Energy-dependent transformation of the catalytic activities of the mitochondrial $F_0 \cdot F_1$-ATP synthase

M.A. Galkin, A.D. Vinogradov*

Department of Biochemistry, School of Biology, Moscow State University, Moscow 119899, Russia

Abstract The ADP(Mg^{2+})-deactivated, azide-trapped $F_0 \cdot F_1$-ATPase of coupled submitochondrial particles is capable of ATP synthesis being incapable of ATP hydrolysis and ATP-dependent $\Delta\bar{\mu}_H$+ generation [FEBS Lett. (1995) 366, 29–32]. This puzzling phenomenon was studied further. No ATPase activity of the submitochondrial particles catalyzing succinate-supported oxidative phosphorylation in the presence of azide was observed when ATP was added to the assay mixture after an uncoupler. Rapid ATP hydrolysis was detected in the same system when ATP followed by an uncoupler was added. Less than 5% of the original ATPase activity was seen when the reaction (assayed with ATP-regenerating system) was initiated by the addition of ATP to the azide-trapped coupled particles oxidizing succinate either in the presence or in the absence of the uncoupler. High ATP hydrolytic activity was revealed when the reaction was started by the simultaneous addition of the ATP plus uncoupler to the particles generating $\Delta\bar{\mu}_H$+. The energy-dependent conversion of the enzyme into latent uncoupler-activated ATPase was prevented by free ADP ($K_i \approx 20$ μM) and was greatly enhanced after multiple turnovers in oxidative phosphorylation. The results suggest that the catalytic properties of $F_0 \cdot F_1$ are $\Delta\bar{\mu}_H$+-dependent which is in accord with our hypothesis on different conformational states of the enzyme participating in ATP synthesis or hydrolysis.

© 1999 Federation of European Biochemical Societies.

Key words: F_1-ATP synthase; Protonmotive force; Oxidative phosphorylation; Mitochondria

1. Introduction

The mitochondrial membrane-bound ATP synthase belongs to the $F_0 \cdot F_1$-type ATPase family of the enzymes which catalyze either $\Delta\bar{\mu}_H$+-utilizing ATP synthesis or $\Delta\bar{\mu}_H$+-generating ATP hydrolysis depending on the particular organism and/or physiological conditions. The minimal catalytic enzyme unit is composed of at least eight different subunits: $3\alpha \cdot 3\beta \cdot \gamma \delta \cdot \epsilon$ F_1 peripheral part bears three (out of a total of six) rapidly exchangeable nucleotide-binding 'catalytic' sites on β-subunits, whereas membrane-spanning F_0 is believed to form $a \cdot b_2 \cdot c_{12}$ proton conducting path [1]. According to the widely accepted alternating binding change mechanism [2,3], the flow of protons through the F_0 part drives the rotation of the γ-subunit inside of the F_1 $3\alpha \cdot 3\beta$ hexamer leading to the ordered change in the substrate/product affinities of the catalytic sites. 2.8 Å resolution structures of bovine heart F_1 [4]

and rat liver F_1 [5] are ideally suited for such a mechanism and the unidirectional ATP-dependent rotation of the γ-subunit within $3\alpha \cdot 3\beta$ hexameric F_1 has been deduced from several indirect studies [6,7] and visualized directly [8]. Three identical (although cooperatively alternating) catalytic site models apparently leave no possibilities other than the substrate/product participation of ATP, ADP, P_i and Mg^{2+} in the reaction catalyzed by the reversible H^+-$F_0 \cdot F_1$-ATPase. On the other hand, it is well documented that both soluble (F_1) and membrane-bound ($F_0 \cdot F_1$) enzymes demonstrate extremely complex pre-steady-state and steady-state kinetics [9,10] which are hardly compatible with such a simple model. Perhaps the most intriguing property of the enzyme is its exceptional sensitivity to a very low (almost stoichiometric) concentration of ADP which dramatically changes the kinetic performance of ATP hydrolysis [11,12]. In fact, the effects of several ligands, such as inhibitory azide [13], stimulating sulfite [13] and phosphate [14], Mg^{2+} [15,16] as well as apparent non-Michaelis kinetics [3] seem to be intrinsically related to either ADP(Mg^{2+})-induced inhibition, or to ADP release-dependent activation.

