The Reconstitution of Microsomal Redox Chains

A COMPARATIVE ANALYSIS OF THE EFFECTIVENESS OF MEMBRANE SELF-ASSEMBLY AND TEMPLATE BINDING OF ELECTRON CARRIERS

By ALEXANDER I. ARCHAKOV, GALINA I. BACHMANOVA, VYACHESLAV M. DEVICHENSKY, IRINA I. KARUZINA, NATALYA S. ZHEREBKOVA, GENNADY A. ALIMOV, GALINA P. KUZNETSOVA and ALEXANDER V. KARYAKIN

Central Research Laboratory of the Second Moscow Medical Institute after N. I. Pirogov, Moscow, U.S.S.R.

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A comparative analysis was made of the effectiveness of three methods for the reconstitution of microsomal electron-transfer chains, namely, self-assembly, incorporation of electron carriers into liposomes (non-specific template) and incorporation into 'ghosts' of microsomal vesicles (specific template). It was shown that when the 'ghosts' of the microsomal vesicles were used as a specific template extra cytochrome *b₅* and NADH-specific flavoprotein were incorporated into them, but cytochrome *P-450* and NADPH-specific flavoprotein were not incorporated into the membrane. As a result of the self-assembly and incorporation into liposomes all the electron carriers were present in the reconstituted membrane. Cytochrome *P-450* reactivation took place and the inactive form, cytochrome *P-420*, was converted into the active form, cytochrome *P-450*. Of the four enzyme hydroxylation systems studied, i.e. NADPH- and NADH-dependent *p*-hydroxylation of aniline, and NADPH- and NADH-dependent *N*-demethylation of dimethylaniline, only the NADH-dependent demethylation of dimethylaniline (60% of the initial value) and NADH-dependent *p*-hydroxylation of aniline (30% of the initial value) were reconstituted by self-assembly. NADPH oxidase and NADH oxidase activities were only properly reconstituted by self-assembly and incorporation into liposomes. In contrast, the NADPH-specific system of peroxidation of unsaturated fatty acids was reconstituted by specific template-binding.

Three methods of reconstitution of the different membrane enzyme systems have been used. The first involves no template being introduced into the system to be reconstituted, and the reconstitution proceeds by means of the self-assembly of solubilized membrane proteins and lipids (Hinkle *et al.*, 1972; Racker, 1972; Razin, 1972).

The second method is based on membrane proteins being incorporated into liposomes and bilayer membranes (Cherry *et al.*, 1971; Hammes & Schullery 1970; Razin, 1972; Rogers & Strittmatter, 1973; Sweet & Zull, 1970). Lipids inherent in these membranes, those isolated from other membranes and even synthetic ones have been used. Liposomes and bilayer membranes prepared from such lipids act as a non-specific template to which the proteins should bind. These methods were successfully used for the reconstitution of mitochondrial redox-chain (Racker, 1972) Ca^{2+} -activated ATPase* of sarcoplasmic reticulum (MacLennan *et al.*, 1971; Martonosi, 1968) and sugar-transporting systems of mycoplasma membranes (Razin, 1972).

The third method is when solubilized proteins bind to the membranes from which they were isolated (Strittmatter *et al.*, 1972), in which case the original membranes with the normal content of these proteins, or those from which the proteins have been removed, are, in fact, specific templates. Enomoto & Sato (1973), Rogers & Strittmatter (1973) and Strittmatter *et al.* (1972) have shown that extra isolated microsomal electron-carrier cytochrome *b₅* can also be bound to microsomal membranes.

In the present work a comparative analysis has been made of the effectiveness of the three methods for the reconstitution of redox chains of microsomal membranes. Reconstitution was carried out from the membrane proteins and lipids solubilized with sodium cholate. 'Ghosts' of microsomal vesicles, obtained by treating the microsomal fraction with a low concentration of a detergent, were used as specific templates. Liposomes prepared from egg phosphatidylcholine were used as non-specific templates.

Materials and Methods

Chemicals

NADPH and cytochrome *c* were obtained from

* Abbreviations: ATPase, adenosine triphosphatase; IDPase, inosine diphosphatase.

C. F. Boehringer, Mannheim, W. Germany; EDTA (disodium salt) and phosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.; Neotetrazolium Blue (sodium salt) was obtained from Chemapol, Praha, Czechoslovakia; dithiothreitol and dialysis sacs (N 250-7) were purchased from Calbiochem, Los Angeles, Calif., U.S.A.; sodium cholate was obtained from Merck, Darmstadt, W. Germany; NADH was obtained from Reanal, Budapest, Hungary. Aniline and potassium ferricyanide were analytical grade (Sojuzkhimreaktiv, Moscow, U.S.S.R.). Dimethylaniline was sublimated at 193°C *in vacuo* and was dissolved in 0.05M-HCl.

Buffers

Tris buffer was obtained from Calbiochem and was adjusted with HCl to pH 7.4.

