

## Effects of Compatible Solutes on Mammalian Cytochrome P450 Stability

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Z. Naturforsch. **52c**, 132–135 (1997);  
received September 18/November 18, 1996

Cytochrome P450, Protein Stability, Osmolytes,  
Ectoine, Sucrose

The sensitivity of pig cytochrome P450c17 (CYP17), an endoplasmic reticulum membrane-bound enzyme, towards heat denaturation (48 °C) was measured by the P450-to-P420 spectral transition indicating conformational labilization of the protein. Both sucrose and glucose have comparable and increasingly protective effects at concentrations ranging from 100 to 800 mM, while ectoine, a novel zwitterionic compatible solute which regulates bacterial osmoadaptation and stabilizes cytoplasmic enzymes, has a strong labilizing effect on CYP17 and favours its proteolytic inactivation possibly by electrostatic derangements. Sucrose or glucose, but not ectoine, can therefore eventually be proposed as compatible stabilizers of cytochrome P450 structures.

### Introduction

The stability of proteins, i.e. their conformational and functional resistance towards stress conditions caused by elevated temperatures or denaturants, depends to a considerable extent on structural properties of the surrounding solvent milieu (Taylor *et al.*, 1995). The ability of an additive to impair or to strengthen solvent interaction with hydrophobic groups in a protein is the crucial determinant of its function as a stabilizing or denaturing compound, respectively. For membrane-associated enzymes, the organizational status of water layers adjacent to the hydrophilic lipid headgroups appears to be of particular importance (Mayer and Hoppert, 1996). Strategies to increase protein stability and solubility during biotechnological processes continuously gain in relevance with respect to correct folding of heterologously

overexpressed recombinant proteins or correct reconstitution of membrane proteins; amongst stabilizers, osmolytic and ionic co-solutes can be differentiated (Schein, 1990; Kolena *et al.*, 1992; Galinski, 1993; Taylor *et al.*, 1995). Analogously to such artificial systems, microorganisms which have to cope with high and varying osmotic stress (halophilic and halotolerant bacteria) synthesize and accumulate intracellular osmolytes in an adjustable manner, and the detection of such substances can be expected to be of biotechnological value (Schein, 1990; Galinski, 1993; Talibart *et al.*, 1994). Since those compounds do not interfere with enzyme activities and metabolic routes, they are usually termed “compatible solutes”. One promising novel and representative class are the ectoines (tetrahydropyrimidines; see Fig. 1A for the ectoine structure), which are abundantly present in chemoheterotrophic eubacteria and can protect cytoplasmic model enzymes (e.g., lactate dehydrogenase) against denaturation by heat, freeze-thaw cycles or freeze-drying (Galinski, 1993).

In the present study, ectoine, sucrose and glucose were comparatively tested for potential modification of heat-induced denaturation of membrane enzymes, using a cytochrome P450 protein (CYP17) as the target. Cytochromes P450 are the hydrophobic oxygen-activating heme protein components of monooxygenase systems, and most mammalian isoforms are associated with the smooth endoplasmic reticulum; CYP17 catalyzes mainly the conversion of gestagens to androgens (Kühn-Velten, 1993). Their denaturation can easily be assessed spectroscopically (Omura and Sato, 1964; Anzenbacher *et al.*, 1982; Hui Bon Hoa *et al.*, 1990; Yu *et al.*, 1995).

### Experimental

Testes were obtained from Göttingen minipigs aged 3 to 4 months at the time of routine castrations. Decapsulated organs were homogenized (Potter-Elvehjem) in a 6-fold volume of TS buffer [20 mM Tris(hydroxymethyl)-aminomethane, 250 mM sucrose, pH 7.4] yielding 55 ml homogenate/testis. Microsomal membrane fractions were prepared by differential centrifugation (pellet of a 45 min at 150,000 xg ultracentrifugation of the postmito-

