Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1557 (2003) 109–117
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Cytochrome *c* is transformed from anti- to pro-oxidant when interacting with truncated oncoprotein prothymosin α

Olga V. Markova, Alexandra G. Evstafieva, Svetlana E. Mansurova, Sergey S. Moussine, Larisa A. Palamarchuk, Mikhail O. Pereverzev, Andrey B. Vartapetian, Vladimir P. Skulachev*

Belozersky Institute of Physico-Chemical Biology, Moscow State University, Khokhlova str. 4, Moscow 1198992, Russia

Received 15 July 2002; received in revised form 20 December 2002; accepted 13 January 2003

Abstract

Many apoptotic signals are known to induce release to cytosol of cytochrome *c*, a small mitochondrial protein with positively charged amino acid residues dominating over negatively charged ones. On the other hand, in this group, it was shown that prothymosin α (PT), a small nuclear protein where 53 of 109 amino acid residues are negatively charged, is truncated to form a protein of 99 amino acid residues which accumulates in cytosol during apoptosis [FEBS Lett. 467 (2000) 150]. It was suggested that positively charged cytochrome *c* and negatively charged truncated prothymosin α (tPT), when meeting in cytosol, can interact with each other. In this paper, such an interaction is shown. (1) Formation of cytochrome *c*·tPT complex is demonstrated by a blot-overlay assay. (2) Analytical centrifugation of solution containing cytochrome *c* and tPT reveals formation of complexes of molecular masses higher than those of these proteins. The masses increase when the cytochrome *c*/tPT ratio increases. High concentration of KCl prevents the complex formation. (3) In the complexes formed, cytochrome *c* becomes autoxidizable; its reduction by superoxide or ascorbate as well as its operation as electron carrier between the outer and inner mitochondrial membranes appear to be inhibited. (4) tPT inhibits cytochrome *c* oxidation by H_2O_2, catalyzed by peroxidase. Thus, tPT abolishes all antioxidant functions of cytochrome *c* which, in the presence of tPT, becomes in fact a pro-oxidant. A possible role of tPT in the development of reactive oxygen species- and cytochrome *c*-mediated apoptosis is discussed.

Keywords: Apoptosis; Cytochrome *c*; Prothymosin α; Reactive oxygen species; Mitochondrion

1. Introduction

For a long time, the sole function of cytochrome *c* was assumed to be electron transfer from cytochrome c_1 to cytochrome *c* oxidase. However, recently, some other (''non-canonical'') functions of cytochrome *c* were elucidated. Cytochrome *c* was found to be involved in the antioxidant system of the cell, operating as a superoxide-oxidizing enzyme that regenerates O_2 from $O_2^{\cdot-}$. This activity was shown to be inherent in the water-soluble, but not membrane-bound, cytochrome *c* [1,2]. Moreover, extramitochondrial cytochrome *c* can also operate as an electron carrier between the external NADH-oxidizing cytochrome b_5 reductase–cytochrome b_5 chain in the outer mitochondrial membrane and cytochrome *c* oxidase in the inner membrane. Such an electron transfer chain shunts $O_2^{\cdot-}$-forming segments of the main respiratory chain (Complexes I and III) provided that (i) cytochrome *c* is desorbed from the inner mitochondrial membrane and (ii) the outer mitochondrial membrane has been broken or becomes permeable for cytochrome *c* [3].

Events (i) and (ii) were found to occur during apoptosis when cytochrome *c* is released from mitochondria to cytosol. Here, cytochrome *c* forms complexes (so-called ''apoptosomes'') with apoptosis protease activating factor-1 (Apaf-1) and dATP. These complexes activate caspase-9, one of the apoptotic proteases, which then activates caspase-3 [2,4–7].

In a recent work of our group, it was found that one of caspase-3 targets is prothymosin α (PT) [8]. This is a small (109 amino acid residues), highly acidic nuclear

Abbreviations: $\Delta\Psi$, transmembrane electric potential difference; Apaf-1, apoptosis protease activating factor-1; PG, polyglutamate; PT, prothymosin α; ROS, reactive oxygen species; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene diamine; tPT, truncated prothymosin α lacking 10 C-terminal amino acid residues

* Corresponding author. Tel.: +7-95-9395530; fax: +7-95-9390338/3181.
E-mail address: skulach@belozersky.msu.ru (V.P. Skulachev).

0005-2728/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2728(03)00003-3

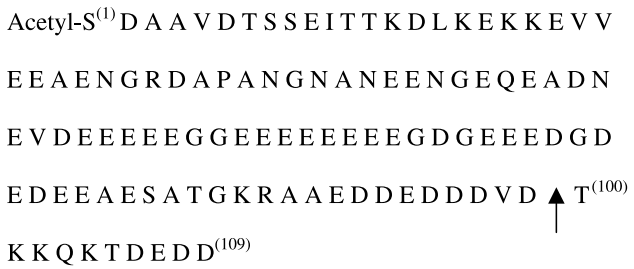

Fig. 1. Sequence of PT. Arrow, peptide bond attacked by caspase 3 when PT is converted to its truncated form (tPT) lacking the nuclear address.

protein found in virtually all mammalian tissues (the main source, thymus). Its function is still obscure. Overexpression of PT in HL-60 cells was shown to stimulate cell division due to shortening of G1 phase [9] and to inhibit differentiation [10]. At an early stage of apoptosis, caspase-3 cleaves from PT 10 C-terminal amino acids involved in formation of the nuclear localization signal (Fig. 1). As a result, truncated protein (tPT) accumulates in cytosol [8].

One can imagine that cytochrome *c* and tPT released from mitochondria and nucleus, respectively, meet and interact in cytosol. Here, we present evidence that cytochrome *c* combines with PT in solution. As a result, complexes of molecular masses higher that those of cytochrome *c* and PT are formed. The complex formation was found to abrogate all the antioxidant functions of cytochrome *c*. Even more, cytochrome *c* complexes formed can operate as pro-oxidants because they become autoxidizable. Such events might be favourable for development of apoptosis.

2. Materials and methods

2.1. Preparation of tPT and blot-overlay cytochrome c–tPT binding assay

For constructing pUC-ProTα(1–99), tPT cDNA excised from pHT15A [8] by *Taq*I digestion and filling-in with Klenow fragment was ligated into the *Sca*I site of pBR322. It was then transferred, as *Bam*HI-filled-in *Sal*I DNA fragment, into *Bam*HI-filled-in *Xba*I-treated pUC19 to produce pUC-ProTα(1–99). For constructing pKT2 plasmid encoding, in successive order, (i) a His tag, (ii) a thrombin cleavage site, (iii) a protein kinase recognition site, and (iv) tPT as a single fusion protein, *Bam*HI–*Hin*dIII fragment from pUC-ProTα(1–99) was ligated into *Bam*HI–*Hin*dIII sites of PKTL1 [11a]. The recombinant protein was isolated from *Escherichia coli* BL21(DE3)/pKT2 cells, radiolabeled with [γ-^{32}P] ATP and protein kinase A (catalytic subunit, Sigma) and further processed as described by Chichkova et al. [11a].

