# Thermostability of membrane enzymes in organic solvents

Guadalupe Ayala, M. Tuena de Gómez-Puyou, A. Gómez-Puyou and Alberto Darszon*

*Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-600, 04510 México DF* and **Departamento de Bioquímica, Centro de Investigación y Estudios Avanzados, Instituto Politécnico Nacional, México DF, Mexico*

Received 11 March 1986

Two multisubunit enzymes of the inner mitochondrial membrane, cytochrome oxidase and the $H^+$-ATPase may be transferred into highly apolar solvents as protein-lipid complexes. At 70° C and an initial water concentration of 13 μl per ml organic solvent (toluene), the half-life of the ATPase was approx. 11 h, whereas that of cytochrome oxidase was about 100 s. Thermostability of cytochrome oxidase could be increased more that 100-times by decreasing the water concentration to 3 μl per ml toluene. At this latter concentration of water the half-life of the ATPase at 90, 80 and 70° C was 5, 48 and 96 h, respectively.

*ATPase* *Cytochrome oxidase* *Enzyme stability* *Organic solvent* *Thermostability* *Water content*

## 1. INTRODUCTION

Enzymes lose their catalytic activity as the temperature is raised. A fundamental step in thermoinactivation is the unfolding of the protein which results from a decrease of the different covalent forces that maintain the native, catalytically active structure [1,2]. Also, it is accepted that water is involved in the maintenance of noncovalent interactions in the enzyme [3–5]. Thus, it appeared of interest to study the thermostability of enzymes that possess highly complex subunit structures under conditions where water is largely substituted by apolar solvents. These studies became possible due to the development of a methodology that allowed the transfer of enzymes into apolar solvents as protein-lipid complexes [6,7]. Previously, Zaks and Klibanov [5] reported that pancreatic lipase suspended in organic solvents withstands 100°C for many hours. Our studies show that mitochondrial $H^+$-ATPase [8] and cytochrome oxidase [9], when transferred to toluene, increase their thermal stability by orders of magnitude. Their thermostability is largely affected by the content of water in the solvent.

## 2. MATERIALS AND METHODS

The transfer of enzymes from submitochondrial particles from bovine heart into toluene was made essentially as in [7], except that the procedure was carried out at room temperature. The turbid organic phase obtained was withdrawn without touching the interphase, placed in 1.7 × 15 cm tubes, and incubated at various temperatures. Liposomes were formed from these extracts at room temperature as described [7]. The ATPase and cytochrome oxidase activities were measured in the liposomes [10,11] and were considered an index of the degree of inactivation of the enzymes in the organic phase.

## 3. RESULTS AND DISCUSSION

Approx. 50% of the protein of submitochondrial particles was extracted into toluene. The activities of the ATPase and cytochrome oxidase in the liposomes were approx. 0.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and 2 $\mu gatom \cdot min^{-1} \cdot mg^{-1}$, respectively. The respective specific activities of the ATPase and cytochrome oxidase in the liposomes were 50% lower and 15% higher than in the particles. The

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/86/$3.50 

reasons for the difference in the specific activities between liposomes and particles have been discussed [7].

Toluene extracts incubated at 70, 80 and 90°C for prolonged times yielded liposomes that possessed ATPase activity (fig.1). The half-life of the enzyme at these temperatures was about 11 h, 4 h and 20 min, respectively. The incubation of submitochondrial particles in 0.25 M sucrose at the above temperatures totally inactivated the ATPase in 2 min, 1 min and 20 s. In contrast to the marked thermostability of the ATPase in toluene, cytochrome oxidase was rapidly inactivated, i.e. total loss of activity was observed at 70°C in less than 2 min.

