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CLASSIFICATION OF NUCLEOTIDE BINDING SITES ON MITOCHONDRIAL F_1-ATPase FROM YEAST

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Summary

Methods are described to classify nucleotide binding sites of the mitochondrial coupling factor F_1 from yeast on the basis of their affinities and stability properties. High affinity sites or states for ATP and related adenine analogs and low affinity sites or states which bind a broad range of different nucleotide triphosphates are found. The results are discussed in terms of a two site, two cycle scheme, where binding of nucleotide at one site facilitates the release of nucleotide at a second site.

Introduction

Mitochondrial ATP-synthesis is a function of the coupling factor 1 moiety (F_1) of the membrane-bound mitochondrial ATP-synthase complex (for review see Refs 1 and 2). For the description of the reaction mechanism of this process an understanding of the nucleotide binding properties of F_1 is essential. Experiments with isolated yeast F_1-ATPase which are reported here demonstrate two different classes of sites or states of nucleotide binding in the isolated enzyme: one class with a high specificity and affinity for ATP, and a second class of low specificity and affinity. Methods are presented to classify

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Abbreviations: F_1-ATPase, ATP phosphohydrolyase (EC 3.6.1.3) (F_1); AMP-PNP, adenylyl imidodiphosphate; ϵ-ATP, ethenoadenosine 5'-triphosphate; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropane sulfonate; Pipes, piperazine-*N*,*N*'-bis(2-ethane sulfonate).

both classes of sites with respect to their nucleotide specificity by measuring nucleotide binding, F_1-ATPase stabilization and steady-state kinetics.

Methods

Mitochondrial coupling factor F_1-ATPase was prepared by the method as given in Ref. 3. The F_1-ATPase activity was determined according to Ref. 4. Nucleotide triphosphates were from Boehringer, and labelled compounds from Amersham; luciferase, luciferin and ϵ-ATP were from Sigma: as judged from a luciferase assay the ϵ-ATP sample contained less than 2% ATP. All other chemicals were of reagent grade.

Nucleotide triphosphate determination with luciferase

An ammonium-sulfate pellet (see Table I) containing about 1 mg of enzyme was dissolved in 250 μl of 50 mM Hepps (potassium salt), 5 mM $MgCl_2$ (pH = 7.8) and centrifuged twice through 1 ml of Sephadex G-25. Appropriate volumes of concentrated nucleotide triphosphate solutions were added to 60 μl of the resulting solution yielding a final concentration of 1 mM, and the mixture was incubated for 10 min at 25° C. Then, 250 μl of buffer were added. The solutions were centrifuged 2 or 3 times through 1 ml of Sephadex G-25, and 30 μl 200 mM EDTA were added. The protein was denatured by injecting the solution into a test tube heated in boiling water, and then quickly cooled down to 10°C. The resulting solutions were used for the ATP determination by the luciferase technique according to Ref. 5.

Results

Binding of nucleotides to F_1-ATPase

To study the binding of [^{3}H]ATP to F_1-ATPase, mixtures of both were subjected to discontinuous gel filtration. The dilution of small molecules was determined by adding micromolar concentrations of labeled sulfate, which only binds with low affinity to F_1-ATPase [6]. After five gel passages the sulfate radioactivity was diluted nearly to background level (Fig. 1a, circles), whereas only a 20-fold dilution for ATP (2000-fold for sulfate) was observed. The F_1-ATPase activity (crosses) declined slightly due to some losses on the gel (about 10%/step). The experiment demonstrates high affinity adenine-nucleotide-binding to F_1-ATPase.

In the same way the adenine nucleotide specificity of the binding with high affinity was studied. Tritium-labeled deoxy-ATP, CTP and ITP, respectively, were mixed with F_1-ATP and $^{35}SO_4^{2-}$ and passed several times through Sephadex G-25 gel. Only the deoxy-adenine nucleotide was tightly bound to F_1-ATPase. This finding was further substantiated by using mixtures of two nucleotide triphosphates in the incubation solution, one carrying a ^{3}H and the other carrying a ^{14}C label. $^{35}SO_4^{2-}$ was omitted from the mixture. Fig. 1b shows an experiment with [^{3}H]ATP and [^{14}C]ITP. The ITP counts (circles) were diluted similar to SO_4^{2-} in the experiment of Fig. 1a. These experiments were repeated with other [^{3}H]nucleotide triphosphate instead of [^{3}H]ATP.

