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Location of Lysine-β162 in Mitochondrial F_1-Adenosinetriphosphatase†

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ABSTRACT: The quenching of the fluorescence of bovine heart F_1-adenosinetriphosphatase labeled specifically at its essential Lys-β162 with 7-chloro-4-nitro-2,1,3-benzoxadiazole (*N*-NBD-F_1) by 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-triphosphate (TNP-ATP) has been studied. Analysis of the fluorescence data in the presence of 1 mM ATP shows that the dissociation constant of TNP-ATP from its first binding site in the covalently labeled enzyme is 250-fold lower than that of ATP, which it replaces in pH 7.0 buffer containing 25% glycerol, and that this binding causes a 54% quenching of the fluorescence of the *N*-NBD label due to energy transfer to the weakly fluorescent TNP-ATP molecule. Computation based on the observed quenching gives a distance of 25.6 ± 0.4 Å between the NBD label and the chromophore of the bound TNP-ATP molecule. Since the distance between the chromophore and the farthest O atom of the bound TNP-ATP is about 16 Å, it seems quite likely that the ϵ-amino group of Lys-β162 is near the γ-phosphate group of the TNP-ATP bound at the catalytic site. Similar measurements in the presence of 1 mM ADP show that the replacement of ADP at the catalytic site by TNP-ATP causes a 49% quenching of the fluorescence of the *N*-NBD label, which gives a distance of 26.5 ± 0.4 Å between the label and the chromophore of the bound TNP-ATP molecule.

Since the determination of the amino acid sequence of the β subunit of a number of F_1-ATPases[1] (Sarate et al., 1981; Kanazawa et al., 1982; Runswick et al., 1983; Kurawski et al., 1982; Krebbers et al., 1982), several essential functional groups have been identified with specific labeling reagents. In the case of F_1-ATPase from bovine heart mitochondria, the amino acid residues with essential functional groups identified in this way include Glu-β199 (Yoshida et al., 1982), Tyr-β197 (Ho & Wang, 1983), Tyr-β311 (Andrews et al., 1984a), Lys-β162 (Andrews et al., 1984b), and Lys-β401 (Sutton & Ferguson, 1985). The available data seem to show that the essential Lys labeled by NBD-Cl is Lys-β162. Although the probable secondary structure of the β subunit has been predicted (Kanazawa et al., 1982), we have little direct information on the locations of the above essential functional groups relative to the substrate bound at the catalytic site.

Labeling one of the three Lys-β162 residues in bovine heart mitochondrial F_1-ATPase with NBD-Cl completely inactivates the enzyme. Is this because the labeled single Lys-β162 is at the catalytic site or because the labeling induces long-range conformation change that inactivates the protein? The question could be answered by determining the location of the *N*-NBD label relative to the bound substrate. In the present work, the quenching of fluorescence of the *N*-NBD label on Lys-β162 of F_1 in the presence of 1 mM ATP by added TNP-ATP has been determined as a function of TNP-ATP concentration. Since the TNP-ATP is bound to the catalytic site (Grubmeyer & Penefsky, 1981) and since the molar ratios of *N*-NBD label to F_1 and bound TNP-ATP to F_1 are both less than 1, it is possible to calculate the distance between the *N*-NBD label and the chromophore of the TNP-ATP in a very simple way directly from the observed efficiency of electronic excitation energy transfer from the single donor group to the single acceptor group in the labeled enzyme.

Experimental Procedures

Materials

2′,3′-*O*-(2,4,6-Trinitrocyclohexadienylidene)adenosine 5′-triphosphate (TNP-ATP) was purchased from Molecular Probes and further purified by chromatography on Sephadex LH-20-100 (Hiratsuka & Uchida, 1973). [^{14}C]NBD-Cl was supplied by Research Products International Corp. It was found to have a specific radioactivity of 77 mCi/mmol (Wang et al., 1986).