We have previously shown that ADP(Mg^{2+}) exposed azide-trapped $F_0 \cdot F_1$-ATPase of coupled submitochondrial particles is fully capable in the ATP synthesis reaction whereas both hydrolytic activity and ATP-dependent $\Delta\bar{\mu}_H$+ generation ceased [17]. This observation was interpreted as the evidence for our original hypothesis [18] on the different reaction pathways (states of the enzyme) in the hydrolase or synthase directions of the steady-state catalytic turnovers. In this report we will show that the mitochondrial $F_0 \cdot F_1$-ATP synthase undergoes $\Delta\bar{\mu}_H$+-dependent transformation of its catalytic activity similar to the phenomenon which has been demonstrated for photosynthetic coupling machinery [19]. We interpret our findings as to suggest that $\Delta\bar{\mu}_H$+-induced metastable ATP synthase conformation of the enzyme exist which is converted to ATPase conformation when $\Delta\bar{\mu}_H$+ is collapsed.

2. Materials and methods

SMP essentially free of protein inhibitor were prepared as described [20] and stored in liquid nitrogen as the suspension (30 mg of protein/ml) in 0.25 M sucrose. Before the experiments the suspension was thawed and diluted (5 mg/ml) at room temperature (18–20°C) with the mixture comprising (final concentration) of 0.25 M sucrose, 10 mM HEPES, 0.1 mM EDTA, 1 mM malonate (potassium salts, pH 8.0), BSA (1 mg/ml) and oligomycin (0.25 nmol/mg of SMP protein). 5 μM ADP, 2.5 mM $MgCl_2$ and 0.1 mM sodium azide (final concentrations) were added to one sample (deactivated SMP [13,15], SMP_D); 5 mM potassium phosphate (pH 8.0) was added to another sample (activated SMP [14], SMP_A). The samples were further incubated for 1 h before the assays for complete activation of succinate dehydrogenase [21], for 'coupling' by substoichiometric oligomycin and for activation of the ATPase activity by P_i [14]. The rate of

*Corresponding author. Fax: (7) (095) 939 3955.
E-mail: adv@biochem.bio.msu.su

ATP synthesis was measured at 30°C continuously as H+-consumption with phenol red as pH indicator ($\Delta A_{557-620}$) [22] in 2 ml of the standard mixture containing: 0.25 M sucrose, 12 mM phosphate, 10 mM succinate, 0.2 mM EDTA (potassium salts, pH 8.0), 2 μM rotenone, 0.1 mM potassium azide, 30 μM phenol red and BSA (1 mg/ml). It was shown in the separate control experiments that 0.1 mM azide had no inhibitory effect on succinate-supported respiration under the conditions employed. MgCl$_2$, ATP and ADP were added as indicated in the legends to the figures. ATPase activity was assayed in the standard reaction mixture (10 mM HEPES and no phosphate were present) at 30°C continuously either as H+-release measured with phenol red [22] or as NADH oxidation at 340 nm with ATP-regenerating system (phospho-*enol*pyruvate, pyruvate kinase and lactate dehydrogenase [9]). No pH changes were detected in course of the aerobic succinate oxidation when ATP synthesis/hydrolysis was blocked by either oligomycin or the uncoupler. 5 μM FCCP or gramicidin D (0.12 μg/ml) were added as the uncouplers where indicated. The same results were obtained with either uncoupler. Our standard SMP preparations show the following catalytic activities (μmol per min per mg of protein) at 30°C: fully activated (by preincubation with P$_i$ [14]) uncoupled ATPase 4–7; uncoupled succinate oxidase 0.9; ATPase (after coupling with substoichiometric oligomycin) 1.2–1.5 (no uncoupler) and 2–2.5 (plus uncoupler); respiratory control with succinate ≥ 2; succinate oxidation-supported ATP synthesis 0.1–0.2. ATPase and ATP synthase activities were more than 95% sensitive to oligomycin. ATP formation was more than 95% sensitive to the uncouplers and oligomycin (no significant adenylate kinase activity was present). We were unable to find any evidence for 'heterogeneity' of our SMP (i.e. the presence of several 'populations' such as tightly coupled, completely uncoupled, right side-out, right side-in, or opened particles) as will be discussed elsewhere. The details of the experiments are described in the legends to the figures.