The figures for the dilution factor ratios (f) were obtained from the slopes

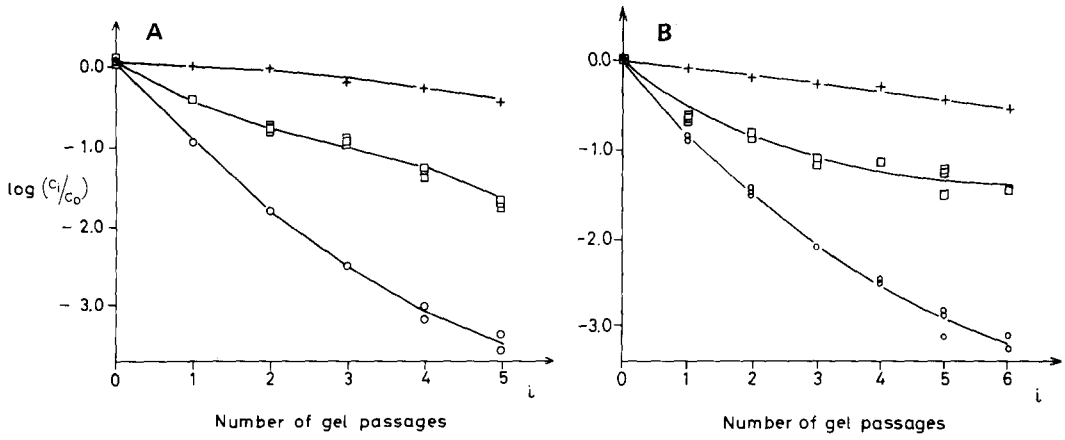


Fig. 1. Dilution of low molecular weight solutes on repeated centrifugation through Sephadex G-25. Methods see legend Table I, enzyme activity (c) expressed in c_i/c_0. Conditions at the start of the experiment: (A) +———+, 3 μM ATPase; □———□, 20 μM [^{3}H]ATP (= 100 000 cpm ^{3}H); ○———○, 15 000 cpm $^{35}SO_4^{2-}$. (B) +———+, 4 μM ATPase; □———□, 50 μM [^{3}H]ATP; ○———○, 40 μM [^{14}C]ITP.

of the experiments as given in Fig. 1a and 1b and outlined in Table I. Dilution factor ratios lower than one mean tighter binding of ^{3}H-labelled nucleotides. From the numbers of Table I as well as Fig. 1a and 1b it can be concluded that binding affinities decrease in the order deoxy-ATP > ATP > CTP > ITP > SO_4^{2-}. The experiments show the specificity of the binding of nucleotide triphosphate, but do not answer the question whether the compounds are bound to F_1-ATPases as triphosphates or after their hydrolysis as disphosphates.

Using the same technique the binding of [^{3}H]ADP to the high affinity sites was studied in the presence of Mg^{2+} and inorganic phosphate. Table I shows that no significant change in the dilution pattern is obtained, indicating that the ADP indeed binds to the high affinity site.

To decide whether adenosine triphosphate binds with high affinity, too, the analog AMP-PNP was used because it is not hydrolyzed by F_1-ATPase [8]. With this compound also tight binding was observed (Table I). This indicates strongly that also adenine nucleotide triphosphates bind to the high affinity site.

The influence of Mg^{2+} on the binding of nucleotides was studied by repeating some of the above-mentioned experiments in the absence of Mg^{2+} in the presence of EDTA. The second column in Table I shows that no significant change is observed as compared to the experiments done in the presence of Mg^{2+} (first column), thus indicating, that Mg^{2+} in the medium is not required for the observed high affinity binding of adenine nucleotides.

Identification of non-exchangeable ATP

Multiple nucleotide binding sites have been demonstrated for a variety of F_1-ATPases from different sources (for review see Ref. 2). It was reported that some of the enzyme-bound adenosine triphosphate is not available for hydrolytic sites and therefore can be liberated as triphosphate after cold-, heat- or acid-denaturation of the enzyme (Ref. 9, and own unpublished data). The

TABLE I

DILUTION FACTOR RATIOS (f) FOR GEL CENTRIFUGATION OF [^{3}H]NUCLEOTIDE TRIPHOSPHATES MEASURED AGAINST A [^{14}C]NUCLEOTIDE TRIPHOSPHATE CONTROL (5—20 μM NUCLEOTIDE TRIPHOSPHATE

