Inactivation of beef heart mitochondrial F_1-ATPase by the 2′,3′-dialdehyde derivatives of adenine nucleotides

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Beef heart mitochondrial F_1-ATPase was inactivated by the 2′,3′-dialdehyde derivatives of ATP, ADP and AMP (oATP, oADP, oAMP). In the absence of Mg^{2+}, inactivation resulted from the binding of 1 mol nucleotide analog per active unit of F_1. The most efficient analog was oADP, followed by oAMP and oATP. Complete inactivation was correlated with the binding of about 11 mol [^{14}C]oADP/mol F_1. After correction for non-specific labeling, the number of specifically bound [^{14}C]oADP was 2–3 mol per mol F_1. By SDS-polyacrylamide gel electrophoresis, [^{14}C]oADP was found to bind covalently mainly to the α and β subunits. In the presence of Mg^{2+}, oATP behaved as a substrate and was slowly hydrolyzed.

F_1-ATPase *Dialnucleotide* *Affinity labeling* *Enzyme inactivation*

1. INTRODUCTION

Nucleotide sites in the F_1 sector of mitochondrial, chloroplastic and bacterial ATPases are currently studied by means of affinity or photoaffinity labeling with nucleotide analogs. These derivatives include the photoactivatable azidonucleotides in which the azido moiety is attached either to the purine ring or to the ribose moiety [1–4], *p*-fluorosulfonyl benzoyl-5′-adenosine [5,6] and the 2′,3′-dialdehyde nucleotides (oAdN). The chemical properties of oAdN have been described [7,8]. The inactivating effect of oATP or oADP was reported in the case of beef heart F_1 [9–11], *E. coli* F_1 [12,13] and *M. phlei* F_1 [14]; however, correlation between binding of oAdN and inhibition was controversial. A typical case is that of beef heart F_1; whereas oADP was reported to bind to the α subunit without inactivation of F_1 [11], oATP in the presence of $MgSO_4$ was found to bind irreversibly to both the α and β subunits and to inactivate F_1 [10]. Because of the potential use of oAdNs as affinity labels, it was decided to study in more detail the kinetics of inactivation of beef heart F_1 by oAdNs and to compare inactivation and binding data.

2. MATERIALS AND METHODS

2.1. *Materials*

Nucleotides, phospho*enol*pyruvate (cyclohexylammonium salt) and pyruvate kinase were purchased from Boehringer Mannheim. [^{14}C]ADP (212 Ci/mol) was obtained from the Service des Molécules Marquées, Commissariat à l'Energie Atomique, Saclay. oAMP, oADP and oATP were prepared by treatment of the corresponding nucleotides with sodium periodate and purified as in [15]; they were stored at −70°C. Their concentrations were determined by measuring the absor-

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Abbreviations: F_1, beef heart soluble mitochondrial ATPase; Tra, triethanolamine; oAdN, 2′,3′-dialdehyde derivatives of ATP, ADP and AMP (oATP, oADP, oAMP)

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bance at 258 nm, using 14900 $cm^{-1} \cdot M^{-1}$ for the absorption coefficient. As these derivatives are not very stable, their purity was routinely checked by thin-layer chromatography on PEI–cellulose, using as a solvent 0.75 M potassium phosphate adjusted to pH 3.5 with concentrated HCl. Since oAdN reacts with PEI–cellulose, 5–50 nmol oAdN were reduced prior to the chromatography with a 10-fold molar excess of $NaBH_4$. Under these conditions, they gave single spots with the following R_F values: reduced oAMP, 0.49 (AMP, 0.44); reduced oADP, 0.43 (ADP, 0.28); reduced oATP, 0.20 (ATP, 0.10). They also migrated as single spots with the following system: 0.1 M LiCl, 1.9 M HCOOH. Soluble coupling factor F_1 was stored at 0–4°C as an ammonium sulfate suspension [16].

2.2. *Modification of F_1 by oAdN*

The required amount of F_1 was centrifuged for 2 min in an Eppendorf 5412 centrifuge, and the pellet was solubilized in 0.1 M Tra buffer (pH 8.0). Residual ammonium sulfate was eliminated by centrifugation-filtration [17] through a Sephadex G-50 (fine) column equilibrated in 0.1 M Tra (pH 8.0). The recovered F_1 in 0.1 M Tra (pH 8.0) was incubated at 30°C with oAdN. At given intervals, aliquots were removed and assayed for ATPase activity.