ATP, ADP (sodium salt), lactate dehydrogenase, HEPES, NADH, gramicidin D, FCCP, and succinate were obtained from Sigma (USA). Phospho-*enol*pyruvate and pyruvate kinase were from Reanal (Hungary). Rotenone was from Ferak (Germany). Other chemicals were of the purest grade commercially available.

3. Results

We have previously shown [17] that ADP(Mg^{2+})-deactivated F$_0$·F$_1$ catalyzes synthesis of ATP in the presence of azide (which is known to prevent the ATP-dependent activation of ATPase [13]) at the same rate as active F$_0$·F$_1$. Fig. 1A demonstrates the actual time-course of ATP synthesis/hydrolysis catalyzed by the deactivated coupled particles. The addition of SMP$_D$ to the mixture containing azide and all the components needed for oxidative phosphorylation resulted

in immediate (no lag phase) H+ consumption which was, as expected, completely prevented by oligomycin (10 nmol/mg of protein) or by an uncoupler (not shown). The addition of the uncoupler about 2 min after multiple turnovers in ATP synthesis stopped the reaction and the subsequent addition of ATP, also as expected [17], resulted in no hydrolytic reaction. Note that the hydrolysis of either a low concentration of ATP (≈ 15 μM, which was formed from ADP and P$_i$ during phosphorylation) or a high concentration of added ATP (1 mM) was prevented, evidently because of the presence of 1 mM ADP (which is much higher than needed to inhibit uncoupled ATPase in the presence of azide). However, when ATP followed by uncoupler was added to the assay mixture after F$_0$·F$_1$ was operating in the direction of ATP synthesis (Fig. 1B), an immediate hydrolysis of ATP started, in spite of the presence of inhibitory ADP and azide. This hydrolytic activity was slowly decreased which is in accord with well documented time-dependent inhibition of coupled or uncoupled ATPase activity by azide [13]. The initial rate of the ATP plus uncoupler-induced hydrolysis (0.5 μmol/min/mg of protein) was equal to that measured as the control values for activated SMP$_A$ (see Section 2) under the same conditions (i.e. in the presence 1 mM ADP, 12 mM P$_i$ and azide) and was completely abolished by oligomycin. The results shown in Fig. 1 suggest that the catalytic activity of F$_0$·F$_1$ (ATP synthase and/or ATP hydrolase) is $\Delta\bar{\mu}_{H^+}$-dependent: when $\Delta\bar{\mu}_{H^+}$ was collapsed by an uncoupler the hydrolysis of added ATP was blocked; on the other hand, 100% of the hydrolytic activity was revealed when ATP and an uncoupler (to stop oxidative phosphorylation) were added. It should be noted that high ATPase activity revealed as depicted in Fig. 1B cannot be explained by an uncoupler-induced release of the $\Delta\bar{\mu}_{H^+}$ back pressure on proton flow: the ATPase activity of the oligomicin-coupled particles as assayed under standard conditions was stimulated by an uncoupler not more than twice (see Section 2 and Fig. 2B).