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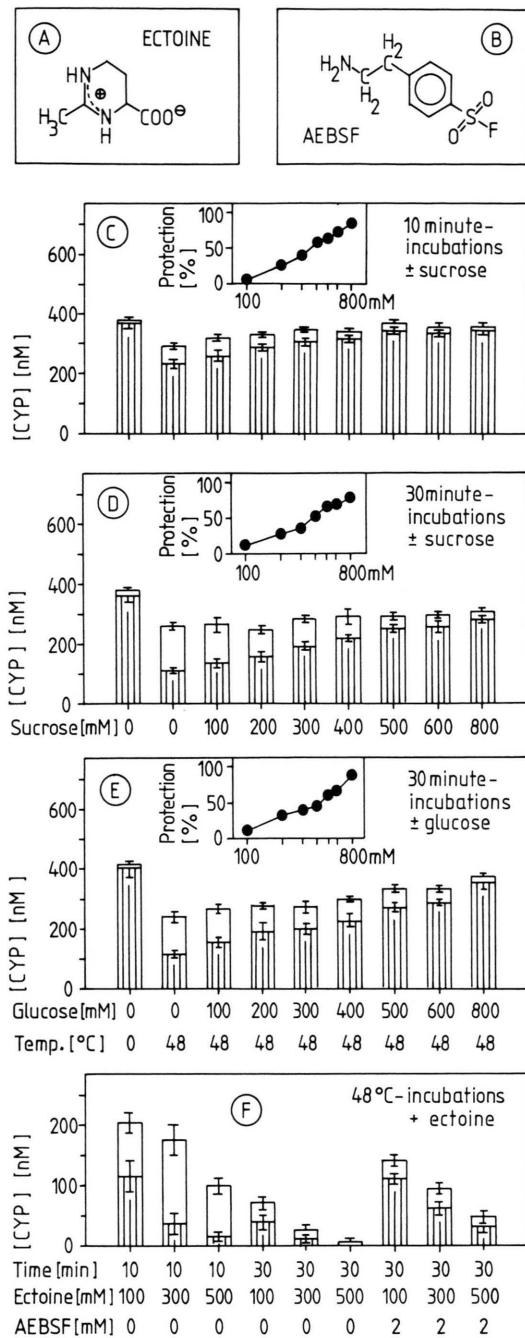


Fig. 1. Effect of compatible solutes on stability of cytochrome (= CYP) P450 (shaded column fractions) and its transition to P420 (white fractions) in pig testis microsomes. A, chemical structure of ectoine. B, chemical structure of the serine protease inhibitor AEBSF. C and D, effects of sucrose addition at temperatures (= Temp.) of 0 °C or 48 °C during 10-minute and 30-minute incubations, respectively. E, effects of glucose addition at 0 °C or 48 °C during 30-minute incubations. F, effects of ectoine without or with protease inhibitor. The insets in panels C, D, and E depict the solute concentration-dependence of CYP17 stabilization (mean from protection against P450 loss and protection against P420 formation; the 100% value corresponds to the differences of the 0 °C and 48 °C data without addition). Basal medium composition was 20 mM sucrose (from the membrane fraction) plus 20 mM Tris; pH was constantly 7.4. Control values (0 °C and 48 °C without osmolytes) shown in panels C and D are also valid for data shown in panel F. Means from duplicate measurements of two preparations and ranges are given; note the different ordinate scales.

chondrial supernatant obtained in a 10 min at 12,000 xg centrifugation of tissue homogenate), rehomogenized in TS buffer (10 ml per testis equivalent) and stored frozen at -40 °C until use (4 weeks at maximum). Pig testis microsomes were used as

the cytochrome P450 source since they contain only one main isoform (P450c17 or CYP17; EC 1.14.99.9 + 4.1.2.30) at relatively high concentrations; problems originating from CYP heterogeneity can thus be avoided (Kühn-Velten, 1993).