Blot-overlay analysis of tPT binding to cytochrome *c* was performed essentially as described previously [11a]. Cytochrome *c* was incubated in the Laemmli sample buffer at 37 °C for 10 min and fractionated by 12% SDS-PAGE alongside molecular weight markers and HeLa cell extract obtained as described by Evstafieva et al. [8]. The protein was electrophoretically transferred to Protran BA83 nitrocellulose membrane (Schleicher & Schuele), visualized by staining with Ponceau S and destained with a binding buffer (20 mM HEPES, pH 6.8, 100 mM NaCl, 0.05% Tween 20). The membrane was blocked by overnight incubation at 4 °C in the blocking solution (0.1% BSA in the binding buffer with or without 200 μM $ZnSO_4$). Binding of ^{32}P tPT (500,000 cpm) to membrane-immobilized proteins was performed in 25 ml of the blocking solution for 3 h at 4 °C. Following incubation, the membranes were washed three times with the binding buffer with or without 200 μM $ZnSO_4$ at 4 °C and autoradiographed.

Human tPT lacking artificially added sequences was isolated from *E. coli* BL21(DE3)/pET-ProTα(1–99) cells [11b] by a phenol extraction procedure and further purified by DEAE-chromatography as described previously for full-length PT [8].

2.2. Analytical ultracentrifugation

Analytical ultracentrifugation of solution containing tPT and cytochrome *c* was employed to estimate apparent molecular masses of the protein complexes [12]. Experiments were made using a Beckman Spinco E ultracentrifuge equipped with a spectrophotometer, multiplexor, and monochromator. Titanium rotor An-Ti-F with two 0.4 ml cells was used (rotation speed, 60,000 rpm, $t^0 = 22$ °C). The incubation medium contained 5 mM MOPS, 200 μM $ZnSO_4$, 0.05% Tween 20, pH 6.8. The sedimentation coefficient of the cytochrome *c*-containing complexes was measured by monitoring light absorbance at 409 nm (the Soret band of cytochrome *c* spectrum) [13].

2.3. Preparation of liver mitochondria; conditions for external NADH oxidation and transmembrane electric potential difference (ΔΨ) measurement

White rats (150–200 g) were used for the preparation of liver mitochondria as described previously [3]. The isolation medium was 250 mM sucrose, 5 mM MOPS–KOH, 2 mM EGTA, pH 7.4. Mitochondria were washed with the same medium supplemented with BSA (3 mg/ml). Protein concentration in the mitochondrial suspension was measured by the Biuret method (using BSA as a standard).

For measuring membrane potential under conditions of external NADH oxidation, mitochondria were incubated in a hypotonic medium [3].

To monitor membrane potential (ΔΨ) in rat liver mitochondria, we used diS-C_3-(5), a ΔΨ-sensitive fluorescent cyanine dye [14]. Measurements were made with a Hitachi MPF-4 fluorimeter. The concentration of the dye was 0.6 μM. Excitation and emission wavelengths were

650 and 710 nm, respectively. An increase in membrane potential resulted in a decrease in diS-C_3-(5) fluorescence.

2.4. Preparation of heart mitochondria and measurement of H_2O_2 generation

Heart muscle mitochondria were isolated from white rats (150–200 g) by a standard method (for details, see Ref. [1]). The isolation medium contained 250 mM sucrose, 0.5 mM EDTA, 10 mM MOPS–KOH, pH 7.4.

For measuring hydrogen peroxide generation by rat heart mitochondria, we used the scopoletin–horseradish peroxidase method [1].

2.5. Measurement of cytochrome c reduction and oxidation

Cytochrome *c* reduction by ascorbate or $O_2^{\cdot -}$ was measured with an SLM Aminco DW-2000 spectrophotometer (550–540 nm light absorbance difference was used). To generate $O_2^{\cdot -}$, xanthine oxidase and hypoxanthine were employed. The incubation medium contained 20 μM hypoxanthine, 5 mM MOPS (pH 6.8). In Fig. 5A, the medium was supplemented with 80 mM sucrose and 0.5 mM EGTA, pH 7.4.

Cytochrome *c* spectra were measured with the SLM Aminco DW 2000 spectrophotometer.

Cytochrome *c* was reduced by dithionite and desalted by pathing through Sephadex G-25 (coarse). Concentration of the reduced cytochrome *c* was measured spectrophotometrically using extinction coefficient $\Delta\varepsilon_{550-540} = 19.9\ mM^{-1}\ cm^{-1}$ for the reduced *minus* oxidized form. Oxidation of reduced cytochrome *c* by O_2 was initiated by adding tPT, polyglutamate (PG), or KCl to solution containing 5 mM MOPS, pH 6.8. In the case of cytochrome *c* oxidation by H_2O_2, horseradish peroxidase was added. In both cases, oxidation of cytochrome *c* was monitored spectrophotometrically similarly to cytochrome *c* reduction.

2.6. Chemicals

Fatty acid-free BSA, MOPS, rotenone, hypoxanthine, xanthine oxidase, NADH, myxothiazol, and PG (13.6 kDa) were from Sigma; sucrose, scopoletin, EGTA, EDTA, DNP, and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene diamine (TMPD) were from Serva; KOH and $MgCl_2$ were from Reakhim. DiS-C_3-(5) was a generous gift of Dr. A. Waggoner. Scopoletin (from GCN) was dissolved in dimethylsulfoxide; other chemicals were dissolved in bidistilled water. Ninety-five percent bovine cytochrome *c* prepared without trichloroacetic acid (Sigma) was kept in a diluted solution (0.6–1.4 μM) for a night before experiment to avoid formation of oligomers [15].