^{3}H-labelled nucleotide	^{14}C-labelled nucleotide	Mg^{2+} (2 mM)	EDTA (2 mM)
ATP	vs. ITP	0.5	0.5
AMP-PNP	vs. ITP	0.5	0.6
CTP	vs. ITP	0.9	1.2
CTP	vs. ATP	—	1.6
deoxy-ATP	vs. ATP	0.9	0.9
ADP + P_i	vs. IDP	0.4	—

$$f = \frac{\Pi(^3H,i) \cdot \Pi(^{14}C,i+1)}{\Pi(^3H,i+1) \cdot \Pi(^{14}C,i)}$$

Π(isotope,i) = count rate for the isotope in the i-th centrifugate. An ammonium sulfate suspension of the F_1-ATPase (1 mg) was spun down at 100 000 × g for 90 min. The resulting pellet was dissolved in 50 mM potassium Hepps buffer (pH = 7.8) containing the appropriate additions (see above) and centrifuged through Sephadex G-25 fine gel equilibrated with buffer according to Ref. 7. To the centrifugate, ^{3}H- or ^{14}C-labelled nucleotides were added and the centrifugation through Sephadex was repeated several times. In some cases $^{35}SO_4^{2-}$ was used instead of the [^{14}C]nucleotide to determine the dilution factor for small molecules. In each centrifugate, the ATPase activity was determined and an aliquot (10 or 20 μl) was taken for scintillation counting. These samples were incubated for 30 min at 60°C with 200 μl of a 1:1 mixture (v:v) of the tissue solubilizer PROTOSOL (New England, Nucl.) and ethanol. Then, 7.0 ml of Quickszint scintillator (Koch and Light) were added. Under these conditions in preliminary experiments high rates of chemiluminescence were found which could be reduced to the background level (coincidence setting) by acidification of the mixture. Therefore 200 μl conc. HCl/ethanol (1:3. v/v) were added before counting. The samples were counted in a Searle Mark III scintillation counter on program 5. The ^{3}H counts were corrected for channel-overlap and background counts. For the study of ADP binding a mixture of [^{3}H]ADP and phosphate was prepared by adding a catalytic amount of F_1-ATPase to [^{3}H]-ATP in the presence of 2 mM Mg^{2+}.

question whether such a tight site is involved in the here observed nucleotide binding was decided by utilizing the high specificity of firefly luciferase emitting photons only with ATP but not with deoxy-ATP, ε-ATP and ITP (Ref. 10, and own unpublished data). Nanomolar quantities of F_1-ATPase were reacted with 1 mM ATP, deoxy-ATP, ε-ATP and ITP, respectively for 2 min. After removal of unbound nucleotide triphosphate by gel-centrifugation in all cases 1.8 ± 0.2 mol/mol ATP were found at the enzyme after denaturation. This result shows that the classical tightly bound ATP [11] is not exchangeable with any of the added nucleotides of the medium under the conditions employed here.

In addition, the fact that no increase of tightly bound ATP was found after reaction with ATP shows, that nucleotide triphosphates in the medium undergo hydrolysis and are finally bound as diphosphates at a high affinity site different from the classical tight site [11] under the conditions used in this experiment.

ATPase stability reflects tight nucleotide binding

It has been described previously that the stability of F_1-ATPase is influenced by tightly bound ligands [12]. To further substantiate the results obtained in the dilution experiments we examined the effect of nucleotide triphosphates

on the stability of F_1-ATPase more closely. The nucleotide specificity of the stabilization was determined by incubating the enzyme in the presence of different nucleotide triphosphates. Low protein concentrations were chosen to augment the experimental sensitivity, and Mg^{2+} was omitted from the buffers to avoid of hydrolysis of nucleotide triphosphate. Fig. 2 shows that there is indeed a marked stabilization of the F_1-ATPase activity by the presence of deoxy-ATP, ATP and ϵ-ATP. However, in the presence of ITP, GTP and CTP the enzyme activity declined as fast as in the absence of any added nucleotide triphosphate. These results demonstrate the same specificity as observed in the dilution studies given above. However, it should be noted that ϵ-ATP has not been included in the dilution experiments. The binding to high affinity sites can be titrated indirectly by measuring the concentration dependence of the rate of F_1-ATPase inactivation. Fig. 3 shows a Hill plot for the stabilization of F_1-ATPase activity by deoxy-ATP, ATP, and ϵ-ATP. Half-maximum stabilization of F_1-ATPase activity was obtained with 6 μM deoxy-ATP or 16 μM ATP. The values agree with the qualitative order of nucleotide triphosphate affinities