Mitochondria were prepared from fresh bovine heart (Low et al., 1963). F_1-ATPase was prepared from frozen mitochondria by Betty Stone in our laboratory and stored as described by Knowles and Penefsky (1972). *N*-[^{14}C]NBD-F_1

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; F_1 or F_1-ATPase, F_1-adenosinetriphosphatase; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; *N*-NBD-F_1, F_1 labeled by NBD-Cl at its essential Lys-β162; *O*-NBD-F_1, F_1 labeled by NBD-Cl at its essential Tyr in its catalytic β subunit; qs, quinine sulfate; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate, also called 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-triphosphate.

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was prepared by the procedure reported previously (Wang, 1984). The radioactively labeled F_1, with a label/F_1 molar ratio n equal to 0.59, was stored under liquid nitrogen in frozen solutions of buffer A (50 mM Hepes–NaOH, pH 7.0, 2 mM EDTA, and 25% glycerol).

Methods

Assays. Protein concentrations were determined by the Coomassie Blue binding method (Bradford, 1976). Radioactivity was assayed by liquid scintillation counting with Aquasol II (New England Nuclear) containing 5% water as the counting liquid. The counting efficiency in this medium was determined to be 92% with [^{14}C]toluene standard supplied by New England Nuclear. The specific radioactivity of the [^{14}C]NBD-Cl was determined to be 77 mCi/mmol by linear extrapolation of the ATPase activity vs. label/F_1 molar ratio plot as described before (Wang, 1985). ATPase activity was assayed by an ATP-regenerating system coupled to the oxidation of NADH at 30 °C.

Fluorescence Measurements. The fluorescence emission of N-[^{14}C]NBD-F_1 under 440-nm exciting light was measured with a Hitachi–Perkin-Elmer fluorescence spectrophotometer (MPF-2A). Calibration measurements show that, after correction for Raman scattering by the medium, the intensity of fluorescence is proportional to the concentration of N-NBD-F_1. A 2-mL solution containing 0.210 mg of N-[^{14}C]NBD-F_1 (0.3 μM) in buffer A was used for each set of fluorescence measurements. One- or two-microliter aliquots of 0.5 or 2.0 mM solution of TNP-ATP in buffer A were added successively with glass micropipets. The solution was mixed with a 1-mL mechanical pipet with polypropylene tip after each addition and assayed for fluorescence after a waiting period of a few minutes.

Correction for the Fluorescence of Free and Bound TNP-ATP. In order to obtain the fluorescence due to N-NBD-F_1, the observed fluorescence of the solution should be corrected for the weak fluorescence due to free and bound TNP-ATP in the solution. A reference solution containing 0.3 μM unlabeled F_1 and 1 mM ATP in buffer A was titrated by the successive additions of 1- or 2-μL aliquots of TNP-ATP solution in exactly the same way as the N-NBD-F_1 sample solution was titrated. For each total TNP-ATP concentration, the observed fluorescence emission by N-NBD-F_1 was obtained as the difference in fluorescence between the sample and the reference solutions.

Correction for the Inner Filter Effect. The exciting light at 440 nm and the emitted light monitored at 520 nm were both attenuated appreciably as the concentration of TNP-ATP was increased. Thus each observed fluorescence intensity was multiplied by an appropriate factor to correct for this inner filter effect. For the sample in a 1 cm × 1 cm quartz cuvette, the exciting light is attenuated approximately by a factor of $10^{-A_{440}/2}$ and the emitted light is attenuated approximately by a factor of $10^{-A_{520}/2}$. Consequently, each observed fluorescence intensity was multiplied by an overall correction factor of $10^{\langle A\rangle}$ due to the inner filter effect, where $\langle A\rangle = (A_{440} + A_{520})/2$.