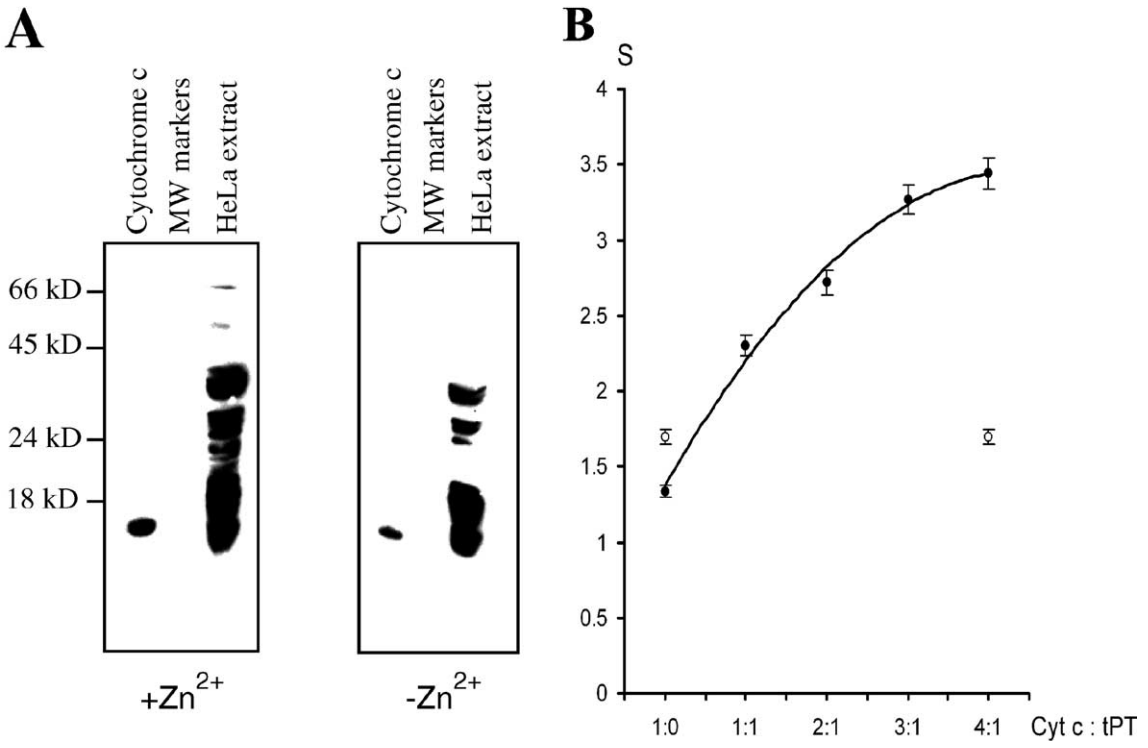

Fig. 2. Complex formation of truncated PT and cytochrome *c*. (A) Blot-overlay tPT–cytochrome *c* binding assay. Horse cytochrome *c* (3 μg), HeLa cell extract (100 μg), and molecular weight markers (BSA, ovalbumin, trypsinogen, and β-lactoglobulin, 3 μg each) were fractionated in duplicate by 12% SDS-PAGE, transferred to nitrocellulose membrane, and incubated with [^{32}P] tPT in the blocking solution for 3 h at 4 °C with (left) or without (right) 200 μM $ZnSO_4$. Following incubation, the membranes were washed and autoradiographed. (B) Sedimentation coefficients (in Svedbergs, S) measured by analytical centrifugation of the cytochrome *c*–tPT mixtures. Cytochrome *c*, 1.4 μM. (For other conditions, see Materials and methods.) Open and closed circles, with and without 0.125 M KCl. Bar, standard error. Results of five experiments.

3. Results

3.1. Formation of complexes of cytochrome c and truncated PT

In Fig. 2A, results of a blot-overlay analysis of the tPT–cytochrome *c* complex formation are given. In this case, ^{32}P-phosphorylated tPT was used (in all other experiments, we employed non-phosphorylated tPT). One can see that in a crude extract from HeLa cells, there are several tPT-binding proteins. Their number increases when Zn^{2+} is added (for Zn^{2+} binding by tPT, see our previous publication [11a]). It is seen that horse heart cytochrome *c* can bind tPT even without Zn^{2+}, but Zn^{2+} seems to increase the binding. With or without Zn^{2+}, tPT was not bound to the marker proteins used (BSA, ovalbumin, trypsinogen, and β-lactoglobulin).

Fig. 2B shows results of analytical ultracentrifugation experiments. The sedimentation of cytochrome *c* and its complexes was monitored in the Soret band of the cytochrome *c* absorbance spectrum. It is seen that addition of tPT caused an increase in the cytochrome *c* sedimentation coefficient, which might be due to formation of cytochrome *c* homo-oligomers or of cytochrome *c*·tPT hetero-oligomers. Calculation of apparent molecular mass revealed that, for example, 1:1 mixture of cytochrome *c* and tPT results in an increase in the apparent molecular mass from 13.75 ± 0.6 up to 29.75 ± 1.9 kDa. This corresponds to conversion of the cytochrome *c* monomer to homodimer or cytochrome *c*·tPT

