ISOLATION AND CHEMICAL COMPOSITION OF THE NADH: SEMIDEHYDROASCORBATE OXIDOREDUCTASE RICH MEMBRANES FROM RAT LIVER

Dieter GEIß* and Hans-Ulrich SCHULZE
Zentrum für Biochemie der Universität Gießen, 63 Gießen Friedrichstrasse 24, G.F.R.

Received 11 October 1975

1. Introduction

The enzymatic reduction of semidehydroascorbate to ascorbate is catalysed by NADH: semidehydroascorbate oxidoreductase (EC 1.6.5.4.) (SOR) [1] which have been studied both in *Neurospora crassa* [2] and in various subcellular particles from many mammalian organs [3], especially in the rat liver microsome fraction [3–6]. It is generally accepted that the microsome fraction usually isolated by differential centrifugation [7] is heterogeneous and contains plasma membranes, Golgi membranes, and endoplasmic membranes as well as low contaminations of mitochondria, lysosomes and peroxisomes.

We have shown recently that the SOR does not sediment with glucose 6-phosphatase, NADH: ferricytochrome b_5 oxidoreductase, cytochrome b_5, NADPH: ferricytochrome *c* oxidoreductase and cytochrome *P*-450 when original microsomes are fractionated by zonal centrifugation, but the SOR is bound to a membrane fraction with an especially low density [8]. This finding is in agreement with the earlier report on our first results on the fractionation of original microsomes by differential centrifugation [8,9].

In the present paper we report the results of experiments carried out in our laboratory indicating that the SOR is localized in a hitherto not identified vesicle fraction.

2. Materials and methods

Cytochrome *c*, 5′-adenosinmonophosphate, glucose 6-phosphate, glycerate 2-phosphate, and reduced pyridine nucleotides were obtained from Boehringer Mannheim GmbH. All the other reagents were of analytical grade. Dehydro-L(+)ascorbate was produced by the method of Staudinger and Weis [10] and trypsin-solubilized cytochrome b_5 was isolated from pig liver microsomes according to the procedure of Strittmatter [11].

Male Wistar rats (AF/Han.) weighing about 200 g and fasted for 12 h were used. Liver microsomes were prepared essentially by the method of Schneider [7] modified as described earlier [8]. Rat liver plasma membranes were isolated by the method of Ray [12] and Golgi membranes were prepared from the liver of ethanol-treated rats using the procedure worked out from Ehrenreich et al. [13]. The isolation and purification of a subcellular fraction composed mainly of SOR-rich membranes were performed by the following combined differential and density gradient centrifugation. Livers from exsanguinated rats were minced thoroughly with scalpels and homogenized mildly in an ice-cold solution (40 ml per 30 g of liver) containing 0.25 M sucrose, 5 mM $MgCl_2$ buffered at pH 7.5 with 5 mM Tris–HCl. The homogenisation was performed in a Teflon–glass Potter–Elvehjem homogenizer (A. H. Thomas, Size C) at low speed (400 rev/min, Multifix constant) with only two complete strokes. All subsequent procedures were conducted at 0 to 4°C. The homogenate was centrifuged at 2300 g_{max} for 20 min in a Spinco ultracentrifuge (rotor SW 25.1). The brownish yellow, upper one-third of the pellet was suspended to 26.5 ml 5 mM Tris–HCl pH 7.5, homogenized (homogenizer size C) with 5 complete strokes at 2800 rev/min, and then 8.5 ml of this suspension (fraction 1), respectively, layered onto the following discontinuous sucrose gradient: 10 ml 1.0 M

*This work contains the Dissertation from D. Geiß

North-Holland Publishing Company – Amsterdam

and 14 ml 0.75 M sucrose. The centrifugation was performed at 90 100 g_{max} for 5 h (Spinco rotor SW 25.1). The membrane fraction at the 0.75 M/1.0 M sucrose interphase was collected with a Pasteur pipette, diluted with 1 mM EDTA, 0.1 M Tris–HCl pH 7.5 (buffer A) to 108 ml and subsequently pelleted at 105 700 g_{max} for 60 min (Spinco rotor 30). The pellets were homogenized (5 full strokes, homogenizer size A, 2800 rev/min) in approx. 3 ml buffer A, adjusted to 41% sucrose by the addition of 70% sucrose, and 4 ml of this preparation (fraction2) were placed in a tube. The sample was overlayed with 8 ml 31.8%, 10 ml 30.4%, and 10 ml 28.4% sucrose. After centrifugation for 10 h at 90 100 g_{max} (Spinco rotor 25.1), the material at the 28.4%/30.4% sucrose interphase was taken out with a Pasteur pipette, diluted with buffer A to 30 ml, centrifuged at 105 700 g_{max} for 60 min (Spinco rotor 30), and the pellet was resuspended to 2.5 ml of buffer A. This sample was designated SOR-rich membrane fraction (fraction 3). All sucrose solutions (w/w) were buffered with 5 mM Tris–HCl pH 7.5 and adjusted by means of Abbe refractometer (Fa. C. Zeiss, Oberkochen, Württ.).