However suggestive the results shown in Fig. 1 were, their straightforward interpretation is somehow obscured by the complexity of the assay system (the simultaneous presence of the substrates and products of oxidative phosphorylation). Thus, it seemed desirable to show unequivocally that F$_0$·F$_1$ is capable of the $\Delta\bar{\mu}_{H^+}$-dependent transformation of its catalytic
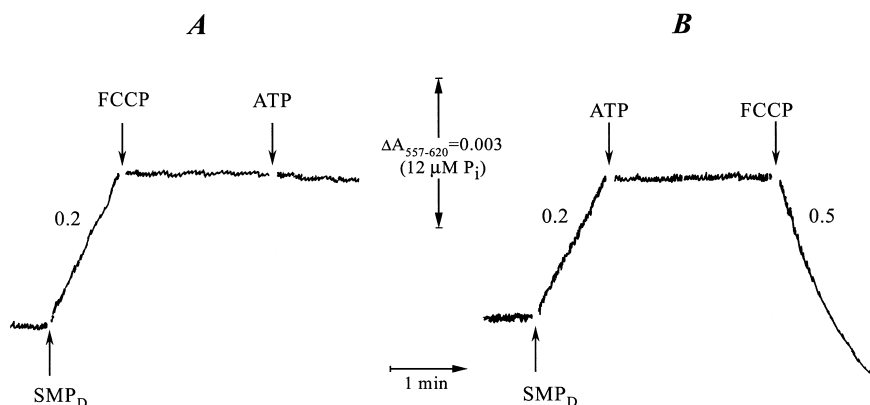

Fig. 1. Time-course of ATP synthesis or hydrolysis catalyzed by the coupled azide-trapped submitochondrial particles oxidizing succinate. The reactions were followed as H+ concentration change as described in Section 2. 1 mM ADP was present in the standard reaction mixture. Instant pH changes induced by the additions of 1 mM ATP (due to ATP·Mg^{2+} complex formation) were compensated. The reactions were started by the additions of 200 μg of SMP$_D$ (protein basis). Figures on the curves correspond to the specific activities of ATP synthesis or hydrolysis (μmol per min per mg of protein). 5 μM FCCP was added where indicated.

A   **B**

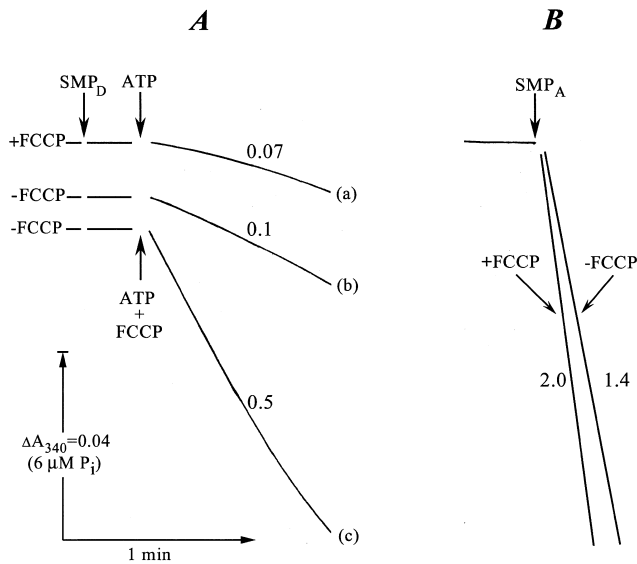Fig. 2. Time-course of ATP hydrolysis in the presence of ATP re-generating system. A: Deactivated SMP_D (50 μg), 1 mM ATP or 1 mM ATP plus 5 μM FCCP were added as indicated by the arrows. 5 μM FCCP was present in the standard reaction mixture (curve a). Figures on the curves correspond to the specific ATPase activities (μmol/min/mg of protein). B: Activated SMP_A (50 μg) were added to the standard reaction mixture containing 1 mM ATP and 5 μM FCCP (where indicated). No phosphate was present in the assay mixture.

Fig. 3. Effect of ADP on the energy-dependent transformation of $F_0 \cdot F_1$-ATPase. The initial rates of ATP hydrolysis were measured with phenol red (see Section 2). Deactivated particles (SMP_D, 200 μg) were preincubated for 30 s in the standard reaction mixture ($MgCl_2$ and phosphate were omitted) containing different concentrations of ADP (as indicated). ATP hydrolysis was initiated by the simultaneous addition of 1 mM ATP, 5 mM $MgCl_2$ and 5 μM FCCP. The arbitrary unit of the activity corresponds to the initial rate of ATP hydrolysis measured after 30 s preincubation in the absence of added ADP. The highest concentration of ADP employed (70 μM) inhibited the initial rate of ATP hydrolysis not more than 20%.