Determination of the Quantum Efficiency of N-NBD-F_1. The quantum efficiency of N-NBD-F_1 was found to be 3.57×10^{-2} by using quinine sulfate with a quantum efficiency (Q_{qs}) of 0.70 (Scott et al., 1970) as a reference standard according to

$$Q_{N\text{-NBD-F}_1} = Q_{qs}(I_{320}/I_{440})(f_{N\text{-NBD-F}_1}/f_{qs})(A_{qs}/A_{N\text{-NBD-F}_1})$$

where I_{320}/I_{440}, which denotes the ratio of light intensities of the xenon lamp at 320 and 440 nm, respectively, was found to be 0.127. The ratio of the total emission output $f_{N\text{-NBD-F}_1}$ of the 0.3 μM N-NBD-F_1 solution to the total emission output f_{qs} of a 50 nM quinine sulfate in 0.1 N H_2SO_4 solution was found to be 1.112 by cutting out and weighing the recorded emission spectra (Cantley & Hammes, 1975). The ratio of the absorbance of the quinine sulfate at 320 nm (A_{qs}) to that of the N-NBD-F_1 solution at 440 nm ($A_{N\text{-NBD-F}_1}$) was found to be 0.361 from experimental values obtained at higher concentrations.

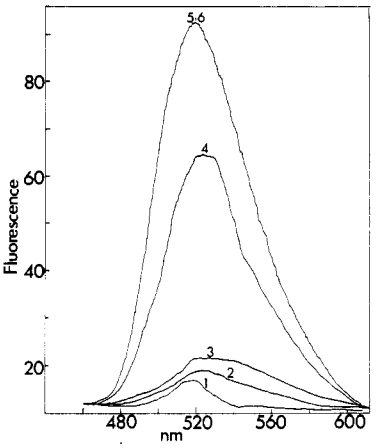

FIGURE 1: Fluorescence spectra of (N-NBD)$_{0.59}F_1$ and F_1 in different solutions. Composition of the solutions: (1) 0.3 μM F_1 + 1 mM ATP in buffer A; (2) 6.0 μM TNP-ATP; (3) 0.3 μM F_1 + 6.0 M TNP-ATP in buffer A; (4) 0.3 μM (N-[^{14}C]NBD)$_{0.59}F_1$ + 6.0 μM TNP-ATP in buffer A; (5) 0.3 μM (N-[^{14}C]NBD)$_{0.59}F_1$ in buffer A; (6) 0.3 μM (N-[^{14}C]NBD)$_{0.59}F_1$ + 1 mM ATP in buffer A. Buffer A contains 50 mM Hepes–NaOH at pH 7.0, 2 mM EDTA, and 25% (v/v) glycerol.

Computation of the Overlap Integral. The overlap integral in Förster's theory of energy transfer

$$J = \left[\int_0^\infty f_D(\nu)\epsilon_A(\nu)(d\nu/\nu^4)\right] \Big/ \left[\int_0^\infty f_D(\nu)(d\nu/\nu^4)\right]$$

where the subscripts D and A refer to donor and acceptor groups, respectively, was computed by the approximate equation that was used by Cantley and Hammes (1975):

$$J = \sum_\lambda f_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda / \sum_\lambda f_D(\lambda)\Delta\lambda$$

By choosing $\Delta\lambda$ = 5 nm and summing over the entire emission spectrum of N-NBD-F_1 in buffer A, it was found that $J = 44.7 \times 10^{-15}$ cm^6/mmol.

RESULTS

The effect of TNP-ATP on the fluorescence spectra of N-NBD-F_1 is illustrated in Figure 1. The binding of TNP-ATP to F_1 increases the fluorescence of the former, but the enhanced fluorescence of bound TNP-ATP at 520 nm (excited at 440 nm) is still much lower than that of an equivalent concentration of N-NBD-F_1. Upon the successive addition of TNP-ATP to a 0.3 μM solution of N-NBD-F_1 in buffer A, the fluorescence of the N-NBD-F_1 solution was found to decrease after each addition. This was because the quenching of the fluorescence of N-NBD-F_1 by bound TNP-ATP was greater than the enhancement of fluorescence due to the added TNP-ATP. The addition of ATP has no effect on the fluorescence spectrum of N-NBD-F_1 in buffer A (Ferguson, et al., 1975; Wang, 1984).

