CYTOCHROMES IN BOVINE LIVER NUCLEAR MEMBRANES

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Received April 16, 1971

SUMMARY

Cytochrome a,a_3 has been detected in highly purified nuclear membrane preparations. Nuclear membranes resemble microsomal membranes in cytochrome b_5 content but differ by the absence of the microsomal cytochrome P-450. Cytochrome a,a_3 content in nuclear membranes is 30% that of mitochondria, and cytochrome b_5 content is 33% of control microsomes.

Earlier studies by Penniall and co-workers (1,2) indicated that rat liver nuclei contained no cytochrome b_5. In contrast cytochrome b_5 has been reported, in rat liver nuclei by Conover and Siebert (3), and more recently by Fleischer et al. (4) in bovine liver nuclei. However, since the analysis was performed with intact nuclei, the localization of cytochrome b_5 could not be definitely ascertained, and the possibility existed that it was merely a microsomal contaminant. Also none of the above investigators were able to detect cytochrome a,a_3, and it was concluded that this cytochrome is absent from liver nuclei (3).

Since cytochromes appear to be universally associated with membrane bound electron transport systems, it is desirable to use membrane fractions highly purified from contaminating membranes to accurately determine the cytochrome content of a particular membrane. To be most meaningful, even trace quantities of contaminating membranes should be carefully evaluated and taken into account in cytochrome estimations. This report attempts such an analysis using highly purified preparations of nuclear membranes.

METHODS

Nuclear membranes were isolated from bovine liver nuclei as previously described (5) with the following modifications. After resuspension of the

DNase treated nuclei in sucrose TKM (0.25M sucrose, 0.05M Tris-HCl, pH = 7.5, 0.025M KCl, 0.005M $MgCl_2$) (6) made 0.5M in $MgCl_2$, the suspension was layered directly on a discontinuous sucrose gradient consisting of 15 ml of 0.5M $MgCl_2$-2.2M sucrose TKM and 15 ml of 0.5M $MgCl_2$-1.6M sucrose TKM. After centrifugation for 30 minutes in the Spinco Sw 25.2 rotor at 25,000 rpm, the purified nuclear membranes were collected at the 0.25M-1.6M sucrose interface, washed in 0.5M $MgCl_2$ sucrose TKM and centrifuged at 104,000xg for 30 minutes. The pellets were then washed in sucrose TKM, centrifuged and finally resuspended in a small amount of sucrose TKM. Mitochondria and microsomes, treated in exactly the same way as nuclei, were isolated from the same homogenate (5).

Mitochondria contamination was estimated by assuming that succinate dehydrogenase activity is exclusively mitochondrial. The % of mitochondrial protein in nuclear membrane was then calculated by comparing its succinate dehydrogenase activity with the mitochondrial value. The assay was performed as previously described (7) according to the procedure of King (8). Microsomal contamination was measured in a similar manner using NADPH-cytochrome c reductase as a marker for microsomal membranes (7) according to Ernster, et al. (9).

Reductions for difference spectra were achieved by addition of a few grains of dithionite. Cytochrome b_5 was estimated from the difference spectrum at 426-410 nm using an extinction coefficient of 160 cm^{-1} mM^{-1} (10).

Difference spectra for cytochrome P-450 were obtained by measuring the difference between the sample reduced with dithionite and treated with carbon monoxide versus the sample reduced with dithionite. The reduction and carbon monoxide treatment were performed under anaerobic conditions by means of Thunberg cuvettes. An extinction coefficient of 91 cm^{-1} mM^{-1} at 450-490 nm was used to calculate P-450.

In order to measure the α band of cytochrome a,a_3, a protein concentration of 9 mg/ml was used, and turbidity reduced by the addition of 0.05 ml of 10% deoxycholate (sodium salt) per ml of suspension. An extinction coefficient of 13.1 cm^{-1} mM^{-1} at 605-630 nm was used in calculating cytochrome a,a_3

content (11). All spectral recordings were obtained on a Shimadzu MPS-50L recording spectrophotometer.

RESULTS

The typical dithionite reduced versus oxidized difference spectrum of nuclear membranes, shown in Figure 1B, reveals absorption peaks at 556, 526 and 426 nm which are identical to those of cytochrome b_5 seen in difference spectra of microsomal membranes (Figure 1A). The nuclear membrane difference spectrum also shows a shoulder at 445 nm which is absent in the microsomes (Figure 1A), but present in mitochondria (Figure 1C). This 445 nm absorption is the characteristic soret band for cytochrome a,a_3. A cytochrome a,a_3 α band however, is not visible in nuclear membranes and is barely visible in mitochondria (Figures 1A and 1C).

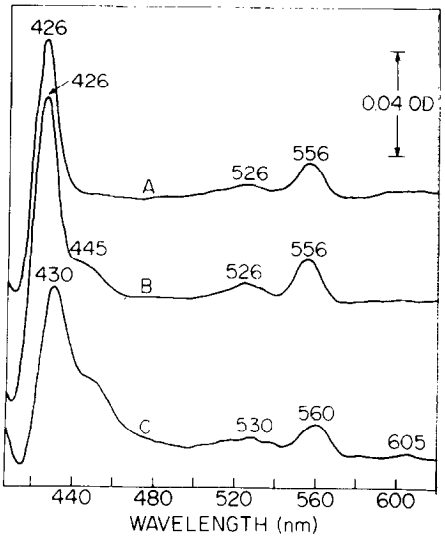

Figure 1. Reduced versus oxidized difference spectra.