2.3. *Assay of ATPase activity*

ATPase activity was measured at 30°C in a reaction medium containing 50 mM Tris–SO_4, 5 mM ATP, 2.5 mM $MgCl_2$, 30 μg/ml pyruvate kinase, 10 mM KCl, 5 mM phospho*enol*pyruvate. The total volume was 0.5 ml and the final pH 8.0. The reaction was started by the addition of an aliquot fraction of the enzyme and stopped after 5 min incubation by addition of 0.1 ml of 2.5 N perchloric acid. Phosphate released was determined colorimetrically [18]. Under these conditions, the specific activity of F_1 was about 80 μmol ATP hydrolysed/min per mg protein. Protein concentration of F_1 solutions was determined by a Coomassie blue G-250 assay [19].

2.4. *SDS-polyacrylamide gel electrophoresis*

Acrylamide gels (7.5%) were used to separate the [^{14}C]oADP-labeled subunits of F_1 [20]. After migration, the gels were stained with Coomassie blue R250. They were frozen in solid CO_2 and sliced into 1-mm slices with a Joyce-Loebl gel slicer. Slices were digested by overnight incubation in 1 ml of 15% H_2O_2 at 60°C and radioactivity was measured by scintillation counting.

3. RESULTS

3.1. *Kinetics of inactivation of F_1 by oAdN*

As shown in fig.1 incubation of F_1 with oATP resulted in a time- and concentration-dependent decrease in ATPase activity; the time course of inactivation followed pseudo-first-order kinetics. Similar kinetics of inactivation was observed for oADP. Unexpectedly, oAMP was also found to inactivate F_1. The enzyme activity could not be

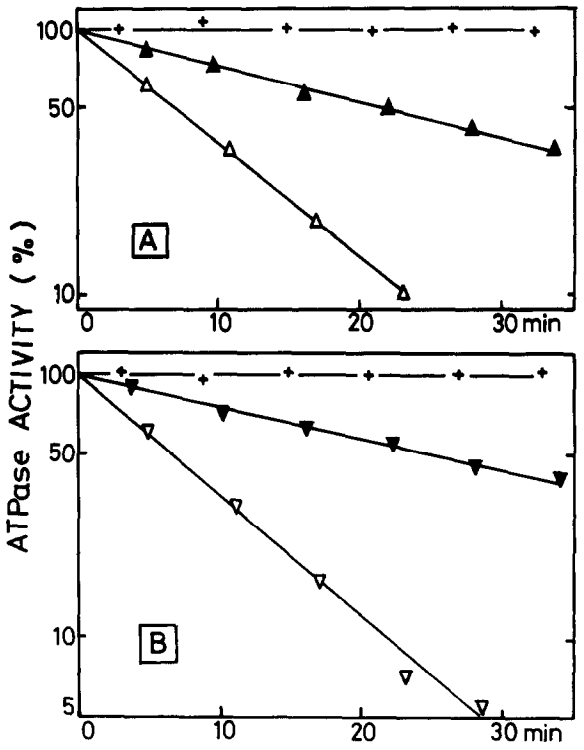

Fig.1. Semi-logarithmic plots of the time course of inactivation of F_1-ATPase by oAMP and oATP. F_1 (0.1 mg/ml) was incubated at 30°C in 0.1 M Tra (pH 8.0), (A) with 0.5 mM (▲) or 3 mM (△) oAMP or (B) with 1 mM (▼) or 5 mM (▽) oATP. Controls without reagents (+) are included. At the indicated times, aliquot samples were immediately diluted 25-fold in an ATPase assay medium for determination of the remaining ATPase activity.

restored by gel filtration on Sephadex G-50 or dilution of the enzyme in a medium free of oAdN, indicating an irreversible inactivation process. Neither the presence of $NaBH_3CN$ (5 mM), a selective reducing agent of Schiff bases [21], during incubation with the oAdN, nor the addition of $NaBH_4$ (10 mM) after incubation, was necessary for inactivation of ATPase. This was also shown for the inactivation of pyruvate kinase and NAD-isocitrate dehydrogenase by oAdN [22,23], and beef heart ATPase [10]. All our experiments were carried out at pH 8.0 since inactivation was about 3-times faster at this pH than at neutral pH.