Animals

Male albino rats (150–200 g) were starved overnight and killed by decapitation. Livers were perfused *in situ* with 1.15% KCl solution, containing 0.2mM-EDTA, 1mM-dithiothreitol, until free of haemoglobin. The tissue was minced and then homogenized in 3 vol. of this solution in a Dounce homogenizer (glass with a Teflon pestle) with 20 strokes. The homogenates were centrifuged for 20min at 14000 g in a MSE High-Speed 18 centrifuge. The microsomal fraction was obtained by centrifugation of the 14000 g supernatant fraction at 150000 g for 90min in a VAC-601 (Janetzki) centrifuge. The microsomal pellets were resuspended in the 100mM-Tris-HCl, containing 0.2mM-EDTA and 1mM-dithiothreitol (TED buffer) to a protein concentration of approx. 20mg/ml. The microsomal protein content was measured by the method of Lowry *et al.* (1951) in the presence of 0.1% sodium deoxycholate with crystalline bovine serum albumin as standard. The content of phospholipids was measured by the methods of Chen *et al.* (1956), and that of RNA by the method of Spirin (1958).

Enzyme assays

The rate of NADPH- and NADH-dependent *p*-hydroxylation of aniline was determined by measuring *p*-aminophenol formation. The incubation medium contained, in a total volume of 1 ml, 40mM-Tris-HCl buffer, 16mM-MgCl₂, 3mM-aniline and 3mM-NADPH plus 1.5mg of microsomal protein or 6mM-NADH plus 3mg of microsomal protein. The incubation was carried out at 37°C with continuous shaking for 20min and 60min for NADPH- and NADH-dependent *p*-hydroxylation respectively. The reaction was terminated by the addition of 15% (w/v) trichloroacetic acid. After centrifugation at

3000 g for 10min in a CLN-2 centrifuge 1.0ml of the supernatant was added to 0.5ml of 10% (w/v) Na₂CO₃ solution, then 1.5ml of 2% (w/v) phenol in 0.2M-NaOH (Hilton & Sartorelli, 1970) was added. The samples were incubated in a water bath at 37°C for 30min. The content of *p*-aminophenol was measured in a Hitachi-124 spectrophotometer at 630nm. The molar extinction coefficient of *p*-aminophenol was 1×10^4 litre·mol⁻¹·cm⁻¹.

The rate of *N*-demethylation of dimethylaniline was determined by measuring formaldehyde formation (Archakov *et al.*, 1974).

The content of cytochrome *b*₅ and cytochrome *P*-450 was determined by the method of Omura & Sato (1964) in a split-beam SF-10 spectrophotometer.

The activities of NADPH-cytochrome *c* reductase and NADH-cytochrome *c* reductase were determined kinetically at 30°C in a Hitachi-124 spectrophotometer, in a thermostatically controlled 1cm-path-length cuvette at 550nm. The incubation medium contained 330μM-NaCN, 50μM-cytochrome *c*, 100mM-Tris-HCl buffer and 100μM-NADH plus 10μg of protein or 100μM-NADPH plus 30μg of protein, in a total volume of 3ml. The molar extinction coefficient of cytochrome *c* (reduced minus oxidized) is 1.85×10^4 litre·mol⁻¹·cm⁻¹ (Dallner, 1963).

NADPH- and NADH-Neotetrazolium Blue reductase activities were measured in a thermostatically controlled 1cm-path-length cuvette at 30°C for 2–3 min in a Hitachi-124 spectrophotometer at 550nm. The incubation medium contained 100mM-Tris-HCl buffer, 20μM-Neotetrazolium Blue and 50μM-NADPH plus 100μg of protein or 50μM-NADH plus 20μg of protein, in a total volume of 3ml. The molar extinction coefficient of oxidized Neotetrazolium Blue is 2.88×10^4 litre·mol⁻¹·cm⁻¹ (Roerig *et al.*, 1972).

NADPH oxidase and NADH oxidase activity was recorded fluorimetrically. The incubation medium contained 100mM-Tris-HCl buffer, 33μM-NADPH or 33μM-NADH, in a total volume of 3ml. The reaction was initiated by the addition of 3mg of microsomal protein. The measurements were made at 30°C in a thermostatically controlled cuvette of a EF-3M fluorimeter supplied with an automatic recording device. Maximum transmittance of the excitation filter was at 366nm and that of the recording filter at 520nm.

The activity of IDPase was determined by the micromethod of Pokrovsky *et al.* (1968) by measuring the rate of P_i formation from IDP.

The rate of NADPH- and ascorbate-stimulated lipid peroxidations was determined by measuring malonic dialdehyde. The incubation medium contained 50mM-Tris-HCl buffer, microsomal protein (0.5mg/ml), 0.012mM-Fe(NH₄)₂(SO₄)₂·6H₂O, 0.4mM-Na₄P₂O₇·10H₂O, 1mM-NADPH or 1mM-ascorbate in a total volume of 1ml. The incubation

was carried out at 37°C for 20 min with continuous shaking. The reaction was interrupted by addition of 30% (w/v) trichloroacetic acid. The colour reaction was performed as described by Archakov *et al.* (1969).