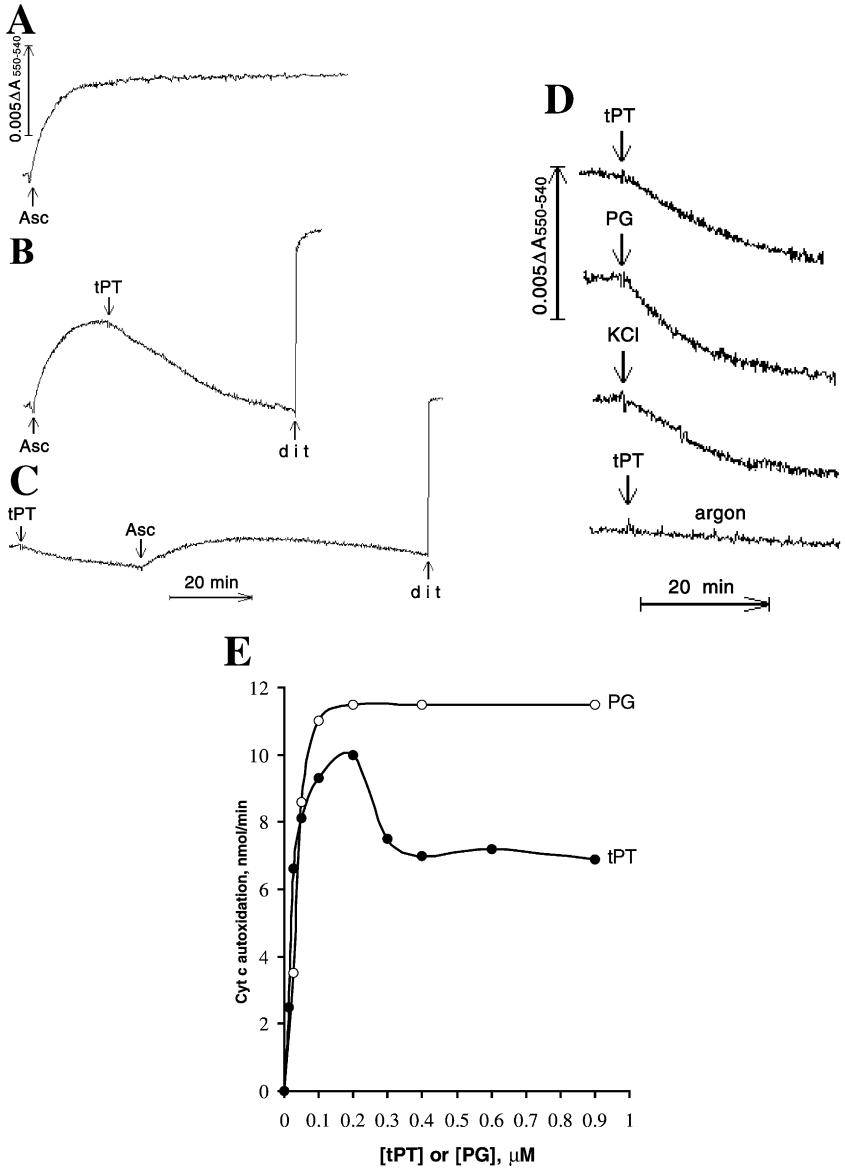

Fig. 3. Truncated PT makes cytochrome *c* autoxidable. Incubation mixture contained 0.6 μM oxidized (A–C) or the dithionite-reduced (D, E) cytochrome *c* and 5 mM MOPS, pH 6.8. In E, the initial rates of cytochrome *c* oxidation are given. Additions, 0.2 μM tPT, 0.2 μM PG, 125 mM KCl, 0.6 μM ascorbate.

heterodimer. The masses increased with increase in the cytochrome *c*/tPT ratio. This effect of tPT was abolished by 0.125 M KCl.

3.2. tPT makes cytochrome c autoxidizable

Reduction, protein/protein interaction, and binding with lipids are known to cause changes in the cytochrome *c* spectrum [15,16]. Some conformational changes (caused by low pH [17]) or interaction with membrane surface turning cytochrome into molten globule-like state [18] result in an increase in the tryptophan residue fluorescence [17]. We found no effect of tPT on cytochrome *c* fluorescence but observed significant changes in its absorption spectrum. These spectral changes clearly indicate oxidation of the cytochrome. Such an effect can be explained assuming that cytochrome *c* complexes formed when tPT was added were autoxidizable. Spectra of (i) the reduced cytochrome *c* under anaerobic conditions and (ii) the oxidized cytochrome *c* under aerobic conditions were not affected by tPT (not shown).

In Fig. 3A–D, kinetics of the cytochrome *c* redox reactions are given. Fig. 3A shows the rate of cytochrome *c* reduction by a small concentration of ascorbate. In Fig. 3B, 0.2 μM tPT was added to 0.6 μM cytochrome *c* partially reduced by ascorbate. This resulted in oxidation of cytochrome *c*, which could be then reduced again by adding dithionite. In Fig. 3C, tPT was added before ascorbate. This resulted in oxidation of small amounts of reduced cytochrome *c* that are present in the cytochrome *c* sample. Subsequent addition of ascorbate reduced cytochrome *c* only slightly. Then cytochrome *c* was spontaneously oxidized. Subsequent dithionite addition caused fast and complete cytochrome *c* reduction. Fig. 3D shows that addition of 0.2 μM tPT to the solution of reduced cytochrome *c* under aerobic conditions results in cytochrome *c* oxidation. In an argon-saturated solution, such an effect was absent. 0.2 μM PG or 125 mM KCl was found to substitute for tPT. In Fig. 3E, the rate of the tPT- or PG-induced oxidation of reduced cytochrome *c* was plotted against the tPT (PG) concentration. One can see that very low tPT or PG concentrations (5×10^{-8} M), which were clearly substoichiometric to the cytochrome *c* concentration (6×10^{-7} M), were competent in cytochrome *c* oxidation. As to KCl, it also induced cytochrome *c* autoxidation but at very much higher concentrations ($C_{1/2} = 10$ mM, not shown).

3.3. tPT inhibits peroxidase-catalyzed oxidation of cytochrome c by H_2O_2

As was shown previously [19a], addition of cytochrome *c* to heart muscle mitochondria oxidizing succinate in State 4 decreased the H_2O_2 level in the medium, measured by the

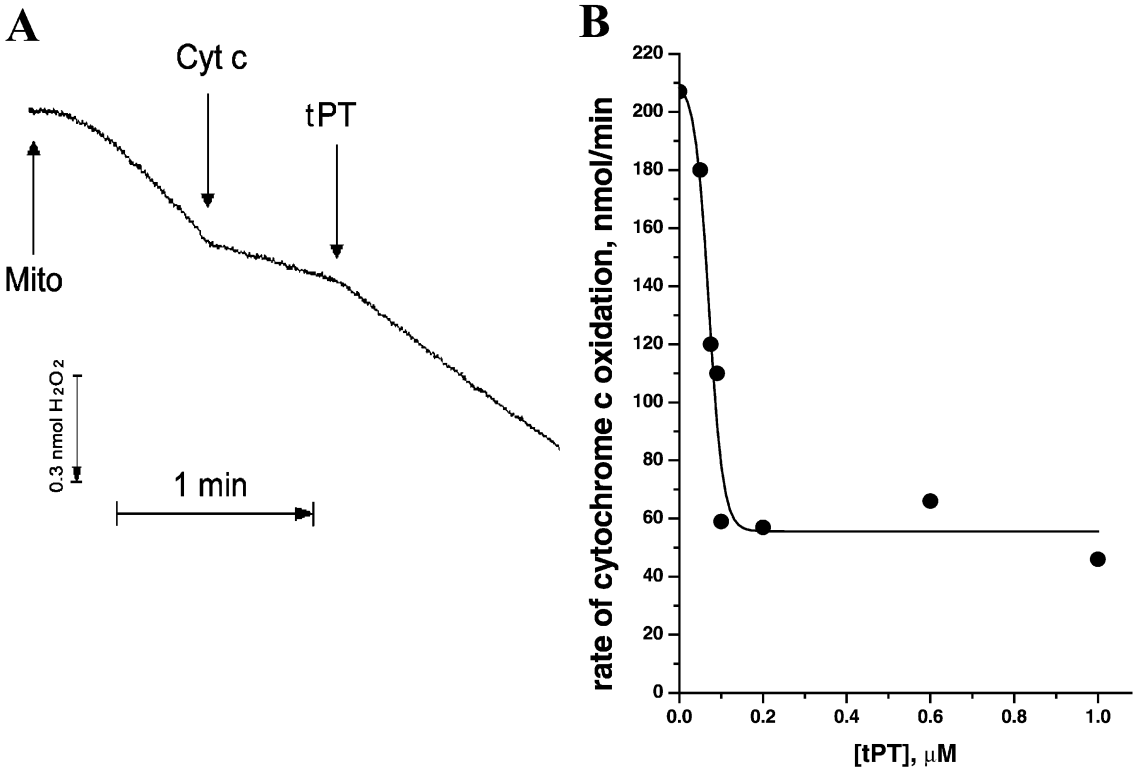

Fig. 4. Effect of tPT in the systems containing cytochrome *c*, scopoletin, and horseradish peroxidase. (A) Measurement of the H_2O_2 production by mitochondria. Incubation medium, 250 mM sucrose, 1 mM EDTA, 10 mM MOPS (pH 7.4), 6 mM succinate, 1.2 μM scopoletin, horseradish peroxidase (9.5 U/ml). Additions: mito, rat heart mitochondria (0.5 mg protein/ml); cyt. *c*, 0.4 μM cytochrome *c*; 1.2 μM tPT. (B) The tPT inhibition of the peroxidase-mediated cytochrome *c* oxidation by H_2O_2. Incubation mixture, 5 mM MOPS (pH 7.4), 1.2 μM scopoletin, horseradish peroxidase (9.5 U/ml), and 0.6 μM cytochrome *c*.

