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The allosteric ATP-inhibition of cytochrome *c* oxidase activity is reversibly switched on by cAMP-dependent phosphorylation

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Abstract In previous studies the allosteric inhibition of cytochrome *c* oxidase at high intramitochondrial ATP/ADP-ratios via binding of the nucleotides to the matrix domain of subunit IV was demonstrated. Here we show that the allosteric ATP-inhibition of the isolated bovine heart enzyme is switched on by cAMP-dependent phosphorylation with protein kinase A of subunits II (and/or III) and Vb, and switched off by subsequent incubation with protein phosphatase 1. It is suggested that after cAMP-dependent phosphorylation of cytochrome *c* oxidase mitochondrial respiration is controlled by the ATP/ADP-ratio keeping the proton motive force Δp low, and the efficiency of energy transduction high. After Ca^{2+}-induced dephosphorylation this control is lost, accompanied by increase of Δp, slip of proton pumping (decreased H^+/e^- stoichiometry), and increase of the rate of respiration and ATP-synthesis at a decreased efficiency of energy transduction.

Key words: Cytochrome *c* oxidase; cAMP-dependent phosphorylation; Respiratory control; Allosteric effector; Protein kinase A; Ca^{2+}-activated protein phosphatase

1. Introduction

According to the chemiosmotic hypothesis [1–3] the mitochondrial respiration is mainly controlled by the proton motive force Δp across the inner mitochondrial membrane. 'Respiratory control', i.e. the stimulation of oxygen consumption of isolated mitochondria by ADP (active, state 3 respiration), followed by its decrease (controlled, state 4 respiration) due to conversion of ADP into ATP, was explained by uptake of ADP into mitochondria, stimulation of the ATP-synthase accompanied by a decrease of Δp, which in consequence stimulates the activity of the three proton pumps of the respiratory chain (NADH dehydrogenase, cytochrome *c* reductase, and cytochrome *c* oxidase) and thus mitochondrial respiration [3].

Recently a second mechanism of respiratory control was found which is independent of Δp and based on the intramitochondrial ATP/ADP-ratio [4–6]. The nucleotides bind to the matrix domain of subunit IV of eukaryotic cytochrome *c* oxidase [7,8] and change at high ATP/ADP-ratios the hyperbolic into sigmoidal kinetics, with half-maximal inhibition of activity at ATP/ADP = 28 [5]. Inhibition of cytochrome *c* oxidase activity at high ATP/ADP-ratios is also found in yeast but not in bacteria [9], lacking the corresponding subunit IV [10]. In substrate titrations the concentration of cytochrome *c* up to which full inhibition of cytochrome *c* oxidase activity by ATP was obtained, however, was found to be variable, depending on the preparation of mitochondria or of the isolated enzyme.

The identification of cAMP-dependent protein kinases in mitochondria [12–14], and the recently described phosphorylation of subunit IV of cytochrome *c* oxidase in mitochondria from rat liver and heart [11], prompted us to investigate cAMP-dependent phosphorylation of cytochrome *c* oxidase and its effect on enzymatic activity. In yeast mitochondria four different protein kinases have been identified, one of which is cAMP-dependent and localized in the intermembrane space [12]. The cAMP-dependent modification of mitochondrial proteins appeared to be very shortlived [13]. cAMP-dependent protein kinases have also been identified in mitochondria of bovine heart [15], of varies rat tissues [16], and of the crustacean Artemia franciscana [17]. One product of cAMP-dependent phosphorylation of mitochondrial proteins from bovine heart was identified as the 18 kDa subunit of mitochondrial NADH dehydrogenase [14].

In a previous paper the increase of ATP-inhibition of solubilized cytochrome *c* oxidase after preincubation of isolated mitochondria with ATP and cAMP was shown [18]. In the present study the allosteric ATP-inhibition of isolated cytochrome *c* oxidase from bovine heart was switched on by cAMP-dependent phosphorylation with protein kinase A and ATP. The ATP-inhibition is reversed by protein phosphatase 1, or by incubation of mitochondria, but not of the isolated enzyme, with Ca^{2+}-ions. Incubation of the isolated enzyme with [γ-^{32}P]ATP, cAMP and protein kinase A resulted in specific labeling of subunits II (and/or III) and Vb.