Since the labeled enzyme contains only 0.59 mol of the fluorescent label per mole of F_1, which is bound covalently to the catalytic β subunit (Wang, 1985), it could be used directly for determining the distance between Lys-β162 (Andrews et al., 1984a) and the bound substrate TNP-ATP, if the molar

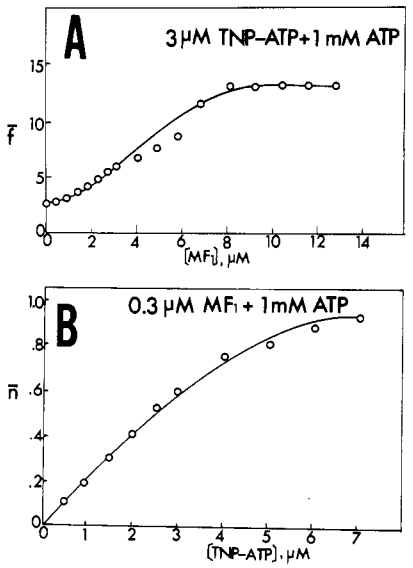

FIGURE 2: Fluorometric measurement of binding of TNP-ATP to F_1 in buffer A containing 1 mM ATP. (A) Corrected molar fluorescence intensity (f) at 545 nm (excitation wavelength 408 nm) of 3.0 μM TNP-ATP in buffer A containing 1 mM ATP and different concentrations of F_1-ATPase. The molar fluorescence increment due to the binding of TNP-ATP to F_1 determined in this way is 3.50×10^6 arbitrary units M^{-1}. (B) Number ($\bar{n}$) of moles of TNP-ATP bound per mole of F_1-ATPase in buffer A containing 0.3 μM F_1 + 1 mM ATP and different concentrations of TNP-ATP.

ratio of bound TNP-ATP to F_1 does not exceed unity.

In aqueous Mg^{2+} buffer at pH 7.5 containing 0.25 M sucrose, each F_1-ATPase binds 2 TNP-ATP molecules with high affinity (Grubmeyer & Penefsky, 1981). By measuring the enhanced fluorescence emission of TNP-ATP when it is bound to F_1, we found that even in buffer A containing 6 μM TNP-ATP, each F_1 binds 1.8 TNP-ATP molecules. But in buffer A containing 1 mM ATP, less than one TNP-ATP molecule is bound to each F_1 up to a total TNP-ATP concentration of 7 μM as shown in Figure 2.

The observed fluorescence emission at 545 nm (excitation at 408 nm) was corrected for the inner filter effect and for the blank due to F_1. Figure 2A shows that this corrected fluorescence intensity increases as the concentration of F_1 in buffer A is raised from 0 to 8 μM while the ligand concentrations are kept constant at [ATP] = 1 mM and [TNP-ATP] = 3 μM. The increased intensity is due to a larger fraction of TNP-ATP bound to F_1 at a higher protein concentration. But for $[MF_1] < 3$ μM the increase is less than what would be expected if all bound TNP-ATP had the same K_d and exhibited the same enhancement in fluorescence intensity upon binding. Actually, in this dilute protein concentration range some F_1 molecules bind more than one TNP-ATP molecule, and the second TNP-ATP molecule is bound with lower affinity (relative to ATP which it displaces) with lower fluorescence enhancement. However, for $[F_1] > 8$ μM, the corrected fluorescence intensity stays constant and is unaffected by further increase in protein concentration. The observation shows that for $[F_1] > 8$ μM each TNP-ATP molecule in the system is already bound exclusively to its own F_1 molecule, and hence its fluorescence will not increase further at higher protein concentrations. By dividing this limiting fluorescence increment by the concentration of TNP-ATP (3 μM), we obtain 3.50×10^6 as the molar fluorescence increment for the binding of TNP-ATP to F_1 in buffer A containing 1 mM ATP.

