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Interaction of cytochrome c_L with methanol dehydrogenase from *Methylophaga marina* 42:

Thermodynamic arguments for conformational change

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Abstract. The reaction of methanol dehydrogenase with cytochrome c_L from *Methylophaga marina* and the reactions of the non-physiological substrates, Wurster's blue and ascorbic acid, with both proteins were studied as a function of temperature (4–32 °C), pressure (1–2000 bar) and ionic strength using the optical high pressure stopped-flow method. The thermodynamic parameters $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$ were determined for all reactions where electron transfers are involved. These data allowed the determination of the Maxwell relationships which proved the internal thermodynamic consistency of the system under study. A conformational change on the cytochrome c_L level was deduced from both breaks in the Arrhenius plots and the variation of the $\Delta V^{\ddagger}$ with temperature.

Key words: Methanol dehydrogenase – Cytochrome c_L – Electron transfer – High pressure stopped-flow – Hydrostatic pressure – High pressure electrophoresis

Introduction

Methanol oxidation in gram negative methylotrophic bacteria is performed by a periplasmatically located quinoprotein methanol dehydrogenase (MDH). MDH is a 2,7,9-tricarboxy-1*H*-pyrrolo-[2,3-*f*]quinoline-4,5-dione (PQQ) dependent enzyme (Duine et al. 1986) and three different redox forms, having distinct absorption spectra, have been identified so far (Duine and Frank 1980; Frank et al. 1988). Several reports indicate that MDH is composed of two different subunits with M = 60–70 kDa and M = 6–10 kDa, respectively, arranged in an $\alpha_2\beta_2$ structure (Nunn et al. 1989; van Spanning et al. 1991; Anderson and Lidstrom 1988) and this has also been shown to be the case for the MDH from *Methylophaga marina* 42 used in the present study (Janvier et al. 1992).

All methylotrophic bacteria contain at least two soluble cytochromes *c*, which are designated as cytochrome c_L and cytochrome c_H according to their isoelectric point (O'Keeffe and Anthony 1980). These isoelectric points are 4.6 and 9.2, respectively, in the case of *Methylophaga marina* 42 (Janvier et al. 1992).

Early, but indirect, evidence indicated that cytochrome c_L was the natural electron acceptor for MDH (Anthony 1982). Direct proof was obtained when it was shown that methanol dependent, albeit slow, turnover takes place in a system containing MDH, cytochrome c_L and horse heart cytochrome *c* as terminal electron acceptor (Beardmore-Gray et al. 1983). This was substantiated by stopped-flow experiments demonstrating rapid electron transfer between MDH and cytochrome c_L (but not cytochrome c_H) from *Hyphomicrobium X* (Dijkstra et al. 1988). A similar electron transfer has been reported between components from other methylotrophic bacteria (see Anthony 1992 for review).

The rate of the reaction between cytochrome c_L and MDH is decreased when the ionic strength is raised, suggesting the involvement of electrostatic forces in some stage of the reaction, possibly the formation of a transient complex. The occurrence of such a complex is likely in view of the observation of saturation kinetics at pH 9 for the reaction between the components isolated from *Hyphomicrobium X* (Dijkstra et al. 1989). Recently, crosslinking experiments have shown that specific lysine residues on MDH and carboxyl groups on cytochrome c_L participate in the formation of the complex (Cox et al. 1992), confirming the electrostatic nature of the interaction.

Interestingly, the electron transfer reaction between cytochrome c_L and MDH from *Methylophaga marina*

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Abbreviations: 4-morpholinepropanesulfonic acid (MOPS), 2-(cyclohexylamino)ethanesulfonic acid (CHES), methanol dehydrogenase (MDH), ethylenedinitrilotetraacetic acid disodium salt (EDTA), bromothymol blue (3′,3″-dibromothymolsulfonephthalein) (BTB), 2,7,9-tricarboxy-1H-pyrrolo-[2,3-*f*]quinoline-4,5-dione (PQQ), mammalian horse heart cytochrome *c* (cytochrome c_{HH})

shows saturation behaviour at pH 7, which might be related to the fact that the pI's are relatively close together (pI for MDH = 6.7, Janvier and Gasser 1987) and therefore the electrostatic interactions should be weak.

Here we study the physiological reaction between MDH and its natural substrate cytochrome c_L because, so far, little is known about the specificity of the interaction between these two proteins, mainly in terms of possible conformational changes. One way to have access to information about conformational (and solvational) changes is to thermodynamically perturb the system under study. As in previous work (Balny et al. 1989; Butz et al. 1988; Heiber-Langer et al. 1992; Heremans 1982; Jaenicke 1981; Marden and Hui Bon Hoa 1982) our approach uses the pressure dependence of kinetic constants in addition to the classical variables temperature and ionic strength. A preliminary study of another PQQ dependent enzyme had shown that the use of pressure and temperature may reveal the structural flexibility of quinoproteins (Frank et al. 1991). Here, owing to the complexity of the system under study, we also examined the behaviour of both reactants with non-natural reagents in order to gain insight into the contribution of each compound to the overall activation volume.

Since hydrophobic interactions play an important role in the stabilization of oligomeric proteins, pressure is expected to disrupt quaternary structures (Weber 1987). To monitor the possible dissociation of MDH with pressure, PAG-electrophoresis under high pressure was performed (Balny et al. 1989; Masson et al. 1990).

Materials and methods

Enzymes, reagents and solutions

MDH and cytochrome c_L were prepared from frozen cell paste of *Methylophaga marina* 42 as previously described (Janvier et al. 1992). Wurster's blue was prepared by oxidation of *N,N,N',N'* tetramethyl-p-phenylene diamine (Michaelis and Gramick 1943). A stock solution of Wurster's blue was prepared by dissolving 3 mg of Wurster's blue in 1 ml of water, sonicating this mixture for 1 min followed by filtration through a 0.2 μm filter. The concentration was determined spectrophotometrically, using $\varepsilon_{612\ nm} = 12\ 700\ M^{-1} \cdot cm^{-1}$. This stock solution (2.8 mM) was stored at −80 °C, and dilutions were freshly prepared before the experiments. 4-morpholinepropanesulfonic acid (MOPS) was obtained from Serva, sodium ascorbate and 2-(cyclohexylamino)ethanesulfonic acid (CHES), horse heart cytochrome c_{HH}, type VI, were from Sigma and all other chemicals from Merck. All other chemicals were analytical grade. Ascorbic acid solutions were freshly prepared every day.