The aerobic redox behaviour of cytochrome *b₅* was measured in a Hitachi-356 dual-wavelength spectrophotometer at $\Delta E_{408-424}$. The incubation medium contained 50 mM-Tris-HCl buffer and 3 mg of protein in a total volume of 3 ml at 25°C. The reaction was initiated by the addition of 7 μ M-NADH or 32 μ M-NADPH.

Egg phosphatidylcholine liposomes were prepared as described by Bangham (1968).

Reconstitution of microsomal redox chains

Preliminary experiments established that 0.15% sodium cholate ruptures the membranes of the microsomal fraction (fraction 1), but does not solubilize the electron carriers (see the legend to Fig. 1 for details). Therefore IDPase was used as a marker enzyme for the matrix of the microsomal vesicles (Karmanov *et al.*, 1972) and cytochrome *b₅* and cytochrome *P-450* served as marker enzymes localized in the membrane (Dallner, 1963).

In the experiments on reconstitution of microsomal redox chains the microsomal fraction had a protein concentration of about 20 mg/ml (fraction 1); 2–3 ml of this fraction was kept as a control and 50 ml (about 1 g of protein) was treated with 0.15% sodium cholate. The pellet obtained after centrifugation at 150 000 *g* for 90 min in an ultracentrifuge VAC-601 (Janetzki) contained closed vesicles formed from ruptured microsomal membranes; it was suspended in 50 ml of TED buffer and designated the 'ghosts' fraction. The total yield of protein in the 'ghosts' fraction was on an average about 600 mg, i.e. 60% of the original protein content of the microsomal fraction. The 'ghosts' fraction (15 ml) was used as specific template for reconstitution. The rest of this fraction (36 ml, 430 mg of protein) was treated with 4% (w/v) sodium cholate and recentrifuged under the same conditions. The supernatant obtained, which contained the solubilized proteins and lipids, was designated the solubilized fraction. The total protein yield of the fraction averaged about 90% of the original protein of the 'ghosts' fraction. The solubilized fraction was dialysed against a solution containing 20 mM-Tris-HCl buffer, 0.2 mM-EDTA, 1 mM-dithiothreitol and 0.1% (w/v) sodium cholate for 22 h at 4°C, to remove the 4% sodium cholate which, when used in such high concentration, dissolves microsomal membranes and liposomes. Addition of 0.1% sodium cholate to the medium is necessary to prevent self-assembly. The ratio of the diffusate to the dialysing solution was 1:30 (v/v).

The solubilized membrane components of this fraction was not sedimented when centrifuged at 150 000 *g* for 90 min, and this solubilized fraction (fraction 2), dialysed against 0.1% sodium cholate, was used as the initial material for reconstitution of microsomal redox chains.

To study the reconstitution of microsomal redox chains, four dialysis systems were used. Dialysis was performed against a solution containing 20 mM-Tris-HCl buffer, 0.2 mM-EDTA and 0.2 mM-dithiothreitol for 22 h at 4°C. The first incubation medium contained 6 ml of 'ghosts' fraction (15 mg of protein/ml) and 55 ml of TED buffer (control mixture). The second system, containing 6 ml of 'ghosts' fraction and 55 ml of solubilized fraction (2 mg of protein/ml), was used for specific template binding. The third contained 55 ml of solubilized fraction and 6 ml of TED buffer for self-assembly. The fourth contained 55 ml of solubilized fraction and 6 ml of a suspension of liposomes (3 mg of phosphatidylcholine/ml) for non-specific binding or liposome binding. The ratio of diffusate/dialysis solution was 1:70 (v/v). After dialysis the reconstituted material was centrifuged at 150 000 *g* for 90 min. The pellets obtained were 'ghosts' (fraction 3), 'ghosts'+solubilized fraction (fraction 4), the pellet of the solubilized fraction (fraction 5) and liposomes+solubilized fraction (fraction 6), which were all suspended in the TED buffer and used for analysis.

The activity and content of carriers and RNA was calculated per mg of protein or per total protein of fraction. The protein and phospholipid contents were calculated per ml and per total volume of fraction.

Electron microscopy

The pellets of membranes, isolated after centrifugation at 150 000 *g* for 90 min, were fixed with OsO₄ by the method of Millonig (1961), dehydrated by increasing concentrations of alcohols and embedded in Araldite. Preparations were stained with uranyl acetate as pellets by the method of Farquhar & Palade (1962) and the slices were stained with lead acetate (Reynolds, 1963), uranyl acetate (sodium salt) (Stempak & Ward, 1964) and lead citrate. The micrographs were prepared in a Hitachi 11-E electron microscope by electro-optical magnification of 20 000 to 180 000.

Results

Fig. 1 shows data on the microsomal fraction treated with different concentrations of sodium cholate. The solubilization of IDPase, the enzyme localized in the matrix of the microsomal vesicles, occurs at a concentration of 0.15% sodium cholate. The rupture of membranes does not follow the solubilization of the membrane enzymes, cytochrome *b₅* and cytochrome *P-450*. This treatment removes

some RNA and protein and a little phospholipid. Thus 0.15% sodium cholate removes the matrix of the microsomal vesicles, the absorbed proteins and some ribosomes. The 'ghosts' fraction prepared from the microsomal membranes was used as the specific template in experiments on reconstitution and as the original material for the preparation of the solubilized fraction.