The number $\bar{n}$ of TNP-ATP molecules bound to each F_1 molecule in a solution of buffer A containing 1 mM ATP can be readily obtained from the observed fluorescence increment, the TNP-ATP concentration, and the molar fluorescence increment obtained from Figure 2A. The values of $\bar{n}$ obtained in this way are plotted against the concentration of TNP-ATP in Figure 2B for solutions containing 0.3 μM F_1 and 1 mM ATP, which shows that the number $\bar{n}$ of TNP-ATP molecules bound to each F_1 never exceeded unity up to [TNP-ATP] = 7.0 μM. Consequently it should be possible to determine unambiguously the distance between the N-NBD label on Lys-β162 and the chromophore of TNP-ATP bound at the catalytic site of F_1 by energy-transfer measurements.

Table I: Fluorescence Values of N-NBD-F_1 in the Presence of 1 mM ATP and Different Concentrations of TNP-ATP[a]

[TNP-ATP] (μM)	f_{obsd}	$10^{\langle A \rangle}$	f	$(f_1 - f)/(f - f_2)$
0.0	77.3	1.0000	77.3	0.000
0.2	73.7	1.0055	74.1	0.082
0.4	70.9	1.0110	71.7	0.154
0.6	69.3	1.0166	70.5	0.194
1.0	66.7	1.0278	68.6	0.263
1.5	63.5	1.0419	66.2	0.360
2.0	60.3	1.0563	63.7	0.478
3.0	54.9	1.0856	59.6	0.726
4.0	50.2	1.1157	56.0	1.024
5.0	47.0	1.1467	53.9	1.267

[a] The observed and corrected fluorescence intensities at 520 nm (f_{obsd} and f) under 440-nm excited light are expressed in arbitrary units. Each value of f_{obsd} was obtained as the difference between the emission intensity of a sample solution containing 0.3 μM N-NBD-F_1 + 1 mM ATP at a given concentration of TNP-ATP in buffer A and that of a reference solution containing 0.3 μM F_1 + 1 mM ATP at the same concentration of TNP-ATP in buffer A at 23 °C. Values in the last column were computed with $f_1 = 77.3$ and $f_2 = 35.2$ (details are given in the text).

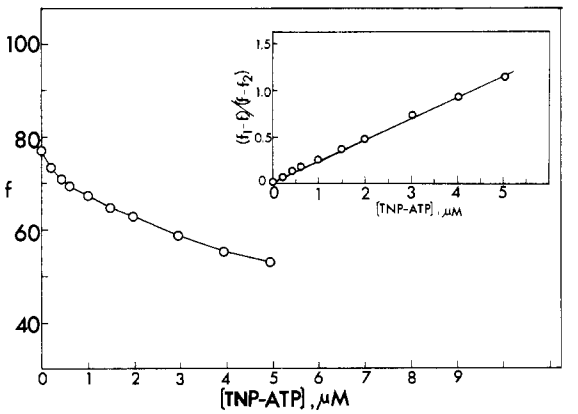

FIGURE 3: Quenching of fluorescence of $(N\text{-NBD})_{0.59}F_1$ by TNP-ATP in buffer A containing 1 mM ATP. Solution compositions are given in the legend of Table I. The inset shows a linear plot according to eq 3.

The observed fluorescence (f_{obsd}) of N-NBD-F_1 in a solution containing TNP-ATP was obtained by subtracting the fluorescence of a reference solution containing the same concentrations of TNP-ATP and unlabeled F_1 from the fluorescence of the sample solution of TNP-ATP and N-NBD-F_1 in the same buffer. Values of f_{obsd} for solutions with the same concentration of N-NBD-F_1 but different concentrations of TNP-ATP are listed in Table I. These values should be corrected for inner filter effect due to the absorption of both the exciting light and emitted light by the TNP-ATP in solution. As discussed under Methods, the correction factor was approximated by $10^{\langle A \rangle}$ where $\langle A \rangle = (A_{440} + A_{520})/2$. Values of $\langle A \rangle$ and the corrected fluorescence values (f) for various concentrations of TNP-ATP are plotted in Figure 3 and are also listed in Table I.