A. Microsomes - 0.49 mg protein/ml
B. Nuclear Membranes - 1.96 mg protein/ml
C. Mitochondria - 2.06 mg protein/ml

In order to demonstrate the α band of cytochrome a,a_3 more clearly, a much higher protein concentration is used as described in Methods. Nuclear membranes (Figure 2B) show absorption peaks at 604 and 556 nm corresponding to the α bands of cytochromes a,a_3 and b_5 respectively. The mitochondrial

absorption peaks are at 604 and 559 nm (Figure 2A), demonstrating the α bands of cytochromes a,a_3 and the mitochondrial b type cytochromes (13). The absence of even a shoulder in the 560 nm region of the nuclear membrane spectrum (Figure 2B) suggests the absence of mitochondrial type b cytochromes in nuclear membranes.

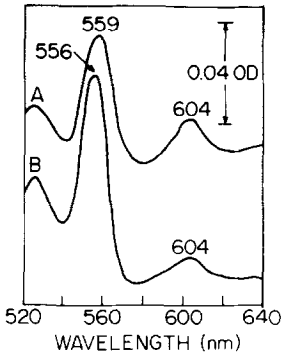

Figure 2. Reduced versus oxidized difference spectra.

A. Mitochondria - 9 mg protein/ml
B. Nuclear Membranes - 9 mg protein/ml

Figures 3A and 3B show the difference spectra for cytochrome P-450 in microsomes and nuclear membranes respectively. The nuclear membranes have a peak at 450 nm, but its size is clearly much less than in microsomes. The microsomes also contain a small amount of P-420 as judged by the peak at 425 nm.

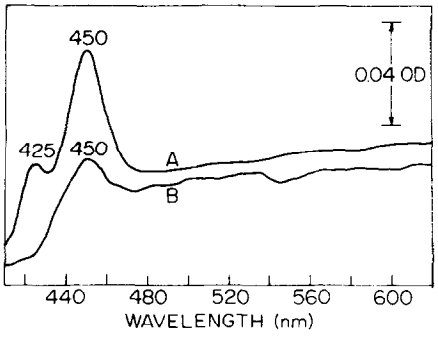

Figure 3. Reduced carbon monoxide versus reduced difference spectra.

A. Microsomes - 0.43 mg protein/ml
B. Nuclear Membranes - 2 mg protein/ml

Table 1

Cytochrome Content of Nuclear Membranes

Assay	Mitochondria	Microsomal Membranes	Nuclear Membranes	Nuclear Membranes Corrected for Contamination
Succinate dehydrogenase[a]	0.099	0.0014(1.4%)[e]	0.0037(3.7%)	
NADPH-cyt c red[b]	-----	0.080	0.0052(6.5%)	
cyt b_5[c]	-----	1.02	0.398	0.332
cyt P-450[c]		1.24	0.055	0
cyt a,a_3[c]	0.152	0	0.051	0.045
cyt oxidase[d]	0.819	0.012	0.284	0.254
$\frac{\text{cyt a,a}_3}{\text{cyt oxidase}}$	0.186	0		0.177

[a] μmoles 2,6-dichlorophenolindophenol reduced/min/mg protein

[b] μmoles cytochrome c reduced/min/mg protein

[c] nmoles cytochrome/mg protein

[d] μmoles 0_2/min/mg protein

[e] % mitochondrial or microsomal protein contamination

No P-420 is detected in nuclear membranes. Further proof that the absorption peaks at 450 nm actually correspond to cytochrome P-450 is obtained by adding deoxycholate to both cuvettes. This shifts the 450 nm peak to a 425 nm peak characteristic of P-420 which is a solubilized form of P-450.

Table 1 presents the cytochrome content of the various membranes on a protein basis. Cytochromes in nuclear membranes are corrected for slight mitochondrial and microsomal contamination by use of appropriate marker enzymes (see Methods).

The corrected values of cytochromes b_5 and a,a_3 are 0.332 and 0.045 nmoles/mg protein respectively. The corresponding value of cytochrome b_5 in microsomes is 1.02 nmoles/mg protein, and of cytochrome a,a_3 in mitochondria is 0.152 nmoles/mg protein. The cytochrome P-450 content of nuclear membranes, 0.055 nmoles/mg protein, is 4.4% of the microsomal value of 1.24 nmoles/mg protein, and is easily accounted for by the estimated 6.5% microsomal protein contamination.

DISCUSSION

Since the corrected values for cytochromes b_5 and a,a_3 show that 83% and 88% of the respective cytochrome can not be accounted for by contamination, it is concluded that cytochrome b_5 and cytochrome a,a_3 are endogenous to nuclear membranes. The cytochrome b_5 value of 0.332 nmoles/mg nuclear membrane protein is nearly twice the value recently reported by Kasper (14), and almost 10 fold higher than reported by Franke *et al.* (15) working with rat liver nuclear membranes.

The very low amount or total absence of cytochrome P-450 in bovine liver nuclear membranes clearly distinguishes nuclear membranes from microsomal membranes which have a P-450 content of 1.24 nmoles/mg protein. Recently Kasper (14) was unable to detect P-450 in 7 out of 10 rat liver nuclear membrane preparations. It is possible that the other three preparations were contaminated with microsomes. Franke *et al.* (15) have reported a small amount of P-450 in rat liver nuclear membranes which was 14% that of control microsomes.

The spectrophotometric studies reported here, showing the presence of

cytochrome a,a_3, are consistent with previous evidence of cytochrome oxidase activity in nuclear membranes (16,17). Moreover the ratio of cytochrome a,a_3 content to cytochrome oxidase activity is similar in mitochondria and nuclear membranes (Table 1).

ACKNOWLEDGEMENTS

The research has been supported by the National Institute of General Medical Science under Grants GM 10741 and 5T1 GM 1195. We are grateful to Dr. W. A. Cramer for use of the Shimadzu spectrophotometer.

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