2. Materials and methods

Cytochrome *c* oxidase of bovine heart was isolated from mitochondrial particles by fractionated ammonium sulfate precipitation in the presence of cholate according to Yoshikawa et al. [19,20]. To remove bound cholate from the nucleotide binding site [7,8] the enzyme was treated overnight at 4°C in a buffer containing 50 mM potassium phosphate, pH 7.4, 1% Tween 20, 5 mM ATP, 200 μM cardiolipin (cardiolipin/cytochrome *c* oxidase molar ratio = 40/1). Subsequent incubation of the enzyme (5 μM heme aa_3) was performed for 20 min at 30°C in the same buffer containing in addition 10 mM PEP (phosphoenolpyruvate), 10 units/ml of PK (pyruvate kinase, Boehringer, Mannheim, Germany), 5 mM $MgSO_4$, and further additions of cAMP (Sigma-Aldrich) and PKA (protein kinase A, from bovine heart, Sigma-Aldrich) as described in the legends to the figures. Measurement of cytochrome *c* oxidase activity (final concentration: 50 nM)

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Abbreviations: PEP, phosphoenolpyruvate; PK, pyruvate kinase; PKA, protein kinase A; PP1, protein phosphatase 1; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

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was performed polarographically [4] at 25°C with 18 mM ascorbate and increasing concentrations of cytochrome *c* (0.21–21 μM) in 50 mM potassium phosphate, pH 7.4, 1% Tween 20, 1 mM EDTA, and 5 mM ATP or 5 mM ADP (+0.05 mM ATP).

The phosphorylated enzyme (2.5 μM heme aa_3) was dephosphorylated by incubation for 10–40 min at 30°C in 50 mM KPi, pH 7.4, 1% Tween 20, 2.5 mM ATP, 5 mM PEP, 5 units/ml of PK, 2.5 mM $MgSO_4$, 25 μM cAMP, 100 units/ml PKA with 500 units/ml PP1 (protein phosphatase 1, rabbit, recombinant subunit C_α, expressed in *Escherichia coli*, Sigma-Aldrich) and 100 μM $MnCl_2$.

Mitochondria were isolated from frozen bovine liver by standard methods in sucrose-medium: 0.25 M sucrose, 20 mM Tris–HCl, pH 7.6, 2 mM EDTA. Mitochondria (0.5 μM heme aa_3, determined spectrophotometrically) were incubated for 20 min in the cold room in 10 ml open Erlenmeyer flasks under shaking in sucrose-medium containing 5 mM $MgSO_4$, 5 mM ATP, 20 mM PEP, 20 units/ml PK in the presence or absence of 50 μM cAMP as indicated in the legends to the figures. When indicated, 1 mM $CaCl_2$ was added and a further incubation for 20 min at 4°C followed. After incubation the mitochondria were solubilized in nine volumes of 1% Tween 20, 50 mM potassium phosphate, pH 7.4, 5 mM $MgSO_4$, 5 mM ATP, 20 mM PEP, 20 units/ml PK and kept for 15 min at 0°C. Measurement of cytochrome *c* oxidase activity was performed polarographically at 25°C with 18 mM ascorbate and increasing concentrations of cytochrome *c* (0.21–21 μM).

To determine the subunits of cytochrome *c* oxidase which are cAMP-dependent phosphorylated by PKA, the enzyme (5 μM) was incubated for 20 min at 30°C in a volume of 50 μl in 50 mM potassium phosphate, pH 7.4, 5 mM $MgSO_4$, 1% Tween 20, with [γ-^{32}P]-ATP (1 μCi, 800 Ci/mmol, ICN), and further additions of cAMP and PKA as described in the legends to the figures. The incubated enzyme was separated into subunits by SDS–PAGE as described by Schägger and von Jagow [21]. The gel was stained with Coomassie blue, dried and autoradiographed.