The equilibrium constant K for the ligand-exchange reaction

$$F_1(ATP)_{n-1}(TNP\text{-}ATP) + ATP \rightleftarrows F_1(ATP)_n + TNP\text{-}ATP$$

may be written as $K = (a - x)(b - x)/(x[ATP])$, where a and b represent the total molar concentrations of the protein and TNP-ATP, respectively, and x represents the concentration of the mixed complex. Since $b \gg x$ in most of the concentration range in the present experiments, we may write

$$(a - x)/x = (K/b)[ATP] \quad (1)$$

The fluorescence f of N-NBD-F_1 is given by

$$af = (a - x)f_1 + xf_2 \quad (2)$$

where f_1 and f_2 represent the fluorescence of N-NBD-F_1 without TNP-ATP bound to it and with TNP-ATP bound to its catalytic site, respectively. Therefore

$$(f_1 - f)/(f - f_2) = b/K[ATP] \quad (3)$$

In the absence of TNP-ATP, f_1 was measured directly to be 77.3 arbitrary units. Since it was not possible to measure f_2 directly in a solution of high TNP-ATP concentration without using large inner filter corrections of dubious validity, eq 3 was solved by the iterative method with assumed values of f_2 until it was found that the equation with $f_2 = 35.2$ was consistent with all the measured values of f within experimental uncertainties. The calculated values of $(f_1 - f)/(f - f_2)$ are listed in Table I and also plotted vs. [TNP-ATP] in the inset of Figure 3. The slope of the linear plot gives $K = 4.0 \times 10^{-3}$ for the above ligand-exchange equilibrium. Since $K = (K_d$ for TNP-ATP$)/(K_d$ for ATP), we may conclude that K_d for the single TNP-ATP bound to N-NBD-F_1 in buffer A at pH 7.0 is 250-fold lower than that for ATP which competes with it for the same binding site. This binding site is the catalytic site, because the bound TNP-ATP is hydrolyzed slowly in the presence of Mg^{2+} and is also a competitive inhibitor for ATP hydrolysis (Grubmeyer & Penefsky, 1981).

The efficiency E of energy transfer from the N-NBD label to the TNP-ATP bound to the catalytic site is given by (Förster, 1948; Haugland & Stryer, 1967)

$$E = (R_0/R)^6/[(R_0/R)^6 + 1] \quad (4)$$

or

$$R = R_0[(1 - E)/E]^{1/6} \quad (5)$$

where R is the distance between the donor and the acceptor groups. R_0 is the distance corresponding to 50% transfer efficiency and is given by

$$R_0 = (9.79 \times 10^3)(\kappa^2 Q J n^{-4})^{1/6} \quad (6)$$

κ is the relative orientation factor of the oscillating dipoles, Q is the quantum yield of N-NBD-F_1 in the absence of TNP-ATP, J is the overlap integral, and n is the refractive index of the medium.

For energy transfer from the N-NBD label to the bound TNP-ATP, the overlap integral, J, has been found to be 44.7×10^{-15} cm^6/mmol and the quantum yield, Q, of the N-NBD label has been found to be 3.51×10^{-2} (reported under Methods). By choosing $\kappa^2 = {}^2/_3$ (the average value for random orientation) and $n = 1.4$, we obtain from eq 6 the value of 26.3 Å for R_0.

Values in Table I and Figure 2 show that $E = 42.1/77.3 = 0.544$. Therefore, eq 5 gives

$$R = 26.3[(1 - 0.544)/0.544]^{1/6} = 25.6 \text{ Å}$$

as the distance between the N-NBD label and the 2′,3′-O-(2,4,6-trinitrocyclohexadienylidyl) group of the TNP-ATP bound at the catalytic site of the labeled F_1-ATPase. The estimated probably error in this distance on the basis of the results of three independent experiments is ±1.4%.

