

Thermostability of respiratory terminal oxidases in the lipid environment

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Received 21 November 1994; revised 7 February 1995; accepted 13 February 1995

Abstract

The effect of the lipid environment on the thermostability of three respiratory terminal oxidases was determined. Cytochrome-*c* oxidase from beef heart and *Bacillus stearothermophilus* were used as representative proteins from mesophilic and thermophilic origin, respectively. Quinol oxidase from the archaeon *Sulfolobus acidocaldarius* represented the model for a extreme thermoacidophilic enzyme. All three integral membrane proteins were tested for their thermal inactivation in detergent and after reconstitution in liposomes composed of phospholipids of *Escherichia coli* or tetraether lipids from *S. acidocaldarius*. When preincubated at 0° C, all three enzymes exhibited biphasic thermal inactivation curves. Data could be analysed according to a two-state model that defines two conformations of the enzyme, differing in their thermostability. Monophasic inactivation curves were observed when the enzymes were preincubated at higher temperatures prior to thermal inactivation. Lipids rendered the beef-heart cytochrome-*c* oxidase and *S. acidocaldarius* quinol oxidase more thermostable as compared to detergent solution. In contrast, the *B. stearothermophilus* oxidase, an intrinsically thermostable enzyme, was as thermostable in detergent as in the reconstituted state. These data suggest that the lipid environment can be an important factor in the thermostability of membrane proteins.

Keywords: Cytochrome-*c* oxidase; Tetraether lipid; Reconstitution

1. Introduction

Microorganisms can be divided into psychrophiles, mesophiles, thermophiles and extreme thermophiles, depending on their growth characteristics at different temperatures. Proteins of thermophiles must be able to maintain structural integrity and catalytic function at elevated temperatures. The (thermo-)stability of globular proteins is considered to be an intrinsic property of the protein. For membrane proteins the lipid environment could in principle contribute to the thermostability due to lipid–protein

interactions [1], but few experimental data support this hypothesis.

To investigate the influence of the lipid environment on the thermostability of integral membrane proteins, we studied the thermostability of respiratory oxidases from different sources in detergent solution and after reconstitution in different lipid environments. The activity of these enzymes after thermo-inactivation can conveniently be measured in a solubilized state by following the rate of oxidation in the presence of a suitable electron donor. Lipids were used from *Escherichia coli*, a mesophilic bacterium and from *Sulfolobus acidocaldarius*, an extreme thermoacidophilic archaeon. The predominant lipid species in the natural lipid mixture of *E. coli* are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin [2]. *S. acidocaldarius* lipids are to an extent of 95% composed of a mixture of tetraethers [3,4]. These tetraethers form a monolayer, in contrast to the diester lipids from *Escherichia coli*, which form a bilayer. The terminal respiratory oxidases studied were cytochrome-*c* oxidase from beef heart and from the

Abbreviations: $\Delta\psi$, Transmembrane electrical potential; ΔpH , transmembrane pH gradient; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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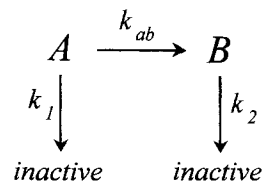
thermophilic bacterium *Bacillus stearothermophilus* [5], and quinol oxidase from *S. acidocaldarius* [6]. This latter enzyme does not reduce cytochrome *c*, but utilizes the physiological electron donor caldariella quinone. The mammalian cytochrome-*c* oxidase contains 13 subunits, whereas the bacterial enzyme contains only 3 subunits. The archaeal enzyme is built up from 4 subunits coded by the *soxABCD* genes [7,8], but a fully functional protein has been purified from the cytoplasmic membrane that consists of only the *soxB* gene product. This protein is homologous to subunit I of beef-heart cytochrome-*c* oxidase. The natural lipid environment of the two cytochrome-*c* oxidases and the quinol oxidase is the bilayer and monolayer, respectively.