3. Results

Incubation of isolated cytochrome *c* oxidase from bovine heart with ATP, PKA and cAMP results in allosteric inhibition of activity, i.e. in a sigmoidal activity/substrate (v/s) relationship with full inhibition of activity up to 4 μM cytochrome *c*, when the ascorbate respiration is measured in the presence of ATP, as shown in Fig. 1 (open triangles). Measurement of activity in the presence of ATP after incubation without cAMP and/or PKA, resulted in partially inhibition of activity and a slightly sigmoidal curve, but lacking full inhibition at low cytochrome *c* concentrations (open circles). These results suggest that the allosteric ATP-inhibition of cytochrome *c* oxidase is predominantly due to cAMP-dependent phosphorylation of the enzyme. Before incubation the enzyme was kept overnight in a buffer containing 5 mM ATP and 200 μM cardiolipin, in order to exchange bound cholate by ATP [7], and to saturate the enzyme with cardiolipin, which is essential for the allosteric ATP-inhibition [4]. PEP and PK were added during incubation in order to keep the ATP/ADP-ratio high. Measurement of activity in the presence of ADP results in hyperbolic v/s curves (closed symbols). After preincubation with cAMP and PKA the hyperbolic curve is slightly higher (closed circles), excluding a direct inhibitory effect of cAMP on the enzyme activity.

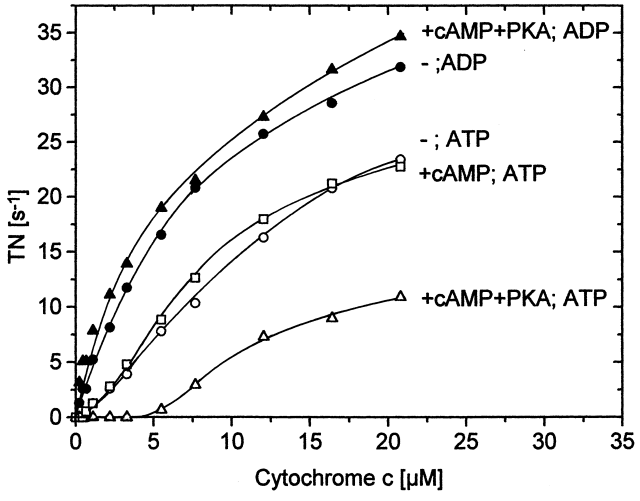

Fig. 1. The allosteric inhibition of cytochrome *c* oxidase activity by ATP is switched on by cAMP-dependent phosphorylation. Cytochrome *c* oxidase, pretreated with ATP and cardiolipin as described in Section 2, was incubated for 20 min at 30°C in 1% Tween 20, 50 mM potassium phosphate, pH 7.4, 5 mM $MgSO_4$, 5 mM ATP, 10 mM PEP, and 10 units/ml PK (circles), with 1 mM cAMP (squares), and 200 units/ml PKA (triangles). The activity was measured as described in Section 2 in the presence of 5 mM ADP (+0.05 mM ATP) (closed symbols) or 5 mM ATP (open symbols). The legends at the curves indicate before the semicolon the additions during incubation, and after the semicolon the presence of ATP or ADP during measurement of activity.

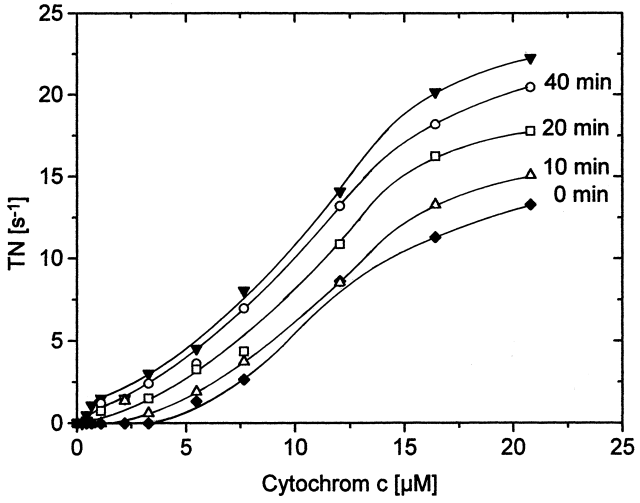

Fig. 2. Reversal of cAMP-dependent induced allosteric ATP-inhibition of bovine heart cytochrome *c* oxidase by incubation with protein phosphatase 1. The cAMP-dependent phosphorylated enzyme was dephosphorylated by PP1 as indicated in Section 2 at 30°C for the indicated times. Closed triangles: isolated cytochrome *c* oxidase only pretreated overnight with ATP and cardiolipin, as described in Section 2. The activity was measured in the presence of 5 mM ATP.