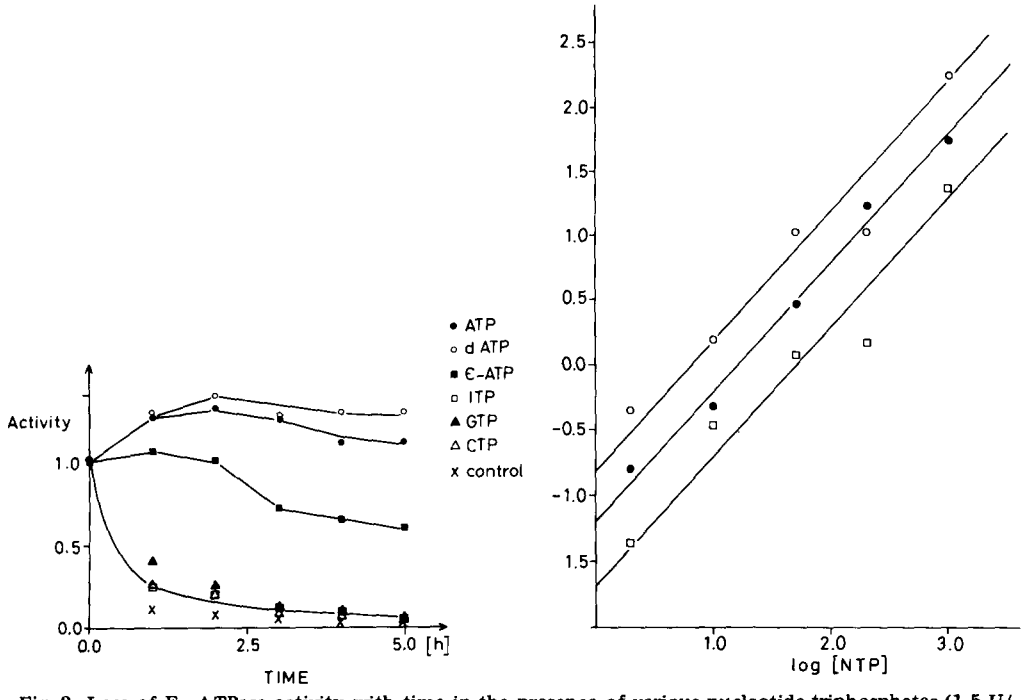


Fig. 2. Loss of F_1-ATPase activity with time in the presence of various nucleotide triphosphates (1.5 U/ml F_1-ATPase at $t = 0$ in 50 mM potassium Hepps, 2 mM EDTA (pH = 7.8). ○———○, deoxy-ATP; ●———●, ATP; ▪———▪, ϵ-ATP; ▫———▫, ITP; ▲———▲, GTP; △———△, CTP (all 2 mM); X———X, control. An ammoniumsulfate pellet of F_1-ATPase (see Table I) was dissolved in 20 mM potassium Hepps, 20 mM potassium Pipes, 2 mM EDTA (pH = 8.0) and centrifuged twice through Sephadex G-25 gel equilibrated with the same buffer. The protein was diluted to 0.5—2.0 U/ml F_1-ATPase with the above buffer containing the appropriate nucleotide concentrations. The solutions were incubated at 25°C. After given time intervals 10 to 20 μl samples were taken for F_1-ATPase assay.

Fig. 3. Hill plot for the protection of F_1-ATPase by deoxy-ATP (○———○), ATP (●———●) and ϵ-ATP (▫———▫). The ordinate values were obtained from $y = \log(v_{6hc} - v_{6ho})/(v_{6hoo} - v_{6hc})$, $v_{6hc} = F_1$-ATPase activity after 6 h incubation at nucleotide triphosphate (NTP) concentration c (in μM).

TABLE II

K_m FOR THE HYDROLYSIS OF NUCLEOTIDE TRIPHOSPHATES BY F_1-ATPase DETERMINED AT ATP CONCENTRATIONS BELOW 0.5 mM.