MOPS (10 mM, pH 7.0) was chosen as buffer salt for all experiments as it did not interfere in the reaction of MDH and cytochrome c_L. Furthermore, the small activation volume of 4.7 ml · mol^{-1} (Kitamura and Itoh 1987) indicates that the pressure dependent variation of the proton activity of MOPS can be neglected in the pressure range used. The use of imidazole (instead of MOPS) resulted in biphasic curves for the absorption change *versus* time for these experiments. Two second order rate constants were calculated: $k_{slow} = (1\ 200 \pm 200)\ M^{-1} \cdot s^{-1}$ and $k_{fast} = (6\ 500 \pm 700)\ M^{-1} \cdot s^{-1}$. The reason for biphasic curves is not known but it is presumably due to an interaction between the buffer salt and the proteins.

Some reactions with Wurster's blue were too slow at pH 7.0, they were performed at pH 9.0, using CHES (10 mM, pH 9.0) as the buffer salt.

For electrophoretic experiments the following chemicals were used: acrylamide "electran" from BDH, methylene bisacrylamide and Triton X100 from Sigma, N,N,N',N'-tetramethylendiamine, ammonium peroxydisulfate and β-alanine from Fluka.

Kinetic equipment

Kinetic measurements under high pressure were performed with equipment developed in our laboratory which were interfaced to Amino DW2 spectrophotometers (Markley et al. 1981; Balny et al. 1987). Data were stored and analyzed as described previously (Balny et al. 1989). At least three measurements were performed at each temperature and pressure.

Kinetic measurements

a) Reduction of cytochrome c_L by MDH. All experiments at atmospheric pressure for the reaction of cytochrome c_L with MDH were examined using the dual wavelength mode of the Aminco spectrophotometer at $A_{419\ nm}$ *minus* $A_{403\ nm}$. These two wavelengths are the maximum and minimum in the difference spectrum of reactants and products which assures the maximum amplitude and allows one to work at very low concentrations of enzyme (i.e. 2 μM cytochrome c_L, 10 μM MDH).

The catalytic site concentration of MDH was calculated using $\varepsilon_{280\ nm} = 132\ 600\ M^{-1} \cdot cm^{-1}$, based on subunits with M = 65 kDa and an $A_{280\ nm}$ (1 mg/ml) = 2.04 determined according to van Iersel et al. (1985). Similarly, an $A_{280\ nm}$ (1 mg/ml) = 2.0 was found for cytochrome c_L from which $\varepsilon_{280\ nm} = 38\ 000\ M^{-1} \cdot cm^{-1}$ and $\varepsilon_{550\ nm} = 21\ 600\ M^{-1} \cdot cm^{-1}$ for the ferrous form could be derived based on M = 19 kDa (van Iersel et al. 1985).

Experiments were performed in the concentration ranges of 1 to 2.5 μM for cytochrome c_L and 3 to 40 μM for MDH. All reactions were in 10 mM MOPS at pH 7.0. The ionic strength was varied by adding NaCl (10 to 200 mM). In some experiments EDTA (5 and 7.5 μM) was used as inhibitor for the overall reaction. Experiments were also performed using cytochrome c_{HH} instead of cytochrome c_L as electron acceptor in order to evaluate the specificity of cytochrome c_L. Measurements were generally carried out at 25 °C. The temperature dependence of the reduction of cytochrome c_L by MDH was studied at atmospheric pressure in the temperature range 4 to 32 °C.

b) Reduction of cytochrome c_L by ascorbic acid. The reaction rate for the reduction of cytochrome c_L by ascorbic

acid was followed using the dual wavelength mode of the Aminco ($A_{419\ nm}$ *minus* $A_{403\ nm}$) in 10 mM MOPS, pH 7.0. The concentrations of cytochrome c_L and ascorbic acid varied in the range 2.5 to 3 μM and 250 to 3 000 μM, respectively. Temperature dependent measurements were performed in the temperature range 4–31 °C. The influence of EDTA (5 μM) on the reaction was studied at 25 °C. The dependence on the ionic strength was measured by adding 100 mM NaCl.

c) Reduction of Wurster's blue by MDH. The reaction rate for the reaction of Wurster's blue with MDH was followed at $A_{612\ nm}$ *minus* $A_{660\ nm}$ using 22.5 μM MDH and 7.5 μM Wurster's blue at pH 9.0 since the reaction was too slow at pH 7.0 (Frank et al. 1988). The reaction was examined in the temperature range 4.5–32 °C using 10 mM CHES, pH 9.0, as a solvent for the MDH and water for the Wurster's blue. After mixing these two solutions the pH remained 9.0 (5 mM CHES).

d) Reaction of Wurster's blue with ascorbic acid. The reaction rate for the reaction of Wurster's blue with ascorbic acid was followed at $A_{612\ nm}$ *minus* $A_{660\ nm}$. The concentrations of Wurster's blue and ascorbic acid were 2.5 μM and 5.5 μM in 10 mM MOPS, pH 7.0 (T=25 °C). Owing to the dead-time of the stopped-flow (≈5 ms) the reaction was too fast to be measured at pH 9.0 where $k_{obs} \gg 20\ s^{-1}$.

Treatment of data

All the kinetic curves were simple exponential functions which obeyed

$$A_{(t)} = X_1 \exp(-t \cdot k_{obs}) + A_e \quad (1)$$

with $A_{(t)}$ and A_e being the actual and end absorption, X_1 the absorption change during the reaction, k_{obs} the observed reaction rate constant, and t the time.

The pressure dependence of the rate constant (k_{obs}) was linear in all experiments, allowing us to calculate activation volumes ($\Delta V^{\neq}$) using the classical equation:

$$(\delta \ln k_{obs}/\delta P)_T = -\Delta V^{\neq}/RT \quad (2)$$

where P is the hydrostatic pressure, T the absolute temperature and R the gas constant (82 ml · atm · K^{-1}, with 1 atm = 101.3 kPa).

