Investigation of the Aurovertin Binding Site of *Escherichia coli* F_1-ATPase by Fluorescence Spectroscopy and Site-Directed Mutagenesis†

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ABSTRACT: (1) Previous mutational analyses have shown that residue βR398 of the β-subunit is a key residue for binding of the inhibitory antibiotic aurovertin to *Escherichia coli* F_1F_o-ATP synthase. Here, we studied purified F_1 from the βR398C and βR398W mutants. ATPase activity in both cases was resistant to aurovertin inhibition. The fluorescence spectrum (λ_{exc} = 278 or 295 nm) of βR398W F_1 showed a significant red-shift as compared to wild-type and βR398C enzymes, indicating that residue βR398 lies in a polar environment. On the basis of this and previous evidence, we propose that aurovertin binding to F_1-ATPase involves a specific charged donor–acceptor H-bond between residue βR398 and the 7-hydroxyl group of aurovertin. (2) The fluorescent substrate analog *lin*-benzo-ADP was shown to bind to βR398W F_1 catalytic sites with the same K_d values as to wild-type F_1, and with the same quenching of the fluorescence of the analog. Fluorescence energy transfer was seen between the βR398W residue and bound *lin*-benzo-ADP. Analysis of transfer efficiency at varying stoichiometry of bound *lin*-benzo-ADP showed that interaction occurred between one βR398W residue and one catalytic-site-bound analog molecule at a distance of approximately 23 Å. The relationships of the aurovertin and catalytic sites in the primary and tertiary structure are discussed.

Aurovertin is an antibiotic which inhibits oxidative phosphorylation and ATPase activity in F_1F_o-ATP synthase (Lardy et al., 1975; Lardy, 1980) as a result of binding tightly to the β-subunit of the enzyme (Satre et al., 1980; Dunn & Futai, 1980). Inhibition is of the "uncompetitive" type (Chang & Penefsky, 1973; Wise et al., 1983). With purified soluble F_1 from *Escherichia coli*, 50% inhibition of ATPase activity occurs at a concentration ~1 μM. A total of 3 mol of aurovertin can bind per mole of F_1. The K_d of binding to isolated β-subunit (with a stoichiometry of 1 mol/mol) is 3–6 μM (Issartel et al., 1983; Issartel & Vignais, 1984).[1]

We have shown recently that residue βR398 of the F_1–β-subunit is a key residue for aurovertin binding. An *E. coli* mutant strain, originally selected for resistance of oxidative phosphorylation to aurovertin (Satre et al., 1978, 1980), was shown to contain the point mutation βR398H (Lee et al., 1989). F_1 from this mutant had normal catalytic activity which was not inhibited by up to 20 μM aurovertin, and no binding of the antibiotic to F_1 or isolated β-subunit occurred at concentrations of aurovertin up to 25 μM (Satre et al., 1980; Issartel et al., 1983; Lee et al., 1989). Subsequently, the mutations βR398C and βR398W were introduced by site-directed mutagenesis and shown to confer similar aurovertin resistance on membrane-bound F_1-ATPase. In each of these two mutants, growth characteristics and catalytic activities of membrane-bound F_1 (ATPase and ATP-driven proton pumping) were similar to wild-type (Lee et al., 1991). In two bacterial species (*Bacillus* PS3 and *Bacillus firmus* OF4) where F_1F_o-ATP synthase activity is aurovertin-insensitive (Hicks & Krulwich, 1990; Kagawa & Nukiwa, 1981), the residue equivalent to βR398 (which is usually strongly conserved across species lines) is phenylalanine.

In this paper, we first examine the fluorescence properties of the βR398W mutant F_1, and from the data obtained, we make a proposal regarding the structure and environment of the aurovertin binding site. Second, we demonstrate that the fluorescent nucleotide analog *lin*-benzo-ADP binds to catalytic sites of the βR398W mutant F_1 with the same characteristics with which it binds to wild-type *E. coli* F_1, as recently described (Weber et al., 1992). Third, using the substituted βR398W residue with catalytic-site-bound *lin*-benzo-ADP as a donor–acceptor pair in fluorescence energy-transfer measurements, we determine the distance of the aurovertin binding site from the catalytic nucleotide binding site.