activity from the ATPase ADP(Mg^{2+})-inhibited state to the state which is also inactive as ATPase, but can be transformed into the latter when $\Delta\bar{\mu}_{H^+}$ is collapsed. This was accomplished by measuring ATPase activity in the presence of ATP-regenerating system (i.e. in the absence of ADP in the assay). Fig. 2A demonstrates the time-course of ATP hydrolysis by coupled SMP_D oxidizing succinate in the presence of azide. Less than 5% of the original (SMP_A) initial rates (Fig. 2B) were revealed either in the presence of absence of an uncoupler in the assay when the reaction was started by the addition of ATP (curves a and b). When the enzyme was pre-exposed to $\Delta\bar{\mu}_{H^+}$ the addition of ATP plus uncoupler resulted in immediate hydrolysis which was slowly decreased presumably because of the time-dependent inhibition by azide (curve c). The $\Delta\bar{\mu}_{H^+}$-dependent activation was instant within the time resolution limit (≈ 5 s).

The degree of the activation measured as depicted in Fig. 2 was about 40% and never reached that observed after multiple turnovers in oxidative phosphorylation as shown in Fig. 1 (more than 90%). Note, that 100% ATPase activity shown in Fig. 1 corresponds to the control value of that measured for SMP_A in the presence of 1 mM ADP and 12 mM P_i (0.5 μmol/min per mg), whereas 100% activity revealed as depicted in Fig. 2 corresponds to the control value of that measured for SMP_A in the absence of ADP and P_i (2 μmol/min per mg). The reason for incompleteness of the activation remains to be explained. One possibility is that simultaneous presence of ADP and P_i at a catalytic site somehow facilitates $\Delta\bar{\mu}_{H^+}$-dependent transformation of the enzyme.

Among several ligands tested free ADP (not Mg·ADP) was found to affect the $\Delta\bar{\mu}_{H^+}$-dependent enzyme transition. When ADP was added to the coupled particles oxidizing succinate before the reaction was initiated by the simultaneous addition of ATP, Mg^{2+} and uncoupler the degree of activation was hyperbolically inhibited with an apparent K_i^{ADP} of 20 μM (Fig. 3).
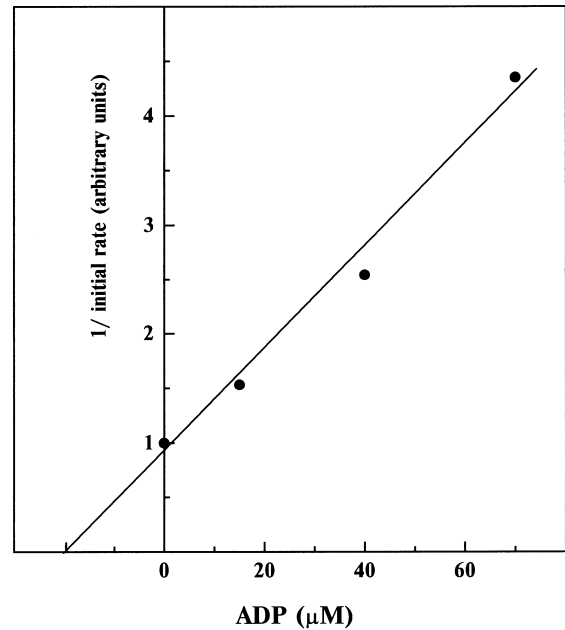
4. Discussion

The effect of $\Delta\bar{\mu}_{H^+}$, other than direct participation in the reaction as the driving force on $F_0 \cdot F_1$-ATP synthase of the photosynthetic organisms is well documented [19,23–26], although neither the mechanism nor the significance for the overall reaction are clearly understood. An obvious regulatory significance of such $\Delta\bar{\mu}_{H^+}$-dependent active/inactive transition is to prevent the futile ATP hydrolysis under the conditions where no light-dependent sources of energy are available. It seems very likely that complex regulatory mechanisms also exist in the mammalian mitochondria as can be exemplified by the strong unidirectional inhibitory effects of ADP [18] or protein inhibitor [27,28]. In this paper we have shown, to our
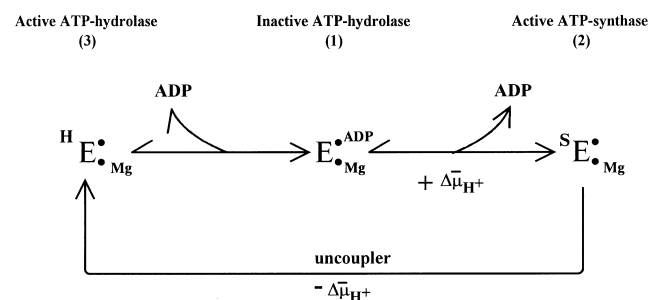