High pressure electrophoresis in capillary gels

Electrophoresis was carried out at different pressures in the range 1 bar to 2 000 bar. The high pressure gel electrophoresis apparatus was described previously (Balny et al. 1989; Masson et al. 1990). The gels were prepared in glass capillaries of 1 mm diameter (Reisfeld et al. 1962). A concentration of 6% of acrylamide was used and in the case of the detection of proteins 1% Triton X100 was added. Triton X100 was not added for enzymatic activity staining as it alters the enzyme activity. In the denaturating experiments a urea concentration up to 6 M was used to dissociate the subunits of the enzyme. Runs were performed in a 350 mM β-alanine buffer adjusted to pH 4.5 with acetic acid. The following conditions were used for the migration: for each capillary 1 to 2 μl of a MDH solution (7.5 mg/1 ml) were used for one experiment and 1 h 30 min for each run at constant intensity (0.3 mA/capillary tube). Runs were performed at 1, 500, 1 000, 1 500 and 2 000 bar at 20 °C. Protein bands were stained using Coomassie Brilliant blue, MDH activity was revealed by soaking the gels in Wurster's blue/methanol/pH 9.0 for 5–10 min. After staining the intensity of proteins bands was determined by densitometric scanning.

Results

a) Reduction of cytochrome c_L by MDH

The reaction of MDH with cytochrome c_L is an electron transfer reaction where the first steps follow the minimum reaction scheme:

$$MDH_{sem} + Cyt\ c_L^{ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [MDH_{sem} - Cyt\ c_L^{ox}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [MDH_{ox} - Cyt\ c_L^{red}] \quad \text{(scheme 1)}$$

with $K_1 = k_1/k_{-1}$ and Cyt c_L^{ox}, Cyt c_L^{red}, MDH_{sem} and MDH_{ox} being the oxidized, reduced and semiquinone forms, respectively. This mechanism has been proposed for other electron transfer reactions (Dijkstra et al. 1989; Heiber-Langer et al. 1992; Heremans et al. 1982). The following equation

$$k_{obs} = k_2 \cdot K_1 \cdot [MDH_{sem}]/(K_1 \cdot [MDH_{sem}] + 1) \quad (3)$$

for the observed rate constant can then be used if one makes two assumptions: 1) the first step of the reaction is

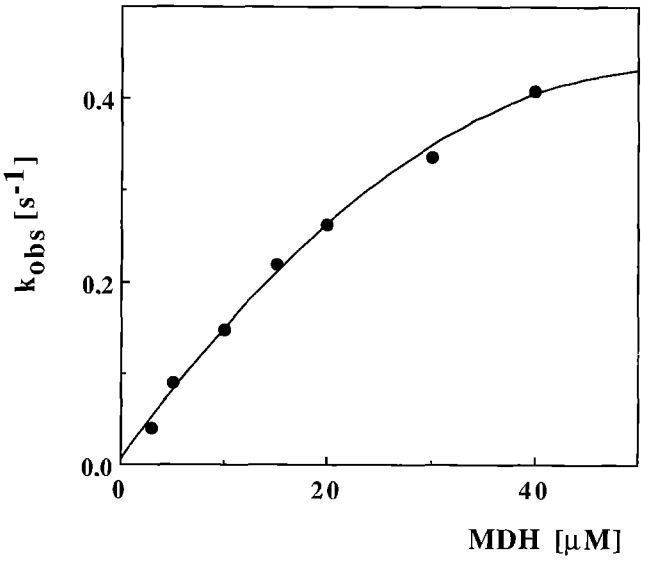

Fig. 1. Dependence of the rate constant on the concentration of MDH. [Cyt c_L] = 1 μM, [buffer] = 10 mM, pH = 7; the temperature was 25 °C. The curve was computer fitted according to (1)

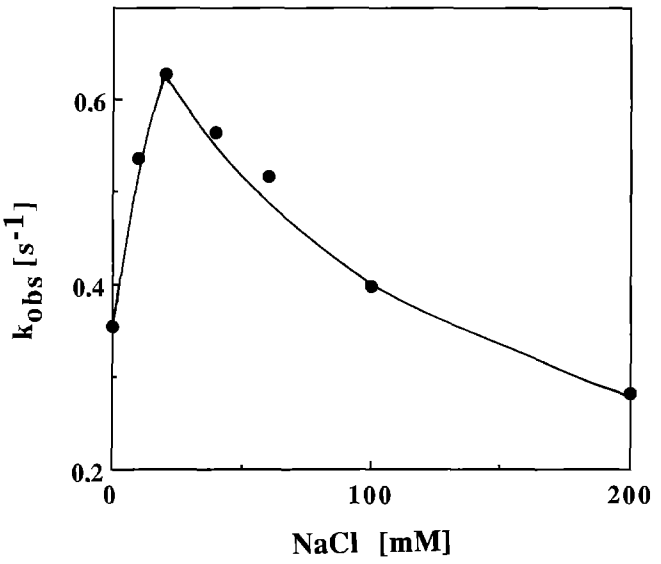

Fig. 2. Dependence of the reaction rate on the ionic strength for the reaction of MDH with cytochrome c_L. Concentrations for MDH, cytochrome c_L and MOPS were 8 μM, 2 μM and 10 mM, the pH was 7. The ionic strength was varied by the addition of NaCl at $T=25$ °C

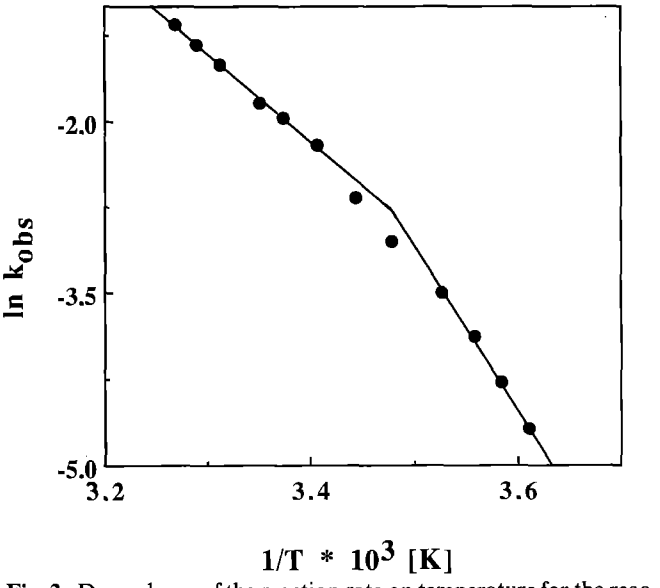

Fig. 3. Dependence of the reaction rate on temperature for the reaction of MDH with cytochrome c_L in 10 mM MOPS, pH 7.0. Concentrations of MDH and cytochrome c_L 10 μM and 2.5 μM respectively

fast compared to the second ($k_1 \gg k_2$) and 2) the backward reaction (k_{-2}) for the reduction step can be neglected.