Liposomes constructed of *S. acidocaldarius* lipids have been shown to form a suitable matrix for the function of exogenous membrane proteins [9,10]. These liposomes are endowed with a low proton permeability and high chemical and mechanical stability [11]. All three oxidases [12,13] have been functionally reconstituted into both tetraether and *E. coli* lipids. This enables us now to compare the thermostability of the three primary proton pumps in the two different lipid environments. The data suggest that the interaction of the protein with the lipid environment can be an important factor in determining the thermostability of an integral membrane protein.

2. Materials and methods

Purification of lipids. *E. coli* phospholipid (*Escherichia coli* L- α -phosphatidylethanolamine, type IX, Sigma) was purified by acetone/diethyl ether extraction as described [14]. Lipids were stored in chloroform under nitrogen at -20°C . *S. acidocaldarius* (DSM 639) cells were grown aerobically in a 50 l fermenter at 80°C in Brock's medium [15] supplemented with 5.8 mM L-glutamic acid, 50 mM K_2SO_4 and 5.8 mM sucrose. Cells were harvested at an A_{546} of about 2, collected by centrifugation and freeze-dried. From the freeze-dried cells a liposomal forming lipid fraction was isolated as described [12]. The lipids were suspended in chloroform/methanol/water (65:25:4, v/v) and stored at 4°C until use.

Preparation of respiratory oxidases containing proteoliposomes. Cytochrome-*c* oxidase (EC 1.9.3.1) from beef heart and *B. stearothermophilus* were isolated and reconstituted as described [12]. Preparations contained 1.23 nmol haem *a* and 20 mg lipid/ml in 50 mM potassium phosphate (pH 7), for beef-heart cytochrome-*c* oxidase and 1 nmol haem *a* and 15 mg lipid/ml in 50 mM potassium phosphate (pH 7), for *B. stearothermophilus* cytochrome-*c* oxidase. Quinol oxidase from *S. acidocaldarius* was isolated as described [13]. The reconstitution procedure was the same as for the two cytochrome-*c* oxidases [12]. The final preparation contained 1.8 nmol haem *a* and 20 mg lipid/ml. Before use, a small sample of the proteo-



Scheme 1. Scheme 1.

liposomes was slowly thawed at room temperature and extruded through 200 nm polycarbonate filters (Avestin, Ottawa, Canada), using a small volume extrusion apparatus (Liposofasttm Basic, Avestin) [16].

Temperature inactivation of the respiratory oxidase. Proteoliposomes containing terminal oxidases or oxidases solubilized in 0.1% (v/v) Lubrol at a concentration of 1–1.5 pmol of haem *a*/ml were stored on ice. Samples of 15 μl were incubated in closed vessels for various times and temperatures. After incubation, the vessels were chilled on ice and the remaining activities were measured at 30°C in the presence of 0.1% (v/v) Lubrol. Beef-heart cytochrome-*c* oxidase activity was measured as the decrease of the adsorption of the α peak of reduced horse-heart cytochrome-*c* using a molar extinction coefficient ($E_{550-540}$) of $19.5\text{ mM}^{-1}\text{ cm}^{-1}$. Horse-heart cytochrome *c* (type III, Sigma) was reduced with dithionite, and excess dithionite was removed by gel filtration via a Sephadex G-25 column. The activity of *B. stearothermophilus* cytochrome-*c* oxidase and *S. acidocaldarius* quinol oxidase was estimated from the oxidation of TMPD using an E_{546} of $8\text{ mM}^{-1}\text{ cm}^{-1}$. Values were corrected for the auto-oxidation of TMPD.

