Tight ATP and ADP binding in the noncatalytic sites of *Escherichia coli* F_1-ATPase is not affected by mutation of bulky residues in the 'glycine-rich loop'

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It is shown that ATP dissociates very slowly ($k_{off} < 6.4 \times 10^5$ s^{-1}, $t_{1/2} > 3$ h) from the three noncatalytic sites of *E. coli* F_1-ATPase and that ADP dissociates from these three sites in a homogeneous fashion with $k_{off} = 1.5 \times 10^{-4}$ s^{-1} ($t_{1/2} = 1.35$ h). Mutagenesis of α-subunit residues R171 and Q172 in the 'glycine-rich loop' (Homology A) consensus region of the noncatalytic sites was carried out to test the hypothesis that unusually bulky residues at these positions are responsible wholly or partly for the observed tight binding of adenine nucleotides. The mutations αQ172G or αR171S,Q172G had no effects on ATP or ADP binding to or rates of dissociation from F_1 noncatalytic sites. $K_{d_{ATP}}$ and $K_{d_{ADP}}$ of isolated α-subunit were weakened by approximately 1 order of magnitude in both mutants. The results suggest that neither residue αR171 nor αQ172 interacts directly with bound nucleotide, and show that the presence of bulky residues per se in the glycine-rich loop region of F_1-α-subunit is not responsible for tight binding in the noncatalytic sites.

ATP binding; ADP binding; F_1-ATPase; *Escherichia coli*; Glycine-rich loop

1. INTRODUCTION

E. coli F_1-ATPase, the terminal enzyme for ATP synthesis by oxidative phosphorylation, has the subunit composition $\alpha_3\beta_3\gamma\delta\epsilon$, is catalytically active as an ATPase, GTPase or ITPase, and binds a total of six mol adenine nucleotide per mol enzyme [1]. Three sites exchange nucleotide rapidly and exhibit broad nucleotide specificity, being able to bind GTP and ITP as well as ATP [2]. These three potential catalytic sites are believed to reside on the three β-subunits (reviewed in [3,4]). The other three sites are specific for adenine nucleotides [2]. These sites appear noncatalytic and their function is unknown [5]. They are believed to reside on the three α-subunits [2,5,6]. Previously we have shown that dissociation of ATP [2] or AMP-PNP [1] from the noncatalytic sites is extremely slow ($t_{1/2} > 3$ h). Here we have examined the rate of ADP dissociation from these sites; the data are germane to considerations as to the likely state of occupancy of the noncatalytic sites in vivo.

The F_1-α-subunit nucleotide-binding domain has been predicted to encompass residues α160–340 approximately [7]. Supporting evidence for this localization has come from affinity-labeling of residue αK201 by the analog pyridoxal-5′-diphospho-5′-adenosine [8,9] and the effects of mutagenesis of residue αK175 [10]. Within the predicted α-subunit nucleotide-binding domain, the amino acid sequence GDRQTGKT occurs at residues α169–176. This sequence is homologous to the 'Homology A' [11] or 'glycine-rich loop' [12] region, now known to be present in numerous, diverse, nucleotide-binding proteins. Studies of EF-Tu, H-*ras*-p21 protein, adenylate kinase and myosin indicate that this region lies very close to the phosphate groups of bound nucleoside triphosphate [13–22].

The glycine-rich loop region of α-subunit is somewhat unusual because it contains bulky residues, whereas this region in other proteins is composed predominantly of small residues (commonly P,G,S). Conceivably, as hypothesized by Fry et al. [12], this might be related, through imposition of conformational inflexibility, to high affinity binding of nucleotide and low rate of exchange by the F_1 noncatalytic sites. Here we have mutagenized residues αR171 and αQ172 to small residues and have assayed binding and release of ATP and ADP by the noncatalytic sites of mutant F_1 and α-subunit to test this hypothesis.

2. MATERIALS AND METHODS

2.1. *Binding and exchange of nucleotides by purified* E. coli F_1

This was done as previously described [1,2]. Briefly the procedure involves passage of F_1 through a 50% (v/v) glycerol-containing column of Sephadex G-25, incubation of the F_1 with radioactive nucleotide, passage through centrifuge columns to allow determination of total bound nucleotide, then addition of 'chase' nonradioactive nucleotide and determination of bound residual radioactive nucleotide at timed intervals. All experiments were done at 23°C in 50 mM Tris-SO_4, 0.5 mM EDTA, 2.5 mM $MgSO_4$, pH 8.0 buffer.

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2.2. *Binding of [α-^{32}P]ATP to α-subunit in dissociated F_1*

This was done by the centrifuge column technique as described in [10]. In this procedure, excess GTP is included to prevent any nucleotide binding to β-subunit in dissociated F_1. ATP dissociation rate (k_{off}) was determined by addition of 20 mM nonradioactive ATP to a preincubated mixture of [α-^{32}P]ATP (50 μM) and dissociated F_1 (0.3 μM) followed by passage through centrifuge columns at timed intervals. k_{on} was calculated from $K_{-d_{ATP}}$ and k_{off}.