Fitting of data to (3) leads to $K_1 = 20 \pm 3 \cdot 10^4\ M^{-1}$ and $k_2 = 0.9 \pm 0.09\ s^{-1}$. The saturation phenomenon is shown in Fig. 1. In all following experiments, we worked at a concentration of MDH lower than 10 μM which is in the nearly linear region of the curve. Thus, the equilibrium and the rate constants cannot be separated and k_{obs} can be described by

$$k_{obs} = k_+ [MDH] \quad \text{with} \quad k_+ = K_1 \cdot k_2$$

(see Balny et al. 1987).

Table 1. Activation parameters for the reaction of MDH with cytochrome c_L. The concentrations of MDH and cytochrome c_L were 10 μM and 2.5 μM, respectively. The reaction was performed in 10 mM MOPS at pH 7.0. Values are presented as mean ± SD

Temperature range [°C]	ΔH^* [kJ · mol^{-1}]	ΔS^* [J · K^{-1} · mol^{-1}]
4–10.5	117 ± 5	140 ± 20
20.5–33	62 ± 2	−54 ± 6

Table 2. Rate constants and activation volumes for the reaction of MDH with cytochrome c_L at different conditions. MOPS 10 mM, pH = 7 was used as buffer, the concentrations of cytochrome c_L and MDH were 2.5 μM and 10 μM, respectively (unless stated otherwise). Values are presented as mean ± SD

Temperature [°C]	Additional compounds	Activation volume [ml · mol^{-1}]	k_{obs} [s^{-1}]
Temperature variation			
4		122 ± 7	0.0254 ± 0.002
10		79 ± 5	0.219 ± 0.02
17		62 ± 5	0.513 ± 0.05
25		54 ± 5	1.065 ± 0.1
32		55 ± 5	0.863 ± 0.08
Ionic strength variation			
25	0 mM NaCl	54 ± 5	1.065 ± 0.1
25	10 mM NaCl	63 ± 5	0.264 ± 0.02
25	100 mM NaCl	70 ± 5	0.244 ± 0.02
Addition of EDTA			
25	0 μM EDTA	54 ± 5	1.065 ± 0.1
25	5 μM EDTA	46 ± 5	0.468 ± 0.04
25	7.5 μM EDTA	47 ± 5	0.199 ± 0.02
Different concentrations of MDH			
25	10 μM MDH	54 ± 5	1.065 ± 0.1
25	20 μM MDH	47 ± 5	1.75 ± 0.2
Different cytochromes			
25	2.5 μM cyto c_L	54 ± 5	1.065 ± 0.1
25	2.5 μM cyto c_{HH}	20 ± 5	0.013 ± 0.003

Table 3. Activation parameters for the reaction of cytochrome c_L with ascorbic acid. The concentrations of cytochrome c_L and ascorbic acid were 3 μM and 3 mM, respectively in 10 mM MOPS at pH 7.0. Values are presented as mean ± SD

Temperature range [°C]	ΔH^* [kJ · mol^{-1}]	ΔS^* [J · K^{-1} · mol^{-1}]
4.0–10.0	38 ± 2	−127 ± 7
18.0–31.0	22 ± 5	−184 ± 20

The effect of ionic strength is shown in Fig. 2. In the first part of the curve the reaction rate rises with increasing ionic strength while it decreases at ionic strength values higher than 25 mM.

The results of the temperature dependent kinetics are represented in Fig. 3 where a break in the Arrhenius plot can be seen. In Table 1 the results for separate fits for the two regions of the experimental points are shown. Upon

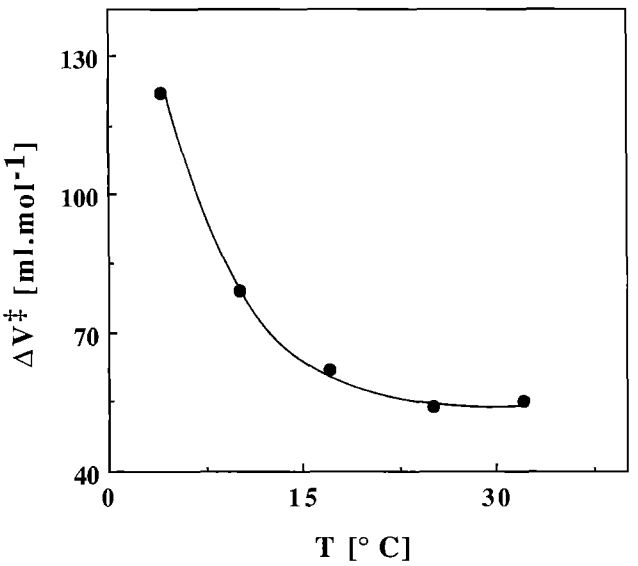

Fig. 4. Temperature dependence of the activation volume for the reaction of MDH with cytochrome c_L in 10 mM MOPS, pH 7.0. [Cyt c_L] = 2.5 μM, [MDH] = 10 μM

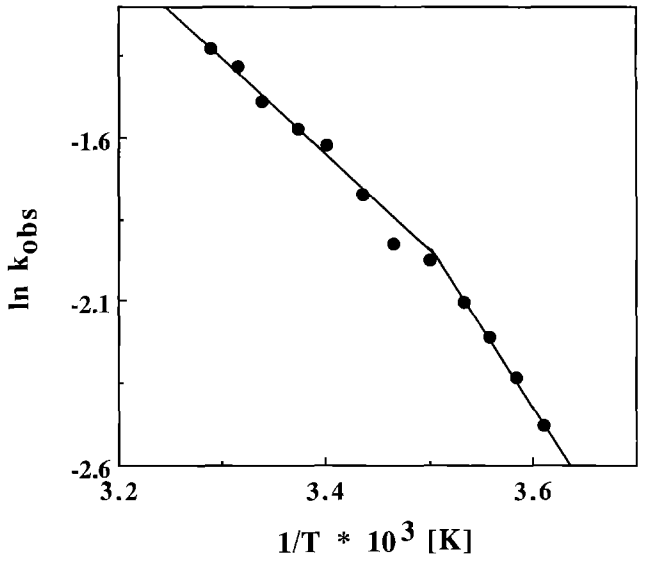

Fig. 5. Dependence of the reaction rate on temperature for the reaction of cytochrome c_L with ascorbic acid in 10 mM MOPS, pH 7.0. The concentrations of cytochrome c_L and ascorbic acid were 3 μM and 3 mM, respectively

extrapolation of the calculated straight lines a crossing at 14 ± 0.5 °C in the Arrhenius plot is obtained.