Triphosphate	K_m (mM)	Reference
ATP	0.08	
	0.045	3
	0.04	6
Deoxy-ATP	0.02	
	0.033	
ϵ-ATP	0.10	
GTP	0.14	
	0.15	3
ITP	0.46	
	0.45	3

as estimated from the direct binding studies decribed above. For ϵ-ATP the half saturating concentration for the stabilization was found to be 50 μM. The above classification of high affinity sites for adenine nucleotides and low affinity sites for other hydrolyzable nucleotide triphosphates corroborates with the results obtained in an analysis of the steady state kinetics. Lineweaver-Burk plots of the steady state rate of F_1-ATPase are biphasic with adenine nucleotides, whereas linear plots are obtained with other nucleotides. For comparison, Table II shows the Michaelis constants for different nucleotide triphosphates, calculated from the low concentration parts ($C_{NTP} < 0.5$ mM) of the Lineweaver-Burk plots as well as older data [3,6]. The values reflect the same order of affinity as found by direct binding assays and stabilization studies as described above, thus suggesting a participation of the high and low affinity sites in soluble F_1-ATPase activity.

Discussion

In F_1-ATPase, derived from several sources, high- and low-affinity nucleotide binding sites have been described and nucleotides have been classified as to their ability to bind to those sites (for review see Ref. 2).

In this paper, we investigated the specificity of F_1-ATPase towards nucleotides by three different methods: by direct binding experiments, by determination of the protection by nucleotides against slow inactivation, and by the kinetics of steady-state nucleotide triphosphate hydrolysis. We have found that the three methods yielded comparable results in terms of nucleotide specificity. Two categories of nucleotides could be distinguished: Nucleotides of one category are bound to F_1-ATPase with high affinity as judged by the slope of the dilution curves (Fig. 1); they also stabilized the enzyme, and showed a high-affinity domain in the kinetics of hydrolysis. Effectively, the nucleotides in this class could be arranged in the order deoxy-ATP, ATP, ϵ-ATP. The other category of nucleotides was easily removed from the enzyme in the dilution experiments and did not stabilize F_1-ATPase at all at concentrations up to 2 mM. Within this class, the affinity for hydrolysis was relatively low ($\approx$1.0 mM).

Nucleotides in this category included CTP, ITP and GTP. Presumably those nucleotides bind to low affinity binding sites.

The stabilization of F_1-ATPase activity by some nucleotide triphosphates can be explained under the assumption that only the enzyme conformation with empty tight sites will loose its activity by dissociation, and thus contribute to the observed rate of inactivation, being a measure for the fraction of empty high-affinity sites. Thus, a measurement of the inactivation in the presence of nucleotides at different concentrations can be used to estimate dissociation constants. For ATP, deoxy-ATP and ϵ-ATP values between 6 and 50 μM were found. ITP, CTP and GTP did not show any influence on the F_1-ATPase stability.

The F_1-ATPase activity in the presence of deoxy-ATP, and, to a smaller extent, ATP and ϵ-ATP, significantly increases in the first few hours. This increase in specific activity can be explained by a very slow exchange [13] of bound nucleotide-diphosphate (acting as a negative ATPase effector [14]) against nucleotide triphosphate (a positive effector [14]). It might be mentioned that for chloroplast F_1-ATPase a nucleotide- and cation-dependent protection of the ATPase against cold dissociation in buffers of high ionic strength has been described [15].

High-affinity sites or -states can also be detected on the basis of the steady-state kinetics of nucleotide-triphosphate hydrolysis in the absence of certain anions such as HSO_3^-, HPO_4^{2-} and SO_4^{2-} [3,6]. A biphasic Lineweaver-Burk plot is obtained for ATP hydrolysis [3,6,16] as compared to a linear one for ITP hydrolysis [3,16].

This biphasic behaviour is indicative of multiple interacting sites, compatible with regulatory sites of high specificity and affinity [6]. The experiments on the release of nucleotides bound with high affinity are consistent with a dual-site scheme [17] also requiring interacting sites. Namely, it was found, that after the first dilution steps, almost no additional nucleotides were released, which may indicate that medium nucleotides are required for exchange. In contrast, it may be infered that the hydrolysis of ITP does not involve multiple interacting sites.

Many properties of F_1-ATPase from various sources apart from those mentioned above show differences between ATP and ITP in the mode of reaction. Yeast F_1-ATPase is inhibited by free Mg^{2+} [12], ITPase is not (unpublished data). ADP inhibits ATP and ITP hydrolysis, IDP inhibits only the ITP hydrolysis of beef heart F_1-ATPase [18]. Methanol stimulates ATP hydrolysis but it inhibits the ITP hydrolysis by beef heart F_1-ATPase [19].

