TIME-DELAYED ONSET OF OXIDATIVE PHOSPHORYLATION INDUCED BY NUCLEOTIDE ANALOGS

Günter SCHÄFER*
Institut für Biochemie, Medizinische Hochschule Lübeck, FRG

Received 25 June 1981

1. Introduction

Hitherto it was unknown how the multiple nucleotide binding sites on coupling factors F1 are related to each other in the process of oxidative phosphorylation [1–5]. Especially the problem of mutual interaction of nucleotide binding sites during subsequent cycles of ADP-phosphorylation, as proposed in the 'alternating site' model [6,7], is unsolved. According to this model the catalytic center comprises two sites which are basically identical, but are functionally interdependent. Although this view would allow a cooperative mechanism, observed kinetics of oxidative phosphorylation normally are of the simple Michaelis-Menten type (ADP = variable substrate), except for one case: this is in presence of certain ADP-analogs, which have been reported from our laboratory to induce sigmoidal v versus [S] plots with n_H 1.5–1.7 [8].

This study shows that oxidative phosphorylation in submitochondrial particles (SMP) reveals a significant lag-phase in presence of the inhibitory analog 3′-*O*-(naphthoyl,1)-ADP. Moreover, the response of the system depends on the sequence of addition of the analog and of ADP, suggesting an equilibrium between two functional states of the enzyme which may be differently stabilized, depending on the ligand present.

2. Methods

Methods for preparation of submitochondrial particles (SMPs), protein determination, synthesis of 3′-analogs of AD(T)P and measurement of oxidative phosphorylation have been detailed [9,10]. Here, ^{32}P incorporation into ATP was used only in a few cases as a parallel control of the luciferase method, which was routinely used to monitor ATP-synthesis directly [11,12]. Submitochondrial particles (10–27 μg/ml) were immersed in a buffer containing 220 mM sucrose, 10 mM Tris, 2.5 mM $MgCl_2$, 4.0 mM P_i, 1.0 mM EDTA (pH 7.4); all measurements were performed at 22°C; 40 mM ethanol, 133 μM NADH and 120 units/ml alcoholdebydrogenase (Boehringer 411523) were present as NADH-regenerating system. The mixture was supplemented with 0.005 μg luciferase (Boehringer 102217, 10^7 units/mg) and 410 μM (−)D-luciferin; total volume was 860 μl. Light output was determined in a LKB-luminometer equipped with a rapid injection device, a Hewlett-Packard digital Voltmeter (3480/82A) and fast analog recorder. In direct recordings light output is given as instrumentation response on a mV scale. Phosphorylation was initiated by injection of small ADP-aliquots to submitochondrial particles respiring with NADH, added 1 min ahead of ADP; 12 μM diadenosinepentaphosphate (AP_5A) were present as inhibitor of myokinase. For quantitative determination of rates calibration by internal standard was used; under the applied conditions light output is proportional to ATP concentration only during the initial phase of the recording. Exact analytical data were obtained from batch incubations (10-times the volume); 500 μl samples were taken at the indicated times; the samples were quenched with 50 μl 3 M trichloroacetic acid, neutralized with KOH–Tris, and after high-speed centrifugation 30 μl aliquots were used for ATP analysis with luciferase under conditions where light output is strictly proportional to ATP [13]; internal standard

* Postal address: Zentrum Biochemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, 3000 Hannover 61, FRG

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

was applied with each individual measurement; the test buffer contained 55 mM Tris·acetate (pH 7.75); EDTA 1.5 mM; 10 mM Mg-acetate; 35 μM D-luciferin; 0.075% BSA; 0.01–0.05 μM luciferase.

3. Results

As shown in former studies, 3′-naphthoyl-ADP is not a substrate of phosphorylation but inhibits with $K_i \leqslant 10^{-7}$ M; this reflects firm binding to a specific site, and stimulated us to investigate its influence on initial phosphorylation kinetics. To resolve initial events during oxidative phosphorylation on a narrow time scale with high sensitivity, ATP synthesis by SMPs was directly monitored by luciferin/luciferase light emission. The respiratory substrate was NADH. Evaluation of initial slopes of ATP-formation *vs* initial ADP concentration gave linear Lineweaver-Burk plots and an app. K_m [ADP] of 15–15 μM in good agreement with other determinations [8,9]. However, added ADP was never completely phosphorylated. Independent of the initial ADP concentration the system reached a steady state of [ATP] at an average phosphorylation potential of $\Delta G_P \sim 10.7$ kcal. This is in contrast to experiments applying an ADP-regenerating hexokinase-trap where ΔG_P is maintained near zero.