In Table 2 the effect of different parameters on reaction rates and volumes is summarized. There is a large effect of the temperature on the activation volume, which rises considerably when the temperature is lowered (see Fig. 4). The influence of the ionic strength is shown in Table 2 where the activation volume increases with increasing salt concentrations. Addition of 7.5 μM EDTA resulted in inhibition of the reaction, whereas the activation volume did not change. Similarly the volume change was found to be independent of the MDH concentration. When another cytochrome c (cyt c_{HH}) was used instead of

Table 4. Rate constants and activation volumes for the reaction of cytochrome c_L with ascorbic acid at different conditions. MOPS 10 mM, pH 7.0 was used as buffer, the concentrations of cytochrome c_L and ascorbic acid (asc) were 3 μM and 250 μM unless stated otherwise. Values are presented as mean ± SD

Temperature [°C]	Additional compounds	Activation volume [ml · mol^{-1}]	k_{obs} [s^{-1}]
Temperature variation			
15	2.5 mM asc	-6.7 ± 1	0.12 ± 0.02
25	2.5 mM asc	-7.0 ± 1	0.18 ± 0.02
25	0.25 mM asc	-6.0 ± 1	0.044 ± 0.004
Addition of EDTA			
25	0 μM EDTA	-6.0 ± 1	0.044 ± 0.004
25	5 μM EDTA	-6.3 ± 1	0.014 ± 0.002
Variation of the ionic strength			
25	0 mM NaCl	-6 ± 1	0.044 ± 0.04
25	100 mM NaCl	-6 ± 1	0.036 ± 0.006

cytochrome c_L, the reaction rate was lower by a magnitude of two, accompanied by a substantially smaller activation volume.

b) Reaction of cytochrome c_L with ascorbic acid

As for the temperature dependence of the MDH/cytochrome c_L reaction, the Arrhenius plot for the reaction of cytochrome c_L with ascorbic acid was not linear. A break was observed at 13 ± 1 °C (see Table 3 and Fig. 5).

The reaction of cytochrome c_L with ascorbic acid showed a small negative activation volume of -6 ± 1 ml · mol^{-1}, which was independent of ascorbic acid concentration (0.25 and 2.5 mM) and temperature (15 and 25 °C). The addition of EDTA resulted in a decrease of reaction rate but did not change the activation volume. Similarly a rise in ionic strength did not affect the activation volume. Results are summarized in Table 4.

c) Reaction of Wurster's blue with MDH

The linear Arrhenius plot for the reaction of Wurster's blue with MDH is shown in Fig. 6 where the fitting gives $\Delta H^{\ddagger} = 51 \pm 1$ kJ · mol^{-1} and $\Delta S^{\ddagger} = -85 \pm 3$ J · K^{-1} · mol^{-1}.

Results as a function of pressure are summarized in Table 5. It can be seen that the activation volume is not affected by the temperature whereas a significant change of $\Delta V^{\ddagger}$ with increasing ionic strength is observed.

d) Reaction of Wurster's blue with ascorbic acid

The pressure dependent k_{obs} values ($k_{obs} = 0.188$ s^{-1} at 1 bar and at 25 °C) for the reaction of Wurster's blue with ascorbic acid led to an activation volume of $\Delta V^{\ddagger} = -43 \pm 4$ ml mol^{-1} for the reaction of Wurster's blue with ascorbic acid in 10 mM MOPS, pH 7.0.

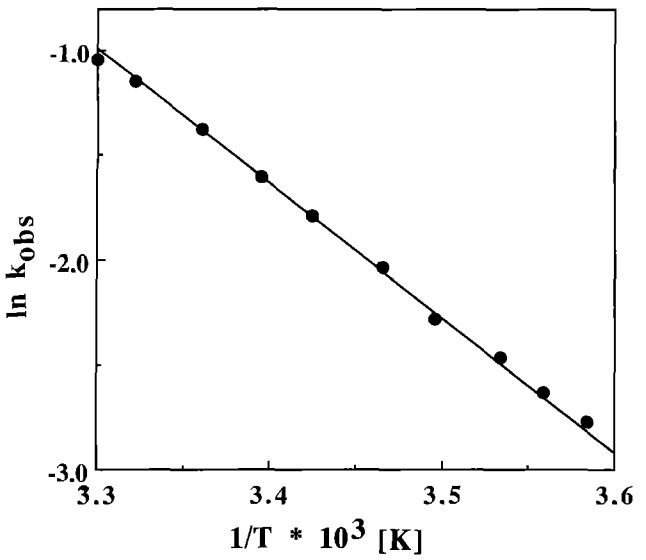

Fig. 6. Temperature dependence for the reaction of MDH with Wurster's blue at pH 9.0 in 5 mM CHES. The concentrations of MDH and WB were 22.5 μM and 7.5 μM, respectively

High pressure electrophoretic measurements

At atmospheric pressure MDH migrated on electrophoresis gels as a single sharp Gaussian band. Upon increasing the pressure, the mobility of the enzyme decreased and at pressures higher than 600 bar a non-symmetrical peak was observed. Staining for enzyme activity showed that the activity is only detectable in the front region of the peak. Up to 2 000 bar the enzyme activity was not affected by the pressure (see Fig. 7).