Plate 1 shows electron micrographs of the fractions obtained. The 'ghosts' fraction contains closed microsomal vesicles, which have no ribosomes and are poorly stained (Plate 1*b* and inset). The membrane

Fig. 1. Solubilization of the components of the microsomal vesicles by treatment with sodium cholate

The incubation mixture contained 20 mg of protein/ml of TED buffer. Detergent was added to a concentration of 0.025–0.5%. The samples were sedimented at 150000 *g* for 90 min and were determined in the following supernatants: protein (10 × mg/ml, ○), phospholipid (mg/ml, △); RNA (mg/mg of protein, ●); cytochrome *b*₅ (nmol/mg of protein, ▲); cytochrome *P*-420 (nmol/mg of protein, ■); IDPase (10² × nmol/min per mg of protein; □).

structures reconstituted from components of the solubilized fraction by self-assembly form a network consisting of fused vesicles (Plate 1*d* and inset); such structures are not formed if reconstitution is from the solubilized fraction with either 'ghosts' or liposomes as specific and non-specific template. In these cases distinctly stained microsomal vesicles with bilayer membranes are formed (Plates 1*e*, inset, and 1*f*, inset). The fraction of multilayer liposomes contains large vesicles surrounded by poorly stained membranes (Plate 1*c*, inset). The addition of the solubilized fraction to this liposome fraction leads to the formation of small vesicles, surrounded by a well-stained bilayer membrane (Plate 1*d*, inset). The same effect of breaking up the liposomal vesicles is induced by the addition of 0.1% sodium cholate, which is the amount of detergent used in the solubilized fraction, but in this case small vesicles surrounded by poorly stained and somewhat thinner membranes were formed.

Table 1 shows the contents of protein, RNA and phospholipid in the fractions of reconstituted membranes. Fraction 3 is the pellet obtained by ultracentrifugation of the dialysed 'ghosts' fraction. It contains about 60% of the total protein of the original preparation. The protein remaining in the supernatant contains no cytochrome *b*₅ or cytochrome *P*-450 or NADPH- and NADH-specific reductases. The membranes reconstituted by self-assembly and by binding to liposomes contain about 30% of the initial protein (the protein in fractions 5 and 6 is calculated on the basis of the total protein in fraction 2). The effectiveness of specific template reconstitution is somewhat greater, amounting to 50% of the initial protein (the protein in fraction 4 is calculated on the basis of the total amount in fractions 2 and 3).

The reconstituted membranes contain more cytochrome *b*₅ than the original microsomal fraction (Table 2). The cytochrome *b*₅ content is greatest in the membrane fraction obtained as a result of binding

Table 1. Content of protein, RNA and phospholipid in fractions obtained after membrane reconstitution

The content of protein, phospholipid and RNA was measured as described in the Materials and Methods section. Results are average values from ten experiments ± S.E.M. Fraction 1, microsomal fraction; fraction 2, solubilized fraction; fraction 3, 'ghosts' fraction; fraction 4, 'ghosts'+solubilized fraction; fraction 5, pellet of solubilized fraction; fraction 6, liposomes+solubilized fraction.

		Fractions					
		1	2	3	4	5	6
Protein	(mg/ml)	20.0 ± 1	2.0 ± 0.2	12.0 ± 1	18.5 ± 1.4	9.2 ± 1	9.0 ± 1
	(total content, mg)		110	48.0	74.0	37.0	36.0
RNA	(μg/mg of protein)	150 ± 13	150 ± 18	85.0 ± 9	65.0 ± 6	40.0 ± 6	37 ± 4
	(total content, μg)		1700	330	510	160	140
Phospholipid	(mg/ml)	7.0 ± 0.6	0.70 ± 0.07	7.0 ± 0.07	14.0 ± 1.4	8.0 ± 0.8	8.5 ± 0.9
	(total content, mg)		38.5	28.0	56.0	32.0	33.0
Phospholipid/protein		0.35	0.35	0.58	0.76	0.87	0.95

EXPLANATION OF PLATE I

Electron micrographs of microsomal fraction and reconstituted membranes

Electron micrographs were taken from thin sections of Araldite-embedded pellets fixed by OsO_4 . Sections were stained with uranyl acetate and examined in a Hitachi-11E electron microscope. (a) The pellet of microsomal fraction $\times 35\,000$ and inset $\times 190\,000$; (b) 'ghosts' of microsomal vesicles $\times 35\,000$ and inset $\times 90\,000$; (c) liposomes $\times 10\,000$ and inset $\times 60\,000$; (d) the pellet of solubilized fraction (self-assembly) $\times 35\,000$ and inset $\times 90\,000$; (e) 'ghosts' + solubilized fraction (the binding with specific template) $\times 35\,000$ and inset $\times 90\,000$; (f) liposomes + solubilized fraction (the binding with non-specific template) $\times 35\,000$ and inset $\times 90\,000$; (g) liposomes treated with 0.1% sodium cholate $\times 35\,000$ and inset $\times 90\,000$.