Analysis of the inactivation curves. Thermal inactivation of the enzymes was analyzed according to the model depicted in Scheme 1. The enzyme exists in two conformations A and B. At 0°C the equilibrium between the two states is completely to state A. At the inactivation temperature the equilibrium is to state B. At the start of the experiment all molecules are in state A. State A inactivates with rate constant k_1 and converts to state B with rate constant k_{ab} . State B inactivates with rate constant k_2 . The fraction of the molecules in state A and B (f_A and f_B , respectively) decreases with the inactivation time according to the following equations

$$f_A = e^{-(k_{ab}+k_1)t}$$

and

$$f_B = \frac{k_{ab}}{k_2 - k_1 - k_{ab}} [e^{-(k_{ab}+k_1)t} + e^{-k_2 t}] \quad (1)$$

The residual activity equals the sum of the fractions of A and B still present at time *t* and is described by

$$\text{res. act.} = \alpha e^{-\lambda_1 t} + (1 - \alpha) e^{-\lambda_2 t} \quad (2)$$

in which

$$\lambda_1 = k_{ab} + k_1 \text{ and } \lambda_2 = k_2 \text{ and } \alpha = 1 + \frac{k_{ab}}{k_2 - k_1 - k_{ab}} \quad (3)$$

The inactivation data were fitted to Eq. 2 using a non-linear fitting procedure. The rate constants in Scheme 1 can be calculated from the values of λ_1 , λ_2 and α .

Other analytical procedures. Haem *a* concentrations were calculated from reduced minus oxidized difference spectra determined with an SLM Aminco DW-2000 spectrophotometer. The extinction coefficients (E_{605}) were: beef-heart cytochrome-*c* oxidase, 24 mM⁻¹ cm⁻¹; *B. stearothermophilus* cytochrome-*c* oxidase, 30 mM⁻¹ cm⁻¹; and *S. acidocaldarius* quinol oxidase, 24 mM⁻¹ cm⁻¹. The orientation of cytochrome-*c* oxidase in proteoliposomes was measured as described [12]. The electrical potential ($\Delta\psi$, inside negative) and pH gradient (ΔpH , inside alkaline) were measured as described [12].

3. Results

3.1. Characteristics of the reconstituted terminal oxidases

Purified cytochrome *c*-oxidases from beef heart and from *B. stearothermophilus*, and the quinol oxidase from *S. acidocaldarius* were reconstituted in both *E. coli* lipids and *S. acidocaldarius* lipids using a detergent dialysis procedure (see Materials and methods). Unilamellar, sized proteoliposomes were obtained by extrusion through 200 nm pore size polycarbonate filters [11]. A number of experimental criteria were used to demonstrate the functional incorporation of the terminal oxidases in the membranes. The orientation of the two cytochrome-*c* oxidases was determined spectroscopically by following the state of reduction of the oxidases in the presence of a membrane permeable (TMPD) and impermeable electron donor (cyto-

chrome *c*) [17]. This technique can not be used for the *S. acidocaldarius* enzyme since no membrane impermeable substrate is available for this enzyme. Beef-heart cytochrome-*c* oxidase reconstituted into *E. coli* or *S. acidocaldarius* lipids was reduced by cytochrome *c* to about 40% of the level observed in the presence of TMPD indicating that 60% of the protein has an inside-out orientation. About 60% of the cytochrome *c* binding sites of the *B. stearothermophilus* cytochrome-*c* oxidase was exposed to the external medium, irrespective the type of lipid used in the reconstitution. Both enzymes thus reconstitute into liposomes in a more or less random orientation. The energy-transducing properties of the reconstituted enzymes are summarized in Table 1. All three enzymes generated a high protonmotive force, i.e., $\Delta\psi$ (inside negative) and ΔpH (inside alkaline) when reconstituted into liposomes. Since the *S. acidocaldarius* quinol oxidase was able to generate a high protonmotive force in the presence of the membrane-permeable electron donor TMPD, the majority of the molecules must be present in a right-side-out orientation. The respiratory control index (RCI), a measure of the back pressure of the generated protonmotive force on oxidase activity, was low for most preparations. These data further demonstrate [12,13] that the enzymes can be functionally reconstituted in both bilayer and monolayer membranes.