The behavior of oAdN can be explained either by the direct formation of a covalent bond between oAdN and F_1:

$$F_1 + \text{oAdN} \xrightarrow{k} F_1\text{-oAdN} \quad (1)$$

where F_1 represents the free enzyme and F_1-oAdN the inactivated enzyme, or by the transient formation of a reversible complex with oAdN prior to the establishment of a covalent linkage according to the following scheme:

$$F_1 + \text{oAdN} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} F_1\cdot\text{oAdN} \xrightarrow{k_2} F_1\text{-oAdN} \quad (2)$$

where $F_1\cdot$oAdN is the reversible complex and F_1-oAdN the covalent complex.

To explore each alternative, the $T_{1/2}$ values were plotted *vs* the reciprocal of oAdN concentration (fig.2). The plots gave straight lines passing through the origin for oAMP, oADP and oATP. The calculated inactivation rate constants were as follows (in $mM^{-1}\cdot min^{-1}$): oAMP, 0.05; oADP, 0.60; oATP, 0.031. The fact that the plots pass through the origin is apparently consistent with the direct formation of a covalent bond between oAdN and F_1 (eq.1). However, based on the available evidence in the literature (cf. [24]), formation of a reversible complex before covalency change, as illustrated in eq.2, appears highly probable in irreversible inhibition and modification reactions; for this to occur, it is required that $k_2 << k_{-1}$.

The above experiments were all carried out in the absence of added Mg^{2+}. With oATP in the presence of Mg^{2+} (fig.2D), a straight line was obtained whose intercept with the ordinate, i.e., at infinite concentration of ATP + Mg^{2+}, corresponded to a $T_{1/2}$ of 5 min. It is difficult to interpret these results, since in the presence of Mg^{2+} oATP was hydrolyzed by F_1 to yield oADP (see below, fig.4). On the other hand, the kinetics of inactivation by oADP were the same in the absence or presence of Mg^{2+} (not shown).

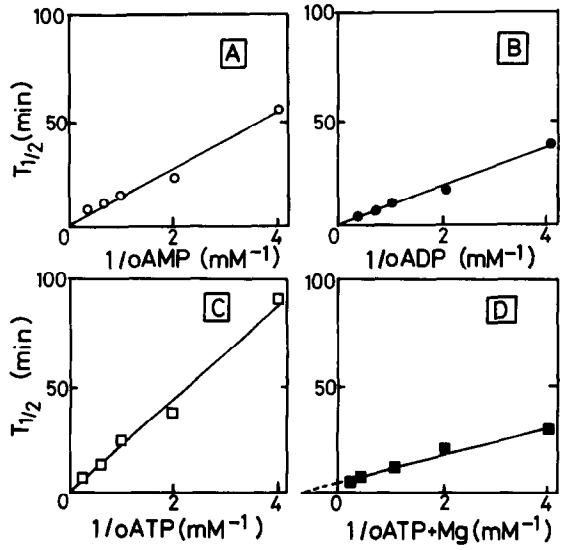

Fig.2. Kinetics of inactivation of F_1-ATPase by oAdN. Data from inactivation experiments similar to those presented in fig.1 were plotted as half-times of inactivation ($T_{1/2}$) *vs* the reciprocal of (A) oAMP (○), (B) oADP (●), (C) oATP (□), and (D) oATP + $MgCl_2$ (■) concentrations.

The plots of $\log(T_{1/2})$ *vs* log(oAdN) as in [25] (experiments carried out in the absence of Mg^{2+}) gave straight lines with slopes close to 1 for oAMP, oADP or oATP suggesting that inactivation is achieved after the binding of only 1 mol oAdN per active unit of F_1 (fig.3). A slope of 0.6 was obtained for oATP in the presence of Mg^{2+}, pointing again to an unusual behavior which may be due to the hydrolysis of oATP.

oADP, the derivative with the highest inactivation potency, was selected for a specificity study. This was based on the protective effect of the F_1 substrates, ATP and ADP during oADP inactivation. ATP and ADP afforded similar protection: $T_{1/2}$ was increased by a factor of 2 in the presence of 3 mM ATP or ADP.