Table 2. Content of cytochromes b_5 , $P-450$ and $P-420$ in fractions obtained after membrane reconstitution

The incubation medium contained 1.0 mg of protein/ml and 100 mM-Tris-HCl buffer in a total volume of 4 ml. For non-enzymic reduction of cytochrome b_5 several crystals of $\text{Na}_2\text{S}_2\text{O}_4$ were added to the cuvette, and for enzymic reduction $7 \mu\text{M}$ -NADH was added. For measurement of cytochrome $P-450$ content CO was blown through the cuvette for 1 min and then several crystals of $\text{Na}_2\text{S}_2\text{O}_4$ were added. Measurements were carried out at 20°C . Results are average values from six experiments $\pm$ S.E.M. The fractions are defined in the text and the legend to Table 1.

	Fractions					
	1	2	3	4	5	6
$\text{Na}_2\text{S}_2\text{O}_4$ -reduced cytochrome b_5 (nmol/mg of protein)	0.45 $\pm$ 0.033	0.53 $\pm$ 0.025	0.61 $\pm$ 0.055	0.81 $\pm$ 0.048	0.72 $\pm$ 0.034	0.66 $\pm$ 0.056
		58.0	30.0	63.0	29.0	25.0
NADH-reduced cytochrome b_5 (nmol/mg of protein)	0.30 $\pm$ 0.030	0.41 $\pm$ 0.028	0.40 $\pm$ 0.025	0.54 $\pm$ 0.037	0.56 $\pm$ 0.047	0.50 $\pm$ 0.029
		45.0	19.0	43.0	22.0	19.0
Cytochrome $P-450$ (nmol/mg of protein)	0.57 $\pm$ 0.031	0.25 $\pm$ 0.024	0.86 $\pm$ 0.041	0.74 $\pm$ 0.056	0.42 $\pm$ 0.038	0.39 $\pm$ 0.04
		28.0	42.0	58.0	17.0	15.0
Cytochrome $P-420$ (nmol/mg of protein)	0	0.59 $\pm$ 0.03	0	0.18 $\pm$ 0.01	0.42 $\pm$ 0.04	0.36 $\pm$ 0.03
	0	65.0	0	14.0	17.0	14.0

of the carriers to the 'ghosts' of the microsomal vesicles (fraction 4). This carrier binds to liposomes somewhat less (fraction 6). The specific content of cytochrome b_5 bound to liposomes is lower than that in the membrane fraction obtained by self-assembly (fraction 5).

Cytochrome $P-450$, in contrast with cytochrome b_5 , was not incorporated into the 'ghosts' of the microsomal membranes (Table 2). Its specific content in fraction 4 is 0.74 nmol/mg, which is lower than that in the 'ghosts' fraction (fraction 3) (0.86 nmol/mg). At the same time the reactivation of this electron carrier is very distinct in the cases of self-assembly and the binding to liposomes. The ratio of cytochromes, $P-420/P-450$, is 2.3 in the solubilized fraction (fraction 2) but is decreased to 1 in fraction 5 (the pellet of the solubilized fraction) and in fraction 6 (liposomes + solubilized fraction).

Table 3 shows the activities of NADPH- and NADH-dependent reductases in the reconstituted membranes. We found that the specific activity of NADPH- and NADH-dependent reductases in fractions 4, 5 and 6 is much higher than that in fractions 1 and 3. However, calculation of the total activities of the reductases in the fraction of reconstituted membranes shows that only with NADH-specific flavoprotein does additional binding occur. The total activity of NADH-dependent reductase in the 'ghosts' + solubilized fraction (fraction 4) is higher than the sum of its activities in fractions 3 ('ghosts') and 5 (the pellet of solubilized fraction).

No complete reconstitution of the activity of the hydroxylation system was observed in any case of incorporation or self-assembly (Table 4). About 10–20% of the initial activity of NADPH-dependent p -hydroxylation of aniline and N -demethylation of dimethylalanine was reconstituted by self-assembly. For NADH-dependent p -hydroxylation of aniline, about 30% of the activity was restored in self-assembly, and for N -demethylation the activity was much higher, i.e. about 60% of the initial value. Incorporation of the electron carriers into the membranes of the microsomal vesicles ('ghosts') was not accompanied by reconstitution of the hydroxylation systems. The specific activities of all the hydroxylases in fraction 4 are less than those in fraction 3. This correlates well with the fact that the terminal component of all the hydroxylation systems, i.e. cytochrome $P-450$, was not incorporated into the 'ghosts' fraction. It is noteworthy that addition of liposomes decreases the effectiveness of self-assembly.

Table 5 shows results on the reconstitution of lipid-peroxidation systems. The activity of the NADPH-dependent lipid-peroxidation system is sharply inhibited in the 'ghosts' fraction, whereas the activity of the ascorbate-dependent system is increased in the same fraction. Both peroxidation systems were reconstituted by the methods used. Therefore the activity

Table 3. *Activity of NADH- and NADPH-specific reductases in fractions obtained after membrane reconstitution*

For experimental details see the Materials and Methods section. Results are means from six experiments $\pm$ S.E.M. The fractions are defined in the legend to Table 1. For each enzyme the top row of values is $10^2 \times$ activity (nmol of reduced acceptor/min per mg of protein) and the bottom row is $10^3 \times$ activity (nmol of the reduced acceptor/min per total protein).

