Catalytic properties of the ATPase on submitochondrial particles after exchange of tightly bound nucleotides under different steady state conditions

Jill A. Myers and Paul D. Boyer

Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, CA 90024, USA

Received 22 August 1983

Energized submitochondrial particles were subjected to high or low [^{3}H]ATP/[^{3}H]ADP ratios, maintained during steady state by a pyruvate kinase or hexokinase regenerating system, respectively. Under both steady state conditions, about 1.4 mol [^{3}H]nucleotide/mol ATPase was retained but considerably more [^{3}H]ATP was retained with the high [^{3}H]ATP/[^{3}H]ADP ratio. The ATPase activity and the oxygen exchange of these differentially labeled SMP were the same, suggesting a lack of control function of non-catalytic tightly bound nucleotides.

ATPase, control *Oxygen exchange* *Mitochondria* *Bound nucleotide*

1. INTRODUCTION

The membrane-bound proton-translocating ATPase of SMP is capable of catalyzing both ATP synthesis and hydrolysis under energized conditions. SMP provide an appropriate system for study of possible control of the ATPase action. This paper presents an assessment of whether tightly bound nucleotides associated with the ATPase may modify its catalytic properties.

An unusual feature of the proton-translocating ATPases is the presence of multiple nucleotide binding sites with different affinities. Present information suggests binding sites on each of the three large α- and β-subunits. Binding sites on either subunit do not have identical properties; negative cooperativity of binding and different nucleotide specificities are apparent. The purified ATPase and the ATPase on coupling membranes have tightly bound ADP and ATP present, as measured by retention of bound nucleotides after separation, precipitation, washing or gel filtration steps. Exchange of tightly bound nucleotides on coupling membanes is promoted by energization capable of driving ATP synthesis (reviews [1–4]).

Although tightly bound nucleotides have sometimes been regarded as entirely non-catalytic, there now appears to be clear evidence that they consist of both catalytic and non-catalytic nucleotides [5–8]. With the mitochondrial ATPase, most tightly bound nucleotides appear to be non-catalytic. Bound nucleotides have been considered by a number of investigators to have a control function. However, demonstration of such a control function is lacking [4]. A tightly bound ADP has been implicated in inhibition of ATPase activity with mitochondrial [7,9,10] and chloroplast [11–14] ATPases. This inhibition could result from an ADP bound at a catalytic site without P_i present [6,15] or from a magnesium-induced binding that is only slowly relieved when conditions favoring ATPase activity are initiated (unpublished).

Abbreviations: SMP, submitochondrial particles; PEP, phosphoenolpyruvate; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; EDTA, ethylenediamine–tetraacetic acid; HEPES, 4-(2-hydroxy-ethyl)-1-piperazine–ethanesulfonic acid, ATPase, mitochondrial proton-translocating adenosine triphosphatase (often called F_1-ATPase)

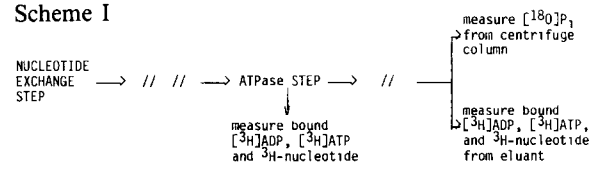
Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

Our objective was to find if different steady-state labeling conditions might demonstrably change the number and nature of tightly bound nucleotides retained on submitochondrial particles. Such differential labeling was achieved. We then tested the ability of the particles to catalyze ATP hydrolysis and oxygen exchange in short-time experiments as sensitive probes of possible control functions. No evidence for any control function of tightly bound nucleotides was obtained.

2. MATERIALS AND METHODS

[^{18}O]PEP was prepared and analyzed mass spectrally as in [16]. [5′,8-^{3}H]ATP was obtained from Amersham (Arlington Heights Il). Heavy beef heart mitochondria [17] were used to prepare SMP [18]. Aliquots were stored in liquid nitrogen until used. ATP synthesis activity ranged from 150–200 nmol.min^{-1}.mg SMP protein^{-1}.