Water is known to be involved in the thermal inactivation of enzymes [3–5]. Using $^3H_2O$, the amount of water in the extracts was estimated (see legend to table 1). This was 13 ± 3 $\mu$l $^3H_2O$ per ml toluene extract. It was assumed that in the preparation of the toluene extracts, $^3H_2O$ distributes uniformly in all water spaces, and that the measured radioactivity corresponded to water. After initiation of incubation at 70°C in 1.7 × 15 cm tubes, the amount of $^3H_2O$ in the extract rapidly diminished to 2–3 $\mu$l per ml in a period of 10 min; this value did not change significantly after 1.5 h (fig.2). The rapid initial elimination of water from toluene extracts proved fundamental for attaining thermostability of enzymes. In fact, it was observed that if the incubation at high temperatures was carried out in 1.3 × 12.5 instead of 1.7 × 15 cm tubes, the ATPase was rapidly inactivated (fig.2); apparently this correlated with a slower evaporation of water from the organic phase (fig.2).

At low temperatures, the amount of water in the toluene extracts could also be decreased by partial evaporation of the organic phase under an $N_2$ current (table 1). This partial elimination of water caused the turbid extracts to become transparent, and provoked a large increase in thermostability of

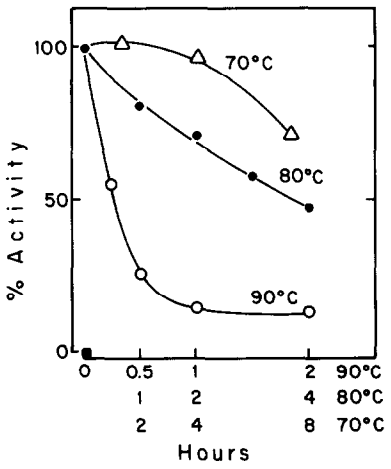


Fig.1. Thermostability of enzyme-lipid complexes in toluene. Toluene extracts were placed in open 1.7 × 15 cm tubes and incubated at the temperatures shown. At various times, the tubes were placed in an ice bath, liposomes were formed and their ATPase activity measured. Cytochrome oxidase activity of liposomes formed from toluene extracts incubated for 2 min at 70°C was also measured (■). The activities of cytochrome oxidase and ATPase of liposomes formed without incubation at high temperatures were considered to be 100%; these activities were 1.9 $\mu$gatom $O \cdot min^{-1} \cdot mg^{-1}$ at 30°C and 0.38 $\mu$mol $\cdot min^{-1} \cdot mg^{-1}$ at 37°C, respectively.

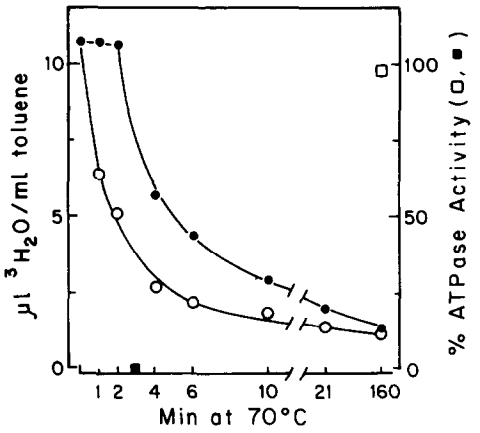


Fig.2. Water concentration in organic extracts and ATPase inactivation. Toluene extracts were prepared as described, except that the sucrose and $CaCl_2$ solutions were made in $^3H_2O$ with the same specific activity (1.9 × $10^7$ cpm/ml). The radioactivity of the toluene extract was assayed to estimate $^3H_2O$ per ml organic phase. Thereafter, 0.8 ml extract was placed in 1.7 × 15 cm (○—○) and 1.3 × 12.5 (●—●) tubes and incubated at 70°C. As indicated aliquots were withdrawn to measure radioactivity. After incubation of the extract at 70°C for 3 min in the small tubes (■) and 160 min in the large tubes (□) liposomes were formed from aliquots of the extract to assay ATPase activity. The results are in % activity remaining; 100% activity was 0.42 $\mu$mol $\cdot min^{-1} \cdot mg^{-1}$ which corresponds to liposomes of toluene extracts that had not been incubated at 70°C.

