and Coon, M. J. (1973) Eur. J. Biochem. 36, 583-592.
13. Murphy, G., Vogel, G., Krippahl, G., and Lynen, F. (1974) Eur. J. Biochem. 49, 443-455.
14. Vygantas, A. M., and Corcoran, J. W. (1974) Fed. Proc. 33, 1233.
15. Gallo, M., Roche, B., and Azoulay, E. (1976) Biochim. Biophys. Acta 419, 425-434.
16. Cerniglia, C. E., and Gibson, D. T. (1978) Arch. Biochem. Biophys. 186, 121-127.
17. O'Haver, T. C., and Green, G. L. (1975) Int. Lab. May/June, 11-18.
18. Butler, W. L. (1979) Methods Enzymol. LVI, 501-515.
19. Balestrieri, C., Colonna, G., Giovane, A., Irace, G., and Servillo, L. (1980) Anal. Biochem. 106, 49-54.
20. Ruckpaul, K., Rein, H., Ballou, D. P., and Coon, M. J. (1980) Microsomes, Drug Oxidations, and Chemical Carcinogenesis, pp. 37-45, Academic Press, New York.
21. Kärenlampi, S. O., Marin, E., and Hänninen, O. O. P. (1981) Biochem. J. 194, 407-413.
22. Yoshida, Y., Aoyama, Y., Kumaoka, H., and Kubota, S. (1977) Biochem. Biophys. Res. Commun. 78, 1005-1010.
23. Stewart, P. R. (1975) Methods Cell Biol. 12, 114.
24. Mok, T. C. K., Rickard, P. A. D., and Moss, F. J. (1969) Biochim. Biophys. Acta 172, 438-449.
25. Baroncelli, V., Boccalon, G., Giannini, I., and Renzi, P. (1979) Mol. Cell. Biochem. 28, 3-6.
26. Ishidate, K., Kawaguchi, K., and Tagawa, K. (1969) J. Biochem. (Tokyo) 65, 385-392.
27. Cartledge, T. G., and Lloyd, D. (1972) Biochem. J. 126, 381-393.
28. Yu, C.-A., and Gunsalus, I. C. (1974) J. Biol. Chem. 249, 107-110.
29. Yoshida, Y., and Kumaoka, H. (1975) J. Biochem. (Tokyo) 78, 785-794.
30. Gilewicz, M., Zacek, M., Bertrand, J.-C., and Azoulay, E. (1979) Can. J. Microbiol. 25, 201-206.
31. Goldstein, J. A., Hickman, P., Bergman, H., McKinney, J. D., and Walker, M. P. (1977) Chem.-Biol. Interactions 17, 69-87.