α-Aspartate 261 Is a Key Residue in Noncatalytic Sites of Escherichia coli F₁-ATPase

(Received for publication, May 15, 1995, and in revised form, June 20, 1995)

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X-ray structure analysis of the noncatalytic sites of F₁-ATPase revealed that residue α-Asp<sup>261</sup> lies close to the Mg of bound Mg-5'-adenylyl-β,γ-imidodiphosphate. Here, the mutation αD261N was generated in Escherichia coli and combined with the αR365W mutation, allowing nucleotide binding at F₁ noncatalytic sites to be specifically monitored by tryptophan fluorescence spectroscopy. Purified αD261N/αR365W F₁-ATPase showed catalytic activity similar to wild-type. An important feature was that, without any resort to nucleotide-depletion procedures, the noncatalytic sites in purified native enzyme were already empty. Binding studies with MgATP, MgADP, and the corresponding free nucleotides showed that Mg-nucleotide binds poorly. The natural ligands at these sites in wild-type enzyme are the Mg-nucleotides and free nucleotides bind poorly. Under conditions where noncatalytic sites were empty, αD261N/αR365W F₁ showed significant hydrolysis of MgATP. This establishes unequivocally that occupancy of noncatalytic sites by nucleotide is not required for catalysis.
forming the plasmids into haploid strain JP2, which contains a and Methods." Then the mutations were expressed by trans-

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Growth of E. coli Cells; Preparation of Membrane Vesicles; Assay of ATPase Activity; Purification of F1; Preparation of Nucleotide-depleted F1; Fluorescence Measurements—These were all as described in Weber et al. (1994).

RESULTS

Effects of the Mutations αK175E, αK175I, and αD261N in Whole Cells and in Membrane Vesicles—The mutations αK175E, αK175I, and αD261N were each combined with the mutation αR365W on plasmids as described under "Materials and Methods." Then the mutations were expressed by trans-

The growth characteristics of each of the three double mutants were studied alongside those of strain AW7 (αR365W single mutation in J P2), an isogenic wild-type strain (pDP34N/P2), and an Unc- strain (pUC118/P2). Table I shows the growth characteristics on succinate plates and the growth yields in limiting (3 mm) glucose liquid medium. Membrane vesicles were also prepared from each strain and assayed for ATPase activity (Table I).

Consistent with previous results, the αR365W single mutant grew well on succinate plates and in limiting glucose medium, and had significant membrane ATPase activity. As we described previously, F1 can be readily purified from this strain and it is similar to wild-type F1 in properties (Weber et al., 1994).

The αK175E/αR365W mutant did not grow on succinate plates and had a growth yield in limiting glucose the same as the Unc- control. Its membrane ATPase activity was not significant. We were unable to prepare F1 from this mutant because in the final step of purification (S-300 gel filtration) there was no protein peak at the position corresponding to F1. With the αK175I/αR365W mutant, growth on succinate plates and in growth yield tests was strong, however, the membrane ATPase activity was low (Table I). We were not able to purify F1 from this mutant either, because again in the S-300 gel filtration step, no protein peak corresponding to F1 was present. Our earlier studies of the αK175E and αK175I single mutants (Rao et al., 1988) showed that the former mutation partially impaired assembly of F1, F0 in the cells, whereas the latter mutation allowed assembly but impaired subunit-subunit interaction, such that both membrane-bound and purified F1 tended to dissociate. We were previously able to purify F1 from the αK175E and αK175I single mutants, but only in low yield. The present results show that combination of the αK175E and αK175I mutations with αR365W exacerbated their effects. Jounouchi et al. (1993) reported that five other mutations at residue α-175 had similar effects, in that they all impaired assembly and/or subunit stability of F1. Yohda et al. (1988) found that the αK175I mutation in Bacillus PS3 F1 impaired subunit interactions. Therefore, it is clear that mutation of residue α-Lys175 interrupts correct assembly of the enzyme and oligomeric stability of F1.

The αD261N/αR365W mutant grew well on succinate plates, had almost normal growth yield, and had 25% of the membrane ATPase activity of the isogenic wild-type strain run alongside (Table I). The membrane ATPase activity was 76% inhibited by dicyclohexylcarbodiimide (150 μM at pH 8.0, 30 °C, for 60 min), which is similar to wild-type. The membrane vesicles showed normal ATP-dependent pH gradient formation when assayed using acridine orange fluorescence quenching as described by Perlin et al. (1983) (data not shown), consistent with the fact that the specific ATPase activity of the membranes from the αD261N/αR365W mutant (~0.8 μmol of ATP hydrolyzed per min/mg of protein) was similar to that of a haploid wild-type strain (Cox et al., 1978).