3.2. Experimental setup of thermal inactivation

Thermal inactivation of the oxidases was studied by following the oxidase activity in time after incubation at different temperatures. To exclude possible problems associated with a changed membrane integrity during inactivation, the oxidase activities were measured after solubilization of the membrane in the non-ionic detergent Lubrol PX100. Oxidase activity was independent of the Lubrol concentration over a wide concentration range as demonstrated in Fig. 1 for the *S. acidocaldarius* quinol oxidase

Table 1
Functional properties of the terminal oxidases reconstituted in liposomes

Terminal oxidase	Lipid composition	ΔpH^b (mV)	$\Delta\psi^c$ (mV)	Oxidase activity (s ⁻¹)	RCI
Beef heart	diester lipids	-50	-181	224	3.1
	tetraether lipids	-58	-95	95	1
<i>B. stearothermophilus</i>	diester lipids	-50	-151	271	1.5
	tetraether lipids	-60	-111	193	1
<i>S. acidocaldarius</i>	diester lipids	- ^a	-90	104	1
	tetraether lipids	-58	-110	112	1

Purified beef-heart cytochrome-*c* oxidase, *B. stearothermophilus* cytochrome-*c* oxidase and *S. acidocaldarius* cytochrome *aa*₃ were reconstituted in liposomes composed of *E. coli* phospholipids or *S. acidocaldarius* tetraether lipids. ΔpH , $\Delta\psi$, the oxidase activity and the respiratory control index (RCI) were measured as described in Materials and methods.

^a Not detectable.

^b In the presence of 500 nM valinomycin.

^c In the presence of 100 nM nigericin.

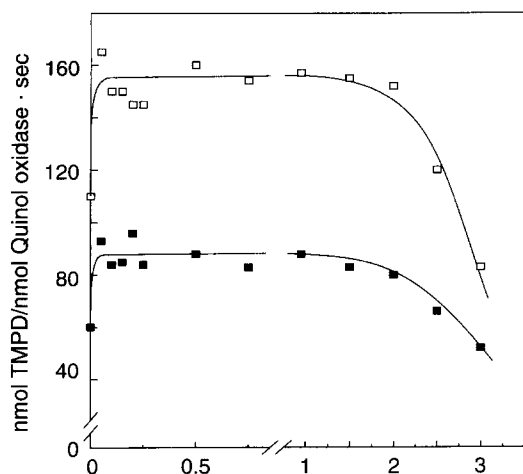


Fig. 1. Effect of Lubrol on the activity of *S. acidocaldarius* quinol oxidase reconstituted in liposomes of *E. coli* lipid. (□) native enzyme, (■) enzyme after inactivation for 2 min at 72°C (50% residual activity).

reconstituted in *E. coli* lipids. Similar results were obtained for the other two oxidases (data not shown) or when the enzyme was thermally inactivated to about 50% of the control activity (Fig. 1). It should be noted that the addition of Lubrol stimulates the enzyme activity to the level expected only when both orientations of the enzyme are accessible to the electron donor.

For thermal inactivation of the oxidases, either reconstituted in lipids, or solubilized in detergent, proteins were first kept on ice. The temperature was then rapidly shifted from 0°C to the inactivation temperature, t_{inact} . After variable time, t_{inact} , samples were chilled rapidly on ice. Oxidase activity was measured before and after the temperature jump. Fig. 2A shows a typical result obtained with this protocol. The inactivation of *B. stearrowthermophilus* cytochrome-*c* oxidase in detergent solution is