	Fractions					
	1	2	3	4	5	6
NADH-cytochrome <i>c</i> reductase	19 $\pm$ 0.75	36 $\pm$ 0.98	20 $\pm$ 1.4	39 $\pm$ 1.2	39 $\pm$ 2.2	42 $\pm$ 2.5
		400	96	300	160	170
NADPH-cytochrome <i>c</i> reductase	1.9 $\pm$ 0.11	1.3 $\pm$ 0.1	1.6 $\pm$ 0.16	2.2 $\pm$ 0.17	2.1 $\pm$ 0.14	2.1 $\pm$ 0.12
		14	7.9	17	8.7	8.3
NADH-ferricyanide reductase	66 $\pm$ 3.5	74 $\pm$ 4.8	74 $\pm$ 3.2	120 $\pm$ 5.4	110 $\pm$ 6.0	140 $\pm$ 7.0
		820	360	900	460	530
NADPH-ferricyanide reductase	1.5 $\pm$ 0.96	1.4 $\pm$ 0.12	1.6 $\pm$ 0.11	2.1 $\pm$ 0.14	2.0 $\pm$ 0.10	2.1 $\pm$ 0.20
		16	7.5	16	11	14
NADH-Neotetrazolium Blue reductase	1.0 $\pm$ 0.07	1.0 $\pm$ 0.063	1.1 $\pm$ 0.10	2.0 $\pm$ 0.15	1.7 $\pm$ 0.085	1.7 $\pm$ 0.14
		11	5.2	15	6.9	6.4
NADPH-Neotetrazolium Blue reductase	0.24 $\pm$ 0.02	0.23 $\pm$ 0.02	0.26 $\pm$ 0.015	0.38 $\pm$ 0.025	0.35 $\pm$ 0.04	0.33 $\pm$ 0.032
		2.5	1.2	2.9	1.4	1.3

of ascorbate-dependent peroxidation in the reconstituted membranes and 'ghosts' fraction is much higher than that of NADPH-stimulated peroxidation. The decrease in the activity of the NADPH-dependent peroxidation system in the 'ghosts' fraction and the reconstituted membranes is not a result of the inhibition of NADPH oxidation (Table 5). The rate of NADPH oxidation in the 'ghosts' fraction is higher than that in the microsomal fraction. NADPH oxidases readily reconstitute by self-assembly and by the binding of electron carriers to a non-specific template. The specific template binding was not accompanied by the additional reconstitution of NADPH oxidase.

NADH oxidase was not reconstituted as well by self-assembly or non-specific template binding (about 60%). The activity of this system was decreased in the 'ghosts' fraction (about 70%). The specific template binding is not accompanied by the additional reconstitution of NADH oxidase. In contrast with NADPH oxidase, about 30% of NADH oxidase activity was observed in the solubilized fraction.

Fig. 2 shows the aerobic redox behaviour of cytochrome *b*₅, reduced with NADH and NADPH in the original microsomal fraction and reconstituted membranes. NADPH-reduced cytochrome *b*₅ is oxidized most slowly in the solubilized fraction ($t_{1/2}$ = 24 min). The oxidation rate of cytochrome *b*₅ in the pellet from the solubilized fraction and the liposomes+solubilized fraction are almost the same ($t_{1/2}$ = 10 min) as that in the microsomal fraction ($t_{1/2}$ = 10 min), but are higher than that in the solubilized fraction.

The NADH-reduced cytochrome *b*₅ in the microsomal fraction ($t_{1/2}$ = 5 min) is oxidized more quickly than that in the 'ghost' fraction ($t_{1/2}$ = 10 min) or

'ghosts'+solubilized fraction ($t_{1/2}$ = 13 min). Cytochrome *b*₅ is oxidized more slowly in the solubilized fraction ($t_{1/2}$ = 28 min) than that in the membrane fraction obtained by self-assembly and non-specific template binding ($t_{1/2}$ = 17 min and 21 min respectively) or than that in the 'ghosts' fraction or 'ghosts'+solubilized fraction ($t_{1/2}$ = 10–13 min).

Discussion

A comparative analysis of the effectiveness of three methods of reconstitution of microsomal redox chains has been made in this work. It has been shown that when the 'ghosts' of microsomal vesicles are used as a specific template, the reconstituted membranes contained 50% of the total protein. Only about 30% of the total protein was sedimented with this fraction of the membranes reconstituted by self-assembly or by binding to liposomes. The lipid/protein ratio in all reconstituted membranes (0.75–0.95) is higher than that in the microsomal (0.35) or 'ghosts' (0.58) fractions.

Electron microscopy clearly demonstrates that the addition of ghosts and liposomes to the solubilized fraction facilitates reconstitution of the distinctly stained vesicles with bilayer membranes.