Investigations on CYP17 stability were performed by incubating 0.4 ml microsomes plus 4.5 ml T buffer (20 mM Tris, pH 7.4) with or without compatible solutes for 10 or 30 min at 48 °C when the first heat-induced conformational transitions are known to occur (Anzenbacher *et al.*, 1982); controls were kept at 0 °C. Sucrose (Merck, Darmstadt),  $\alpha$ -D-glucose (Serva, Heidelberg), or ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid; Bitop Gesellschaft für biotechnische Optimierung, Witten; structure in Fig. 1A) were added as potential stabilizers; in some experiments, final pH values were varied, or AEBSF (Pefabloc<sup>R</sup> SC; 4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochloride; Boehringer, Mannheim; structure in Fig. 1B) was added as an irreversible serine protease inhibitor. After incubation, samples were chilled on ice, and P450 and P420 (the active and inactive species, respectively) concentrations were quantified by the CO-dithionite-reduced versus dithionite-reduced difference spectroscopy (Omura and Sato, 1964) using an UV300 photometer (Shimadzu, Kyoto).

## Results and Discussion

Exposure of CYP17 to an environmental temperature of 48 °C in the absence of osmolytes resulted in the expected time-dependent denaturation which was evident from the increasing P450-to-P420 transition (Fig. 1C–D). There was also a slight diminution of total CYP17 (P450 plus P420) under this condition which could be attributed to proteolytic digestion of the inactive P420 form. The P450-to-P420 spectral transition is a sensitive marker for changes in conformational integrity of the heme protein, since it reflects a labilization of the iron-thiolate bond linking the prosthetic group to the apoprotein. Such a transition can be caused by partial apoprotein unfolding; it can be achieved *in vitro* by a number of reagents and conditions including heat and can be attenuated by the addition of cysteine, glycerol, or spermine (Omura and Sato, 1964; Anzenbacher *et al.*, 1982; Hui Bon Hoa *et al.*, 1990; Yu *et al.*, 1995).

It is established for the first time in this note that addition of sucrose and glucose exhibited a distinct, concentration-dependent protective effect which was half-maximal at about 450 mM glucose or 350 mM sucrose and almost complete with 800

mm, i.e. heat-denaturation to P420 and enzyme loss were nearly abolished (Fig. 1C–E). Though it is not clear whether these beneficial effects occur directly via modulation of solvent/protein interactions and/or indirectly via solvent-membrane interactions (Kolena *et al.*, 1992; Taylor *et al.*, 1995), the observation as such may be important for improvement of stable folding of cytochromes P450 when these versatile catalysts are used in environmental degradation processes under otherwise unfavourable conditions.

In contrast, ectoine promoted a sharp rise in the P450-to-P420 transformation even at relatively low concentrations and, especially after 30 minutes, a nearly complete loss of CYP17 enzyme (Fig. 1F). The latter was partly reversed by addition of a serine protease inhibitor, but this action was only important at low ectoine levels present (Fig. 1F). Higher concentrations of ectoine as well as pH variations (ranging from 6.2 to 7.8) did not significantly change this pattern, though some P450-to-P420 transition occurred spontaneously (probably by cysteinate protonation) at low pH values (data not shown). This CYP17 labilization in the presence of ectoine contrasts both with its clearly protective effects on a number of other (cytoplasmic) enzymes and bacterial cells (Galinski, 1993; Talibart *et al.*, 1994) and with CYP17 protection by sucrose and glucose. As it has previously been established that P420 is easily proteolytically digested by membrane-bound protease systems (Zhukov *et al.*, 1993), the partial stabilization by ectoine of a CYP17-degrading proteolytic activity can therefore not be excluded. Since ectoine is probably not a CYP17 ligand due to its zwitterionic properties (Kühn-Velten, 1993), and since polarity of additives per se does not necessarily induce cytochrome P450 labilization (for instance, KCl, spermine and cysteine are stabilizers) (Hui Bon Hoa *et al.*, 1990), the molecular mechanisms underlying the observed discrepancies remain to be elucidated; structural as well as electrostatic aspects of the membrane and protein microenvironment should obviously be considered.

## Acknowledgements

This study was financially supported by the Deutsche Forschungsgemeinschaft (SFB 351 A6). I thank Dr. S. Nefen for providing us with pig testes obtained from routine castrations.

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