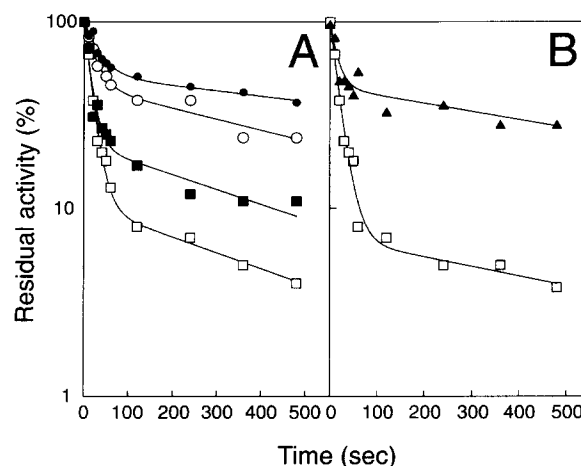


Fig. 2. Inactivation of *B. stearrowthermophilus* cytochrome-*c* oxidase solubilized in detergent. A. Residual activity after inactivation at 60 (●), 65 (○), 70 (■) and 75°C (□). For inactivation the enzymes were transferred for various times from 0°C to the indicated temperature, and subsequently chilled at 0°C. B. Residual activity after inactivation at 75°C with (▲) and without (□) preincubation at 40°C for 10 min.

clearly biphasic. A fast inactivation ($t < 60$ s) is followed by a much slower component. The contribution of the fast phase seems to increase with higher inactivation temperatures. This behaviour is consistent with a two-state model as presented in Scheme 1. At 0°C, the enzyme molecules are in a state (A) which is different from the native state (B) to which the enzymes adapts at ambient temperatures. In response to a temperature jump, two competing events may occur, (i) inactivation of state A (k_1), and (ii) transition to the more thermostable state B (k_{ab}). State B inactivates with rate constant k_2 . The down jump to 0°C will bring all molecules that are not inactivated back to state A. Due to the different temperature dependencies of

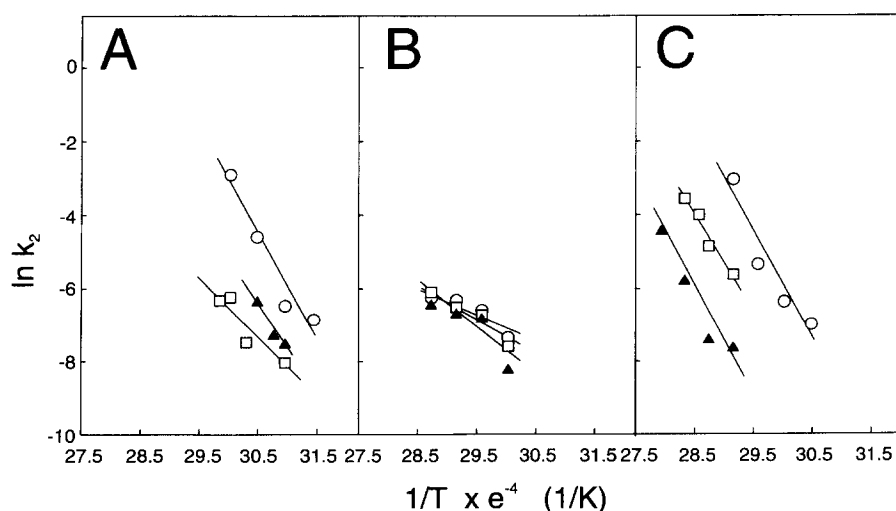


Fig. 3. Arrhenius plots of rate constant k_2 . The inactivation of the enzymes was determined in detergent solution (○), reconstituted in *E. coli* phospholipids (□) and *S. acidocaldarius* tetraether lipids (▲). A: Beef-heart cytochrome-*c* oxidase, B: *B. stearrowthermophilus* cytochrome-*c* oxidase, and C: *S. acidocaldarius* quinol oxidase.