scopoletin–horseradish peroxidase method. We tested tPT in this system and found that it strongly lowered such a cytochrome *c* effect (Fig. 4A). Further analysis revealed that apparent cytochrome *c* inhibition of H_2O_2 formation by mitochondria is in fact due to competition of reduced cytochrome *c* with scopoletin for the peroxidase-mediated H_2O_2 reduction. In line with observation by Nicholls [19b], it was found that H_2O_2 oxidizes cytochrome *c* if horseradish peroxidase is present. The peroxidase-catalyzed cytochrome *c* oxidation by H_2O_2 proved to be inhibited by tPT. The $C_{1/2}$ value of tPT inhibition was of the same order of magnitude as $C_{1/2}$ of the tPT-mediated oxidation of cytochrome *c* by O_2, that is, about 5×10^{-8} M (Fig. 4B).

3.4. tPT inhibits cytochrome c reduction by $O_2^{\cdot-}$

Fig. 5 shows cytochrome *c* reduction by superoxide formed as a result of oxidation of hypoxanthine by xanthine oxidase. It is seen that this process is almost completely suppressed by either tPT or PG. Addition of 0.125 M KCl (Fig. 5A), 5 (Fig. 5A) or 3 (Fig. 5B) mM $MgCl_2$ strongly decreased the tPT (PG) inhibition. Without tPT, these salts lowered the rate of cytochrome *c* reduction only slightly.

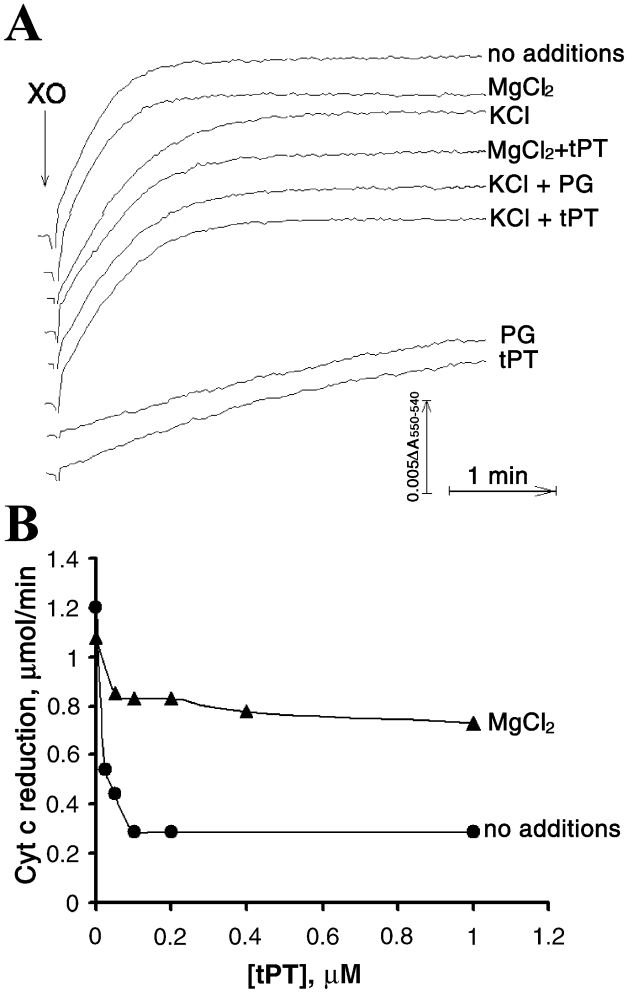

Fig. 5. Effects of tPT and PG on reduction of cytochrome *c* by superoxide. Incubation mixture, see Materials and methods. In B, initial rates of the cytochrome *c* reduction are given. Addition, xanthine oxidase (4×10^{-3} U/ml), 5 (A) or 3 (B) mM $MgCl_2$, 125 mM KCl, 0.2 μM tPT (A), 0.2 μM PG (A).

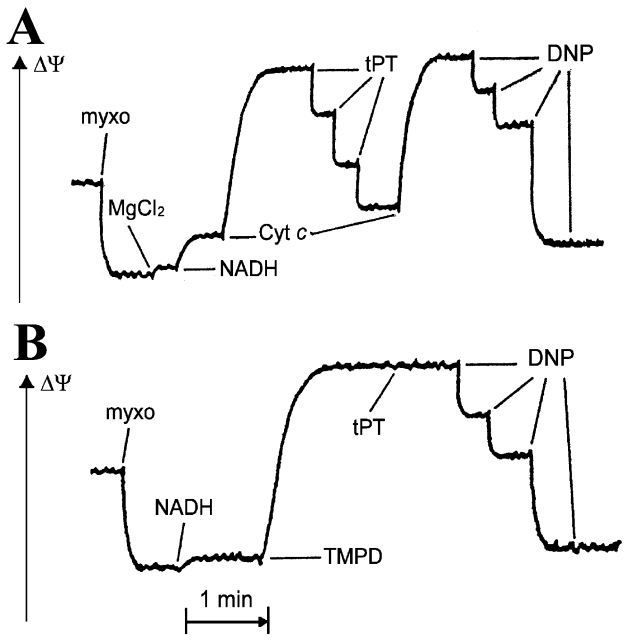

Fig. 6. Effects of tPT on ΔΨ generation coupled to external NADH oxidation by rat liver mitochondria. Incubation medium, 80 mM sucrose, 0.5 mM EGTA, 5 mM MOPS, 0.6 μM DiS-C3-(5), 3 μM rotenone, pH 7.4, 0.5 mM NADH, 3 mM $MgCl_2$, rat liver mitochondria (0.4 mg protein/ml). Additions, 0.7 μM myxothiazol, 0.1 μM cytochrome *c*, 0.1 μM tPT, 4 μM 2,4-*p*-dinitrophenol (DNP), and 20 μM TMPD.