Scheme I



The experimental design is depicted in scheme I. Two slashes (//) represent passage through a centrifuge–Sephadex column [19]. Low blanks, as measured by the retention of [^{3}H]nucleotides in the SMP fraction, were obtained by passage through two consecutive columns. Solutions of SMP needed to contain >1 mg/ml to avoid loss of protein when passed through centrifuge columns.

The energized nucleotide exchange mixture, which allowed exposure to different steady state [^{3}H]ATP/[^{3}H]ADP ratios, contained (final conc.): 20 mM HEPES (pH 7.5), 150 mM sucrose, 6 mM $MgCl_2$, 25 mM K^+-succinate, 5 mM P_i, 1 mM carboxyatractyloside and 167 μM [^{3}H]ATP (about 10^4 cpm/nmol). To maintain a high [^{3}H]ATP/[^{3}H]ADP ratio some samples also contained an ATP regenerating system of 0.5 mg pyruvate kinase/ml and 10 mM PEP; to maintain a low [^{3}H]ATP/[^{3}H]ADP ratio some samples also contained an ADP regenerating system of 0.5 mg hexokinase/ml and 10 mM glucose. The reaction was initiated by the addition of SMP to a final concentration of 2 mg/ml. After 5 min the sample was placed on ice for 30 s, the SMP passed through two centrifuge columns and bound [^{3}H]nucleotides measured. A value of 0.42 nmol ATPase/mg SMP protein was assumed [20]. Carboxyatractyloside, a potent inhibitor of nucleotide translocation across the mitochondrial membrane, was included to minimize passage of medium [^{3}H]nucleotides into the vesicle interior and the consequent interference in tightly bound nucleotide measurement.

The reaction mixture for oxygen exchange and ATPase measurements contained (final concentration): 20 mM HEPES (pH 7.5), 150 mM sucrose, 2 mM $MgCl_2$, 50 mM KCl, 500 μM [^{18}O]PEP, 200 μg pyruvate kinase/ml, 2.5 μg S-13/ml and 25 μM ADP. After 1 min (to allow conversion of the ADP to [γ-^{18}O]ATP), the SMP-containing eluant from the second centrifuge column was added. The ATPase was quenched by the addition of 10 μl 125 mM EDTA after about 100 nmol [^{18}O]P_i had been produced (20 s). Highly enriched [^{18}O]P_i was added, the dilution of which allowed calculation of P_i formed and thus the ATPase activity. The quenched reaction mixture was passed over a third centrifuge column.

Protein was determined by the Lowry method with bovine serum albumin as a standard [21]. To estimate the relative SMP content of the centrifuge column eluants solutions were diluted into 6 M guanidine hydrochloride and the absorbance of the cytochrome Soret band measured at 416 nm.

3. RESULTS

The levels of tightly bound nucleotides exchanged and retained after 5 min of oxidative phosphorylation are shown in table 1. About 1.4 mol [^{3}H]nucleotide/mol ATPase was present with the different steady state conditions. When the [^{3}H]ATP/[^{3}H]ADP ratio was kept high by the pyruvate kinase regenerating system the retained nucleotides were essentially equally divided among [^{3}H]ADP and [^{3}H]ATP. There was good agreement between the sum of the retained [^{3}H]ADP and [^{3}H]ATP measured separately and the measured total retained [^{3}H]nucleotide. When the [^{3}H]ATP/[^{3}H]ADP ratio was kept low by the hexokinase regenerating system, the amount of retained [^{3}H]ADP was similar but the amount of retained [^{3}H]ATP was much smaller. The sum of the re-

Table 1

Retention of tightly bound [^{3}H]nucleotides after exposure of energized SMP under steady state conditions to high and low [^{3}H]ATP/[^{3}H]ADP ratios

Regenerating System	mol [^{3}H]nucleotide / mol ATPase	mol [^{3}H]ADP / mol ATPase	mol [^{3}H]ATP / mol ATPase
Pyruvate kinase	1.36 (1.26 – 1.65)	0.72	0.66
Hexokinase	1.36 (1.27 – 1.55)	0.70	0.12