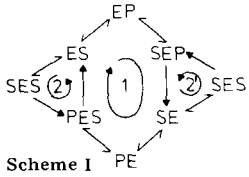
These and our observations together indicate that there are at least two classes of nucleotide binding sites and two different pathways or enzyme states for nucleotide-triphosphate hydolysis, associated with these: one with a high and another with a low specificity for nucleotide triphosphates.

On the other hand the nucleotide specificity for the high-affinity sites described here for soluble F_1-ATPase corresponds well to that of energy-dependent reactions of beef heart submitochondrial particles [20], such as ATP—P_i exchange and reversed transhydrogenase. The phosphorylation of nucleotide diphosphates, however, shows a less pronounced nucleotide specificity.

Most of the data discussed here can be explained by the reaction scheme for

nucleotide-triphosphate hydrolysis by mitochondrial F_1-ATPase shown below (cf. also Ref. 17), where E is enzyme, P, product (NDP + P_i), and S, substrate (NTP). Cycle 1 shows high affinity and high specificity for nucleotide triphosphates (NTP). For symmetry reasons two cycles (2 and 2') are shown representing low specificity predominant at high nucleotide concentrations. The scheme also illustrates that the enzyme always has bound nucleotides. Exchange of tightly bound nucleotides (cycle 1) needs exogeneous nucleotides, as given in the sequence EP → SEP → SE.

It is tempting to discuss the mechanism shown in Scheme I with the results



Scheme I

of a recently published experiment [21] which shows that more than three oxygen atoms from water can be incorporated into inorganic phosphate after ATP hydrolysis, thus demonstrating that ATP formation and hydrolysis can occur on isolated F_1-ATPase. In Scheme I cycle 1 would explain excess oxygen exchange by reversal of the step ES ⇌ EP because at low substrate concentrations EP → SEP is slow. At high substrate concentrations the latter reaction will become fast thus reducing oxygen exchange. The relationship of this scheme with the mechanism of ATP synthesis has to be further established by additional experimentation.

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References

1 Kozlov, I.A. and Skulachev, V.P. (1977) Biochim. Biophys. Acta 463, 29—89
2 Harris, D.A. (1978) Biochim. Biophys. Acta 463, 245—273
3 Takeshige, K., Hess, B., Böhm, M. and Zimmermann-Telschow, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1605—1622
4 Pullmann, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322—3329
5 Lundin, A., Rickardsson, A. and Thore, A. (1976) Anal. Biochem. 75, 611—620
6 Recktenwald, D. and Hess, B. (1977) FEBS Lett. 76, 25—28
7 Neal, M.W. and Florini, J.R. (1973) Anal. Biochem. 55, 328—330
8 Adolfsen, R., McClung, J.A. and Moudrianakis, E.N. (1975) Biochem. 14, 1727—1735
9 Rosing, J., Harris, D.A., Kemp, A. and Slater, E.C. (1975) Biochim. Biophys. Acta 376, 13—26
10 Lee, R.T., J.L. Denburg, and W.D. McElroy (1970) Arch. Biochem. Biophys. 141, 38—52
11 Harris, D.A., Radda, G.K. and Slater, E.C. (1977) Biochim. Biophys. Acta 459, 560—572
12 Recktenwald, D. (1978) Ph.D. Thesis, University Bochum, F.R.G.
13 Adolfsen, R. and Moudrianakis, E.N. (1976) Arch. Biochem. Biophys. 172, 425—433
14 Slater, E.C., Kemp, A., van der Kraan, J., Muller, J.L.M., Roveri, O.A., Verschoor, G.J., Waagevoord, R.J. and Wielders, J.P.M. (1979) FEBS Lett. 103, 7—11
15 Posorske, J. and Jagendorf, A.T. (1976) Arch. Biochem. Biophys. 177, 276—283
16 Schuster, S.M., Eberl, R.E. and Lardy, H.A. (1975) J. Biol. Chem. 250, 7848—7853
17 Kayalar, C., Rosing, J. and Boyer, P.D. (1977) J. Biol. Chem. 252, 2486—2491
18 Schuster, S.M., Gertschen, R.J. and Lardy, H.A. (1976) J. Biol. Chem. 251, 6705—6710
19 Schuster, S.M. (1979) Biochemistry 18, 1162—1167
20 Harris, D.A., Gomez-Fernandez, J.C., Klungdyr, L. and Radda, G.K. (1978) Biochim. Biophys. Acta 504, 364—383
21 Choate, G.L., Hutton, R.L. and Boyer, P.D. (1979) J. Biol. Chem. 254, 286—290