3.5. Effect of tPT on ΔΨ generation mediated by cooperation of external and internal respiratory chains in liver mitochondria

When the outer mitochondrial membrane is disrupted or becomes cytochrome *c*-permeable, mitochondria of liver, kidney, and some other tissues appear to be competent in respiration with external NADH as a substrate. This process is mediated by NADH–cytochrome b_5 reductase and cytochrome b_5 in the outer membrane and cytochrome *c* oxidase in the inner membrane of mitochondria. The link between the two mitochondrial membranes is provided by shuttling of soluble cytochrome *c* which oxidizes cytochrome b_5 on the outer surface of the outer mitochondrial membrane and reduces cytochrome oxidase on the outer surface of the inner membrane. The operation of cytochrome oxidase is coupled with membrane potential generation, which can be easily measured [3].

We incubated rat liver mitochondria in a hypotonic medium (80 mM sucrose) to induce matrix swelling and disruption of the outer membrane. The medium was supplemented with 3 mM Mg^{2+} (to cause desorption of endogenous cytochrome *c*) as well as with rotenone and

myxothiazol (to inhibit Complexes I and III of the main respiratory chain).

As seen in Fig. 6A, addition of 0.1 μM cytochrome *c* after NADH to such a system results in generation of $\Delta\Psi$, which proved to be tPT sensitive. Three additions of tPT (0.1 μM each) inhibited almost completely the $\Delta\Psi$ generation. Subsequently, one more addition of 0.1 μM cytochrome *c* restored $\Delta\Psi$ which could be completely dissipated by uncoupler 2,4-*p*-dinitrophenol. Fig. 6B shows that tPT was ineffective when TMPD substituted for cytochrome *c* as an electron carrier between the outer and inner mitochondrial membranes. This means that the tPT inhibition is inherent in the system where soluble cytochrome *c* is required and cannot be accounted for by uncoupling or arrest of the membrane-linked electron transfer steps.

4. Discussion

It is well known that cytochrome *c* easily combines with numerous proteins, that is, cytochrome oxidase, cytochrome c_1, cytochrome b_5, cytochrome *c* peroxidase [15,16,21], hinge protein of Complex III [20], heat shock protein 27 [22], Apaf-1 [23], and some others. Cytochrome *c* also interacts with phospholipid micelles and membranes and, as a result, changes its conformation [17]. Conformational changes of cytochrome *c* were also shown to accompany development of apoptosis at its early stage [24]. Most of the interactions are caused by the positively charged region in the cytochrome *c* molecule, namely, the cluster of Lys and Arg residues (so-called ‘‘crown’’) surrounding the entrance to heme. When cytochrome *c* is desorbed from the membrane, this cationic ‘‘crown’’ acquires the possibility to interact with polyanions. tPT seems to be a good partner for such an interaction. tPT is a very acidic protein with regions where negatively charged amino acid residues are clustered. At low (nonphysiological) pH, the PT molecule acquires some elements of globular structure, but at pH 7–8, it is fully unstructured. In the nucleus, PT is known to contact histones [25]. Activation of caspase-3 during apoptosis results in formation of tPT lacking the nuclear address, so tPT accumulates in cytosol [8]. As to cytochrome *c* normally separated from cytosol by the outer mitochondrial membrane, it is released to the cytosol at the preceding stage of apoptosis. In cytosol, cytochrome *c* and tPT can meet each other and perhaps interact.

The above data indicate that such an interaction really takes place when cytochrome *c* and tPT are dissolved in a solution of low ionic strength. As a result, complexes of molecular mass larger than cytochrome *c* and tPT are formed. It has been known for a long time that (a) polyanions induce cytochrome *c* autoxidation and (b) cytochrome *c* di- and oligomers, in contrast to monomers, are autoxidizable [15,26–29]. These effects may be responsible for the tPT-induced autoxidation of cytochrome *c* revealed in our experiments. Such reasoning was confirmed by the fact that PG could effectively substitute for tPT as a polyanion inducing cytochrome *c* autoxidation (Fig. 3D). Some difference between tPT and PG was revealed only when concentration dependencies of the effect were studied (Fig. 3E). Low concentrations of these two polyanions (cytochrome *c*/polyanion ratio $\geq$3:1) were found to affect cytochrome *c* in a similar way. However, at higher polyanion concentrations (the ratio $\leq$2:1), the rate of cytochrome *c* autoxidation always decreased to values equal to about 70% of the maximal in the case of tPT and did not decrease in the case of PG. This effect was revealed when dithionite- (Fig. 3E) or ascorbate- (not shown) reduced cytochrome *c*’s were used.

One more remarkable feature of the studied phenomenon was that some effect of both tPT and PG could be revealed at cytochrome *c*/polyanion ratios as high as 48:1. Both these observations might be accounted for assuming that (i) one polyanion molecule initiates formation of an oligomer composed of many cytochrome *c* monomers and (ii) tPT·cytochrome *c* heterodimer, but not PG·cytochrome *c* heterodimer, shows 30% lower autoxidation activity than cytochrome *c* oligomer.

In contrast to oxidation of cytochrome *c* by O_2 which is activated by tPT, its oxidation by the system H_2O_2–peroxidase is inhibited by tPT (Fig. 4B). This effect is responsible for a tPT-induced reversal of apparent inhibition by cytochrome *c* of mitochondrial H_2O_2 production (Fig. 4A). Cytochrome *c* was found to decrease a horseradish peroxidase-mediated scopoletin response in the H_2O_2-producing mitochondria. This effect proved to be superoxide dismutase-resistant and was assumed to be a cytochrome *c*-induced inhibition of H_2O_2 formation in mitochondria [19a]. The present study revealed that this cytochrome *c* effect can be explained by competition of reduced cytochrome *c* and scopoletin for the peroxidase-mediated H_2O_2 reduction. Cytochrome *c* added to mitochondria can be reduced by the respiratory chain of organelles with damaged outer membrane, which are always present as a contamination to native mitochondria.

One more effect of tPT was observed when reduction of oxidized cytochrome *c* by $O_2^{\cdot -}$ was studied. It was found that low tPT concentrations inhibited reduction of cytochrome *c* by the $O_2^{\cdot -}$-producing system composed of hypoxanthine and xanthine oxidase [$C_{1/2}$, about 5×10^{-8} M tPT; maximal inhibition, five times (Fig. 5)]. The $C_{1/2}$ value, in fact, coincided with those for stimulatory effect of tPT on the cytochrome *c* oxidation by O_2 and its inhibitory effect on the cytochrome *c* oxidation by H_2O_2 in the presence of peroxidase.