Samples containing the nucleotide exchange mixture described in section 2 were incubated in the absence of SMP with aeration at 30°C for 1 min; the reaction was initiated with SMP, added to final concentration of 2 mg/ml in a final volume of 400 μl. The regenerating systems were as described in section 2. After 5 min the samples were cooled on ice for 30 s, then a 325 μl aliquot was transferred to a 2.5 ml centrifuge column and spun. A 300 μl aliquot of that eluant was immediately placed on a second 2.5 ml centrifuge column and spun. One aliquot of that eluant was counted to determine total bound [^{3}H]nucleotide. Protein was removed from a second aliquot by perchloric acid addition and the amount of [^{3}H]ADP and [^{3}H]ATP determined as in [22]. The numbers in parentheses are ranges observed

tained [^{3}H]ADP and [^{3}H]ATP was much less than the total retained [^{3}H]nucleotide measured; this may have been due to retained [^{3}H]AMP.

For test of catalytic characteristics a short reaction time seemed essential to avoid a marked change in the levels of tightly bound nucleotides. A considerable portion of the bound [^{3}H]nucleotide is replaced or exchanged either during the 20 s period of the catalytic assay or the subsequent column separation or both (table 2). A control showed that this loss or exchange is not due to the EDTA quench (not shown). Although the total amount of the retained [^{3}H]nucleotide decreased during this catalytic assay, the differences in labeling patterns remained.

Also shown in table 2 are the oxygen exchange ATPase measurements. The observed and predicted distributions of the [^{18}O]P_i species formed are given in fig.1. Both the catalytic parameters and the ^{18}O distributions were essentially the same for SMP with different levels of retained nucleotides.

Table 2

Oxygen exchange and ATPase activity of SMP with different levels of tightly bound [^{3}H] nucleotides retained

Regenerating system	After nucleotide labeling step	after ATPase step			Catalytic properties	
	mol [^{3}H]nucleotide / mol ATPase	mol [^{3}H]nucleotide / mol ATPase	mol [^{3}H]ADP / mol ATPase	mol [^{3}H]ATP / mol ATPase	O/P	ATPase nmol / min.mg
Pyruvate kinase	1.26	0.48	0.24	0.20	1.76	488
Hexokinase	1.55	0.44	0.11	0.02	1.69	492

The nucleotide exchange step is described in the legend to table 1. A 275 μl aliquot of the eluant of the second centrifuge column was put into the ATPase assay mixture described in section 2, which had been at 30°C for 1 min to convert the ADP into [^{18}O]ATP. The final volume was 400 μl. After 20 s the reaction was quenched by the addition of 10 μl 125 mM EDTA with vortexing. Highly enriched [^{18}O]P_i was added as a standard. A 325 μl aliquot was put onto a new 3 ml centrifuge column. The amount of bound [^{3}H]nucleotide retained in the eluant was determined as in table 1. To determine the number of water oxygens/phosphate released, O/P, the [^{18}O]P_i was eluted from the centrifuge column with 3 ml 500 μM NH_4Cl in 100 μM NH_4OH, and isolated and analyzed as in [23,16]. The amount of P_i produced enzymically was measured by the dilution of a mass spectral peak of the added highly enriched [^{18}O]P_i