The activities of the following enzymes were measured as described previously: 5′-nucleotidase (EC 3.1.3.5) [14], Na^+-K^+-Mg^{++} ATPase (EC 3.6.1.4) [15], glucose 6-phosphatase (EC 3.1.3.9) [16], NADPH: ferricytochrome *c* oxidoreductase (EC 1.6.99.?) [17], NADH: ferricytochrome b_5 oxidoreductase (EC 1.6.2.2) [4], NADH: semidehydroascorbate oxidoreductase (EC 1.6.5.4) [4], succinate dehydrogenase (EC 1.3.99.1) [18], and acid phosphatase (EC 3.1.3.2) [19].

Protein was assayed by the biuret method of Bode et al. [20]. The phospholipids were extracted with chloroform/methanol 2:1 (v/v) [21], separated by one-dimensional t.l.c. [22] and determinated by inorganic phosphate analysis [23]. Electron micrographs were obtained with a Siemens Elmiskop 101; experimental details are given in [24].

3. Results and discussion

Table 1 shows the results of a typical purification experiment. About 2 mg protein was recovered in the SOR-rich membrane fraction from about 6 g homogenate protein obtained from 30 g wet weight of rat liver. The specific activity of SOR in this purified fraction was increased about 50-fold over the homogenate with significant differences (40- to 60-fold enrichment with specific activities from 720 to 1060 mU/mg protein, respectively) dependent on the homogenisation procedure and the collection of the friable brownish yellow pellet which is suspended as fraction 1. Other details of the purification procedure appeared less critical. In general a very good total recovery of protein and enzymic activities after centrifugation was obtained.

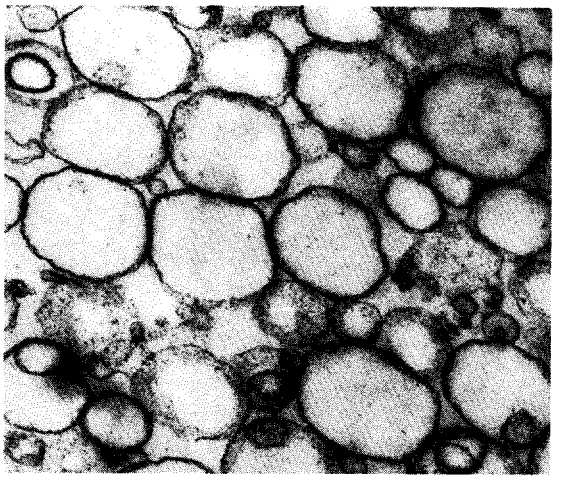

Fig.1. Electron micrograph of purified SOR-rich membranes characterized by large empty vesicles (final magnification X 24 570).

Table 2 summarizes the distribution of several enzymatic activities in some subcellular fractions of rat liver. The specific activities of generally accepted specific organelle marker enzymes in the original microsomes, plasma membranes, and the unknown membrane fraction were compared. It can be seen that purified material with very low activities of the marker enzymes which were measured can be obtained by the procedure described in Materials and methods. Moreover, this unknown membrane fraction is highly enriched with respect to the SOR. The purification ratio of this enzyme was usually about 30 over the original microsomes. On the other hand pure plasma membranes separated by the method of Ray [12] and Golgi-membranes prepared with the technicque used by Ehrenreich et al. [13] show little (5 mU/mg

Table 1
Purification of SOR-rich membranes from rat liver homogenate

Step	Protein	total	NADH: Semidehydroasc.-oxidored Sp. activity		Total		5'-Nucleotidase Sp. activity		Total		Glucose 6-phosphatase Sp. act.		Total	
	mg	%	mU/mg	ratio	Unit	%	mU/mg	ratio	Unit	%	mU/mg	ratio	Unit	%
Homogenate	6060	100	18	1.0	109.1	100	45	1.0	272.7	100	111	1.0	672.7	100
Fraction 1	606	10	37	2.1	22.4	20.5	52	1.2	31.5	11.6	118	1.1	71.5	10.6
Fraction 2	8.8	0.15	322	17.9	2.8	2.6	104	2.3	0.9	0.3	240	2.2	2.1	0.3
Fraction 3 (SOR-rich membranes)	2.2	0.04	850	47.2	1.9	1.7	110	2.4	0.24	0.09	93	0.8	0.2	0.03
Total recovery		87			84				81				90	

The results presented were obtained from a single homogenate, and are representative of 20 similar experiments. Recoveries represent the sum of the concentrations of a component in all fractions (see Materials and methods) relative to the concentration in the crude homogenate. SOR is expressed as NADH oxidized, phosphatases are expressed as P_i released.