Cytochrome *b*₅ and NADH-specific flavoprotein effectively bind to microsomal membranes in contrast with cytochrome *P*-450 and NADPH-specific flavoprotein which do not bind to them. This seems to be due to the lower affinity of the two latter electron carriers for microsomal membranes. The affinity of the NADH-specific chain carriers for microsomal membranes is very high as demonstrated by experiments with isolated enzymes (Rogers & Strittmatter, 1973; Strittmatter *et al.*, 1972).

Table 4. Activity of hydroxylases in fractions obtained after membrane reconstitution

Experimental details are given in the text (see the Materials and Methods section). Results are means from five experiments $\pm$ S.E.M. The fractions are defined in the legend to Table 1.

	Fractions					
	1	2	3	4	5	6
NADPH-dependent <i>p</i> -hydroxylation of aniline (nmol of <i>p</i> -aminophenol/min per mg of protein)	0.64 $\pm$ 0.05	0	0.70 $\pm$ 0.03	0.40 $\pm$ 0.02	0.070 $\pm$ 0.006	0.065 $\pm$ 0.005
		0	34	33	2.9	2.6
NADPH-dependent <i>N</i> -demethylation of dimethylaniline (nmol of formaldehyde/min per mg of protein)	11 $\pm$ 0.98	0	9.6 $\pm$ 0.38	6.1 $\pm$ 0.42	2.1 $\pm$ 0.18	1.7 $\pm$ 0.12
		0	490	510	90	67
NADH-dependent <i>p</i> -hydroxylation of aniline (nmol of <i>p</i> -aminophenol/min per mg of protein)	0.060 $\pm$ 0.05	0	0.10 $\pm$ 0.08	0.076 $\pm$ 0.004	0.030 $\pm$ 0.001	0.029 $\pm$ 0.001
		0	4.8	6.3	1.2	1.1
NADH-dependent <i>N</i> -demethylation of dimethylaniline (nmol of formaldehyde/min per mg of protein)	0.88 $\pm$ 0.10	0	0.92 $\pm$ 0.03	0.81 $\pm$ 0.05	0.61 $\pm$ 0.01	0.49 $\pm$ 0.02
		0	47	70	26	20

Table 5. Activity of NADPH- or ascorbate-dependent systems of lipid peroxidation and NADH- or NADPH-oxidation rate in fractions obtained after membrane reconstitution

Experimental details are given in the text (see the Materials and Methods section). Results are means from five experiments $\pm$ S.E.M. The fractions are defined in the legend to Table 1.

	Fractions					
	1	2	3	4	5	6
NADPH-dependent peroxidation of lipid (nmol of malonic dialdehyde/min per mg of protein)	2.2 $\pm$ 0.25	0.13 $\pm$ 0.04	1.3 $\pm$ 0.12	1.9 $\pm$ 0.2	1.7 $\pm$ 0.22	1.7 $\pm$ 0.22
		14	62	140	63	61
Ascorbate-dependent peroxidation of lipid (nmol of malonic dialdehyde/min per mg of protein)	2.2 $\pm$ 0.08	0.05 $\pm$ 0.006	2.7 $\pm$ 0.30	4.5 $\pm$ 0.31	3.9 $\pm$ 0.35	4.4 $\pm$ 0.50
		0.35	130	330	140	160
NADPH-oxidase (nmol of NADPH/min per mg of protein)	4.2 $\pm$ 0.31	0	5.8 $\pm$ 0.45	4.2 $\pm$ 0.28	3.7 $\pm$ 0.26	3.5 $\pm$ 0.18
		0	270	290	120	110
NADH-oxidase (nmol of NADH/min per mg of protein)	3.5 $\pm$ 0.18	0.80 $\pm$ 0.05	2.5 $\pm$ 0.17	2.3 $\pm$ 0.14	1.8 $\pm$ 0.11	2.0 $\pm$ 0.10
		88.0	100	160	59	63

Fig. 2. Spectrophotometric aerobic redox behaviour of cytochrome b_5 in microsomal and reconstituted fractions

Incubation medium contained 50mM-Tris-HCl buffer and 3mg of protein in a total volume of 3.0ml. The measurements were made at 25°C. The reaction was initiated by the addition of 32 μ M-NADPH (a) or 7 μ M-NADH (b) to the incubation medium. $\circ$, Microsomal fraction; $\square$, solubilized fraction; $\triangle$, 'ghosts' fraction; $\blacksquare$, 'ghosts'+solubilized fraction; $\bullet$, pellet of solubilized fraction; $\blacktriangle$, liposomes+solubilized fraction. Arrow indicates the addition of $\text{Na}_2\text{S}_2\text{O}_4$.

It should be emphasized that in our work these electron carriers were bound much less effectively. It seems reasonable to assume that when the solubilized fraction, and not isolated carriers, was used as the initial material for the reconstitution, some competition for the binding sites of the template occurs between proteins or proteins and lipids and this fact may explain the smaller quantity of cytochrome b_5 and NADH-specific flavoprotein being bound to the template.

Reconstitution of membranes by self-assembly or by binding to liposomes led to the reactivation of cytochrome $P-420$ to $P-450$, which may be interpreted

as being a result of the removal of detergent by dialysis and incorporation of haemoprotein into hydrophobic regions of these reconstituted membranes.