Table 2
Inactivation parameters of the terminal oxidases in different environments

Terminal oxidase	Environment	$E_a(k_1)$ (kJ/mol)	$T_{\ln k_1 = -4}$ (°C)	$E_a(k_2)$ (kJ/mol)	$T_{\ln k_2 = -6}$ (°C)	$k_{ab} \pm \text{S.D.}$ (s ⁻¹)
Beef heart	detergent	12	— ^a	237	50	0.044 ± 0.015 (3) ^e
	diester lipids	191	59	138	64	0.013 ± 0.001 (3)
	tetraether lipids	79	48	208	56	0.022 ± 0.002 (4)
<i>B. stearothermophilus</i>	detergent	86	62	68	76	0.013 ± 0.004 (4)
	diester lipids	— ^b	74 ^c	90	75	0.004 ± 0.001 (2)
	tetraether lipids	— ^b	73 ^c	105	76	0.004 ± 0.002 (2)
<i>S. acidocaldarius</i>	detergent	— ^b	66 ^c	235	60	0.004 ± 0.002 (2)
	diester lipids	— ^d	— ^d	213	69	— ^f
	tetraether lipids	— ^d	— ^d	225	79	— ^f

Activation energies (E_a) were estimated from the slope of the Arrhenius plots ($\ln k$ versus $1/T$, see Fig. 3 for the k_2 data). Inactivation temperatures are defined as those temperatures at which $\ln k_1$ and $\ln k_2$ take the values of -4 and -6 , respectively. k_{ab} values derived from the inactivation curves at different temperatures were averaged.

^a Temperature dependence too small to allow extrapolation; k_1 ranged from 0.039 (45°C) to 0.055 s⁻¹ (60°C).

^b Inactivation rate constant available at only two inactivation temperatures.

^c Values from interpolation between two inactivation temperatures.

^d Inactivation curves did not allow an accurate estimation of k_1 .

^e Number of k_{ab} values from different inactivation temperatures.

^f Inactivation curves did not allow an accurate estimation of k_{ab} .

rate constants k_1 and k_{ab} , the contributions of the fast and the slow phase in the inactivation curves vary. This inactivation model is supported by the results presented in Fig. 2B. Preincubation of *B. stearothermophilus* cytochrome-*c* oxidase at a temperature at which inactivation is negligible results in a decrease of the contribution of the fast inactivation phase (compare \square and \blacktriangle). After fitting the data to Eq. (2), values for λ_1 (0.051 s⁻¹ and 0.062 s⁻¹) and λ_2 (0.0012 s⁻¹ and 0.0010 s⁻¹) were found to be the same within experimental error. These two parameters represent

both inactivation rates. The value of α , representing the relative contribution of the two processes, decreases from 0.93 to 0.55 . Apparently, during the preincubation period, a fraction of the molecules has converted from state A to state B, resulting in a different distribution over the two states at the start of the inactivation experiment. In another experiment the enzyme was preincubated for 200 s at 60°C , resulting in a residual activity of about 0.65 (Fig. 2A, \bullet) after which the inactivation was monitored at 65 , 70 and 75°C . The inactivation curves were mono-exponen-