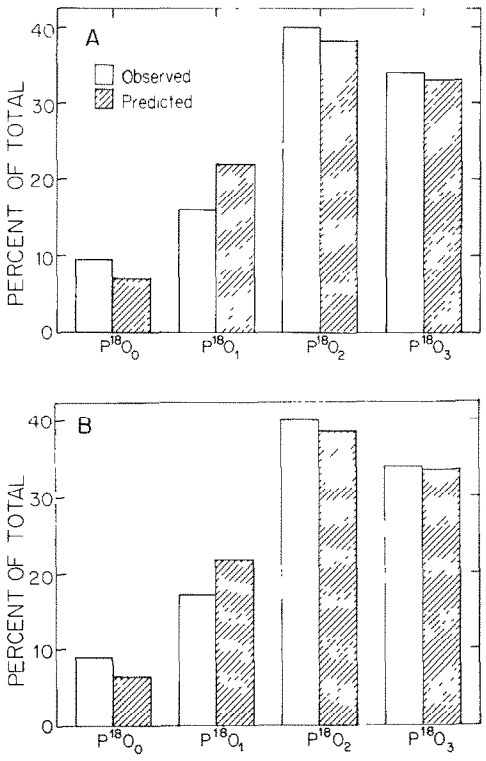


Fig.1. Experimental and theoretical patterns of $[^{18}O]P_i$ species formed during ATP hydrolysis by SMP with different levels of tightly bound nucleotides. The solid bars represent the observed distribution of the different $[^{18}O]P_i$ species from the samples shown in table 2. The open bars represent the distribution predicted for a single catalytic pathway for the observed O/P values: (A) distributions for the sample with the pyruvate kinase regenerating system; (B) distributions for the sample with the hexokinase regenerating system.

4. DISCUSSION

The results in table 1 show that the nature of the tightly bound nucleotides retained with SMP after a period of oxidative phosphorylation can be modified by the relative amounts of ATP and ADP in the reaction medium. The conditions used were such that the particles were exposed largely to medium [^{3}H]ADP or largely to medium [^{3}H]ATP during a 5 min period of oxygen uptake. The prominent difference in the labeling pattern is the presence of considerable tightly bound [^{3}H]ATP when the medium contained principally [^{3}H]ATP and low levels of such bound [^{3}H]ATP when the medium contained principally [^{3}H]ADP. Also, the particles exposed to the medium with a low [^{3}H]ATP/[^{3}H]ADP ratio had appreciably bound nucleotide present that was not [^{3}H]ADP or [^{3}H]ATP.

These differences in bound nucleotide labeling were present at the beginning and to a lesser extent at the end of the 20 s catalytic assay period (table 2). Thus, any appreciable effect of differences in tightly bound nucleotides on net reaction velocity or oxygen exchange should have been evident. No effect was observed. The catalytic activities were measured at an ATP concentration where the velocity and exchange rates are sharply dependent upon medium ATP concentration. This condition was chosen as one likely to be sensitive to any control effects.

Additional indication of the lack of regulatory effects of tightly bound nucleotides comes from the measurements of the distribution of $[^{18}O]P_i$ species formed (fig.1). These, as in earlier studies with the submitochondrial ATPase [24] and ATPase purified from mitochondria [25], and chloroplasts [23] were as expected for catalysis by only one reaction pathway. Individual ATPase molecules in SMP likely do not carry equal amounts of tightly bound ADP and ATP on the α-subunits. If nucleotides on the α-subunit affect the rate constants governing oxygen exchange, heterogeneous distributions of $[^{18}O]P_i$ species from SMP containing different amounts of bound nucleotide would be anticipated. The slight amount of heterogeneity that is observed, however, may be due to experimental error. Also, the possibility exists that there is a small amount of heterogeneity due to hindered rotation of the phosphoryl group, as has been proposed to occur in ATP synthesis by SMP [26]. Nevertheless, the ^{18}O distributions of $[^{18}O]P_i$ formed by SMP containing different amounts and types of nucleotides are the same, consistent with a lack of control function of noncatalytic tightly bound nucleotides.

The lack of any observed effect of tightly bound nucleotides on the catalytic properties of the mitochondrial ATPase does not mean that tightly bound nucleotides have no control effects on this reaction. However, at the present time convincing evidence of such a function is lacking. A possibility that merits consideration is that a control function could be exerted by loosely bound nucleotides on

the α-subunits, monitoring the concentrations of ADP and ATP in the physiological range.