3.2. *oATP as a substrate of F_1*

In the presence of 2.5 mM Mg^{2+} and 5 mM

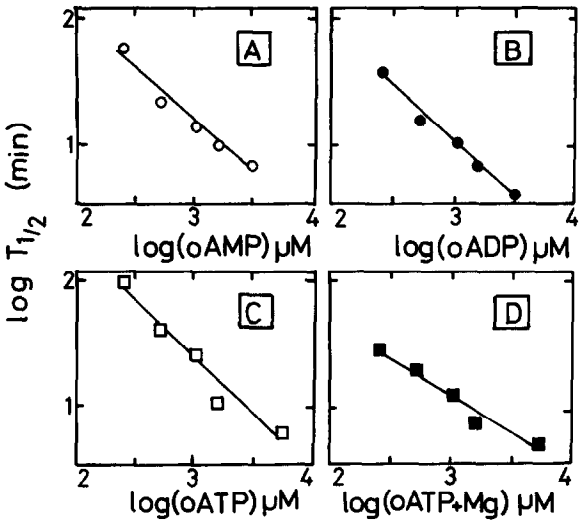

Fig.3. Determination of the number, n, of reactive oAdN per active site of ATPase. The data of fig.2 were plotted as log ($T_{1/2}$) *vs* the logarithm of (A) oAMP (○), (B) oADP (●), (C) oATP (□), and (D) oATP + $MgCl_2$ (■) concentrations. Slope values: n = 0.82 for oAMP; n = 0.85 for oADP; n = 0.90 for oATP and n = 0.60 for (oATP + $MgCl_2$).

oATP the rate of hydrolysis of oATP was 2% of that measured under similar conditions with ATP and $MgCl_2$. As shown in fig.4, the rate of release of P_i decreased after 10 min, due to the irreversible inactivation by oATP and its hydrolysis product, oADP. Like ADP, which inhibits ATP hydrolysis,

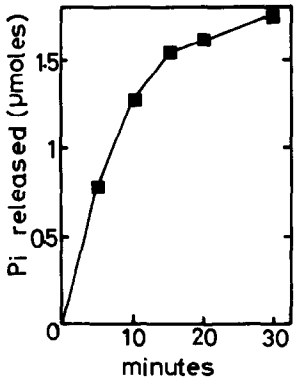

Fig.4. Hydrolysis of oATP by F_1. F_1 (0.14 mg) was incubated at 30°C in 1 ml of 0.1 M Tra, 5 mM oATP, 2.5 mM $MgCl_2$ (pH 8.0). At the indicated times samples were supplemented with 10% (w/v; final concentration) trichloroacetic acid, and P_i released was determined.

oADP may also inhibit hydrolysis of oATP. It should be noted that the classical spectrophotometric assays using phospho*enol*pyruvate and pyruvate kinase as regenerating system cannot be used since oADP is not a substrate of pyruvate kinase [22].

3.3. *[^{14}C]oADP binding to F_1-ATPase*

Incorporation of [^{14}C]oADP into F_1, as measured by the centrifugation gel-filtration technique [17], was linearly related to the loss of ATPase activity. By extrapolation, complete inactivation of F_1 was attained when about 11 mol [^{14}C]oADP had bound per mol F_1 (fig.5). SDS-polyacrylamide gel electrophoresis (fig.6) showed that the covalently bound [^{14}C]oADP was distributed among the α, β, and γ subunits. Specific labeling was determined by comparing incorporation with [^{14}C]oADP alone and in the

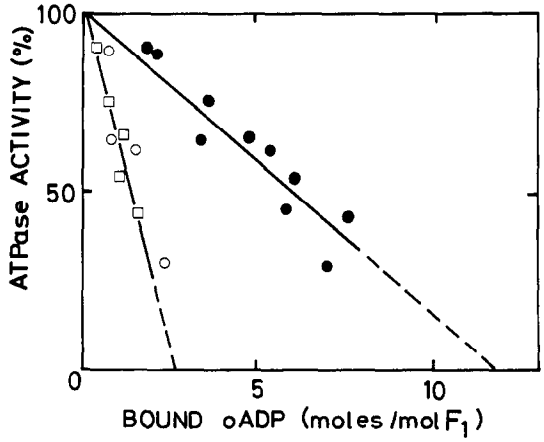

Fig.5. Correlation of inactivation of F_1 with incorporation of [^{14}C]oADP. F_1 (0.8 mg/ml) was incubated at 30°C with 0.08 mM [^{14}C]oADP, 0.1 M Tra (pH 8.0). Aliquots were removed at intervals and subjected to chromatography-centrifugation on Sephadex G-50 columns equilibrated in 50 mM Tris–HCl (pH 8.0) before being assayed for ATPase activity and [^{14}C]oADP incorporation (●). At the same incubation times, non-specific labeling with [^{14}C]oADP was also determined under the same conditions, but in the presence of 10 mM ADP or 10 mM ATP. The difference between the total [^{14}C]oADP incorporation and the [^{14}C]oADP bound in the presence of 10 mM ADP or 10 mM ATP represents the specific labeling; (○) specific ADP binding and (□) specific ATP binding.