Fig. 4. Schematic illustration of the energy-dependent transformation of $F_0 \cdot F_1$-ATP synthase. The presence of bound Mg^{2+} (not as a constituent of the nucleotide·Mg^{2+} complexes participating as true substrates in ATP synthesis or hydrolysis) is indicated ([16,31] and our unpublished results). ^HE and ^SE stand for hydrolase and synthase conformations, respectively. See text for other explanations.

knowledge, for the first time that the mitochondrial $F_0 \cdot F_1$-ATP synthase is also subjected to $\Delta\bar{\mu}_{H^+}$-dependent transition. In a brief discussion we would like to focus on the problem of the ATP hydrolysis/synthesis reversibility rather than on the regulatory significance of this phenomenon. Our interpretation of the findings reported here is illustrated schematically as depicted in Fig. 4. ADP(Mg^{2+})-inhibited ATPase (form 1) is capable of $\Delta\bar{\mu}_{H^+}$-driven, ADP-dependent transformation into form 2 which is also inactive in ATP hydrolysis, being the ATP synthase conformation of the enzyme. When $\Delta\bar{\mu}_{H^+}$ is collapsed ATP synthase is rapidly transformed into the active ATPase state (form 3) which is subjected to ADP(Mg^{2+})-dependent inhibition by azide or activation by sulfite [13] or by P_i [14]. Only form 2 is capable of $\Delta\bar{\mu}_{H^+}$-driven ATP synthesis. Note that according to the scheme $\Delta\bar{\mu}_{H^+}$ has a dual role in oxidative phosphorylation: it serves as a driving force for the overall ATP synthesis catalyzed by ATP synthase and as an effector which maintains an appropriate enzyme conformation. The microreversibility principle usually quantitatively expressed in enzymology as Haldane relations would certainly hold for either the reaction of ATP synthesis as catalyzed by form 2 or for the reaction of ATP hydrolysis as catalyzed by form 3, but not for both operating simultaneously. According to the scheme the strong stimulating effect of uncouplers on the ATPase activity in coupled mitochondria is not due to the prevention of $\Delta\bar{\mu}_{H^+}$ back pressure in the reaction catalyzed by form 2, but rather to the rapid form 2 → form 3 transformation. Since ATP-dependent $\Delta\bar{\mu}_{H^+}$ generation is well known phenomenon ('coupled reversible' ATPase can be exemplified by several uncoupler sensitive ATP-dependent reactions such as reverse electron transfer, transhydrogenase reaction, or ion accumulation) an obvious question arises whether these reactions are catalyzed by form 2 or form 3. Our present working hypothesis is that ATPase in form 3 is involved and ATP as a ligand prevents $\Delta\bar{\mu}_{H^+}$-dependent transition form 1 → form 2.

In conclusion it is pertinent to note that the unidirectional effect of ADP on the mitochondrial $F_0 \cdot F_1$-ATPase reported by us two decades ago [18], and which is the key point of further conceptual development seems to be a general phenomenon for the proton-translocating ATPases. Recently, V_1-ATPase of plasma membrane of thermophilic eubacterium *Thermus thermophilus* has been reported to be sensitive to high affinity inhibitory ADP and the ADP(Mg^{2+}) inactivated $V_0 \cdot V_1$-ATPase was active in light-driven ATP synthesis when incorporated into bacteriorhodopsin containing proteoliposomes [29]. The direct effect of azide on proton-translocating ATPase activity of $V_0 \cdot V_1$-ATPase of clathrin-coated vesicles from calf brain [30] seems also relevant to the present discussion.