Properties of Purified F1 from αD261N/αR365W Mutant—Purified αD261N/αR365W mutant F1 was obtained in low yield (0.025 mg/g wet weight cells), but showed normal chromatographic profile on S-300 gel filtration, and normal subunit composition in SDS gels. The Vmax of ATPase activity was 20 units/mg, with Km(MgATP) = 90 μM, and kcat/Km = 1.41 × 106 M−1 s−1, i.e. similar to wild-type and αR365W F1. The pH dependence of ATPase activity between pH 6.0 and 8.5 was assayed and was similar to that of wild-type and αR365W F1, indicating that the oligomeric stability of the enzyme was not impaired by the αD261N mutation in this pH range. Above pH 8.5 the mutant enzyme did appear to show loss of activity, possibly due to subunit dissociation. All the assays reported below were done at pH ≤ 8.5.

Yohda et al. (1988) found previously that the αD261N mutation (in Bacillus PS3) did not significantly affect subunit assembly when the mutant α-subunit was reconstituted into an αβγ subcomplex. Compared to the wild-type subcomplex, the mutation caused nearly 5-fold reduction in Vmax of ATPase activity of the mutant αβγ subcomplex, and abolished apparent negative cooperativity that was evident in the wild-type in Lineweaver-Burk plots. The effect on Vmax was larger than was seen here in intact F1, but comparisons are difficult because both the species and enzyme forms are different. In our ATPase assays, with the intact F1 enzymes from wild-type, αR365W, or αD261N/αR365W, plots of V versus [S] showed no deviation from simple monophasic Michaelis-Menten kinetics between 5 and 100% of Vmax. We did not scrutinize the kinetic behavior at very low substrate concentrations.

The fluorescence properties of the purified mutant F1 were interesting. It may be recalled that in the αR365W single mutant F1 as purified ("native F1") the noncatalytic sites are

| Mutant | succinate plates | growth yield | membrane ATPase activity |
|--------|-----------------|--------------|----------------------|
| Wild-type | ++ + + + + | 100 | 100% |
| Unc | - | 50 | NS* |
| αR365W | ++ + + + + | 119 | 65% |
| αK175E/αR365W | - | 50 | NS |
| αK175I/αR365W | ++ | 94 | 3.0 |
| αD261N/αR365W | ++ + + + + | 95 | 25 |

* Specific activity of wild-type (pDP34N/P2) = 3.2 μmol of ATP hydrolyzed per min/mg.

NS, not significant.
essentially filled with endogenous adenine nucleotide and the tryptophan fluorescence spectrum ($\lambda_{em} = 295 \text{ nm}$) is the same as for wild-type $F_1$, because fluorescence of the $\alpha$-Trp$^{365}$ residues is fully quenched (Weber et al., 1994). When $\alpha$R365W $F_1$ is depleted of nucleotide by gel filtration in 50% (w/v) glycerol-containing buffer, the marked fluorescence signal of the $\alpha$-Trp$^{365}$ residues is revealed. In contrast, in the case of $\alpha$D261N/$\alpha$R365W purified $F_1$, the fluorescence of the $\alpha$-Trp$^{365}$ residues was already fully apparent even in the native $F_3$, without requiring nucleotide depletion (Fig. 1). This showed that the $\alpha$D261N mutation had a strong effect, and that in native $F_3$ from the $\alpha$D261N/$\alpha$R365W mutant, the noncatalytic sites were empty. Addition of high concentrations of nucleotide quenched the fluorescence of the $\alpha$D261N/$\alpha$R365W mutant $F_1$ almost down to the level of the wild-type enzyme, and it is reasonable to ascribe the residual difference between the signal of quenched $\alpha$D261N/$\alpha$R365W mutant $F_1$ and wild-type in Fig. 1 to the presence of a small amount of contaminating protein in the mutant enzyme.

Binding of Free ATP or MgATP to Noncatalytic Sites of Purified $\alpha$D261N/ $\alpha$R365W $F_1$—The fluorescence signal of the $\alpha$-Trp$^{365}$ residues was used to characterize binding of nucleotides to noncatalytic sites in the mutant enzyme. Fig. 2A and B, show titration of native $\alpha$D261N/$\alpha$R365W $F_1$ with MgATP and free ATP, together with titration of nucleotide-depleted $\alpha$R365W $F_1$ for comparison. The titration with free ATP was done as described earlier (Weber et al., 1994) in buffer containing EDTA. For the MgATP titration we had to modify the conditions used earlier (Mg/ATP ratio of 4/10 and a maximal MgSO$_4$ concentration of 2.5 mM) because the binding affinity for MgATP was very low. Here we used 25 mM MgSO$_4$ in the buffer and titrated with ATP up to 25 mM. Binding parameters were calculated by fitting theoretical curves to the data and calculated $K_d$ values are tabulated in Table II.