Under denaturating conditions, i.e. in the presence of urea in the gels in order to promote subunit dissociation, three peaks were observed at atmospheric pressure. The most mobile peak showed an intense activity up to 1.5 M urea whereas the slower peaks detected by protein staining showed no activity. When pressure was applied to the system, activity in the first peak was detected up to a concentration of 1 M urea at 1 000 bar whereas at 2 000 bar only a small activity was observed at 1 M urea.

Discussion

Studies at atmospheric pressure

As shown in the Results section the dependence of the observed rate constant on the MDH concentration can be described by a hyperbolic function leading to the values $K_1 = 20 \pm 3 \cdot 10^4\ M^{-1}$ and $k_2 = 0.9 \pm 0.09\ s^{-1}$ (see Fig. 1). These values can be compared with results for the MDH/cytochrome c_L system from *Hyphomicrobium X*: $K_1 = 0.8 \pm 0.2 \cdot 10^4\ M^{-1}$ and $k_2 = 0.33 \pm 0.07\ s^{-1}$ (Dijkstra et al. 1989). The equilibrium constant K_1 for our system is far larger than that in the *Hyphomicrobium X* system whereas the rate constant is only three times higher. In spite of the relatively small difference in the pI of MDH (6.7) and cytochrome c_L (4.6), compared to other systems

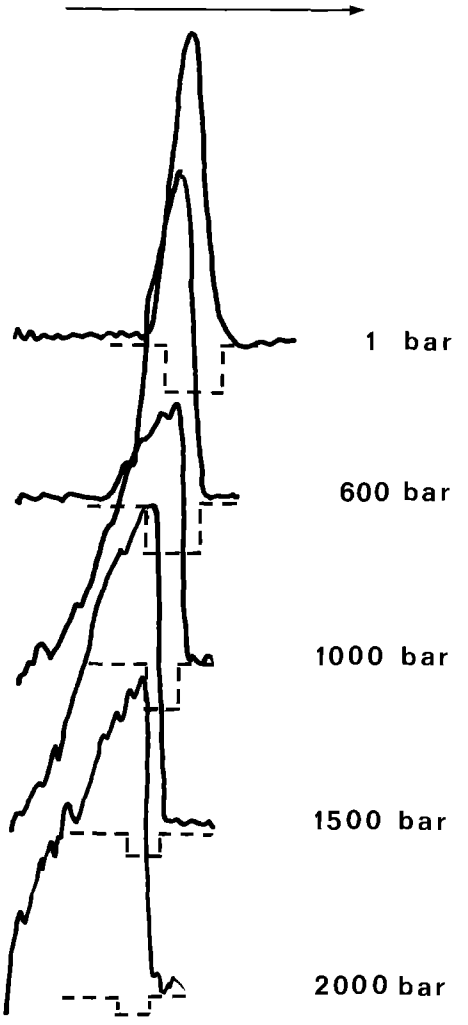

Fig. 7. Electrophoretic patterns of MDH at atmospheric pressure and under high pressure up to 2 000 bar. 1 μl of MDH solution (7.5 μg) was subjected to electrophoresis in 1 mm internal diameter capillary gels. The total polyacrylamide concentration was 6% and the running buffer was 350 mM β-alanine/acetic acid, pH 4.5. Runs were carried out at constant intensity (0.3 mA/gel) for 90 min. Continuous curves correspond to proteins. Dashed line are for enzyme activity: the active enzyme zones were shown on gels as colorless zones (dashed line wells)

Table 5. Rate constants and activation volumes for the reaction of Wurster's blue with MDH at different conditions in 5 mM CHES, pH 9.0. The concentrations of MDH and Wurster's blue were 22.5 μM and 7.5 μM, respectively unless stated otherwise. The Wurster's blue was dissolved in water before mixing. Values are presented as mean ± SD

Temperature [°C]	Additional compounds	Activation volume [ml · mol^{-1}]	k_{obs} [s^{-1}]
Temperature variation			
15		−19.5 ± 1	0.667 ± 0.07
25		−22.3 ± 1	0.452 ± 0.04
Variation of the ionic strength			
25	0 mM NaCl	−22.3 ± 1	0.452 ± 0.04
25	10 mM NaCl	−17.5 ± 1.5	0.423 ± 0.04
25	100 mM NaCl	−13.0 ± 1	0.381 ± 0.04
25	200 mM NaCl	−14.0 ± 1	0.289 ± 0.03

(Arciero et al. 1991), this shows a rather great affinity in the MDH/cytochrome c_L system under study, which might be interpreted in terms of a higher specificity.

The reaction showed a marked effect of the ionic strength upon the reaction rate. Initially, up to 20 mM NaCl, the reaction was accelerated by increasing ionic strength, higher salt concentrations resulted in a slower reaction. Similar behaviour was also observed for the above-mentioned *Hyphomicrobium X* system. This phenomenon is difficult to interpret. The descending part of the curve reflects a behaviour typical for electrostatic interactions between the two proteins as shown for other systems (Arciero et al. 1991; Cusanovich et al. 1988; Heiber-Langer et al. 1992). For the ascending part of the curve, it could be suggested that extensive protein conformation and/or hydration changes occur during the binding at low salt concentration. A similar explanation has been proposed for enzyme-substrate binding in the carbamylation reaction of butyrylcholinesterase (Masson and Balny 1990).

The temperature effect on the MDH – cytochrome c_L reaction shows a break in the Arrhenius plot at 14 ± 0.5 °C. Similarly for the reaction between cytochrome c_L and ascorbic acid a break at 13 ± 1 °C was observed, whereas a linear Arrhenius plot was obtained for the MDH/Wurster's blue reaction.

As discussed by Biosca et al. (1984) for lipid-free protein systems a break in the Arrhenius plot can be due to: 1) a temperature induced protein conformational change, 2) a change in the rate-limiting step at a critical temperature, 3) a modification of the solvent structure at a critical temperature. In this context we should also consider "breaks" observed in the Montpellier laboratory for two other electron transfer reactions. The first concerned the reaction of two-band cytochrome c_1^{2+} with cytochrome c_{HH}^{3+} in 40% ethylene glycol where a break was observed between −15 °C and −21 °C (Kim et al. 1987). In this case the break was thought to result from a shift in the equilibrium between two conformations of cytochrome c_1. The second example concerned the intramolecular electron transfer reaction which takes place between the cytochrome P460 – containing site and a series of *c* hemes in hydroxylamine oxidoreductase of *Nitrosomonas* (reaction achieved in 40% ethylene glycol) where a break occurring at approximately 0 °C was observed. This was interpreted as a modification of the physical orientation of electron transfer centers by temperature, a phenomenon magnified by the application of high pressure (Balny and Hooper 1988). Since we worked in water and in a limited temperature range, modification of the solvent structure is not expected.