Whereas in absence of the inhibitory analog ATP-formation starts immediately (time resolution 1 s) after ADP addition, 1 min preincubation of SMPs with extremely low concentrations of 3′-naphthoyl-ADP produces a pronounced delay before a steady rate of ATP-synthesis is established (fig.1). Extrapolation of the linear part of the trace to the abscissa yields an intercept, defined as lag-time τ. Duration of the lag-phase depends on the concentration of the inhibitor (table 1, top).

To exclude the possibility of artifacts which may result from effects of the inhibitor on luciferase, several efforts have been taken to verify this surprising result. Fig.2 shows a trace of ATP-synthesis obtained by direct monitoring, overlayed with the results of ATP-analysis from two independent batch-incubations run in parallel. Samples have been taken at the indicated times and were processed as in section 2. This result confirms the occurance of a lag-phase which could be further confirmed by ^{32}P incorporation from $^{32}P_i$ into ATP. In addition, ATP synthesis from phosphoenol–pyruvate and ADP in

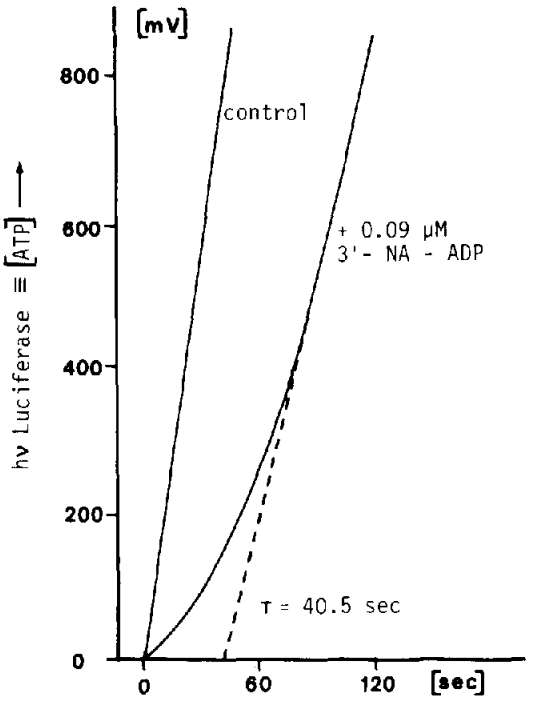


Fig.1. Direct recording of light output as a measure of ATP-synthesis in submitochondrial particles. Left: control; phosphorylation initiated by injection of 14 μM ADP; conditions as in section 2. Right: 0.09 μM 3′-naphthoyl-ADP were added 1 min prior to ADP.

Table 1

Rel. v [ox. phos.]	τ [s]	Conditions
100	0	Control ≙ 79 nmol/mg . min 14.6 μM ADP
45	16–21	+ NA-ADP 0.07 μM (a)
30	40–49	+ NA-ADP 0.15 μM (a)
16–21	53–66	+ NA-ADP 0.2 μM (a)
100	0	+ NA-ATP 0.2 μM (a)
98–102	0	+ NA-ATP 0.45 μM (a)
45	0	+ NA-ATP 22.0 μM (a)
42	0	+ NA-ADP 0.06 μM (b)
35	0	+ NA-ADP 0.13 μM (b)
17	0	+ NA-ADP 0.22 μM (b)

Effect of 3′-naphthoyl-ADP (NA-ADP) and 3′-naphthoyl-ATP (NA-ATP) on lag time of oxidative phosphorylation: (a) inhibitors were added 1 min before addition of ADP to respiring SMPs; (b) the inhibitor was added 20–30 s after ADP. All data were obtained with the same preparation of SMPs, giving a rate of 79 nmol ATP/min . mg at 14.6 μM ADP in the non-inhibited state; relative rates of the inhibited system refer to the the linear phase following the lag time (cf. fig.1)