Higher [tPT] was necessary to inhibit cytochrome *c*-mediated electron transport between the outer and inner membranes of rat liver mitochondria (Fig. 6). Here, $C_{1/2}$ proved to be about 2×10^{-7} M tPT at cytochrome *c* concentration equal to 1×10^{-7} M (instead of 5×10^{-8} M tPT at 6×10^{-7} M cytochrome *c* when three other tPT effects were studied). This might be a result of the fact that in the experiments on mitochondria, 3 mM $MgCl_2$ was added to lower nonspecific binding of cytochrome *c* to

mitochondrial membranes. In the non-mitochondrial systems studied, 125 mM KCl and 3 mM $MgCl_2$ prevented the tPT inhibition. However, in these systems, excess of tPT failed to overcome the effect of the ions (see, e.g., Fig. 5B). One more reason for requirement of higher [tPT] in the mitochondrial experiments may well be that cytochrome *c* affinities to cytochrome b_5 and cytochrome oxidase are higher than that to tPT. As a result, cytochrome b_5 and cytochrome oxidase can effectively compete with tPT for cytochrome *c* at low [tPT].

Effects of the low molecular mass ions are noteworthy. It may be directed on both positively charged cytochrome *c* and negatively charged tPT. In the case of the tPT inhibition of the cytochrome *c* reduction by $O_2^{\cdot -}$, it was tPT that was most probably the target because $MgCl_2$ proved to be much more efficient than KCl. The same was true for Zn^{2+}–K^+ interplay when formation of the cytochrome *c*·tPT complex was studied (Fig. 2). Here, Zn^{2+} proved to be favorable while K^+ unfavorable for the complex formation. On the other hand, autoxidation of cytochrome *c* was shown to be induced not only by tPT or PG but also by high concentration of KCl (Fig. 3D). In this case, it was cytochrome *c* that was most probably the target for Cl^- (concerning complexes of cytochrome *c* with Cl^- and other low molecular mass anions, see Ref. [29]). According to Alberty [30], change in ionic strength from 0 to 0.1 M shifts the standard redox potential of cytochrome *c* from 0.25 to 0.22 V, that is, in the direction favorable for one electron reduction of O_2 by cytochrome *c*. However, our attempt to find an effect of tPT on the cytochrome *c* redox potential failed.

At least three aspects should be taken into account when antioxidant functions of cytochrome *c* are considered.

(1) Cytochrome *c* is a necessary component of the main respiratory chain resulting in four electron reduction of O_2 to H_2O, a process maintaining intracellular [O_2] at a low level unfavorable for one electron reduction of O_2 to $O_2^{\cdot -}$ [31].
(2) When desorbed to the intermembrane space, cytochrome *c* can operate as an enzyme oxidizing $O_2^{\cdot -}$ or reducing H_2O_2 [1,2]. In the latter case, a peroxidase is involved. In yeast, cytochrome *c* peroxidase is localized in the intermembrane space [2]. In this paper, the same activity was found to be inherent in horseradish peroxidase. Perhaps, some animal peroxidase(s) are also competent in cytochrome *c* oxidation by H_2O_2.
(3) During apoptosis, cytochrome *c* releases to cytosol due to breakage of the outer mitochondrial membrane or an increase in its permeability to proteins [2,4–7]. Under these conditions, cytochrome *c* can carry out a by-pass (via NADH–cytochrome b_5 reductase and cytochrome b_5) of Complexes I and III, that is, of those steps of the respiratory chain where $O_2^{\cdot -}$ can be formed [2,3].

All the effects listed should decrease [reactive oxygen species (ROS)] which are known to be involved in the apoptotic cascades. Hence, cytochrome *c* should be regarded as an *antiapoptotic* component.

On the other hand, it is well established that cytochrome *c*, when released from mitochondria to cytosol, possesses a very important *proapoptotic* function, being combined with Apaf-1. This event initiates the caspase 9–caspase 3 cascade [2,5,6,32].

It seems obvious that apoptosis should be stimulated if antioxidant properties of cytosolic cytochrome *c* are somehow suppressed. This might be done by tPT which, like cytochrome *c*, appears in the cytosol during apoptosis [8].

(i) Cytochrome *c* binding by tPT should decrease the concentration of free cytochrome *c* in cytosol, which, in turn, should decrease respiratory conversion of O_2 to H_2O and, hence, increase intracellular [O_2].
(ii) tPT strongly inhibits both $O_2^{\cdot -}$-oxidizing and H_2O_2-reducing activities of cytochrome *c*. Even more, cytochrome *c*·tPT complex appears to be competent in $O_2^{\cdot -}$ generation.
(iii) tPT inactivates the cytochrome *c*-mediated electron transfer from the outer to inner mitochondrial membrane.

The above data show that tPT can transform cytochrome *c* from anti- to pro-oxidant. However, the rate of direct reduction of O_2 to $O_2^{\cdot -}$ by cytochrome *c*·tPT complex is low. Under conditions used, it is about 10 nmol cytochrome *c* per minute. Therefore, it hardly really contributes to ROS formation in the cell. Moreover, an effect similar to that of tPT is produced by physiological concentrations of KCl (Fig. 3D). As to inhibition by tPT of the $O_2^{\cdot -}$ oxidation activity of cytochrome *c*, it cannot be reproduced by KCl or $MgCl_2$. On the other hand, these salts reverse the tPT inhibition (Fig. 5). This can well be explained by prevention by KCl of cytochrome *c* binding with tPT (Fig. 2B). $MgCl_2$ failed to prevent tPT inhibition of $\Delta\Psi$ generation mediated by the added cytochrome *c* if the cytochrome *c*/tPT ratio was 1:3.

One might suggest that it is just tPT that abolishes various antioxidant activities of cytochrome *c* during apoptosis. In favor of such a suggestion is that all the effects described require very low (submicromolar) concentration of both tPT and cytochrome *c*. However, a serious limitation of this assumption consists in that physiological concentrations of KCl or $MgCl_2$ prevent formation of the cytochrome *c*·tPT complex and abolish majority of the above effects. One can speculate that in vivo, there are some factor(s) stabilizing the cytochrome *c*·tPT complex and preventing decomposing effects of KCl and $MgCl_2$. An alternative possibility consists in that some other polyanions are formed during apoptosis, which combine with cytochrome *c* in a KCl ($MgCl_2$)-resistant fashion.

In this context, it should be noted that PT stimulates ROS production by leukocytes [33], an effect which should be proapoptotic. On the other hand, Rodriguez et al. [10] reported that PT antisense induces apoptosis in HL-60 cells.

It seems possible that effects of tPT depend upon its level in cytosol. Below some critical concentration, it might increase [ROS] by the above described mechanisms but above it, tPT might compete with Apaf-1 for cytochrome *c* and inhibit apoptosis.

Acknowledgements

The authors are grateful to Dr. A. Waggoner for a sample of Dis-C_3-(5) and to Ms. N. Magretova and Mr. P. Kalmykov for assistance in the analytical centrifugation experiments.

This study was supported by Grants RBO-863 and RBO-864 of the Ludwig Institute for Cancer Research, 00-15-97799 of the Russian Foundation for Basic Research, and KO 597 of the Russian Federal Program "Integration of Science and Education".