Concerning the reaction of cytochrome c_L with ascorbic acid, where a break in $\Delta H^{\neq}$ is also observed, a minimal reaction scheme similar to that for MDH/cytochrome c_L can be proposed:

$$\text{Asc} + \text{Cyt } c_L^{ox} \overset{K_1}{\rightleftharpoons} [\text{Asc} - \text{Cyt } c_L^{ox}] \overset{k_2}{\rightleftharpoons} [\text{Deh. Asc} - \text{Cyt } c_L^{red}] \quad \text{(scheme 2)}$$

In this case (see Eq. (3)) the expression for k_{obs} is:

$$k_{obs} = k_2 \cdot K_1 \cdot [\text{Asc}]/(K_1 \cdot [\text{Asc}] + 1) \quad (4)$$

The temperature dependence of the reaction rate k_{obs} can then be calculated as follows:

$$\frac{\partial \ln k_{obs}}{\partial 1/T} = -\frac{\Delta H_{obs}^{\neq}}{R} = -\frac{\Delta H_2^{\neq}}{R} - \frac{1}{1 + K_1 [\text{Asc}]} \frac{\Delta H_1^{\circ}}{R} \quad (5)$$

where ΔH_1° is the reaction enthalpy for the first equilibrium (K_1) and $\Delta H_2^{\neq}$ the activation enthalpy for the reduction step (k_2). Thus, only a measurement of the temperature dependence of the reaction as a function of ascorbic acid concentration would allow the determination of both enthalpies. Unfortunately such a determination requires large amounts of material at the precision needed for this study and it was impossible to perform it. Thus it was not possible to determine which rate constant (k_1 or k_2) governs the non-linearity of the observed $\Delta H_{obs}^{\neq}$.

So far neither a temperature-induced protein conformational change nor a change in the rate-determining step can be definitely ruled out. However, at this point, in view of the narrow range of temperature explored and the similar sharp temperature breaks observed (and not a "jump", see Biosca et al. 1984) for both the reduction of cytochrome c_L by MDH and by ascorbic acid, it can be postulated that a modification of the conformation of cytochrome c_L is a plausible interpretation of the non-linear Arrhenius plots observed here. Similar interpretations have been reported for a certain number of soluble enzymes (Massey et al. 1966; Masson and Balny 1986).

Studies at high pressure

Exposure of MDH to high pressure did not result in irreversible inactivation, since after release of the pressure the initial reaction with cytochrome c_L was restored. This does, however, not rule out the possibility that pressure might induce a splitting of the $\alpha_2\beta_2$ structure of MDH into catalytically active α β structures.

Electrophoretic experiments showed that high pressure leads to a decrease in the mobility of the enzyme which migrates as a single peak even at 2 000 bar where its activity is still measurable (at least in the front part of the peak). This indicates that MDH is maintained in its active tetrameric structure in the pressure range explored.

The volume changes accompanying the reaction of MDH with cytochrome c_L are large whatever the temperature (see Tables 1 and 2). Then we obtained values for $\Delta H^{\neq}$, $\Delta S^{\neq}$ and $\Delta V^{\neq}$ which describe the properties of the activated state $[\text{MDH}^{ox} - \text{cyt } c_L^{red}]^{\neq}$. However, if we admit that cytochrome c_L undergoes a conformational change to cyt c_L' a second activated state, $[\text{MDH}^{ox} - \text{cyt } c_L'^{red}]^{\neq}$ may be considered. In the two regions of both sides of the Arrhenius plot the thermodynamic values fit to Maxwell's relationships:

$$(\partial \Delta V^{\neq}/\partial T)_p = -(\partial \Delta S^{\neq}/\partial p)_T \quad (6)$$

and

$$(\partial \Delta H^{\neq}/\partial p)_T = \Delta V^{\neq} - T(\partial \Delta V^{\neq}/\partial T)_p \quad (7)$$

(see Table 6). This merely shows that for the two parts of the Arrhenius plots, the results are internally consis-

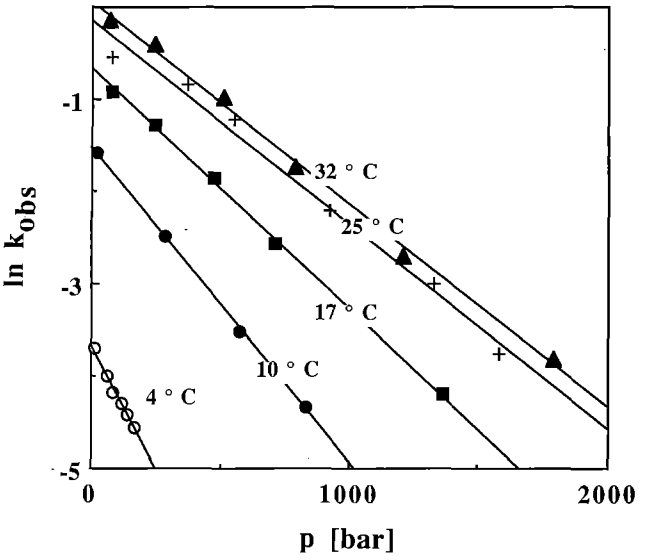

Fig. 8. Pressure dependence of the reaction rate for the reaction of MDH with cytochrome c_L at different temperatures. Conditions see Table 2 and Fig. 4

Table 6. Temperature coefficients of activation volume and pressure coefficients of activation entropy and enthalpy for k_{obs} for the reaction of MDH with cytochrome c_L for the two different temperature ranges. Values are presented as mean ± SD

Coefficients		4–10 °C	17–25 °C
$(\partial \Delta V^{\neq}/\partial T)_p$	$(ml \cdot mol^{-1} \cdot K^{-1})$	-7.2 ± 3.5	-1 ± 1.6
$-(\partial \Delta S^{\neq}/\partial p)_T$	$(ml \cdot mol^{-1} \cdot K^{-1})$	-11.0 ± 3	-3 ± 1
$(\partial \Delta H^{\neq}/\partial p)_T$	$(ml \cdot mol^{-1})$	$3\,300 \pm 1\,000$	860 ± 500
$\Delta V^{\neq}$ $-T(\partial \Delta V^{\neq}/\partial T)_p$	$(ml \cdot mol^{-1})$		
Temperature	4 °C	$2\,100 \pm 500$ 17 °C	350 ± 500
	10 °C	$2\,100 \pm 500$ 25 °C	350 ± 500

tent, the correlation being a mathematical consequence of the definitions of thermodynamic functions (Balny and Travers 1989).