References

[1] Senior, A.E. (1988) Physiol. Rev. 68, 177–231.
[2] Repke, K.R.H. and Shön, R. (1974) Acta Biol. Med. Germ. 33, K27–K38.
[3] Boyer, P.D. (1993) Biochim. Biophys. Acta 1140, 215–250.
[4] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Nature 370, 621–628.
[5] Bianchet, M.A., Hullihen, J., Pedersen, P.L. and Amzel, M. (1998) Proc. Natl. Acad. Sci. USA 95, 11065–11070.
[6] Duncan, T.M., Bulygin, V.V., Zhou, Y., Hutcheon, M.L. and Cross, R.L. (1995) Proc. Natl. Acad. Sci. USA 92, 10964–10968.
[7] Sabbert, D., Engelbrecht, S. and Junge, W. (1996) Nature 381, 623–625.
[8] Noji, H., Yasuda, R., Yoshida, M. and Kinosita, K. (1997) Nature 386, 299–302.
[9] Vasilyeva, E.A., Fitin, A.F., Minkov, I.B. and Vinogradov, A.D. (1980) Biochem. J. 188, 807–815.
[10] Jault, J.-M. and Allison, W.S. (1994) J. Biol. Chem. 269, 319–325.
[11] Fitin, A.F., Vasilyeva, E.A. and Vinogradov, A.D. (1979) Biochem. Biophys. Res. Commun. 86, 434–439.
[12] Vasilyeva, E.A., Minkov, I.B., Fitin, A.F. and Vinogradov, A.D. (1982) Biochem. J. 202, 9–14.
[13] Vasilyeva, E.A., Minkov, I.B., Fitin, A.F. and Vinogradov, A.D. (1982) Biochem. J. 202, 15–23.
[14] Yalamova, M.V., Vasilyeva, E.A. and Vinogradov, A.D. (1982) Biochem. Int. 4, 337–344.
[15] Moyle, J. and Mitchell, P. (1975) FEBS Lett. 56, 55–61.
[16] Bulygin, V.V. and Vinogradov, A.D. (1991) Biochem. J. 276, 149–156.
[17] Syroeshkin, A.V., Vasilyeva, E.A. and Vinogradov, A.D. (1995) FEBS Lett. 366, 29–32.
[18] Minkov, I.B., Vasilyeva, E.A., Fitin, A.F. and Vinogradov, A.D. (1980) Biochem. Int. 1, 478–485.
[19] Gräber, P. (1994) Biochim. Biophys. Acta 1187, 171–176.
[20] Kotlyar, A.B. and Vinogradov, A.D. (1990) Biochim. Biophys. Acta 1019, 151–158.
[21] Kotlyar, A.B. and Vinogradov, A.D. (1984) Biochim. Biophys. Acta 784, 24–34.
[22] Chance, B. and Nishimura, M. (1967) Methods Enzymol. 10, 641–650.
[23] Junge, W. (1970) Eur. J. Biochem. 14, 582–592.
[24] Bickel-Sandkötter, S. and Strotmann, H. (1981) FEBS Lett. 125, 188–192.
[25] Du, Z. and Boyer, P.D. (1989) Biochemistry 28, 873–879.
[26] Krab, K., Bakel, R.H.A., Scholts, M.J.C. and van Walraven, H.S. (1993) Biochim. Biophys. Acta 1141, 197–205.
[27] Harris, D.A. and Das, A.M. (1991) Biochem. J. 280, 561–563.
[28] Vasilyeva, E.A., Panchenko, M.V. and Vinogradov, A.D. (1989) Biokhimiya (USSR) 54, 1490–1498 (in Russian).
[29] Yokoyama, K., Muneyuki, E., Amono, T., Mizutani, S., Yoshida, M., Ishida, M. and Ohkuma, S. (1998) J. Biol. Chem. 273, 20504–20510.
[30] Vasilyeva, E.A. and Forgac, M. (1998) J. Biol. Chem. 273, 23823–23829.
[31] Bulygin, V.V., Syroeshkin, A.V. and Vinogradov, A.D. (1993) FEBS Lett. 328, 193–196.