References

[1] S.S. Korshunov, V.P. Skulachev, A.A. Starkov, FEBS Lett. 416 (1997) 15–18.
[2] V.P. Skulachev, FEBS Lett. 423 (1998) 275–280.
[3] M.E. Bodrova, V.I. Dedukhova, E.N. Mokhova, V.P. Skulachev, FEBS Lett. 435 (1998) 269–274.
[4] X. Liu, J. Yang, X. Wang, Cell 86 (1996) 147–157.
[5] J. Yang, X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Cai, T.-I. Peng, D.P. Jones, X. Wang, Science 275 (1997) 1129–1132.
[6] R.M. Kluck, E. Bossy-Wetzel, D.R. Green, D.D. Newmeyer, Science 275 (1997) 1132–1136.
[7] R.M. Kluck, S.J. Martin, B.M. Hoffman, J.S. Zhou, D.R. Green, D.D. Newmeyer, EMBO J. 16 (1997) 4639–4649.
[8] A.G. Evstafieva, G.A. Belov, M. Kalkum, N.V. Chichkova, A.A. Bogdanov, V.I. Agol, A.B. Vartapetian, FEBS Lett. 467 (2000) 150–154.
[9] C. Wu, A. Shiau, C. Lin, Life Sci. 61 (1997) 2091–2101.
[10] P. Rodriguez, J. Vinuela, J. Gomez-Marquez, Cell Death Differ. 6 (1999) 3–5.
[11a] N.V. Chichkova, A.G. Evstafieva, I.G. Lyakhov, A.S. Tsvetkov, T.A. Smirnova, R.N. Karapetian, E.M. Karger, A.B. Vartapetian, Eur. J. Biochem. 267 (2000) 4745–4752.
[11b] E.A. Sukhacheva, A.G. Evstafieva, T.V. Fateeva, V.R. Shakulov, N.A. Efimova, R.N. Karapetian, Y.P. Rubtsov, A.B. Vartapetian, J. Immunol. Methods 266 (2002) 185–196.
[12] C. Chervenka, A Manual of Methods for the Analytical Ultracentrifuge, Spinco Division of Beckman Instuments, Palo Alto, CA, 1969.
[13] D. Dreifelder, Physical Biochemistry: Applications to Biochemistry and Molecular Biology, Freeman, New York, 1976.
[14] C. Laris, D.P. Bahr, R. Chaffee, Biochim. Biophys. Acta 376 (1975) 415–425.
[15] E. Margoliash, A. Schejter, Adv. Protein Chem. 21 (1966) 113–286.
[16] G.R. Moore, G.W. Pettigrew, Cytochromes *c*, Evolutionary, Structural and Physico-Chemical Aspects, Springer Verlag, Berlin, 1990.
[17] N. Sanghera, T.J. Pinheiro, Protein Sci. 9 (2000) 1194–1202.
[18] V. Bychkova, O. Ptitsyn, Biochemistry 35 (1996) 6058–6063.
[19a] S.S. Korshunov, B.F. Krasnikov, M.O. Pereverzev, V.P. Skulachev, FEBS Lett. 462 (1999) 192–198.
[19b] P. Nicholls, in: B. Chance, R.W. Estabrook, T. Yonetani (Eds.), Hemes and Hemoproteins, Academic Press, New York, 1966, pp. 307–318.
[20] M. Okazaki, S. Ohta, Biochem. Biophys. Res. Commun. 243 (1998) 131–136.
[21] V.P. Skulachev, Membrane Bioenergetics, Springer, Berlin, 1988.
[22] J.-M. Bruey, E. Solary, C. Garrido, et al., Nat. Cell Biol. 2 (2000) 645–652.
[23] C. Purring, H. Zou, X. Wang, G. McLendon, J. Am. Chem. Soc. 121 (1999) 7435–7436.
[24] R. Jemmerson, J. Liu, Biochemistry 38 (1999) 3599–3609.
[25] T. Papamarcaki, O. Tsolas, FEBS Lett. 345 (1994) 71–75.
[26] E. Margoliash, J. Lustgarten, J. Biol. Chem. 237 (1962) 3397–3405.
[27] A. Schejter, S.C. Glauser, P. George, E. Margoliash, Biochim. Biophys. Acta (1963) 641–643.
[28] P. Person, A.S. Fine, P.T. Mora, H. Zipper, J. Biol. Chem. 240 (1965) 3159–3164.
[29] L.C. Petersen, R.P. Cox, Biochem. J. 192 (1980) 687–693.
[30] R.A. Alberty, Arch. Biochem. Biophys. 389 (2001) 94–109.
[31] V.P. Skulachev, Q. Rev. Biophys. 29 (1996) 169–202.
[32] J. Cai, J. Yang, D.P. Jones, Biochim. Biophys. Acta 1366 (1998) 139–149.
[33] A. Aliverti, D. Galaris, O. Tsolas, Arch. Biochem. Biophys. 321 (1995) 108–114.

Further reading

NOTE ADDED IN PROOF

While our paper was under consideration by the BBA Editorial Board, an article by Jiang et al. [1] was published. It was reported that prothymosin α specifically inhibits apoptosome formation from added cytochrome *c*, dATP, and endogenous Apaf-1 in HeLa cell extract. In the presence of prothymosin α, 1 mM dATP was required for apoptosome formation, while in its absence, 0.01 mM dATP proved to be quite sufficient. Elimination of prothymosin α expression by RNA interference sensitized HeLa cells to ultraviolet-induced apoptosis. The above observations can be explained by the cytochrome *c*–prothymosin α interaction described in our paper and may indicate that such an interaction takes place in vivo. Quite recently, we found that dATP inhibited the tPT-induced cytochrome *c* autoxidation, 100 mM ATP being ineffective. The dATP effect reached its maximum at 25 mM. An in vivo antiapoptotic effect of prothymosin α revealed in Ref. [1], in line with recent observations by Rodriguez et al. (Ref. [10] in our paper) and by Evstafieva et al. [2], is a feature typical for some oncoproteins. As was shown by Orre et al. [3], expression of prothymosin α in fibroblasts results in increased proliferation, loss of contact inhibition, anchorage-independent growth, and decreased serum dependence.

[1] X. Jiang, H.-E. Kim, H. Shu, Y. Zhao, H. Zhang, J. Kofron, J. Donnelly, D. Burns, S.-C. Ng, S. Rosenberg, X. Wang, Science 299 (2003) 223–226.
[2] A.G. Evstafieva, G.A. Belov, Y.P. Rubtsov, M. Kalkum, B. Joseph, N.V. Chichkova, E.A. Sukhacheva, A.A. Bogdanov, R.F. Pettersson, V.I. Agol, A.B. Vartapetian, Exp. Cell Res. (2003) (in press).
[3] R.S. Orre, M.A. Cotter, C. Subramanian, E.S. Robertson, J. Biol. Chem. 276 (2001) 1794–1799.