Table 1

Effect of water concentration in toluene extracts on thermostability of the ATPase and cytochrome oxidase

| Initial $^3H_2O$/ml toluene ($\mu$l) | Temperature (°C) | Half-life | |
|---|---|---|---|
| | | Cytochrome oxidase | ATPase |
| 13 | 70 | <3 min | 11 h |
| 3 | 70 | 4 h | 96 h |
| 3 | 80 | 50 min | 48 h |
| 3 | 90 | 7.5 min | 5 h |

The water content of toluene extracts (13 $\mu$l) was measured as in fig.2. The starting water content of the extracts was diminished to 3 $\mu$l evaporating them under an $N_2$ current to about half of their original volume which was restored with toluene. The two extracts were incubated at the temperatures shown. Aliquots were withdrawn at various times to assay ATPase and cytochrome oxidase activity. During incubation toluene was added to compensate evaporation

both cytochrome oxidase and ATPase (table 1). This decrease in water content caused about a 100-times increase in the half-life of cytochrome oxidase (see data at 70°C, table 1).

These results suggest that in organic solvents, all enzymes acquire thermostability provided that bulk water is eliminated. Nevertheless, even at initial low water concentrations in the extracts, the ATPase was more stable than cytochrome oxidase; after 2, 8 and 60 h at 90, 80 and 70°C, respectively, 100% ATPase activity remained, whereas in these conditions cytochrome oxidase was inactivated (not shown).

We believe that water that is removed from the system as in the experiment of table 1 is directly involved in the rapid formation of incorrect structures, and thus in enzyme inactivation. In addition there also seems to exist a definite amount of water that is not as readily removed; at least part of this water could correspond to protein-bound water that may prove essential for maintenance of the native protein structure [15]. In agreement with Zaks and Klibanov [5], it was found that enzymes in organic solvents are very stable at high temperatures. However, the system described here differs from that of those authors in that the enzymes in the solvent exist as protein-lipid complexes and do not sediment at low centrifugal forces (see methodology). Although we have not explored whether, under our conditions, the enzymes possess catalytic activity in the solvent, as do other enzymes [5,12,14], it is remarkable that the highly complex multi-subunit ATPase and cytochrome oxidase [8,9] acquire high thermostability when transferred to toluene.

## ACKNOWLEDGEMENTS

This work was supported by grants from the Organization of American States, CONACYT (México) and the Ricardo J. Zevada Foundation.

## REFERENCES

[1] Kausman, W. (1959) Adv. Protein Chem. 14, 1–63.
[2] Tanford, C. (1968) Adv. Protein Chem. 23, 121–282.
[3] Ahern, T.J. and Klibanov, A.M. (1985) Science 228, 1280–1284.
[4] Klibanov, A.M. (1983) Adv. Appl. Microbiol. 29, 1–28.
[5] Zaks, A. and Klibanov, A.M. (1984) Science 224, 1249–1251.
[6] Darszon, A., Phillip, M., Zarco, J. and Montal, M. (1978) J. Membrane Biol. 43, 71–90.
[7] Ayala, G., Nascimento, A., Gómez-Puyou, A. and Darszon, A. (1985) Biochim. Biophys. Acta 810, 115–122.
[8] Capaldi, R.A., Malatesta, F. and Darley-Usmar, V.M. (1983) Biochim. Biophys. Acta 726, 135–148.
[9] Hatefi, Y. (1985) Annu. Rev. Biochem. 54, 1015–1069.
[10] Barranco, J., Darszon, A. and Gómez-Puyou, A. (1981) Biochem. Biophys. Res. Commun. 100, 1402–1408.
[11] Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329.
[12] Takahashi, K., Nishimura, H., Yoshimoto, T., Sart, Y. and Inada, Y. (1984) Biochem. Biophys. Res. Commun. 121, 261–270.
[13] Inada, Y., Nishimura, H., Takahashi, K., Yoshimoto, T., Saha, A.R. and Saito, Y. (1984) Biochem. Biophys. Res. Commun. 122, 845–850.
[14] Zaks, A. and Klibanov, A.M. (1985) Proc. Natl. Acad. Sci. USA 82, 3192–3196.
[15] Douzou, P. and Petsko, G.A. (1984) Adv. Protein Chem. 36, 245–361.