Table II: Fluorescence Values of N-NBD-F_1 in the Presence of 1 mM ADP and Different Concentrations of TNP-ATP[a]

[TNP-ATP] (μM)	f_{obsd}	f	$(f_1 - f)/(f - f_2)$
0.0	69.3	69.3	0.000
0.2	67.4	67.8	0.047
0.4	65.3	66.0	0.107
0.6	63.0	64.1	0.183
1.0	60.8	62.5	0.251
1.5	58.0	60.4	0.353
2.0	55.3	58.4	0.472
3.0	51.8	56.2	0.625
4.0	47.9	53.4	0.875
5.0	44.3	50.8	1.195

[a] Experimental conditions are the same as those for Table I, except that 1 mM ADP was used instead of 1 mM ATP. Values in the last column were computed with $f_1 = 69.3$ and $f_2 = 35.3$.

The data from similar measurements with TNP-ATP solutions containing 1 mM ADP are summarized in Table II. A similar quantitative treatment of the values in Table II gives 26.5 ± 0.4 Å as the distance from the N-NBD label to the TNP-ATP bound at the catalytic site and an equilibrium constant $K = 4.2$ for the ligand-exchange reaction

$$F_1(ADP)_{n'-1}(TNP\text{-}ATP) + ADP \rightleftarrows F_1(ADP)_{n'} + TNP\text{-}ATP$$

Discussion

Measurements of energy transfer in a derivatized F_1-ATPase often yield only the average distances because of the presence of multiple binding sites. This average distance would be equal to the actual distance if the three binding sites are equivalent. But if the β subunits of F_1 are functionally distinct (Wang, 1985), the actual distances could be different, and hence there could be an infinite number of sets of the three individual distances that give the observed transfer efficiency. In such a case, it would be more informative to prepare a derivatized enzyme that has less than one specifically attached donor group and binds less than one acceptor substrate molecule per F_1 so that the distance between this donor-acceptor pair can be determined unambiguously by energy-transfer measurements.

In the present work the number of specific N-[^{14}C]NBD labels per F_1 is only 0.59, which is, within experimental uncertainties, also equal to the fractional inhibition of the ATPase activity of the control F_1 sample. By maintaining the concentration of ATP at 1 mM in the medium, it is also possible to keep the number of TNP-ATP bound at the catalytic site less than 1 per F_1 molecule. Thus the distance between the fluorescent N-NBD label and the chromophore of the bound TNP-ATP can be determined by measuring energy transfer from the single donor group to the single acceptor group.

One may raise the question of whether the binding of TNP-ATP to the catalytic site could also quench the fluorescence of N-NBD label due to ligand-triggered change in protein conformation in addition to direct quenching due to Förster type of energy transfer. The answer is almost certainly to be negative for the following reasons. In the absence of TNP-ATP, ATP itself has no effect on the fluorescence of N-NBD-F_1 (Figure 1, curve 6). Although ADP does quench the fluorescence of N-NBD-F_1 due to ligand-induced change in protein conformation, concentration-dependence studies show that the responsible ADP molecules are bound to second and possibly third sites with K_d values

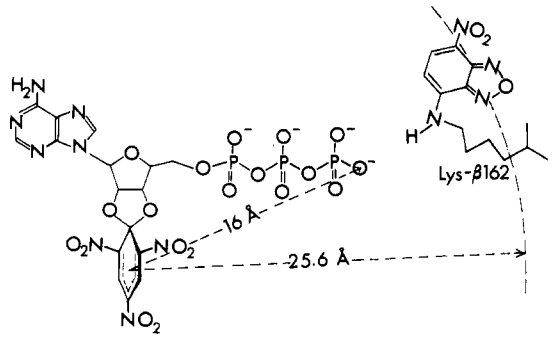

FIGURE 4: Location of *N*-NBD label relative to bound TNP-ATP in *N*-NBD-F_1. The sphere of possible positions of the fluorescent label covalently attached to Lys-β162 are indicated by a large circular arc.