In Fig. 1 high concentrations of cAMP were used (1 mM) in order to show possible unspecific effects of cAMP. In other experiments, where the concentration dependency of cAMP was investigated, incubation with 5 μM cAMP for 20 min at 30°C induced already a sigmoidal curve with 50% inhibition at 20 μM cytochrome *c*. Incubation with 50 μM cAMP resulted in the same allosteric ATP-inhibition as that obtained with 1 mM cAMP in Fig. 1 (data not shown).

In order to prove that the allosteric ATP-inhibition is due to reversible phosphorylation of cytochrome *c* oxidase, the enzyme was incubated with protein phosphatase 1 after preincubation with ATP, PKA and cAMP, as presented in Fig. 2. With increasing incubation time the allosteric ATP-inhibition is abolished, and results after 40 min in almost the same v/s kinetics, as measured with the untreated enzyme. This result

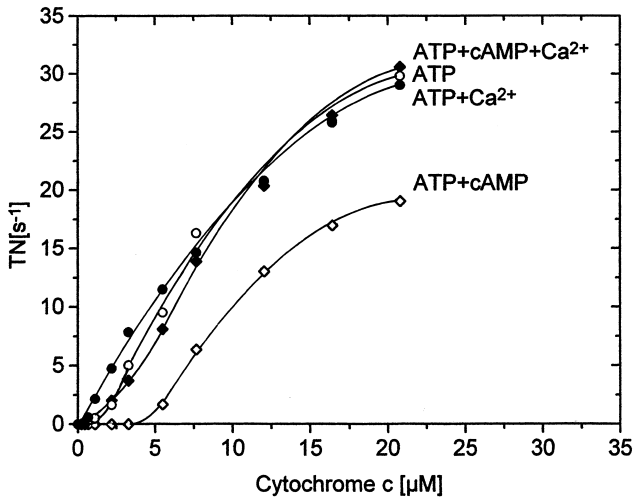

Fig. 3. Ca^{2+}-ions abolish the allosteric ATP-inhibition of cytochrome *c* oxidase in mitochondria, but not with the isolated enzyme. Mitochondria from bovine liver were treated as described in Section 2, and the activity of the solubilized mitochondria was measured in the presence of ATP and an ATP-regenerating system with ascorbate and increasing concentrations of cytochrome *c*. Before solubilization the mitochondria were incubated with ATP and an ATP-regenerating system (circles), and in addition 1 mM cAMP (diamonds). Closed symbols indicate a second incubation for 20 min at 0°C with 1 mM $CaCl_2$.

indicates that the allosteric ATP-inhibition is induced by reversible cAMP-dependent phosphorylation.

In mitochondria the allosteric ATP-inhibition of cytochrome *c* oxidase is reversed by a Ca^{2+}-activated protein phosphatase, as demonstrated in Fig. 3. Isolated bovine liver mitochondria were preincubated with ATP in the presence or absence of cAMP. An aliquot of the samples was further incubated for 20 min at 0°C with 1 mM $CaCl_2$. Incubation of mitochondria with ATP and cAMP induces the allosteric ATP-inhibition of the ascorbate respiration of Tween 20-solubilized cytochrome *c* oxidase with full inhibition up to 4 μM cytochrome *c* (open diamonds). The following incubation of mitochondria with $CaCl_2$ abolished the ATP-inhibition but resulted in a slightly sigmoidal curve (closed diamonds), which is similar to that obtained after incubation of mitochondria only with ATP (open circles). Mitochondria preincubated only with ATP and then with $CaCl_2$ result in a hyperbolic titration curve (closed circles). Ca^{2+}-ions do not interact directly with cytochrome *c* oxidase, as was shown by incubation of the preincubated enzyme with 1 mM $CaCl_2$. After preincubation of the bovine heart enzyme with ATP, cAMP and PKA, a sigmoidal v/s relationship with full inhibition of activity up to 4 μM cytochrome *c* was measured, which was not changed after subsequent incubation for 20 min at 30°C with 1 mM $CaCl_2$ (data not shown).