2.3. *Site-directed mutagenesis and expression of mutant F_1-α-subunit*

This was done as described in [10]. A synthetic 20-mer oligonucleotide was used to generate the αQ172G mutant, in which codon GGG (glycine) replaced wild-type CAG (glutamine). Subsequently a mixed oligonucleotide, containing NCT instead of the usual CGT codon at residue 171, and containing also the GGG codon at residue 172, was used to generate the double mutants R171A,Q172G; R171P,Q172G;R171S,Q172G; and R171T,Q172G.

3. RESULTS

Fig. 1A shows, in confirmation of previous work [2], that normal *E. coli* F_1 binds almost 6 mol of [α-^{32}P]ATP/mol F_1, of which 3 mol/mol dissociates rapidly, and 3 mol/mol dissociates extremely slowly in the presence of 'chase' nonradioactive ATP or ADP ($t_{1/2} > 3$ h, $k_{off} < 6.5 \times 10^{-5}$ s^{-1}). Fig. 1B shows that [γ-^{32}P]ATP behaves in exactly the same manner as [α-^{32}P]ATP in terms of rate of release from the three slowly-dissociating sites. Fig. 1C shows that [^{3}H]ADP loads to the same extent as ATP (up to 6 mol/mol) but the rate of exchange of ADP out of the three slowly exchangeable sites is faster. A more detailed study of ADP dissociation was done (not shown), in which data points were obtained down to 0.5 mol ADP remaining bound per mol F_1, and non-linear regression analysis of a plot of [ADP bound] vs time, demonstrated that all three sites behaved in equivalent fashion with $t_{1/2}$ = 1.35 h, $k_{off} = 1.5 \times 10^{-4}$ s^{-1}. Fig. 1D shows that 50 mM GTP prevented ADP binding to catalytic but not to noncatalytic sites, and the apparent $k_{off_{ADP}}$ from noncatalytic sites in Fig. 1D was the same as in Fig. 1C. Kironde and Cross [23] found a dissociation rate constant 4×10^{-4} s^{-1} for dissociation of ADP from a noncatalytic site of mitochondrial F_1 in the presence of Mg ions, similar to the value seen here.

As described in section 2, five mutant strains were generated by site-directed mutagenesis, containing respectively the mutation αQ172G alone, and then αQ172G in combination with A, P, S, or T substituted for residue αR171 in the F_1-α-subunits. These 5 strains had normal growth characteristics on succinate plates or in limiting glucose medium, showing that in vivo ATP synthesis by oxidative phosphorylation was normal. Membrane vesicles were prepared from each strain. The αR171A,Q172G; αR171P,Q172G; and αR171T,Q172G mutants showed low NADH-induced pH-gradient formation (measured by acridine orange fluorescence quencing) and lower-than-normal membrane ATPase activity. These data indicate there was partial loss of F_1 from the membranes, implying that

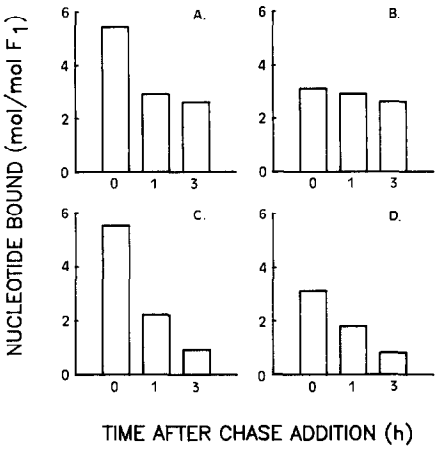

Fig. 1. Binding of nucleotide to normal F_1 and subsequent exchange of bound nucleotide. Normal F_1 which had been chromatographed on Sephadex G-25 in 50% (v/v) glycerol-containing buffer [1] was preincubated for 2–2.5 h with [2-^{3}H]ADP (1.25 mM), [α-^{32}P]ATP (1.5 mM), or [γ-^{32}P]ATP (1.5 mM) in 50 mM Tris-SO_4, 0.5 mM EDTA, 2.5 mM $MgSO_4$, pH 8.0 at 23°C. Free and bound nucleotides were separated by the centrifuge column technique and the amount of bound radioactive nucleotide was determined (time zero). Nonradioactive 'chase' ADP (2.5 mM) was added and at timed intervals, samples were passed through centrifuge columns to determine remaining bound radioactive nucleotide. (A) F_1 incubated with [α-^{32}P]ATP. (B) F_1 incubated with [γ-^{32}P]ATP. (C) F_1 incubated with [2-^{3}H]ADP. (D) as in (C), but the pre-incubation medium also contained 50 mM GTP. Similar results were obtained using ATP (2.5 mM) added at time zero.

the mutations caused some structural instability. The αQ172G and αR171S,Q172G mutants were structurally-normal as shown by normal membrane ATPase activity (±DCCD), high NADH-induced pH-gradient formation and normal ATP-driven proton-pumping.

