Enzyme Assay in Microsomes Below Zero Degrees
(ethylene glycol/electron transfer/rats/liver)

PASCALE DEBEY, CLAUDE BALNY, AND PIERRE DOUZOU
Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France
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ABSTRACT Reactions of a membrane-bound multi-

enzyme complex (electron-transport chain of rat-liver

microsomes) suspended in aqueous-organic solvent used

as antifreeze at temperatures below 0° were studied. In

the presence of a relatively high concentration of ethylene

glycol, electron transfer can still be observed and some

individual and sequential enzyme assays can be per-

formed over a wide range of temperatures below 0°.

Many soluble flavin and heme enzymes retain activity in

aqueous-organic solvents, which can then be used as “anti-

freeze” over a wide range of temperatures below zero degrees

(1). Under such conditions, enzyme reactions are slowed
down by the effect of the organic solvent itself at room

temperature and by the effect of cooling, according to the

Arrhenius expression. One of us studied the rate of a reaction

catalyzed by a peroxidase under these conditions by use of

cycles of cooling and heating (2). This communication reports

similar experiments with membrane-bound enzymes that

belong to a multienzyme complex, in order to investigate

their individual activities as well as the sequential steps of

the reactions. We chose to study the drug-metabolizing system

of the endoplasmic reticulum of hepatocytes (rat-liver

microsomes). Our goal was first to define the conditions

required for spectrophotometry at temperatures below 0°,

then to assay the activity of each membrane-bound and solubilized

enzyme at various temperatures, and finally to observe the

activity of the whole system at these temperatures in order to

elucidate new information about its still controversial way of

functioning.

MATERIALS AND METHODS

Preparation of Microsomal Samples. Liver microsomes were

obtained from male Wistar Albino rats, by the method of

Ernst and Coll (3). The rats, weighing 130–160 g, were

starved 24 hr before they were killed by sectioning the carotid

artery. The livers were removed, rinsed with 0.25 M sucrose,
cut into small pieces, and homogenized in a Potter tube with a

Teflon pestle (0.1-mm clearance). The homogenate was

centrifuged at 10,000 × g for 10 min at 4°. The micro-
somes were then precipitated by centrifugation at 105,000 ×
g at 4° for 1 hr in a Spincuo model L2 ultracentrifuge.
The pellets were rinsed with 0.25 M sucrose and suspended in
0.25 M sucrose to a final concentration of 70 mg of protein
per ml. This stock suspension was kept at 4° and used the
same day or the day after. Preparations kept more than 2
days were never used.

NADPH cytochrome c reductase (EC 1.6.2.3) was isolated
by trypsin digestion and purified by a method slightly
modified from that of Omura et al. (4). The enzyme was
stored at 4° and used within 10 days after preparation; no
significant change in enzyme activity was observed during
this time. Proteins were measured by the method of Lowry
et al. (5); purified bovine-serum albumin (twice crystallized)
was used as standard. The concentration of the purified
flavoprotein was calculated on the basis of ε = 11.3 × 10³
M⁻¹ cm⁻¹ at A₆₈₀ nm (6).

Preparation of Aqueous–Organic Suspensions. Several
organic solvents miscible with water (ethylene glycol, gly-
cerol, dimethylformamide, and dimethylsulfoxide) were used to
prepare “antifreeze” suspensions for studies at tempera-
tures below 0°. Ethylene glycol was selected, based on our
own experience with soluble enzymes and on several studies
on microsomal suspensions. The stock microsomal suspension
was first diluted in 0.05 M Tris-HCl (pH 7.5)–0.15 M KCl;
ethylene glycol was then slowly added at 4° to a final volume
percentage of 10–50%. This procedure avoids the precipita-
tion that occurs when the stock suspension is diluted directly with
the aqueous–organic mixture, and no denaturation or precipi-
tation occurred during the 2–3 hr after mixing.

In experiments performed at temperatures below 0°, equal
volumes of water and ethylene glycol were used. This mixture
freezes at about −45° and presents a low viscosity, compared
to other aqueous–organic mixtures (for example glycerol–
water).