NADH-dependent N -demethylation of dimethylaniline and p -hydroxylation of aniline were reconstituted by self-assembly and by binding to liposomes by 60 and 30% respectively. The NADPH-dependent hydroxylation systems were reconstituted by not more than 10–20%. Probably this difference may be explained by different rate-limiting steps occurring in these reactions. This is supported by Schenkman's observation on the temperature-dependence of ethylmorphine-, aminopyrine- and aniline-hydroxylation reactions (Schenkman, 1972) and by the results obtained by Archakov *et al.* (1974).

The binding of NADH-specific carriers to the 'ghosts' of microsomal vesicles was not accompanied by reconstitution of NADH-dependent hydroxylation activity. This phenomenon may be explained by the fact that cytochrome $P-450$, an enzyme which is indispensable for all hydroxylation reactions, is not bound to the 'ghosts' of microsomal membranes.

The reconstitution of different hydroxylation activities from solubilized NADPH-cytochrome c reductase, cytochrome $P-450$ and lipid (Lu & West, 1972; West & Lu, 1972; Lu *et al.*, 1971, 1972a,b) was not accompanied by the formation of complexes from these carriers. Hydroxylation activity was reconstituted only in the supernatant obtained by sedimentation at 105 000 g for 60 min; no hydroxylation activity was observed in the pellet (Autor *et al.*, 1973). Thus in the above work reactivation of cytochrome $P-450$ hydroxylase did not lead to reconstitution of the NADPH-specific redox chain of the membranes of the endoplasmic reticulum.

Unlike for the hydroxylases all the reconstitution methods were effective for the lipid-peroxidation systems. The activity of the ascorbate-dependent peroxidation was higher in the reconstituted membranes and 'ghosts' than that in the microsomal fraction. Addition of the 'ghosts' and liposomes to the solubilized fraction facilitates reconstitution. It appears reasonable therefore to assume that the liposomes and 'ghosts' in these cases are a source of additional substrates, e.g. unsaturated fatty acids. The low rate of NADPH-dependent peroxidation in the 'ghosts' fraction is noteworthy. The specific template binding of the electron carriers and lipids increases the activity of this system. The NADPH-dependent peroxidation was effectively reconstituted by self-assembly (about 80% of the microsomal activity). Addition of non-specific template (liposomes) does not change the effectiveness of this reconstitution.

Fluorimetric-analysis data on the NADPH- and NADH-oxidation rate and spectrophotometric redox behaviour of NADPH- and NADH-reduced cyto-

chrome b_5 show that the NADPH oxidase is most active in the 'ghosts' fraction and is reconstituted to a large extent in the pellet from the solubilized fraction and in the liposomes+solubilized fractions. Comparison of these results with those in Tables 4 and 5 shows the different degrees of reconstitution of the NADPH oxidase and NADPH-dependent hydroxylases. The effective reconstitution of the NADPH oxidase is not accompanied by the same amount of reconstitution of the NADPH-specific hydroxylases. The reconstitution of NADPH oxidase and NADPH dependent lipid peroxidation correlate well in cases of self-assembly and non-specific template binding. However, such a correlation is not observed in the 'ghosts' or the 'ghosts'+solubilized fraction. NADPH oxidase activity in fraction 1 is the same as that in fraction 4 and lower than that in fraction 3. NADPH dependent peroxidation activity is higher in fraction 1 than in fraction 3. Additional reconstitution of NADPH-lipid peroxidation by specific template binding is not accompanied by the same amount of reconstitution of NADPH oxidase.

NADH oxidase activity can be used as one of the most sensitive tests for membrane reconstitution. Treatment of the microsomal fraction with a low concentration of detergent decreases NADH oxidase activity. Specific template binding does not lead to the reconstitution of this enzyme. Self-assembly and non-specific template binding reconstitute only about 60% of its activity. In these fractions the oxidation rate of NADH-reduced cytochrome b_5 is faster than that in the solubilized fraction but is much slower than that in the original microsomal fraction.

Thus from these results one may assume that the NADH-specific carriers (NADH-specific flavoprotein and cytochrome b_5) may be bound to the microsomal and 'ghosts' fractions and effectively incorporated into the membranes reconstituted by self-assembly and non-specific template binding. Therefore activation of NADH-specific reductases is observed, but NADH oxidase activity is not reconstituted. The carriers of NADPH-specific chains (flavoprotein and cytochrome $P-450$) did not bind to the specific template. However, these carriers were incorporated into the reconstituted membranes by self-assembly and non-specific template binding. As no additional binding of NADPH-specific flavoprotein and cytochrome $P-450$ to the 'ghosts' of the microsomal vesicles was observed, not a single hydroxylase could have been reconstituted by this method. In contrast, NADPH-lipid peroxidation was reconstituted by specific template binding. This system and NADPH oxidase were effectively reconstituted by self-assembly and non-specific template binding. A distinct reactivation of cytochrome $P-450$ in these cases was not accompanied by reconstitution of hydroxylases.

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