For the MDH/cytochrome c_L reaction a large effect of the temperature on the activation volume was observed. This temperature dependent behaviour can be divided into two effects. In the temperature range from 17 to 32 °C, the activation volume is nearly constant ($\Delta V^{\neq} \approx 57\ ml \cdot mol^{-1}$) while it increases dramatically in the region from 10 to 4 °C (see Table 2 and Fig. 4). The rather constant value in the higher temperature range might reflect that in this temperature region the interactions between the two proteins are not altered. In the low-temperature region there is a change by a factor of 2 between 4 and 10 °C, suggesting that in this region, the protein which governs the interaction is subjected to a modification of its conformation or state of hydration.

The change in activation volume may be explained by conformational changes associated with cytochrome c_L. As already discussed above, the temperature dependence showed a non-linearity for the reaction of cytochrome c_L with MDH as well as with ascorbic acid, while the Arrhenius plot is linear for the reaction of MDH with Wurster's blue. From the temperature dependence alone (because of the limited number of measurements) it could not be clearly defined whether this change is due to a conformational change. Variation of $\Delta V^{\neq}$ as a function of temperature is an additional argument which strengthens the above-mentioned hypothesis of a conformational change occurring on the level of cytochrome c_L. We should also point out that there are no temperature dependent variations of the activation volumes when either MDH or cytochrome c_L react with non-physiological compounds.

Another interesting feature concerns the variation of $\Delta V^{\neq}$ with ionic strength (in the range 0–25 mM NaCl). In both MDH/cytochrome c_L and MDH/Wurster's blue reactions, the activation volume rises when the ionic strength is increased, whereas the $\Delta V^{\neq}$ of the reaction of cytochrome c_L/ascorbic acid remains constant (see Fig. 6). This should be compared with the increase in the reaction rate with ionic strength and supports the hypothesis of a conformational change of MDH at ionic strength lower than 25 mM NaCl.

Molecular significance of the activation volume in protein-protein electron transfer

Generally, it is difficult to give a clear molecular interpretation of $\Delta V^{\neq}$ in enzymic reactions. To go further we have 1) performed experiments on MDH and cytochrome c_L with non natural reagents and 2) tried to calculate the global activation volume, knowing activation volumes of related reactions.

Indeed, for the thermodynamic variable ΔH one can get the value of enthalpy for an unknown reaction if the ΔH values for related reactions are known.

Here, a similar approach could not be undertaken for the activation volume of the reaction of MDH with cytochrome c_L as not all reactions necessary for such a calculation were measurable. A first difficulty resulted from the fact that for experimental reasons the different reactions were measured at different pH values. Furthermore it was impossible to determine the k_{obs} value and the activation volume for the reverse reaction of Wurster's blue and ascorbic acid. Therefore only a limited estimation could be performed which nevertheless showed a large discrepancy between the estimated and the measured value for the reaction of MDH and cytochrome c_L whereas for the non-physiological reaction between cytochrome c_{HH} and MDH estimated and measured value were in agreement. This might be a hint for a more specific interaction between the physiological cytochrome c and MDH.

Another way to check the validity of the $\Delta V^{\neq}$ determination is the study of the pressure dependence of k_{obs} (and the associated $\Delta G^{\neq}$) at different temperatures. We obtained a series of converging lines (see Fig. 8). Although the extrapolation to negative pressures has no physical meaning, such behaviour is predicted by the general equation ($\Delta G^{\neq} = f(T, P)$ (Morild 1981). The physical significance of the point of convergence remains obscure and depends on the reference state of $\Delta G^{\neq}$. However, it is a

zone where $(\partial \Delta G^{\neq}/\partial T)_p = \Delta S^{\neq} \approx 0$. This was already observed in the Montpellier laboratory for other systems (Balny et al. 1987; Balny and Hooper 1988; Balny and Travers 1989). The present results once more demonstrate the strong influence of T on $\Delta V^{\neq}$ associated with proteins reactions, compared to that observed for simple chemical systems (Asano and LeNoble 1978).

Conclusion

Although there is a real difficulty in assigning a clear biochemical counterpart to a given $\Delta V^{\neq}$, the present data show that a combination of pressure and the more classical temperature and ionic strength studies allow a better understanding of electron transfer reactions. The thermodynamic results of this study strongly support the hypothesis that electron transfer between MDH and cytochrome c_L proceeds via conformation changes of the two proteins: 1) MDH at low ionic strength and 2) cytochrome c_L whatever the ionic strength.

For these experiments, we must point out that data obtained using high pressure permit one to complete the observations performed at atmospheric pressure. This dual approach was already reported for other systems (Balny and Hooper 1988; Clery et al. 1992).

We tried to use the same additivity principles used for $\Delta H^{\neq}$ but for different reasons these could not be applied to our reaction system. Nevertheless an estimation showed that the results seem to fit for the model system with cytochrome c_{HH} whereas in the MDH/cytochrome c_L system a great discrepancy is seen. It is concluded that this discrepancy is due to specific interactions between the two proteins which do not take place in the reaction with the non-physiological cytochrome *c*. The role of the cytochrome c_L during the electrostatic interaction between the two proteins seems specific. However, we cannot exclude a solvation modification too, as was previously shown for the hydroxylamine oxidoreductase/cytochrome c_{HH} system (Heiber-Langer et al. 1992). In the present experiments, the solvation effect could be masked by large changes in conformation. This is another example of the pressure modulation of protein-protein electron-transfer which could be of practical interest in the biotechnological application of high pressure on biochemical materials (Balny et al. 1992a, b).

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