The induction of the sigmoidal kinetics of cytochrome *c* oxidase after preincubation with cAMP and PKA in the presence of ATP is at least partly due to phosphorylation of subunits II (and/or III) and Vb, as shown in Fig. 4. The isolated enzyme was preincubated with [γ-^{32}P]ATP, cAMP and PKA. After SDS–PAGE the Coomassie blue stained gel shows in the high molecular weight range various bands of PKA (Fig. 4, lane 1). In the autoradiography (lanes 4, 6, 8) these bands are heavily labeled, due to self-phosphorylation of the enzyme [22], even in the absence of cAMP (lane 6). Labeling of subunit Vb and to a smaller extent subunit II (and/or III) is clearly dependent on the presence of cAMP and maximal at a concentration of 50 μM cAMP. In contrast, a cAMP-independent labeling of subunit I cannot be excluded.

4. Discussion

The results of this paper demonstrate that cAMP-dependent phosphorylation of cytochrome *c* oxidase with protein

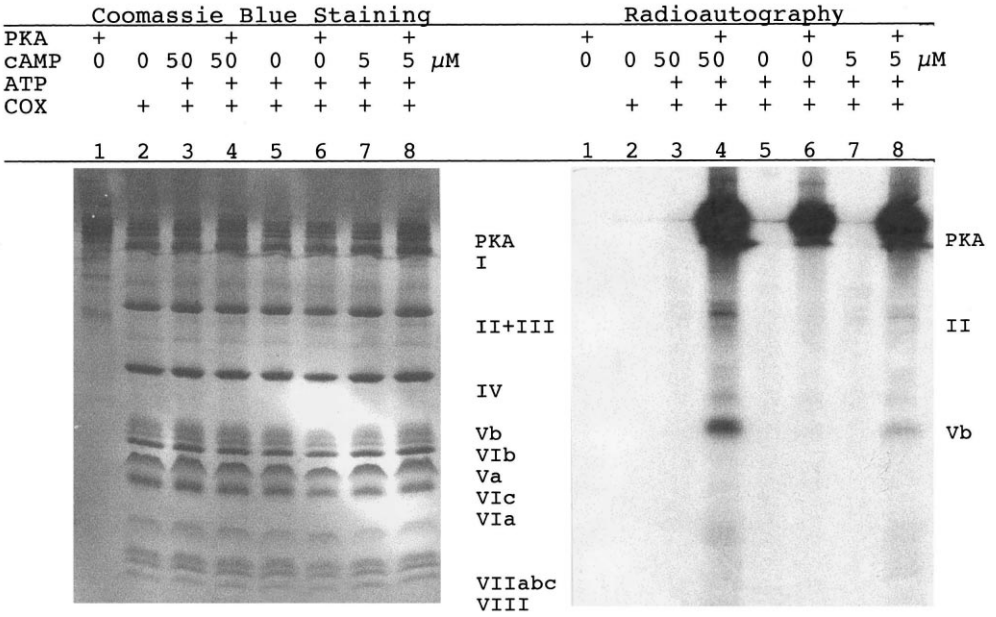

Fig. 4. cAMP-dependent labeling of cytochrome *c* oxidase with [γ-^{32}P]ATP and PKA. Isolated cytochrome *c* oxidase from bovine heart was incubated as described in Section 2 with or without [γ-^{32}P]ATP, cAMP and/or PKA as indicated in the upper panel. The samples were separated by SDS–PAGE, and the gel was stained with Coomassie blue. After drying, the gel was radioautographed.

kinase A switches on the allosteric inhibition of activity at high intramitochondrial ATP/ADP-ratios [4–6]. Similar results are obtained by incubation of isolated mitochondria with cAMP using an endogenous protein kinase (see [18] and Fig. 3). Phosphorylation of the enzyme apparently does not influence the activity in the presence of ADP (Fig. 1). The ATP-inhibition can be switched off by dephosphorylation of the isolated enzyme with protein phosphatase 1. The mitochondrial protein phosphatase which switches off the allosteric ATP-inhibition, is activated by Ca^{2+}-ions, as concluded from abolishing the ATP-inhibition by incubation of mitochondria with $CaCl_2$. The Ca^{2+}-ions, however, do not directly interact with cytochrome *c* oxidase. Furthermore, full inhibition of activity up to 10 μM cytochrome *c* was obtained after preincubation of mitochondria with potassium fluoride [18], an unspecific inhibitor of protein phosphatases [23].