2 or more orders of magnitude higher than that for the catalytic site (Wang, 1984). Since in the present work, the average number of molecules bound to each F_1 is less than 1, the lower affinity auxiliary or regulatory sites can only be occupied by ATP (Table I) of ADP (Table II), which maintains the enzyme in a fixed conformation state for $0 \leq$ [TNP-ATP] ≤ 5 μM.

The possible positions of the *N*-NBD label relative to the structure of the TNP-ATP molecule bound at the catalytic site of F_1-ATPase is illustrated in Figure 4. Inasmuch as the distance from the chromophore to the farthest O atom of TNP-ATP is about 16 Å, it seems quite possible for the ε-amino group of Lys-β162 to be hydrogen-bonded to the γ-phosphate group of the bound substrate as suggested previously (Ting & Wang, 1980). Furthermore, since the *N*-NBD-F_1 was prepared through a direct transfer of NBD label from Tyr-β311 to Lys-β162 in the dark at pH 9, we may infer that Tyr-β311 is near Lys-β162 although these residues may or may not be attached to the same β subunit. But observations on the reaction of *O*-NBD-F_1 with *N*-acetyl-L-cysteine reported in the following paper (Wang et al., 1986) indicate that the labeled Tyr-β311 is at the catalytic site that binds inorganic phosphate. Consequently the NBD-labeled Lys-β162 must also be at or near the catalytic site.

The possible positions of the *N*-NBD label shown in Figure 4 indicate only the approximate location of Lys-β162, since this essential functional group could have moved slightly after it is labeled. On the other hand, because of the inverse sixth power dependence of energy-transfer efficiency on distance, the uncertainty in the above calculated value of R due to the arbitrarily assumed averaged value $\langle\kappa^2\rangle = {}^2/_3$ is not as large as it might appear. For flat donor and acceptor groups with large polarizabilaity in the X and Y directions but much smaller polarizability in the Z direction, simple geometric considerations show that when illuminated with unpolarized light, it is not possible either for all the dipole–dipole pairs to be mutually perpendicular so that $\kappa^2 = 0$ or for all of them to be mutually parallel in an end-to-end arrangement so that $\kappa^2 = 4$. It is possible to have all the dipole–dipole pairs to be mutually parallel in a side-by-side arrangement that would give $\kappa^2 = 1$. Substitution of this value of κ^2 in eq 6 gives $R_0 = 28.2$ Å, and hence $R = 27.9$ Å in 1 mM ATP solution. The possibility that half of the dipole–dipole pairs are mutually perpendicular and the other half of the dipole–dipole pairs are mutually parallel in a side-by-side arrangement would give an average value of $\kappa^2 = {}^1/_2$, which would give $R_0 = 26.0$ Å and $R = 24.4$ Å in 1 mM ATP solution. But none of these possible revised values is sufficiently different from 25.6 ± 0.36 Å to alter the main conclusion that Lys-β162 and Tyr-β311 are probably both at or near the catalytic site of F_1-ATPase.

Why do *O*-NBD-F_1 and *N*-NBD-F_1 still bind ATP or ADP if Tyr-β311 and Lys-β162 are indeed at or near the catalytic site? Figure 4 suggests that the location of the NBD label may not prevent the binding of ATP or ADP, although it could increase the K_d values appreciably. This change in K_d will have no significant effect on the computed correction for the fluorescence due to TNP-ATP (or TNP-ADP) used in the present work for two reasons. First, the correction is always less than 5% of the observed fluorescence in the present experiments. Second, the equilibrium constant K for the binding of TNP-ATP to the enzyme in 1 mM ATP solution is actually equal to $K_{ATP}/K_{TNP\text{-}ATP}$, where K_{ATP} and $K_{TNP\text{-}ATP}$ represent the K_d values of ATP and TNP-ATP, respectively, bound to the catalytic site. Since the NBD label affects K_{ATP} and $K_{TNP\text{-}ATP}$ similarly, their ratio K will remain essentially unaffected by the labeling.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3; L-Lys, 56-87-1.

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