MgATP was seen to bind with the same affinity to all three noncatalytic sites of $\alpha$R365W $F_1$ (Fig. 2A; Table II, first line), similar to previous results (Weber et al., 1994). In $\alpha$D261N/ $\alpha$R365W $F_1$, MgATP binding affinity was drastically reduced (Fig. 2A, Table II, first line). In all likelihood the affinity was reduced by the same factor at all three sites, although as seen in Fig. 2A it was only possible to fill ~2 sites under the experimental conditions. Free ATP was seen to bind with the same affinity to all three sites in $\alpha$R365W $F_1$ (Fig. 2B, open circles); however, for $\alpha$D261N/$\alpha$R365W $F_1$ a better fit was obtained assuming one site of higher and two sites of lower affinity (Fig. 2B, closed circles). Nevertheless, as is clear from inspection of Fig. 2B the two enzymes were not actually very different in overall behavior toward free ATP. The calculated $K_d$ values are given in Table II, second line. It is obvious from Fig. 2 that free ATP bound with much weaker affinity than MgATP in $\alpha$R365W $F_1$, but in contrast, free ATP bound with similar or slightly higher affinity than MgATP in $\alpha$D261N/$\alpha$R365W $F_1$.

Binding of Free ADP or MgADP to Noncatalytic Sites of Purified $\alpha$D261N/ $\alpha$R365W $F_1$—MgADP and free ADP binding were also studied in native $\alpha$D261N/$\alpha$R365W and nucleotide-depleted $\alpha$R365W mutant $F_1$ preparations following the same procedures as for free ATP and MgATP above. The binding curves are shown in Fig. 3. A and B. In each case all three noncatalytic sites behaved identically, and the calculated binding parameters are given in Table II, third and fourth lines. It is seen that MgADP bound much more weakly to $\alpha$D261N/ $\alpha$R365W $F_1$ than to $\alpha$R365W $F_1$ (Fig. 3A), and that in $\alpha$R365W $F_1$, free ADP bound more weakly than MgADP (Table II, third and fourth lines). However, free ADP binding to the $\alpha$D261N/ $\alpha$R365W $F_1$ showed, somewhat unexpectedly, a relatively high affinity (Table II, fourth line). Possible reasons for this effect


**DISCUSSION**

We studied nucleotide binding to the noncatalytic sites of E. coli F1-ATPase using the fluorescence signal of the genetically engineered residue α-Trp<sup>365</sup> as a direct probe of noncatalytic site nucleotide occupancy (Weber et al., 1994). Residues α-Lys<sup>175</sup> and α-Asp<sup>261</sup> are both known to lie close to bound nucleotide in noncatalytic sites (Abrahams et al., 1994). The double mutations αK<sub>175/L365W</sub>, αK<sub>175/L365W</sub>, and αD261N/L365W were constructed. Unfortunately, purified F<sub>1</sub> could not be obtained from the first two mutants because of impaired assembly and oligomeric instability. This appears to be a general feature of mutations at the α-Lys<sup>175</sup> locus. However, purified F<sub>1</sub> was obtained from the αD261N/L365W mutant, and it provided valuable information about the noncatalytic sites.

From the nucleotide-binding data described under "Results" we can draw three conclusions. First, removal of the Asp carboxyl at position α-261 by the Asp → Asn mutation greatly reduces noncatalytic site affinity for MgATP and MgADP (Figs. 2A and 3A), indicating that the α-Asp<sup>261</sup> carboxyl interacts directly with the Mg moiety of bound Mg-nucleotide. This is consistent with the x-ray structure (Abrahams et al., 1994). Second, it is apparent that this interaction is the primary determinant of the preference of noncatalytic sites for Mg-nucleotides, and provides a large component of the overall binding energy. From the <em>K<sub>n</sub></em> values for MgATP and MgADP in Table II it is apparent that in combination residue α-Asp<sup>261</sup> and the Mg of the Mg-nucleotide provide about 3.2 kcal/mol of the MgATP binding energy, and that the corresponding value for MgADP is about 2.5 kcal/mol. Third, since Asp is the residue at position α-261 in wild-type, the natural ligands will be MgATP and/or MgADP.

It was interesting that free ADP was bound with higher affinity by the αD261N/L365W enzyme than by the αR365W enzyme (Fig. 3B). A possible explanation is that free ADP and the α-Asp<sup>261</sup> carboxyl in the noncatalytic sites of the αR365W enzyme undergo mutual electrostatic repulsion, and thus the Asp → Asn mutation can assist binding in this situation. This was not the case, however, with free ATP, which bound overall with relatively low affinity to both enzymes (compare Fig. 2B with Fig. 3B). The presence of two mutations in the binding site in the αD261N/L365W enzyme places limitations on the interpretations of these findings.