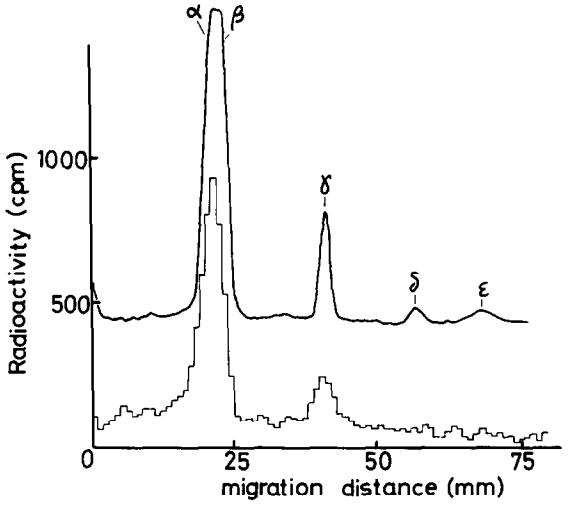

Fig.6. SDS-polyacrylamide gel electrophoresis of F_1 inactivated by [^{14}C]oADP. F_1 was inactivated with 0.08 mM [^{14}C]oADP up to 30% inactivation. After removal of unbound [^{14}C]oADP by centrifugation-filtration on Sephadex G-50 equilibrated in 50 mM Tris–HCl (pH 7.5), the enzyme (48 μg protein) was subjected to SDS-polyacrylamide gel electrophoresis. The scan at 660 nm and the ^{14}C radioactivity distribution profile are shown.

presence of an excess of ADP or ATP [12]. The difference corresponded to the specific [^{14}C]oADP binding; in the present case, 2–3 mol oADP/mol F_1 were found to bind specifically (fig.5). This difference method was, however, not accurate enough to be applied to the determination, by gel electrophoresis, of the subunits that bind [^{14}C]ADP specifically.

4. DISCUSSION

oAdNs have been used as affinity labels of different F_1 species. In such studies, it has to be borne in mind that oAdNs are unstable reagents that have a tendency to decompose to give an adenine-containing compound and a phosphate residue [10]. It is clear therefore that the purity of oAdNs has to be checked before use in inactivation studies. As reported in [10], oATP in the presence of Mg^{2+} inactivates beef heart F_1 and binds covalently to the α and β subunits. This work extends these data by showing that oADP and oAMP inhibit F_1 more efficiently than oATP. The inactivating effect of oAMP was unexpected since AMP is not a substrate for F_1. The inactivating effect of oAMP may be unspecific; on the other hand, it is possible that the opening of the ribose ring results in the extension of the oAMP molecule which then mimicks ADP and is recognized by the active site of F_1. Except for oATP, Mg^{2+} did not play a significant role in the kinetics of inactivation. In the presence of Mg^{2+}, oATP was a substrate for F_1. Hydrolysis of Mg-oATP by F_1 is therefore a complicating factor in the evaluation of inactivation data.

Concomitantly with the inactivation by [^{14}C]oADP, 2–3 mol [^{14}C]oADP were found to bind specifically per mol beef heart F_1. This is in contrast with the data obtained with other inactivating reagents like nitrobenzofurazan [26], dicyclohexylcarbodiimide [27] and efrapeptin [28] which fully inactivate beef heart F_1 upon binding of 1 mol reagent per mol F_1. [^{14}C]oADP bound covalently to the α, β and γ subunits; however, the radioactivity was mostly localized in the α and β subunits. This is in agreement with binding data reported for [^{14}C]oATP in the presence of Mg^{2+} [10], but in contrast with other results showing that [^{14}C]oADP binds to the α subunit of beef heart F_1, with a saturation plateau of 1 mol [^{14}C]oADP/mol F_1 without alteration of ATPase activity [11]. On the other hand, incorporation of 1 mol oATP per mol F_1 of *M. phlei* at the level of the α subunit was reported to inactivate ATPase activity fully. A different situation was encountered with the binding of o-etheno-ATP to the β subunit of beef liver F_1 [29]. Although the behavior of oAdNs with respect to F_1 may depend on the origin of the enzyme and experimental conditions, it is clear that a factor of complexity which has to be carefully assessed in the evaluation of binding and inactivation data is the instability of oAdNs.

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