In a previous study the allosteric ATP-inhibition of cytochrome *c* oxidase could be measured with the enzyme as isolated [4]. In subsequent studies, however, variable degrees or even no allosteric ATP-inhibition at all was found with different preparations of the isolated bovine heart enzyme (not shown), indicating the dependence of the allosteric ATP-inhibition on yet undefined specific conditions during isolation of mitochondria and/or the enzyme, leading to different degrees of dephosphorylation. A rapid dephosphorylation of cytochrome *c* oxidase follows also from the observation that cAMP-dependent modification of mitochondrial proteins appears to be very shortlived [13], and by the previously unsuccessful phosphorylation of cytochrome *c* oxidase subunits after incubation of inner mitochondrial membranes or the isolated bovine heart enzyme with protein kinase A and [γ-^{32}P]ATP [14,24]. A rapid dephosphorylation of cytochrome *c* oxidase could also explain why the allosteric ATP-inhibition has not been found previously in mitochondria or with the isolated enzyme.

The variable degrees of allosteric and non-allosteric ATP-inhibition of isolated cytochrome *c* oxidase suggest several sites of phosphorylation contributing to different degrees of ATP-inhibition. A cAMP-independent phosphorylation of subunit IV, to which ATP binds and induces the allosteric ATP-inhibition [4], has been described by Steenaart and Shore [11]. Here we identified by labeling with [γ-^{32}P]ATP subunits II (and/or III) and Vb as cAMP-dependent phosphorylated subunits. cAMP-dependent protein kinases were identified in the intermembrane space of mitochondria [12,13] and in the mitochondrial matrix [15], based on the observation that phosphorylation of mitochondrial proteins could be inhibited by atractylate [25]. The mechanism of translocation of cytosolic cAMP into the mitochondrial matrix, however, remains to be identified.

The correlation between cAMP-dependent labeling of subunits II (and/or III) and Vb and the increase of allosteric ATP-inhibition suggests the involvement of these subunits in the mechanism of allosteric ATP-inhibition. The crystal structure of bovine heart cytochrome *c* oxidase [26] indicates close vicinity between subunit Vb and the matrix domain of subunit IV, rendering conformational interactions feasible. The recent identification of a cAMP-sensitive association of the regulatory subunit (RIα) of a cAMP-dependent protein kinase A with the matrix-oriented subunit Vb of cytochrome *c* oxidase of human HeLa cells [27] supports the role of phosphorylated subunit Vb in the allosteric ATP-inhibition. The postulated allosteric mechanism of ATP-inhibition, based on cooperativity of the two binding sites for cytochrome *c* in the two monomers of the enzyme [4], probably via the cytosolic-oriented subunit VIb [26], could also involve phosphorylation of subunit II at its cytosolic side. In fact, partial induction of allosteric ATP-inhibition was found after incubation of the reconstituted enzyme with cAMP, ATP and PKA, by which the enzyme is only phosphorylated from the cytosolic side (unpublished results).

The possible physiological consequences of the reversible phosphorylation of cytochrome *c* oxidase has already been discussed in detail [18,28]. Control of respiration by the intramitochondrial ATP/ADP-ratio via the phosphorylated enzyme is assumed to keep the mitochondrial proton motive force Δp low, whereas hormone-induced Ca^{2+}-dependent dephosphorylation results in loss of respiratory control by the ATP/ADP-ratio, accompanied by increased respiration and Δp. Without respiratory control by the ATP/ADP-ratio the rate of respiration is limited by the proton motive force at higher values of Δp. The increased Δp causes slip of proton pumping in cytochrome *c* oxidase (i.e. decreased H^+/e^- stoichiometry), but not in the bc_1-complex [30–33] (for review see [29]), accompanied by increased $-\Delta G^{o'}$. The physiological meaning of the abolition of ATP-inhibition by Ca^{2+}-induced dephosphorylation could thus be to increase Δp and slip in cytochrome *c* oxidase, and thus to stimulate the rate of respiration and ATP-synthesis via lowered efficiency.