For some time we have propounded the view that F<sub>1</sub> catalysis is not dependent upon occupancy of the noncatalytic sites (Perlin et al., 1984; Wise and Senior, 1985), although this idea has been challenged (Bullough et al., 1988; Milgrom et al., 1990, 1991; Allison et al., 1992; Harris, 1993). In a recent paper we provided evidence showing that rapid rates of catalysis were achieved in αR365W F<sub>1</sub> under conditions where less than one
noncatalytic site was filled by nucleotide, on average, per enzyme molecule (Weber et al., 1994). The data in this paper reaffirm our conclusion, by demonstrating unequivocally that the αD261N/αR365W enzyme, with empty noncatalytic sites, still hydrolyzes MgATP.

The αD261N/αR365W mutant strain grew normally on succinate plates and in limiting glucose medium, showing that rates of oxidative phosphorylation in vivo were normal. Thus, it might appear that oxidative phosphorylation occurs in this strain without any requirement for the noncatalytic sites to be occupied by nucleotide. It could be argued that the proton gradient (Δp) alters the behavior of the αD261N/αR365W noncatalytic sites in membrane-bound enzyme, such that they are induced to bind nucleotide, although this seems unlikely given the clear structural rationale for the mutational effect. No doubt, however, the αD261N/αR365W mutant will be helpful in seeking answers to this question, and also to the wider question of the possible regulatory or modulating roles of the noncatalytic sites under the variety of growth conditions encountered by E. coli in vivo.

REFERENCES
Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621-628
Allison, W. S., Jault, J. M., Zhuo, S., and Paik, S. R. (1992) J. Bioenerg. Biomembr. 24, 469-477
Al-Shawi, M. K., Parsonage, D., and Senior, A. E. (1988) J. Biol. Chem. 263, 19633-19639
Bullough, D. A., Brown, E. L., Saario, J. D., and Allison, W. S. (1988) J. Biol. Chem. 263, 14053-14060
Cox, G. B., Downie, J. A., Gibson, F., and Radlik, J. (1978) Biochem. J. 170, 593-598
Garrett, N. E., and Penefsky, H. S. (1975) J. Biol. Chem. 250, 6640-6647
Harris, D. A. (1993) FEBS Lett. 316, 209-215
Hyndman, D. J., Milgrom, Y. M., Bramhall, E. A., and Cross, R. L. (1994) J. Biol. Chem. 269, 28871-28877
Jault, J. M., and Allison, W. S. (1993) J. Biol. Chem. 268, 1558-1566
Jououchi, M., Maeda, M., and Futai, M. (1993) J. Biochem. (Tokyo) 114, 171-176
Milgrom, Y. M., and Cross, R. L. (1993) J. Biol. Chem. 268, 23179-23185
Milgrom, Y. M., Ehler, L. L., and Boyer, P. D. (1990) J. Biol. Chem. 265, 18725-18728
Milgrom, Y. M., Ehler, L. L., and Boyer, P. D. (1991) J. Biol. Chem. 266, 11551-11558
Perlín, D. S., Cox, D. N., and Senior, A. E. (1983) J. Biol. Chem. 258, 9793-9800
Perlín, D. S., Latchney, L. R., Wise, J. G., and Senior, A. E. (1984) Biochemistry 23, 4998-5003
Rao, C., Pagan, J., and Senior, A. E. (1988) J. Biol. Chem. 263, 15957-15963
Senior, A. E., Lee, R. S. F., Al-Shawi, M. K., and Weber, J. (1992) Arch. Biochem. Biophys. 297, 340-344
Vandegar, M., Weber, M., Hutton, C., and Batt, C. (1988) Gene (Amst.) 65, 129-133
Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945-951
Weber, J., and Senior, A. E. (1995) J. Biol. Chem. 270, 12653-12658
Weber, J., Wilke-Mounts, S., Lee, R. S. F., Grell, E., and Senior, A. E. (1993a) J. Biol. Chem. 268, 20126-20133
Weber, J., Lee, R. S. F., Wilke-Mounts, S., Grell, E., and Senior, A. E. (1993b) J. Biol. Chem. 268, 6241-6247
Weber, J., Wilke-Mounts, S., Grell, E., and Senior, A. E. (1994) J. Biol. Chem. 269, 11261-11268
Wise, J. G., and Senior, A. E. (1985) Biochemistry 24, 6949-6954
Wise, J. G., Duncan, T. M., Latchney, L. R., Cox, D. N., and Senior, A. E. (1983) Biochem. J. 215, 343-350
Yohda, M., Ohta, S., Hisabori, T., and Kagawa, Y. (1988) Biochim. Biophys. Acta 933, 156-164