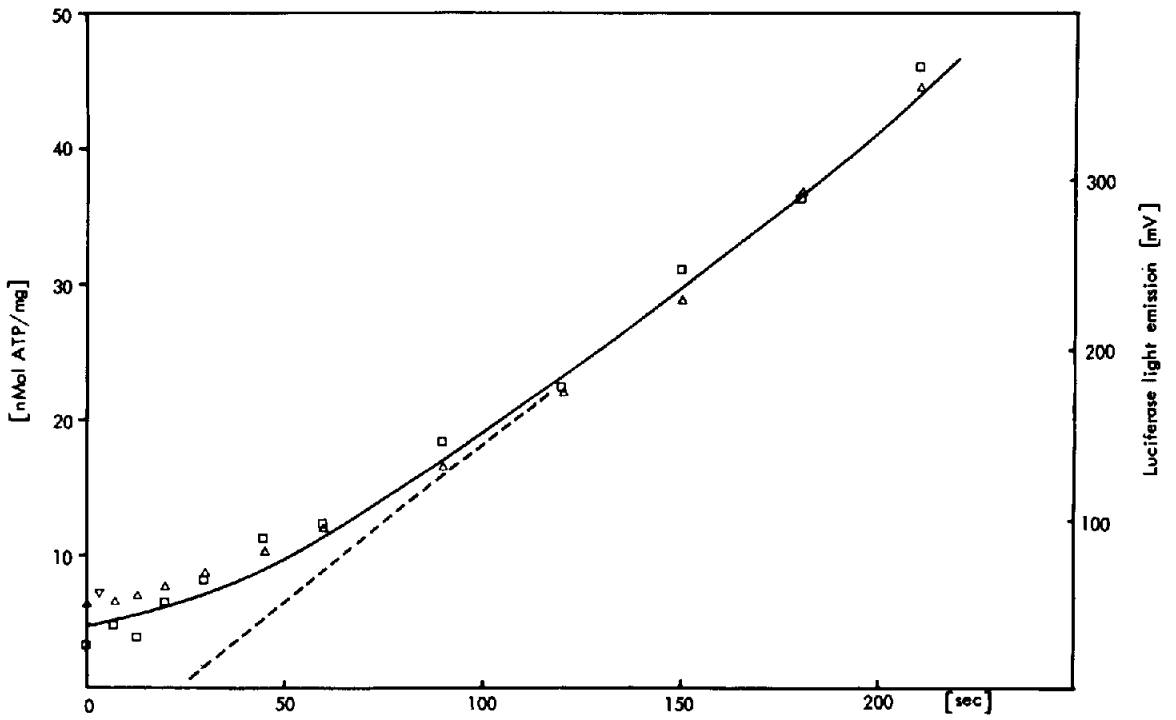
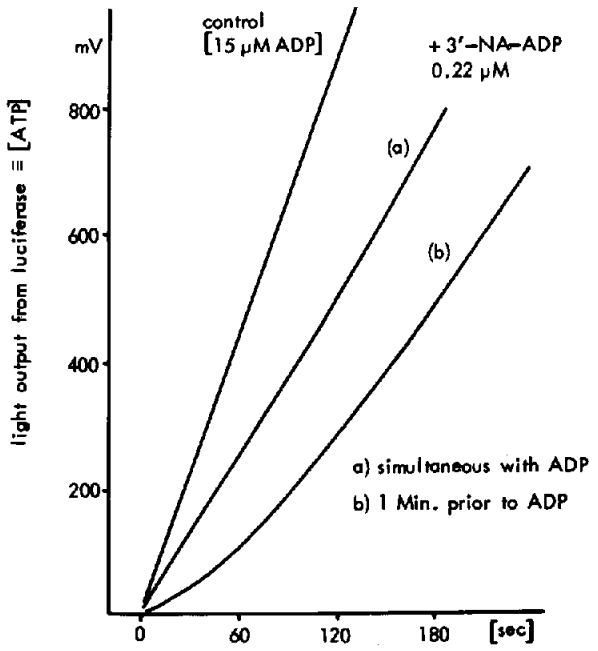


Fig.2. Overlay of direct monitoring of ATP synthesis by luciferase with results from individual sampling of 2 independent batch-incubations as in section 2. Preincubation for 1 min with 0.2 μM 3′-naphthoyl-ADP; start of phosphorylation by 14.5 μM ADP.



presence of pyruvate kinase has been used as a model system, monitoring [ATP] by luciferase. When 3′-naphthoyl-ADP was applied to the latter system under corresponding conditions, no lag-phase could be detected, also indicating that the lag is a specific effect of the inhibitor on the dynamics of F_1-ATPase, but not an effect on the coupled assay system.

Regarding properties and possible origin of the observed lag-phase of phosphorylation the following results become important: As shown in fig.3, the lag-phase τ was significantly shorter or almost absent when ADP and the inhibitory analog were added simultaneously to SMPs instead of preincubation with the inhibitor. The lag-phase was also absent when 3′-naphthoyl-ADP was added to the running system, i.e., after ADP, when some ATP had accumulated already (table 1, bottom).

Fig.3. Influence of the sequence of addition of ADP and the inhibiting analog on occurance of the lag-phase. Conditions as in fig.1, except that the inhibitor was added simultaneously with (a) or 1 min after ADP (b).