Materials and Methods

Materials

Aurovertin B was obtained from Sigma (St. Louis). Aurovertin D was a generous gift of Professor R. B. Beechey (Aberystwyth). *lin*-Benzo-ADP was synthesized as described by Leonard et al. (1976, 1978); its concentration in aqueous solutions was determined spectrophotometrically using an extinction coefficient of 9750 M^{-1} cm^{-1} at 331 nm (Leonard et al., 1976).

Methods

Strains of E. coli. Strains containing the βR398W or βR398C mutation were described previously (Lee et al., 1991). The mutations were transferred to plasmid pDP34 (Maggio et al., 1988) using an *Xma3–KpnI* restriction fragment in order to overexpress mutant F_1. As a source for wild-type F_1, strain SWM1 (Rao et al., 1988) was used.

F_1 Purification and Characterization. Purified soluble F_1-ATPase was obtained as described by Weber et al. (1992).

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[1] As described in Linnett and Beechey (1979) and Linnett et al. (1983), the aurovertins actually comprise a family of closely-related compounds. Previous work on *E. coli* F_1F_o-ATP synthase used aurovertin B or D, which had very similar inhibitory characteristics.

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Purity and subunit composition were checked by gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970). Protein concentrations were determined using the Bio-Rad protein assay (Bradford, 1976), with bovine serum albumin as standard. The molecular mass of F_1 was taken to be 382000 Da (Senior & Wise, 1983). ATPase activities were measured as described by Duncan and Senior (1985), using final F_1 concentrations of ≥26 nM and incubating for 1–5 min. The linear dependence of P_i release with time was seen under these conditions.

Fluorescence Techniques. The fluorescence measurements were performed in a SPEX Fluorolog 2 spectrofluorometer at 23 °C. With the exception of the aurovertin B binding experiments, which were carried out as described by Wise et al. (1981), a buffer containing 50 mM Tris–sulfate/2.5 mM $MgSO_4$, pH 8.0, was used. F_1 was preequilibrated with the buffer by passage of 0.1-mL aliquots through 1-mL Sephadex G-50 centrifuge columns (Penefsky, 1977). Emission spectra were corrected by comparing the technical spectra of tyrosine and tryptophan with the corrected data as given by Eftink (1991). Quantum yields for F_1 Trp residues were determined by the comparative method of Parker and Rees (1960), using a value of 0.13 (Eftink, 1991) for free tryptophan used as a standard.

Equilibrium Binding Analysis. The number of binding sites and dissociation constants for binding of *lin*-benzo-ADP to purified F_1 were determined as described by Weber et al. (1992) using the decrease of the fluorescence of the nucleotide analog (λ_{exc} = 332 nm, λ_{em} = 388 nm) as the signal.

Energy-Transfer Measurements. To determine the distance between the Trp residue at position β398 and enzyme-bound *lin*-benzo-ADP from the efficiency of resonance energy transfer [for a review, see Stryer (1978)], the difference between the fluorescence emission spectrum of βR398W F_1 and that of wild-type F_1 (λ_{exc} = 295 nm; see Figure 1B) was considered to represent the fluorescence emission spectrum of the energy donor. The absorption spectrum of the acceptor, the bound nucleotide analog, was measured on a Beckman DU-70 spectrophotometer. The critical transfer distance, R_0 (at which the efficiency of the energy transfer is 50%), was calculated as described by Stryer (1978). Values for N (refractive index) = 1.4 (Fairclough & Cantor, 1978), κ^2 (orientation factor) = 0.667 (Stryer, 1978; Haas et al., 1978), and Q_0 (quantum yield of the donor in absence of acceptor) = 0.12 (see Results) were used for this calculation, which yielded a value R_0 = 21.5 Å for the donor–acceptor pair under consideration. Measurements of the efficiency of energy transfer were based on experiments in which *lin*-benzo-ADP was added to βR398W F_1. When the *lin*-benzo-ADP binding reaction had reached equilibrium ($t \leq 30$ min), the Trp emission spectrum was recorded and corrected for the contribution of *lin*-benzo-ADP emission. Correction for inner filter effects due to *lin*-benzo-ADP absorption was based on experiments in which binding of the analog was prevented by preincubation with a large excess of ATP. In order to correct for a small amount of energy transfer between Trp residues other than βW398 and bound *lin*-benzo-ADP, parallel experiments were performed with wild-type F_1. The apparent energy-transfer efficiency, E_{app} (see Figure 3), was determined from the quantum yield of the donor in absence (Q_0) and presence (Q_T) of the energy acceptor at the binding stoichiometry given by the experimental conditions:

$$E_{app} = 1 - Q_T/Q_0 \quad (1)$$

In order to determine the distance between donor and acceptor from the energy-transfer efficiency (E), the *lin*-benzo-ADP binding stoichiometry had to be taken into account according to the equation:

$$E = E_{app} \frac{3[\text{enzyme(total)}]}{[\text{ligand(bound)}]} \quad (2)$$

The concentration of bound ligand was calculated from the total concentrations of enzyme and ligand in the respective experiment using the equilibrium binding parameters as given under Results.

Results

General Properties of the βR398C and βR398W Mutant Purified F_1. Purified F_1 from the βR398C mutant had a specific ATPase activity equal to that of wild-type enzyme; βR398W F_1 had 85% of wild-type activity. In each mutant, F_1-ATPase activity was unaffected by 10 μM aurovertin B or D. No change in aurovertin B fluorescence was seen on addition of either mutant F_1, indicating that no binding of aurovertin B occurred. Both mutant enzymes eluted with the same molecular size as wild-type F_1 on a Sephacryl S-300 gel filtration column, and showed the same subunit composition as the wild-type enzyme on SDS gels.

Fluorescence Properties of βR398W Mutant Purified F_1. Wild-type *E. coli* F_1 contains a total of nine Trp residues, comprising one in each α-subunit, one in each β-subunit, two in the γ-subunit, and one in the δ-subunit (Walker et al., 1984). The βR398W mutant enzyme therefore contains a total of 12 tryptophans, with 2 in each β-subunit. The fluorescence emission spectra of wild-type and βR398W mutant F_1 are given in Figure 1; the spectra of the βR398C mutant enzyme were identical to those of the wild-type. Upon excitation at 278 nm (Figure 1A), where the emission spectra show the contributions of Tyr and Trp residues, introduction of the three additional Trp residues led to a red-shift of the emission maximum from 324 nm in wild-type F_1 to 331 nm in the βR398W mutant. Upon excitation at 295 nm (Figure 1B), which selectively reveals tryptophan fluorescence, the emission maximum of the wild-type enzyme was at 330 nm and that of the βR398W mutant at 337 nm. As can be seen from the difference spectrum (Figure 1B), the red-shift was due to the fact that the three genetically-engineered Trp residues in position β398 exhibit an emission maximum at 341 nm. These data indicate that, on average, the environment of the Trp residues in wild-type F_1 is rather nonpolar whereas that of the additional Trp residues in the βR398W mutant is fairly polar [see, e.g., Eftink (1991)]. Therefore, we may conclude that the aurovertin binding site is, at least in the vicinity of residue βR398, rather polar. It may be noted that the amino acid sequence immediately adjacent to residue βR398 contains numerous charged and/or polar residues (Walker et al., 1984). The average quantum yield of the Trp residues in the wild-type enzyme was estimated to be 0.10; that of the Trp residues in position β398 was 0.12. The latter value resembles closely that for tryptophan in aqueous solution [0.13; see Eftink (1991)].

The quantum yield and wavelength position of the emission maximum (at λ_{exc} 278 nm or at λ_{exc} 295 nm) were not affected when ADP or ATP (both ~300 μM) or P_i (up to 7.5 mM) was added to either wild-type or βR398W mutant F_1. These findings suggest that there is no direct interaction of residue β398 with ligands bound to the catalytic site and moreover that the local environment of this residue is apparently not altered, on time average, as a result of conformational changes consequent upon substrate binding or turnover.