Enzyme Assays. Reduction of cytochrome b₅ or P-450 was
measured in a Cary 15 spectrophotometer whose temperature
was kept constant (at any temperature) with a precision of
±0.01° (7). The baseline was recorded at room (20°), as well
as at low, temperature. The reaction was started by addition of
50 μl of a refrigerated solution of the reductant (NADPH,
NADH, or Na₂S₂O₄) containing 50% ethylene glycol. Tem-
perature equilibrium was achieved in less than 30 sec after
mixing.

Reduction of cytochrome b₅ was recorded by measurement of
the difference in absorbance between 424 and 450 nm
rather than between 424 and 409 nm, because of the lower
light scattering at 450 nm. The two measurements give the
same results. As will be seen below, there is no enzymic re-
duction of P-450 (by NADPH or NADH) below −5°; there-
fore, there is no interference between the spectrum of reduced
P-450 and that of reduced cytochrome b₅ at 424 nm, even
under anaerobic conditions.

After completion of the reaction, the sample was warmed to
20° and cooled again, and spectra of the fully reduced cyto-
chrome b₅ at room and low temperatures were recorded. This procedure allows one to measure the percentage of reduction at any temperature and to calculate reaction rates after taking into account the temperature-dependent contraction of the solvent and changes in the refractive index. The same procedure was used for reduction of P-450, except that the suspension was first vigorously bubbled with CO and then transferred into the spectrophotometer cell. The reduction was recorded by measurement of the difference in A between 450 and 490 nm.

Activities of NADPH and NADH cytochrome c reductase in the microsomes were measured at 550 nm as described (3). The reaction medium contained 0.05 M Tris·HCl (pH 7.5)–0.15 M KCl–0.33 mM KCN with or without organic solvent. The final concentrations of the reactants in the medium were 50 μM cytochrome c and 100 μM NADPH or NADH. The reaction was started at 27°C by addition of 20 μl of a diluted microsomal suspension (final concentration about 60 μg of protein per ml for NADPH cytochrome c reductase and 6 μg of protein per ml for NADH cytochrome c reductase). The activity of isolated NADPH cytochrome c reductase was measured under the same conditions of concentration and solvents. Reactions at different temperatures were performed the same day; aliquots from the same preparation of microsomes were used.

Reagents. The disodium salt of NADH, cytochrome c (equine heart), and 2,6-dichlorophenol-indophenol were obtained from Calbiochem; NADPH was obtained from Sigma. Sodium dithionite and ethylene glycol were purchased from Merck. All the products were used without further purification.

Solutions were made in 0.05 M Tris·HCl (pH 7.5)–0.15 M KCl or in a mixture of organic solvent and Tris·HCl buffer; all mixtures were freshly used. Sodium dithionite was dissolved in a mixture of 0.1 M NaOH and ethylene glycol in the chosen volume ratio; the mixture had been previously deoxygenated vigorously by bubbling with N₂.

RESULTS AND DISCUSSION

Solvent Effect at Normal Temperatures. As shown in Fig. 1, the rates of reduction of endogenous cytochrome P-450 and exogenous cytochrome c by NADPH cytochrome c reductase are markedly decreased as the concentration of added ethylene glycol to the aqueous buffered microsomal suspensions at 27°C is increased.

It can be seen in Fig. 1 that a similar, though slightly smaller, decrease occurs when cytochrome c is reduced by the soluble NADPH cytochrome c reductase (Fig. 1, 2, and 3). In both cases, the solvent effect is totally reversible by dilution with water. This solvent effect was detected in many other experiments with soluble flavoproteins and hemoproteins performed in our laboratory and might be due to a reversible modification of the tertiary or quaternary structure of flavoproteins and hemoprotein that “cripple” enzyme activity. A similar partial “inhibition” was previously reported in the mitochondrial respiratory chain by Tyler and Estabrook who used various organic solvents; this inhibition was explained in terms of the accessibility of membrane cytochromes to the solvent, which displaced water at the active center (8, 9).

We reached the same conclusion for the microsomal enzyme assembly, since the electron transfer from NADPH cytochrome c reductase to cytochrome P-450 and to the exogenous cytochrome c is influenced to the same extent by the solvent. However, the solvent effect on the reduction of cytochrome c is similar when NADPH cytochrome c reductase is bound to membrane or solubilized. It is more difficult to determine how the solvent might affect the enzyme activity. Moreover, the above results do not indicate that the solvent viscosity was one of the causes of the decrease in reaction rates. This parameter might play a role at temperatures below 0°C.