Table 2
Enzyme profiles of some subcellular rat liver fractions

Marker enzyme for the subcellular structure	Activity	Specific activity (mU/mg protein) Microsomal fraction	Plasma membranes	ratio	SOR-rich membranes	ratio
Lysosomes	Acid phosphatase	6	–		4	0.7
Mitochondria, inner membranes	Succinate dehydrogenase	7	7	1.0	4	0.6
outer membranes / Endoplasmic membranes	NADH: Ferricytochrome b_5 oxidoreductase	215	14	0.07	560	2.6
	Glucose 6-phosphatase[a]	500	45	0.09	85	0.17
	NADPH: Ferricytochrome *c* oxidoreductase	420	50	0.12	70	0.17
Plasma membranes	5′-Nucleotidase[a]	75	850	11.3	115	1.5
	Na^+, K^+, Mg^{++}-ATPase[a]	75	–		134	1.8
SOR-rich membranes	NADH: Semidehydroascorbate-oxidoreductase	31	5	0.16	910	29.4

SOR and all phosphatases are expressed as in table 1. Succinate dehydrogenase is expressed as nmoles 2,6-dichlorophenolindophenol reduced/min/mg protein. Cytochrome reductases are expressed as nmoles cytochrome reduced/min/mg protein.

[a]Assayed at 37°C, all others assayed at 30°C. The ratios are given with respect to the specific activity of the microsomal fraction (original microsomes) taken as 1. Typical results which are representative of 10 to 20 similar experiments.

Table 3
Phospholipid content and composition of subcellular rat liver fractions

Components	Microsomal fraction		SOR-rich membranes	
	Specific content[a]	%	Specific content[a]	%
Total phospholipids	872	100	1165	100
Sphingomyelin (S)	40	4.6	69	5.9
Phosphatidyl choline (PC)	507	58.1	617	53.0
Phosphatidyl serine + (PS+ Phosphatidyl inositol PI)	126	14.5	210	18.0
Phosphatidyl ethanolamine (PE)	199	22.8	269	23.1

Typical results which representative of 5 similar experiments.
[a]nmoles phospholipid-P/mg protein.

protein) and almost no (about 1 mU/mg protein) activity, respectively. Furthermore, preliminary investigations have indicated that SOR is probably not localized neither in the nuclear envelope isolated by the procedure worked out from Franke et al. [25].

Compared to the endoplasmic marker enzymes NADH: ferricytochrome b_5 oxidoreductase which is likewise bound to the endoplasmic reticulum shows some activity in the SOR-rich membrane fraction. It may merely be an index of the amount of the outer mitochondrial membranes present in this fraction but more probable is that SOR catalyses also slow NADH-linked reduction of cytochrome b_5 as we assumed earlier [2,4].

The SOR-rich membrane fraction can be additionally differentiated from original microsomes, most of which is derived from endoplasmic reticulum, by its higher specific phospholipid content expressed as nmoles of lipid-P per mg of protein (table 3). The phospholipid composition of both membranes is also different. The values for original microsomes given in table 3 are in good agreement with those previously found in our and other laboratories [5,26,27]. As already shown in table 2, the SOR-rich membrane fraction is contaminated only very slightly by other cytoplasmic membranes; their phospholipid composition should therefore be really representative. In contrast to the original microsomes, SOR-rich membranes contain relativly more PS + PI and relativly less PC. Plasma membrane was found to have a high content (19%) of S, the major phospholipid beeing PC (35%), PE and PS + PI representing 20% and 17% of the total, respectively [28,29]. Lysosomal membrane has a similar phospholipid composition [29]. SOR-rich membranes, however, are markedly richer in PC and contain essentially less S.

It should be mentioned here that the presence of S in the SOR-rich membrane fraction, which nearly parallels that of the 5'-nucleotidase activity during the purification procedure, reflects possibly the slightly contamination of the SOR-rich membrane preparation by plasma membranes and perhaps lysosomal membranes. Therefore, the SOR-rich membrane probably does not contain a significant amount of S as lipid constituent.

Electron microscope examination of fractions containing purified SOR-rich membranes revealed a predominance of large, approximately circular, and empty, smooth surfaced vesicles ranging in size from 300 to 800 nm without any apparent contamination with structures typical of the plasma membrane or Golgi apparatus. In addition, smaller and more complicated profiles are seen which apparently represent oblique and tangential sections at various levels and angles through these vesicles. Some of the very small vesicles may be fragmentated large vesicles which converted again or, however, they may be derived from other intracellular sources, although it is not possible to make a definite identification.