Fluorescence Energy Transfer from Residue βW398 to Bound lin-Benzo-ADP. The fluorescence response of βR398W mutant F_1 upon addition of the fluorescent nucleotide analog

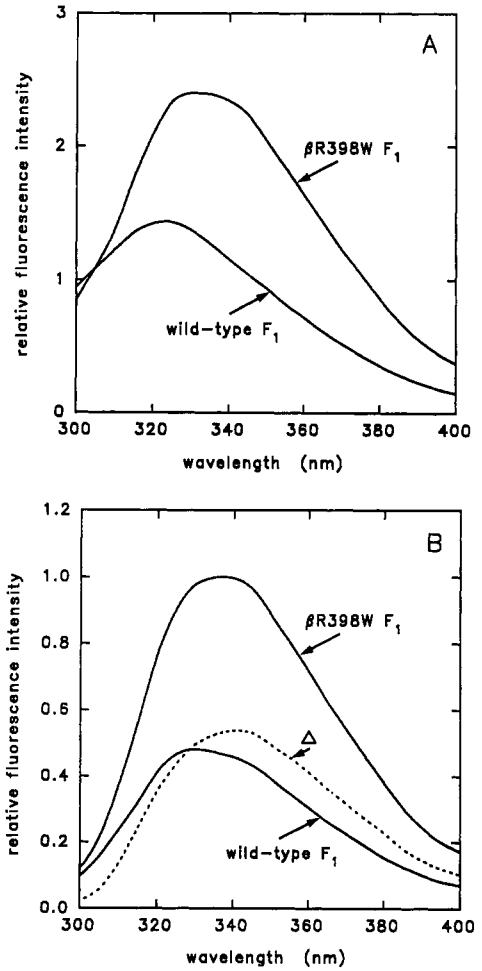

FIGURE 1: Fluorescence emission spectra of wild-type and βR398W mutant *E. coli* F_1. The protein concentration of both enzyme samples was 0.75 μM. (A) λ_{exc} 278 nm; (B) λ_{exc} 295 nm. In (B), the difference spectrum (βR398W F_1 minus wild-type F_1, marked by Δ) is given. For further details, see Methods.

lin-benzo-ADP was of interest because the absorption spectrum of this analog overlaps the tryptophan emission spectrum, and it had been previously shown that *lin*-benzo-ADP is able to occupy the three catalytic nucleotide binding sites on wild-type *E. coli* F_1 (Weber et al., 1992). Analysis of resonance energy transfer between the two fluorophores (Förster, 1948; Stryer, 1978), with the Trp residues as donor and bound *lin*-benzo-ADP as acceptor, could offer the possibility of obtaining information about the spatial relationship between aurovertin and catalytic nucleotide binding sites. Preliminary experiments showed that the tryptophan fluorescence of βR398W mutant F_1 was significantly reduced upon addition of *lin*-benzo-ADP whereas, under identical conditions, that of the wild-type enzyme was only marginally affected. As the properties of *lin*-benzo-ADP and -ATP as ligand or substrate for the *E. coli* F_1-ATPase were very similar to those of ADP and ATP [Weber et al., 1992; for the mitochondrial enzyme, see Kauffman et al. (1978) and Weber et al. (1990)], it was not likely that the fluorescence decrease of the βR398W mutant observed in the presence of *lin*-benzo-ADP, but not in the presence of ADP (see above), was due to a process other than resonance energy transfer.

For evaluation of energy-transfer data (see Methods), the binding stoichiometry of the acceptor in the respective experiment had to be known. Therefore, we determined the equilibrium parameters for binding of *lin*-benzo-ADP to βR398W mutant F_1. As with wild-type enzyme, the emission

Table I: Binding of *lin*-Benzo-ADP to Wild-Type and βR398W Mutant *E. coli* F_1

F_1	F^a	$K_{d1}{}^b$ (μM)	K_{d2} and $K_{d3}{}^b$ (μM)
wild-type (βR398)[c]	0.22	0.20	5.5
βR398W mutant	0.19	0.25	5.4

[a] F is the relative fluorescence intensity (λ_{exc} 332 nm, λ_{em} 388 nm) of bound *lin*-benzo-ADP, with the value for the free analog (in buffer) set at 1.00. [b] The dissociation constants K_d were determined as described in the text. [c] The data for wild-type F_1 were taken from Weber et al. (1992).