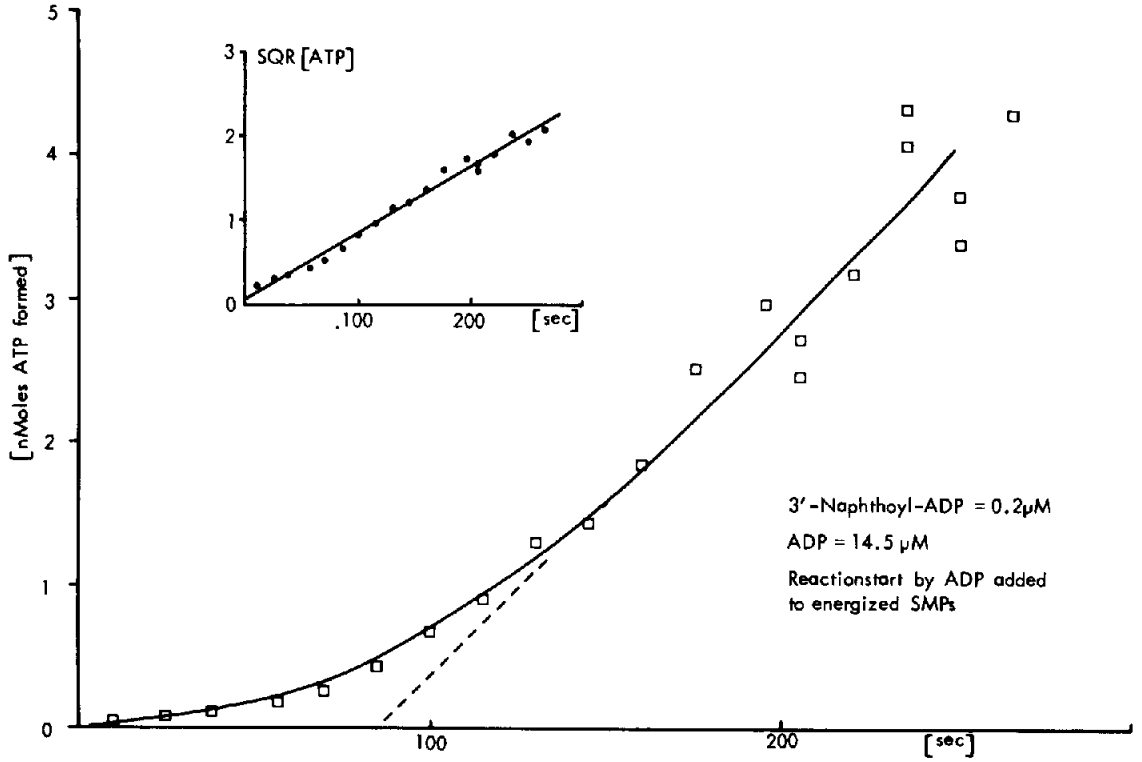


Fig.4. Time course of ADP phosphorylation after 1 min preincubation with 0.2 μM 3'-naphthoyl-ADP; data from batch-incubation (cf. section 2); total protein was 30 μg. The insert gives a plot of [ATP] *vs t*.

Attempts for kinetic analysis of the lag-phase have shown that it does not reflect a simple exponential relaxation phenomenon; it rather follows a power function as depicted in the insert of fig.4. The data give the results of a batch-experiment and were linearized by plotting ATP-formation over the square of time. This behaviour exhibits strong similarities to an autocatalytic process, suggesting that the enzyme is successively activated with progress of time; that means, however: with increasing accumulation of ATP as the product of the reaction. Therefore the influence of ATP on the duration of the lag-phase has been directly tested. Results from the experiment of fig.5 illustrate that sub-μM levels of ATP added together with ADP drastically shorten the apparent lag phase of SMPs which beforehand have been preincubated with 3'-naphthoyl-ADP.

With respect to specificity of the effect previous findings were confirmed that the corresponding ATP-analog is a much weaker inhibitor of phosphorylation (table 1) than the ADP-analog [9,10]. Moreover, even after prolonged preincubation with inhibiting concen-