Soluble F_1 was purified from the latter two mutants, and was found to be normal in subunit composition and oligomeric stability. Eadie-Hofstee plots and pH-dependence of the ATPase activities of both mutant F_1 preparations were the same as normal, as were Arrhenius plots over the temperature range 14–45°C. Binding and dissociation of ATP and ADP were assayed following the procedures used for normal F_1 as described in Fig. 1, with determination of residual bound radioactive nucleotide being performed at 30 min, 1 h, 2 h, 3 h and 3.5 h after addition of chase nonradioactive nucleotide. Plots of [bound residual nucleotide] vs time for both mutant and normal F_1 were superimposable, showing that the mutations did not affect total binding or rate of release of ATP or ADP to or from the F_1 noncatalytic sites.

Binding of radioactive ATP to α-subunit in dissociated F_1 was measured as in section 2. The results (Table I) showed that $K_{d_{ATP}}$ of α-subunit was increased by approximately 1 order of magnitude by the mutations, due to change in both k_{on} and k_{off} rates. We also assayed both $K_{d_{ATP}}$ and $K_{d_{ADP}}$ by following the degree of protection from trypsin proteolysis that these

Table I

Characteristics of ATP-binding to purified normal α-subunit and to mutant and normal α-subunits in dissociated F_1[a]

Source of α-subunit	K_{dATP} (μM)	k_{off} (s^{-1})	k_{on} ($M^{-1} \cdot s^{-1}$)
Purified normal α-subunit[b]	0.3	nd[c]	nd[c]
Dissociated normal F_1	0.5	0.008	1.4×10^4
Dissociated αQ172G F_1	7.0	0.018	2.6×10^3
Dissociated αR171S,Q172G F_1	9.0	0.026	2.9×10^3

[a]Binding data were analyzed using nonlinear regression analysis ([26] and FITB kindly provided by Dr B. Simon, University of Texas). K_{dATP} was calculated as described by Gutfreund [27] since it was of the same order of magnitude as α-subunit concentration in the binding assay.

[b]Prepared as in [24].

[c]nd = not determined here. Dunn [25] determined a k_{off} of 0.0035 s^{-1} for purified normal α-subunit under similar conditions to those used here. Taking a value of K_{dATP} = 0.1 μM [24] or 0.3 μM (determined here) this would yield k_{on} values of 3.5×10^4 $M^{-1} \cdot s^{-1}$ or 1.2×10^4 $M^{-1} \cdot s^{-1}$, respectively, for normal α-subunit

nucleotides afforded the α-subunit in dissociated F_1, as described previously [10]. The trypsin protection experiments (not shown) demonstrated that both K_{dATP} and K_{dADP} were weakened by approximately one order of magnitude by the mutations, and also that the nucleotide binding-specificity of α-subunit (ATP>ADP>>>GTP,ITP,AMP) was unaffected by the mutations.

4. DISCUSSION

Isolated F_1-α-subunit is known to bind ATP and ADP with high affinity (K_{dATP} = 100 nM, K_{dADP} = 1 μM [24] and is most likely assembled into F_1 as α · ATP complex or α · ADP complex. The rates of dissociation of ATP and ADP from the noncatalytic sites of F_1 found here are very slow, and it follows from the data that these sites would be continually occupied by adenine nucleotide in vivo, with ATP being the preferred ligand. If it is assumed that k_{on} for ATP binding to F_1 noncatalytic sites is the same as k_{on} for ATP binding to isolated α-subunit (calculated as 3.5×10^4 $M^{-1} \cdot s^{-1}$ from [25] then the K_{dATP} of F_1 noncatalytic sites would be <2 nM.

We attempted here to perturb this tight binding by mutagenesis of the bulky residues αQ172 and αR171 in the glycine-rich loop region of F_1-α-subunit. Although the mutations did affect nucleotide binding to α-subunit, the effects were small, implying that residues αQ172 and αR171 do not normally interact directly (e.g. by hydrogen-bonding or charge-charge interaction) with bound nucleotide. In contrast, previous mutagenesis of nearby residue αK175 to I or E had large effects on nucleotide-binding to α-subunit [10]. The small effects seen here in α-subunit could be due to an increased flexibility of the glycine-rich loop caused by the substitution of small residues for R171 and Q172, as suggested previously [12]. However, it was clear that this effect was masked in F_1 since the mutations had no effect on the dissociation of nucleotide from the noncatalytic sites of intact F_1. The presence of bulky residues in the glycine-rich loop of the α-subunit is not therefore primarily responsible for the very slow exchange rate out of the F_1 noncatalytic sites.

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