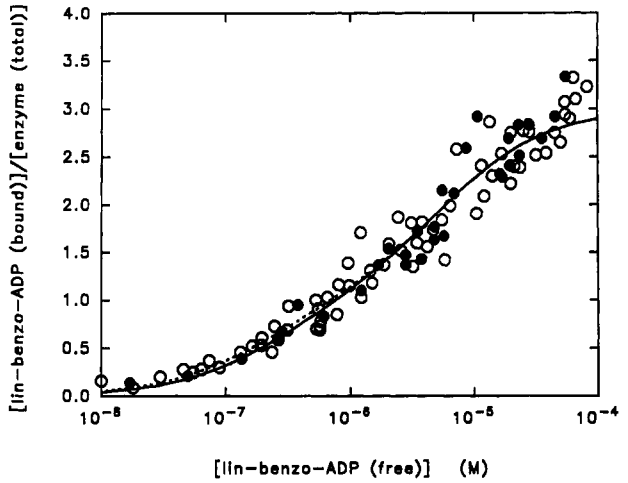

FIGURE 2: Titration of wild-type and βR398W mutant *E. coli* F_1 with *lin*-benzo-ADP. Experimental details are given under Methods. The lines are computer-generated fits; the calculated K_d values are given in Table I. The data for the wild-type enzyme are taken from Weber et al. (1992). (●—●) βR398W; (○---○) wild-type F_1.

spectrum of *lin*-benzo-ADP (λ_{exc} 332 nm) maintained its wavelength position upon binding; however, the fluorescence intensity was reduced by about 80% as compared to that of the free *lin*-benzo-ADP [Table I; see also Weber et al. (1992)]. On the basis of this signal, which is due to interaction of the fluorophore, i.e., the modified adenine moiety, with the Tyr residue in position β331 (Weber et al., 1992), several titration experiments were performed. The resulting binding curve is shown in Figure 2 from which it is clear that the binding characteristics of the mutant βR398W enzyme did not differ significantly from those of wild-type F_1. At free ligand concentrations around 50 μM, the binding stoichiometry was approximately 3 mol of *lin*-benzo-ADP/mol of F_1. Dissociation constants were determined by fitting of theoretical binding curves to the experimental data by nonlinear least-squares analysis, assuming a model with three independent binding sites, two of them being identical. The fit could not be improved by assuming a model with three different sites. The resulting K_d values are given in Table I. For wild-type F_1, it had been described (Weber et al., 1992) that a 200–300-fold excess of ATP over *lin*-benzo-ADP was able to displace rapidly all bound analog, as judged from the increase of the *lin*-benzo-ADP fluorescence intensity up to the value for the free analog. In the study presented here, this displacement could be shown by using a different signal. At similar concentration ratios, ATP rapidly restored the intensity of the tryptophan fluorescence of βR398W mutant F_1 which had been preequilibrated with *lin*-benzo-ADP back to the value for the free enzyme.

The efficiency of energy transfer was measured at protein concentrations of 0.4–1.6 μM and ligand concentrations of 1–35 μM, thus covering nearly the complete range of possible

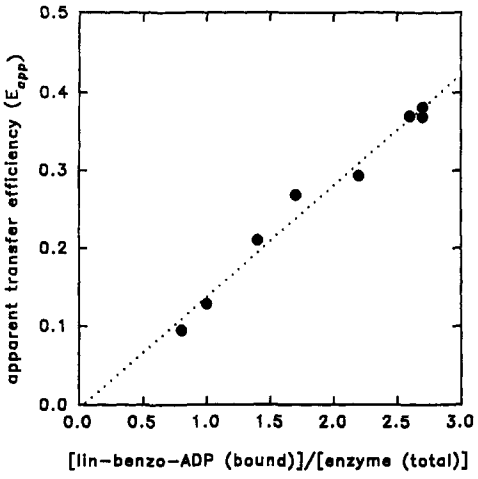

FIGURE 3: Energy transfer between Trp in position β398 and bound *lin*-benzo-ADP. The apparent transfer efficiency, E_{app}, is plotted as a function of the binding stoichiometry. For details, see Methods and the text. The dotted line was obtained from least-squares linear regression analysis.