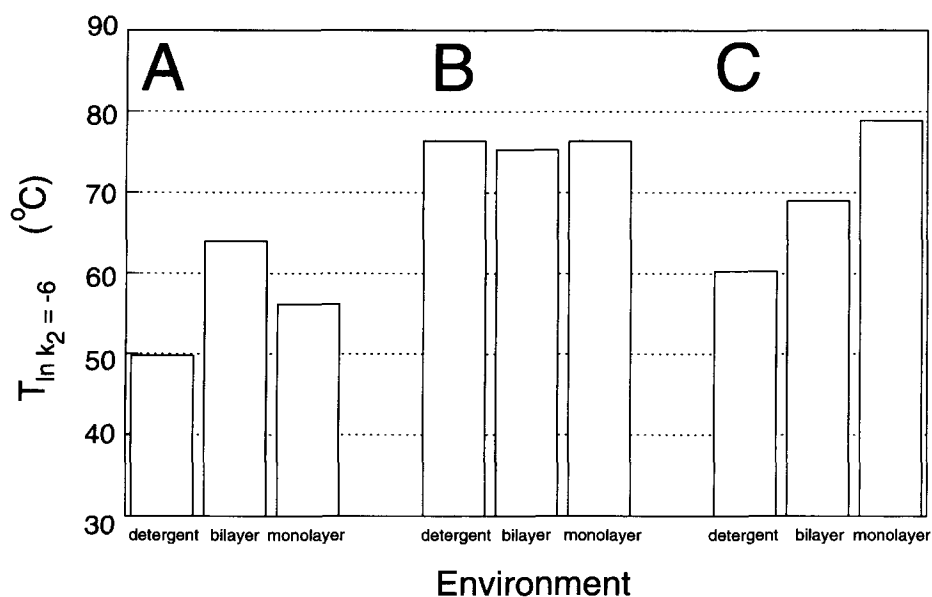


Fig. 4. Inactivation temperatures of state B of the terminal oxidases. Inactivation temperatures were determined for the enzyme suspended in detergent solution, and reconstituted in phospholipid bilayer and tetraether monolayer, respectively. (A) Beef-heart cytochrome *c*-oxidase; (B) *B. stearothermophilus* cytochrome *c*-oxidase, and (C) *S. acidocaldarius* quinol oxidase.

tial and all extrapolated back to 0.65 residual activity, indicating that during the preincubation period all molecules were converted to state B (not shown).

3.3. Analysis of the inactivation studies

The thermostability of the three terminal oxidases reconstituted in *E. coli* lipids, *S. acidocaldarius* lipids, and solubilized in detergent was studied according to the protocol described in the previous section. The inactivation curves of all three oxidases were biphasic in all environments. The fast inactivation phase (λ_1) of *S. acidocaldarius* quinol oxidase in the two lipid environments amounted to maximally 20%, which did not allow an accurate estimate of k_1 and k_{ab} . The data were analyzed by Arrhenius plots (Fig. 3 and Table 2). A complete data set is available for the inactivation of state B, the conformation at ambient temperatures (k_2) (Table 2). In Fig. 3, the data for the three terminal oxidases are plotted using the same $1/T$ range for the x -axis. The stability of the enzymes increases in the order beef heart, *B. stearothermophilus* and *S. acidocaldarius*, as is immediately evident from the shift of the curves from right to left in the three plots. The effect of the environment on the stability of the *B. stearothermophilus* enzyme is negligible. This is clearly not the case for the other two enzymes, which are most stable when embedded in a lipid membrane (compare \circ with \blacktriangle and \square). In both cases, the natural environment, i.e., the bilayer for the beef-heart enzyme and the monolayer for the *S. acidocaldarius* enzyme, results in the highest stability. A quantitative measure for the thermostability was defined by the temperature at which the inactivation rate constant reaches a certain value. With the established definition of 50% inactivation in 10 min it was not possible to compare the inactivation of the three enzymes accurately without extensive extrapolation of the data. Therefore, a level of inactivation was chosen from the curves in Fig. 3 that required only minimal extrapolation. A comparison of the inactivation kinetics of the different enzymes could be made by estimating the temperature at which $\ln k_2 = -6$ (Table 2). This corresponds to a half-time of inactivation of 4.66 min. The above-described effects of the environment on the thermostability of the oxidases is immediately evident from the presentation of the inactivation temperatures in Fig. 4.

Incomplete data sets are available for k_1 and k_{ab} (Table 2). A comparison of the inactivation kinetics was now made by estimating the temperature at which a value of $\ln k_1 = -4$ was reached, i.e., a half-time of inactivation of 0.63 min. Beef-heart cytochrome-*c* oxidase in conformation A was rather unstable in detergent solution with a remarkably low activation energy of 12 kJ/mol. Both in state A and state B, the enzyme is most stable in a conventional phospholipid bilayer. The thermostability of *B. stearothermophilus* cytochrome-*c* oxidase in state A is independent of the lipid environment as was observed for

state B. However, state A seems to be destabilized in detergent solution. The rate constants for the transition between states A and B (k_{ab}) did not change significantly over the inactivation temperature ranges chosen in the experiments. The average values listed in Table II suggest that the rate constants k_{ab} are lower in a lipid environment than in detergent micelles.