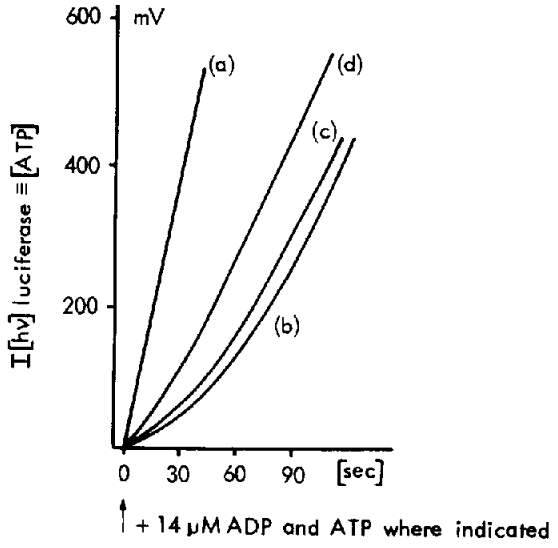


Fig.5. Effect of ATP on lagtime of inhibited oxidative phosphorylation: (a) control, no inhibitor; τ = 0 s; (b) + inhibitor: 3'-NA-ADP 0.22 μM; τ = 52 s; 2 min preincubation; (c) (b) + 0.35 μM ATP together with ADP; τ = 36 s; (d) (b) + 0.76 μM ATP together with ADP; τ = 16.5 s; in section 2.

trations of 3′-naphthoyl-ATP a lag-phase could never be observed; the resulting inhibition occurred immediately and was of a simple competitive type. Thus, the lag is specifically induced by the ADP-analog.

4. Discussion

Several studies made it obvious that solubilized F_1-ATPase from various organisms exhibits time-delayed onset of activity depending on the pattern of nucleotides present [14–16]. This was interpreted by a transition between different forms of the enzyme preceding catalytic activity. Non-linear kinetics in the direction of ATP-synthesis by membrane bound F_1-ATPase reported first by our studies, demonstrating that 3′-naphthoyl-ADP and a similar α-P-acylated analog induce cooperativity and suggest the participation of more than one nucleotide binding site in catalysis of oxidative phosphorylation. This is strongly supported by the present experiments, revealing a lag-time of oxidative phosphorylation which occurs when membrane-bound ATP-synthetase (F_1) has been exposed to the inhibitory analog prior to addition of ADP. The observed lag periods (12–50 s) are orders of magnitude above relaxation times expected for regular conformational changes, and are incompatible also with relaxation times discussed for the dissociation of the ATPase inhibitor protein from membrane bound F_1 [17].

It has also been excluded in independent experiments that the inhibiting analog is slowly removed by phosphorylation or metabolic conversion. Therefore, alternate explanations for the lag-time have to be sought.

Taking into account, that preceding or simultaneous addition of ADP largely prevents occurance of the lag phase, one has to assume that the inhibitory analog binds very tightly to a form of the enzyme accessible only before ADP or ATP were available as alternate ligands.

This interpretation is tentatively summarized in fig.6. The upper line represents the normal competitive case, corresponding to the situation when the inhibitor is added to the running system or together with ADP. k_2 signifys the quasi-irreversible step of oxidative phosphorylation; during continuous input of energy into the open system it is assumed to be $>>k_{-2}$.

In the lower line another form of the enzyme ϵ is

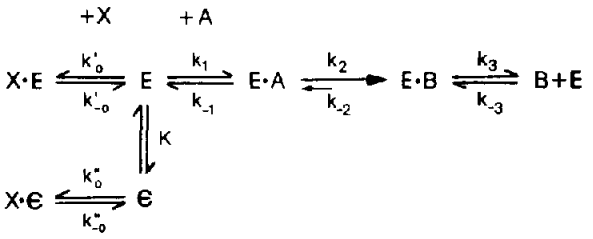


Fig.6. Tentative scheme of a mechanism describing the participation of two forms E and ϵ of the enzyme (F_1) in ligand interactions. Detailed description in the text.

introduced, not capable of catalyzing oxidative phosphorylation, but capable of nucleotide binding. This form is in equilibrium with the active form E as described by the equilibrium constant K. Interaction of both forms with the inhibitory ligand X is expressed by the rate constants k'_o and k''_o, respectively.

According to the above experiments one may assume that the inhibitory analog (when added first) traps the enzyme in state ϵ which would induce a lag-phase during very slow dissociation of the complex $[X \cdot \epsilon]$. From the diminishing effect of ATP on the lag time one may further conclude that the inhibiting ligand is displaced from ϵ by ATP, rather than the ADP. That would be in line also with the quasi-autocatalytic behaviour of the system under the described conditions.

Besides competition for the catalytic center on the activated enzyme (E) it may be that a high affinity site for the analog can be assigned to the activation step ($\epsilon \rightarrow E$) which manifests itself by the observed lag time. The significant characteristic of the above view, a ligand triggered dynamic equilibrium between two forms of the enzyme, may be specific for F_1 residing on the 'intact' membrane. Whether there is a possible relation to functional or conformational states of the enzyme as proposed in the 'alternating site' model [6,7] has to be elucidated by further investigation.

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