lin-benzo-ADP binding stoichiometries. The apparent energy-transfer efficiency E_{app}, which is not corrected for incomplete occupation of the catalytic binding sites by the energy acceptor *lin*-benzo-ADP, is plotted versus the binding stoichiometry in Figure 3. As can be seen, a direct proportionality exists between the two parameters; thus, after correction (see Methods), the transfer efficiency (E) is independent of the binding stoichiometry. This indicates that only one of the three Trp residues in position β398 is able to efficiently transfer energy to a particular acceptor molecule. Otherwise, E would be expected to decrease with an increase of the binding stoichiometry, because upon binding of a second or (especially) a third *lin*-benzo-ADP molecule per F_1, these ligands would have to compete for transfer energy from the same donor residue(s). From the data points given in Figure 3, the average value for the transfer efficiency E was determined to be 0.42. As the critical transfer distance R_0 for the donor–acceptor pair under consideration was calculated to be 21.5 Å (see Methods), the distance between residue β398 in the aurovertin binding site and the base moiety of a nucleotide bound to the catalytic site is approximately 23 Å.

Discussion

Structure of the Aurovertin Binding Site. Aurovertins B and D have almost identical biological properties, and differ chemically in that aurovertin D is hydroxylated at the C-2 position (Linnett & Beechey, 1979). Aurovertin A, which is known to be much less effective than aurovertin B or D as an inhibitor of F_1-ATPase (Linnett & Beechey, 1979), is acetylated at the 7-hydroxyl position, and acetylation of the 7-hydroxyl in aurovertin B greatly reduced the inhibitory activity (Satre et al., 1980). Aurovertins B and D, but not A, retain tightly-bound H_2O on crystallization from organic solvents and in $CHCl_3$ solution, suggesting that the 7-OH group is involved in the tight H_2O binding (Linnett et al., 1983). These authors state that "high affinity for water may relate to the mechanism of inhibition of ATPase and ATP synthase activities by the aurovertins". The data presented here show that at least the part of the aurovertin binding site around residue β398 in F_1 is polar, consistent with this earlier work.

Chang and Penefsky (1973) studied fluorescence properties of aurovertin dissolved in solvents of increasing hydrophobicity or viscosity. Whereas hydrophobicity did not markedly enhance fluorescence, viscous solvents did. They also studied the polarization of aurovertin fluorescence in viscous solvents, or when bound to F_1. There was a marked increase in polarization of aurovertin fluorescence on binding of the antibiotic to F_1, and the fluorescence spectrum was similar to that seen in glycerol at 5 °C. Hence, these workers concluded that the enhanced fluorescence seen on binding of aurovertin to F_1 is due to increased "rigidity" of bound fluorophore, rather than to nonpolarity of the binding site.

With these previous findings in mind, we present here a proposal for the structure of the aurovertin binding site in F_1. The mutational analyses and sequence homologies described above clearly implicate residue βR398 as a key residue for aurovertin binding. Charge alone appears not to be a determining factor in binding since aurovertin is uncharged at pH 6–8 (Chang & Penefsky, 1974), consistent with its structure (Linnett & Beechy, 1979), and the βR398H mutant showed the same lack of inhibition of ATPase activity by aurovertin at pH 6.0 or 8.5 (Lee et al., 1989). Bound aurovertin appears to be markedly impaired in rotational mobility as discussed above. We propose therefore that residue βR398 forms a specific hydrogen bond with bound aurovertin, to the 7-OH group in aurovertins B and D. Loss of a charged donor–acceptor H-bond by mutagenesis of F_1 residue βR398 or by acetylation of aurovertin at the 7-OH position would remove 3–4 kcal/mol of interaction energy (Fersht, 1988). This would be sufficient to raise the K_d of the *E. coli* F_1–aurovertin complex from ~1 μM into the range 160–860 μM, i.e., sufficient to confer "aurovertin-resistance" upon *E. coli* F_1 under experimental conditions previously reported in the literature, where the maximal aurovertin concentration tested was 20–25 μM. Our proposal therefore explains "aurovertin-resistance" in βR398 mutants. It should be remarked that we do not exclude the involvement of other residues in the binding of aurovertin; indeed, this would be expected from the arguments presented above.