4. Discussion

The aim of the present study was to investigate the effect of the lipid environment on the thermal inactivation of respiratory oxidases. Different strategies exist for membrane proteins. As for globular proteins, they can be intrinsically stable, but enhanced stability may also be provided by protein/lipid interactions. Our data show that the intrinsic thermostability of a membrane protein is not only determined by intramolecular and/or intermolecular interactions, but that the interaction of the protein with the lipid environment can significantly contribute to the thermostability.

To quantitate the number of active molecules after thermal inactivation, the residual fraction of oxidase activity was calculated from the ratio of activity after and before inactivation. Oxidase activities were measured in detergent solution to minimize any effect of membrane structure during the activity measurements. Lubrol is a moderate detergent that supports the activity of all three oxidases. All three enzymes exhibited biphasic thermal inactivation curves when analysed after incubation at 0°C. Monophasic inactivation was observed when the enzyme was preincubated at higher temperatures. This suggests that the enzyme adopts a conformation at 0°C that renders it more susceptible for denaturation after a sudden switch to high temperatures or 'heat shock'. We have termed this conformation at 0°C, state A. Most thermophilic proteins are cold-labile, and denature irreversibly at low temperatures. However, state A is reversibly converted into state B at moderate temperatures. The transition of state B to A may represent some loss of the structural integrity of the enzymes due to for instance subunit separation or local unfolding.

The thermostability of the enzymes in their physiological conformation (state B) has been analyzed in more detail (see Figs. 3 and 4). This state may be more relevant for the conformational stability of the enzyme than state A. Thermostability of both the mammalian and the archaeal oxidase is enhanced when the enzyme is embedded in a lipid environment as compared to the detergent-solubilized state. The stability of both enzymes appears to be the highest in the lipids that resemble most closely the native environment. These data demonstrate that integral membrane proteins may gain further thermostability by interacting with their lipid environment. This effect is not observed with the *B. stearothermophilus* cytochrome-*c* oxi-

dase. In this case, the enzyme does not appear to benefit from the lipid environment in obtaining a greater thermostability. Only after incubation at 0° C (in state A), the enzyme inactivates more readily in detergent solution. The thermostability of *B. stearothersophilus* cytochrome-*c* oxidase in its own lipids has been studied before, and it was noted that the stability of the enzyme in the reconstituted and solubilized state is the same [5]. In contrast, it has been suggested that the membrane associated alkaline phosphatase of *B. stearothersophilus* becomes more thermostable when bound to membranes [19]. The *B. stearothersophilus* lipids differ from *E. coli* phospholipids mainly in their acyl-chain composition in that they bear a relatively high amount of saturated acyl chains, while the proportion of branched-chain fatty acids is low [18]. This may provide the proper rigidity to the membrane at 65° C [18].

In conclusion, our data demonstrate that terminal oxidases may gain additional thermostability from the interaction with the lipid environment, but that this attribute is not shared by all enzymes. At least two out of three oxidases behaved in this way. However, this is not a priori the case, since the lipid environment conferred little thermostability to the *B. stearothersophilus* cytochrome-*c* oxidase. This may reflect a fundamental difference in the mechanism by which these enzyme gain thermostability. The high stability of the *B. stearothersophilus* cytochrome-*c* oxidase in detergent makes this enzyme an excellent choice for crystallization trials.

Acknowledgements

This research was supported by the Foundation for Technical Sciences (S.T.W.) with financial aid from the Netherlands Organization for Scientific research (N.W.O.),

and the European Community BIOTECH programme on Extremophiles BIO2-CT93-0274.

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