In summary the variability of the SOR-rich membrane preparation obtained appears rather small and we can conclude that it is a reasonably representative and on biochemical as well as on morphological grounds, highly purified membrane fraction. Furthermore, our experimental data demonstrate that in rat liver SOR is definitely localized in hitherto not

identified membranes. It should be mentioned that the particles derived from these membranes and isolated by us could be identical with the large empty vesicles of uncertain origin, which are scattered among the heavy Golgi fraction described by Ehrenreich et al. [13]. Studies of the distribution of several enzymes in rat hepatic cells obtained essentially by the method of Berry and Friend [30], which are in progress, suggest that SOR does not occur in parenchym cells but in non-hepatocytes.

Acknowledgements

We are greatly indebted to Professor Dr Hj. Staudinger for his continued interest and for many helpful discussions and to Professor Dr R. Lange (Anatomisches Institut, University of Gießen) for excellent help in electron microscopy. We thank Miss R. Kannler for skilful technical assistance. This investigation was supported by the Deutsche Forschungsgemeinschaft (Schu 54/5).

References

[1] Kersten, H., Kersten, W. and Staudinger, Hj. (1957) Biochim. Biophys. Acta 24, 222.
[2] Schulze, H.-U., Schott, H.-H. and Staudinger, Hj. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1931–1942.
[3] Staudinger, Hj., Krisch, K. and Leonhäuser, S. (1961) Ann. N. Y. Acad. Sci. 92, 195–207.
[4] Schulze, H.-U., Gallenkamp, H. and Staudinger, Hj. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 809–817.
[5] Schulze, H.-U. and Staudinger, Hj. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 309–317.
[6] Schulze, H.-U., Gallenkamp, H. and Staudinger, Hj. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 391–406.
[7] Schneider, W. C. (1948) J. Biol. Chem. 176, 259–266.
[8] Schulze, H.-U. and Staudinger, Hj. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1659–1674.
[9] Schulze, H.-U., dissertation, Gießen 1965.
[10] Staudinger, Hj. and Weis, W. (1964) Hoppe-Seyler's Z. Physiol. Chem. 337, 284–285.
[11] Strittmatter, P. (1967) Methods in Enzymol. 10, 553.
[12] Ray, T. K. (1970) Biochim. Biophys. Acta 196, 1–9.
[13] Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, P. and Palade, G. E. (1973) J. Cell Biol. 59, 45–72.
[14] Michell, R. H. and Hawthorne, J. N. (1965) Biochem. Biophys. Res. Commun. 21, 333–338.
[15] Wallach, D. F. H. and Kamat, V. P. (1966) in: Methods in enzymology (Colowick, S. P. and Kaplan, N. O., eds.) Vol. VIII p. 164–172, Academic Press, New York.
[16] Harper, E. (1962) in: Methoden der enzymatischen Analyse (Bergmeyer, H.-U. ed.) p. 788–792, Verlag Chemie, Weinheim/Bergstr.
[17] Masters, B. S. S., Williams, jr., C. H. and Kamin, H. (1967) Methods in Enzymol. 10, 565–573.
[18] Green, D. H., Mii, S. and Kohout, P. M. (1955) J. biol. Chem. 217, 551–567.
[19] Wattiaux, R. and De Duve, C. (1956) Biochem. J. 63, 606–608.
[20] Bode, Ch., Goebell, H. and Stähler, E. (1968) Z. Klin. Chem. Klin. Biochem. 6, 418–422.
[21] Folch, J., Lees, M. and Sloane Stanley, G. H. (1957) J. biol. Chem. 226, 497–509.
[22] Parker, F. and Peterson, N. F. (1965) J. Lipid Res. 6, 455–460.
[23] Bartlett, G. R. (1959) J. biol. Chem. 234, 466–468.
[24] Schulze, H.-U., Pönnighaus, J. M. and Staudinger, Hj. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1195–1203.
[25] Franke, W. W., Deumling, B., Ermen, B., Jarasch, E. and Kleinig, H. (1970) J. Cell. Biol. 46, 379–395.
[26] Dallner, G. and Ernster, L. (1968) J. Histochem. Cytochem. 16, 611–632.
[27] Colbeau, A., Nachbaur, J. and Vignais, P. M. (1971) Biochim. Biophys. Acta 249, 462–492.
[28] Ray, T. K., Skipski, V. P., Barclay, M., Essner, E. and Archibald, F. M. (1969) J. biol. Chem. 244, 5528–5536.
[29] Henning, R., Kaulen, H. D. and Stoffel, W. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 1191–1199.
[30] Berry, M. N. and Friend, D. S. (1969) J. Cell. Biol. 43, 506–520.