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References

[1] Mitchell, P. (1961) Nature 191, 144–148.
[2] Mitchell, P. (1966) Biol. Rev. 41, 445–502.
[3] Nicholls, D.G. and Ferguson, S.J. (1992) Bioenergetics 2, Academic Press Limited, London, pp. 82–87.
[4] Arnold, S. and Kadenbach, B. (1997) Eur. J. Biochem. 249, 350–354.
[5] Arnold, S. and Kadenbach, B. (1999) FEBS Lett. 443, 105–108.
[6] Kadenbach, B. and Arnold, S. (1999) FEBS Lett. 447, 131–134.
[7] Napiwotzki, J., Shinzawa-Itoh, K., Yoshikawa, S. and Kadenbach, B. (1997) Biol. Chem. 378, 1013–1021.
[8] Napiwotzki, J. and Kadenbach, B. (1998) Biol. Chem. 379, 335–339.
[9] Follmann, K., Arnold, S., Ferguson-Miller, S. and Kadenbach, B. (1998) Biochem. Mol. Biol. Intern. 45, 1047–1055.
[10] Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) Nature 376, 660–669.
[11] Steenaart, N.A.E. and Shore, G.C. (1997) FEBS Lett. 415, 294–298.
[12] Müller, G. and Bandlow, W. (1987) Yeast 3, 161–174.
[13] Müller, G. and Bandlow, W. (1987) Z. Naturforsch. 42, 1291–1302.
[14] Papa, S., Sardanelli, A.M., Cocco, T., Speranza, F., Scacco, S.C. and Technikova-Dobrova, Z. (1996) FEBS Lett. 379, 299–301.
[15] Burgess, J.W. and Yamada, E.W. (1987) Biochem. Cell Biol. 65, 137–143.
[16] Schwoch, G., Trinczek, B. and Bode, C. (1990) Biochem. J. 270, 181–188.
[17] Vallejo, C.G., Seguido, A.M. and Fernandez-Renart, M. (1997) Arch. Biochem. Biophys. 339, 9–16.
[18] Kadenbach, B., Bender, E., Reith, A., Becker, A., Hammerschmidt, S., Lee, I., Arnold, S. and Hüttemann, M. (1999) J. Anti-Aging Med. 2, 255–264.
[19] Yoshikawa, S., Choc, M.G., O'Toole, M.C. and Caughey, W.S. (1977) J. Biol. Chem. 252, 5498–5508.

[20] Yoshikawa, S., Tera, T., Takahashi, Y. and Tsukihara, T. (1988) Proc. Natl. Acad. Sci. USA 85, 1354–1358.
[21] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[22] Pearson, R.B. and Kemp, B.E. (1991) Methods Enzymol. 200, 62–81.
[23] Mackenzie III, C.W., Bulbulian, G.J. and Bishop, J.S. (1980) Biochim. Biophys. Acta 614, 413–424.
[24] Sardanella, A.M., Technikova-Dobrova, Z., Scacco, S., Speranza, F. and Papa, S. (1995) FEBS Lett. 377, 470–474.
[25] Sardanella, A.M., Technikova-Dobrova, Z., Speranza, F., Mazzocca, A., Scacco, S. and Papa, S. (1996) FEBS Lett. 396, 276–278.
[26] Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1996) Science 272, 1136–1144.
[27] Yang, W.-L., Iacono, L., Tang, W.-M. and Chin, K.-V. (1998) Biochemistry 37, 14175–14180.
[28] Kadenbach, B., Hüttemann, M., Arnold, S., Lee, I., Mühlenbein, N. and Bender, E. (1999) Free Radic. Biol. Med., in press.
[29] Murphy, M.P. (1989) Biochim. Biophys. Acta 977, 123–141.
[30] van Dam, K., Shinohara, Y., Unami, A., Yoshida, K. and Terada, H. (1990) FEBS Lett. 277, 131–133.
[31] Papa, S., Capitanio, N., Capitanio, G., De Nitto, E. and Minuto, M. (1991) FEBS Lett. 288, 183–186.
[32] Capitanio, N., Capitanio, G., De Nitto, E., Billani, G. and Papa, S. (1991) FEBS Lett. 288, 179–182.
[33] Capitanio, N., Capitanio, G., Demarinis, D.A., De Nitto, E., Massari, S. and Papa, S. (1996) Biochemistry 35, 10800–10806.