Absorption Spectra at Temperatures below 0°C. At room temperature, addition of ethylene glycol to aqueous buffered suspensions of microsomes causes a slight decrease in the usual light scattering, indicating that there is no precipitation of microsomes. For instance, the ΔA 450–700 nm of an oxidized suspension of 3 mg/ml of protein decreases by 0.05 in the presence of 50% ethylene glycol. In addition, cooling to temperatures below 0°C induces an expected sharpening and hyperchromicity of the absorption maxima, increasing the precision of recordings and kinetic measurements. Of course, exogenous compounds, such as cytochrome c, used to test the functioning of microsomal enzymes, similarly benefit under the same recording conditions.

Thus, the macroaddition of ethylene glycol as well as study of the suspensions at temperatures below 0°C permit and eventually facilitate the recording of absorption spectra and kinetics. On another hand, these conditions alter the speed of the reactions and can strongly slow down some of them according to the observations reported below.
Enzyme Assay at Temperatures below 0°C. We recorded by spectrophotometry the individual functioning of the two flavoproteins, their action upon cytochrome c and cytochrome b5, and the reduction of cytochrome P-450 between +20°C and -45°C. We obtained the following results: the reduction rate of exogenous cytochrome c by both NADPH and NADH reductases, already decreased in the normal range of temperatures by addition of ethylene glycol, is practically zero just above 0°C, whereas the reduction of cytochrome P-450 is then unobservable (too slow) at about -5°C.

In the meantime, the reduction of cytochrome b5 both by NADPH and NADH reductases is recordable down to -25°C, thus indicating that the interruption of the reduction of cytochrome P-450 at about -5°C is due to the rate-limiting electron transfer from NADPH cytochrome c reductase to cytochrome P-450.

The fact that NADPH and NADH reductases are still reactive towards cytochrome b5 in a rather large span of temperatures below 0°C was used to record reduction kinetics: The reduction of cytochrome b5 by NADPH cytochrome c reductase is a single first-order reaction (Fig. 2a), and the Arrhenius plot of the rate constant is a straight line between -7°C and -20°C.

With the experimental conditions used, it was impossible to record reduction kinetics above -7°C so as to check the eventual irreproducibility of the rate constant (10) as well as the rapid fall-off in the Arrhenius plot already reported with many enzyme systems studied around 0°C (11).

Reduction of cytochrome b5 by NADH cytochrome c reductase previously studied in normal conditions of media and temperature by stopped-flow experiments (12) has been recorded in the above conditions and appears to be biphasic down to -21°C (Fig. 2b), where the Arrhenius plot of the higher rate constant shows a sharp discontinuity.

Up to now, we have no satisfactory explanation either of the biphasic reaction or of the monophasic one. The biphasic reduction recorded down to -21°C might be due to the two different transfer processes already postulated from experiments in normal conditions (13), and the monophasic reduction occurring at -21°C and below might be due to a viscosity effect hindering one transfer process, as well as to a conformational change of the functional protein or (and) to a phase transition of the membrane similar to that reported by Stein and coworkers upon certain membranes at temperatures below 0°C (14). On another hand, the slower phase of the reduction is surprisingly independent of temperature (see Fig. 2b), and we cannot provide any satisfactory explanation.

P-450 remained chemically reducible well below the temperature arresting its enzymic reduction. Moreover it was observed that the fixation of carbon monoxide upon ferrocytochrome was faster than the reduction of ferricytochrome, which remains rate limiting at each temperature (unpublished data). The fact that cytochrome P-450 is chemically reducible down to -20°C permits us to foresee valuable experiments below 0°C that could provide new information about the intermediates of the reaction cycle of P-450.

CONCLUSION

Reactions of microsomal enzymes can be studied in aqueous-organic solutions that serve as “antifreeze” at temperatures below 0°C, by a method already applied to several isolated enzymes (1). Recently Chance successfully studied some electron-transfer processes in mitochondria under similar conditions (15), and we can expect that other membrane-bound enzymes could be studied in this way.

Nevertheless, one must keep in mind the possible effect of structural integrity on enzyme activity and the possible influence of the solvent on this integrity, as well as the fact that a large decrease in temperature might influence the activity and sequence of enzyme reactions. During the present work,
it was reassuring to note that the normal behavior of several reactions was still exhibited under the present unusual conditions.