Distance between the Aurovertin Binding Site and the Catalytic Site. The fluorescence energy-transfer measurements revealed that at all binding stoichiometries of *lin*-benzo-ADP the same efficiency (E) was seen, indicating a distance of approximately 23 Å between a catalytic site and an aurovertin site. It is pertinent to ask whether this is the distance between *lin*-benzo-ADP bound in the catalytic site of one β-subunit and the tryptophan at residue 398 in the same β-subunit or in a different β-subunit. Recent electron microscopy (Gogol et al., 1989a,b) and X-ray crystallography studies (Bianchet et al., 1991) of F_1 suggest a hexagonal arrangement of F_1 subunits, in which each β-subunit is spatially separated by intervening α-subunits from the two other β-subunits. From the X-ray crystallography studies, it might be argued that in some regions the distance between two β-subunits is less than 23 Å. However, in all scenarios where a hypothetical site A on one β-subunit comes within ≤23 Å of a hypothetical site B on a second β-subunit, the distance from site A to site B on the first β-subunit would also be ≤23 Å. As shown under Results, the data strongly suggested that energy transfer occurred from only one donor site to any given acceptor site. Therefore, the most likely interpretation is that we are measuring here a 23-Å *intra*subunit fluorescence energy transfer between binding sites for the catalytic nucleotide and aurovertin on the same β-subunit.

Location of the Catalytic Nucleotide Binding Site and the Aurovertin Binding Site in the β-Subunit Sequence. Secondary structure prediction (Duncan et al., 1986; Senior, 1988) suggests that a region of β-subunit sequence comprised of residues β140–335, approximately, may form a β-sheet

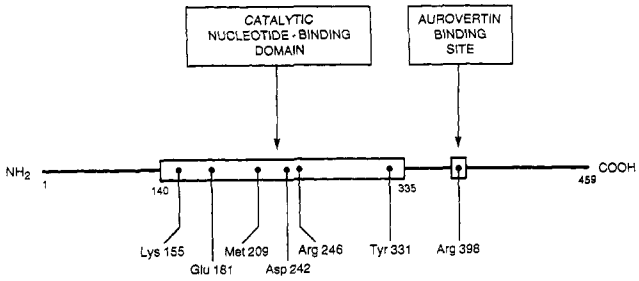

FIGURE 4: Location of functional regions in the β-subunit primary sequence. Residues Lys-155, Glu-181, etc. are some of the sites of mutagenesis, chemical inactivation, or (photo)affinity labeling which have served to define the location of the catalytic nucleotide binding domain [reviewed in Senior (1988) and Lee et al. (1991)].

structure typical of nucleotide binding domains, in this case consisting of six parallel β-strands with five intervening α-helices. Extensive mutagenesis experiments and (photo)affinity-labeling experiments have confirmed that this is the location of the catalytic nucleotide binding domain [reviewed in Senior (1988) and Lee et al. (1991)]. Figure 4 shows the location of the catalytic nucleotide binding domain in the β-sequence, in relation to the aurovertin binding site. The residues marked (Lys-155, Glu-181, etc.) are some of the well-studied sites of chemical modification, (photo)affinity labeling, or mutagenesis which have helped to define the catalytic site. The aurovertin binding site is shown centered on residue β398. These two defined functional regions of β-subunit were shown in this work to be separated by approximately 23 Å in the tertiary structure. This is consistent with the evidence mentioned in the introduction that aurovertin is an "uncompetitive"-type inhibitor of F_1-ATPase, and also with the finding that aurovertin and nucleotide may be bound contemporaneously to isolated β-subunit (Issartel & Vignais, 1984).

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