The decline in tightly bound [^{3}H]nucleotides after the 20 s assay may result from exchange with nonlabeled nucleotides, the replacement of small amounts of catalytic nucleotides, or a dissociation promoted by increased ionic strength [27]. The nature of the tightly bound ^{3}H-labeled component that is not ATP or ADP but is retained with particles initially exposed for 5 min to a low [^{3}H]ATP/[^{3}H]ADP ratio is not known. This substance might be [^{3}H]AMP, resulting from an adenylate kinase-like reaction of the particles. There is some evidence for the occurrence of such a reaction [28]. Further study of the chemical nature and properties of the material appears warranted.

ACKNOWLEDGEMENTS

This work was supported by USPHS grant GM 11904. J.A.M. was a recipient of USPHS training grant GM 17285. We are indebted to Kerstin Stempel for ^{18}O analyses. The [^{18}O]PEP was prepared by Dr Clifford C. O'Neal and the S-13 was a gift from Dr P.C. Hamm of Monsanto.

REFERENCES

[1] Harris, D.A. (1978) Biochim. Biophys. Acta 463, 245–273.
[2] Penefsky, H.S. (1979) Adv. Enzymol. 49, 223–280.
[3] Cross, R.L. (1981) Annu. Rev. Biochem. 50, 681–714.
[4] Senior, A.E. and Wise, J.G. (1983) J. Membr. Biol. 73, 105–124.
[5] Feldman, R.I. and Sigman, D.S. (1982) J. Biol. Chem. 257, 1676–1683.
[6] Smith, L.T. and Boyer, P.D. (1983) J. Biol. Chem. in press.
[7] Vasilyeva, E.A., Minkov, I.B., Fitin, A.F. and Vinogradov, A.D. (1982) Biochem. J. 202, 9–14.
[8] Grubmeyer, C., Cross, R.L. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12092–12101.
[9] Roveri, O.A., Muller, J.L.M., Wilms, J. and Slater, E.C. (1980) Biochim. Biophys. Acta 589, 241–255.
[10] Di Pietro, A., Godinot, C. and Gautheron, D.C. (1981) Biochemistry 20, 6312–6317.
[11] Bruist, M.F. and Hammes, G.G. (1982) Biochemistry 21, 3370–3377.
[12] Carmeli, C. and Lifshitz, Y. (1972) Biochim. Biophys. Acta 267, 86–95.
[13] Dunham, K.R. and Selman, B.R. (1981) J. Biol. Chem. 256, 212–218.
[14] Bar-Zvi, D. and Shavit, N. (1980) FEBS Lett. 119, 68–72.
[15] Vinogradov, A.D., Vasilyeva, E.A. and Yalamova, M.V. (1982) Rep. Eur. Bioenerget. Cong. 2, 113–114.
[16] O'Neal, C.C. jr., Bild, G.S. and Smith, L.T. (1983) Biochemistry 22, 611–617.
[17] Smith, A.L. (1967) Methods Enzymol. 10, 81–86.
[18] Beyer, R.E. (1967) Methods Enzymol. 10, 186–194.
[19] Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
[20] Harris, D.A., Radda, G.K. and Slater, E.C. (1977) Biochim. Biophys. Acta 459, 560–572.
[21] Lowry, O.H., Rosebrough, A.L., Farr, L.H. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[22] Smith, D.J. and Boyer, P.D. (1976) Proc. Natl. Acad. Sci. USA 73, 4314–4318.
[23] Kohlbrenner, W.E. and Boyer, P.D. (1983) J. Biol. Chem. in press.
[24] Mitchell, R.A., Lamos, C.M. and Russo, J.A. (1980) Biochim. Biophys. Acta 592, 406–414.
[25] Hutton, R.L. and Boyer, P.D. (1979) J. Biol. Chem. 254, 9990–9993.
[26] Hackney, D.D. (1983) Curr. Top. Cell. Regul. in press.
[27] Leimgruber, R.M. and Senior, A.E. (1976) J. Biol. Chem. 251, 7110–7113.
[28] Horner, R.D., Froehlich, J.P. and Moudrianakis, E.N. (1983) J. Biol. Chem. 258, 5618–5622.