Intersubunit Interactions in Proton-Translocating Adenosine Triphosphatase As Revealed by Hydrogen-Exchange Kinetics†

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ABSTRACT: The rates of hydrogen–deuterium exchange in the peptide groups of the α and β subunits and the α–β subunit complex of proton-translocating adenosine triphosphatase from the thermophilic bacterium PS3 were examined. The exchange was found to be much slower in the isolated β subunit than in the isolated α subunit. This has been taken as indicating that the structure of the β subunit is tighter than that of the α subunit. Adenosine 5′-triphosphate (ATP) causes tightening of a relatively tight portion of the α subunit and of a relatively loose portion of the β subunit. When the α and β subunits are brought into contact, tightening of the α subunit, but not the β subunit, occurs. The effect of ATP on the structure of the β subunit is more pronounced in the presence of the α subunit than in its absence. These findings support the idea proposed previously that the α subunit has an allosteric site and the β subunit a catalytic site and that the conformation of the β subunit is controlled by the α subunit.

Proton-translocating ATPase[1] catalyzes the terminal step of the synthesis of ATP in oxidative phosphorylation (Mitchell, 1976; Boyer et al., 1977; Kagawa, 1978). The soluble portion of the ATPase, F_1, is composed of five subunits (α, β, γ, δ, and ϵ) (Knowles & Penefsky, 1972; Penefsky, 1974). The ATPase activity was inhibited by chemical modification of the β subunit (Deters et al., 1975; Ferguson et al., 1976). Several different nucleotide binding sites in F_1–ATPase have been reported (Garrett & Penefsky, 1975; Harris, 1977). The kinetic experiments have indicated that this ATPase is a kind of allosteric enzyme (Ebel & Lardy, 1975), but the molecular mechanism of the synthesis and utilization of ATP are not yet understood. Thus it is important to obtain more detailed information on the nucleotide–subunit interaction and subunit–subunit interactions of this ATPase.

Yoshida et al. (1975, 1977a,b) succeeded in purification of the stable subunits of TF_1–ATPase from the thermophilic bacterium PS3 and in the reconstitution of TF_1 from the purified subunits. This ATPase and its subunits are so stable that their physical properties can be examined. Recently, we reported studies on the properties of the purified subunits and on nucleotide binding to the purified subunits (Yoshida et al., 1979; Ohta et al., 1980). We found that the purified α and β subunits both have nucleotide binding sites and that the α subunit has an allosteric site but the β subunit has a catalytic one.

Studies on the kinetics of hydrogen-isotope exchange are useful for obtaining information on conformational changes of protein molecules (Hvidt & Nielsen, 1966; Englander, 1975; Ohta et al., 1977). For instance, Ryrie & Jagendorf (1972) used a tritium–hydrogen exchange method to demonstrate a conformational change of chloroplast F_1 induced by illumination. Conformational changes of chloroplast F_1 and TF_1 induced by nucleotide binding have also been reported (Nabedryk-Viala et al., 1977; Ohta et al., 1978). Recently, a computerized Fourier transform infrared spectrometer was found to be useful for detecting a small difference in the hydrogen–deuterium exchange kinetics of two protein samples (Nakanishi & Tsuboi, 1976; Ohta et al., 1978).

Here, we report studies on the kinetics of hydrogen–deuterium exchange of purified α and β subunits of TF_1 from the thermophilic bacterium PS3 and suggest that subunit–subunit interactions are essential for conformational changes of these subunits.

Experimental Procedure

Materials. The α and β subunits of TF_1 from the thermophilic bacterium PS3 were prepared by the method of Kagawa & Yoshida (1979) and purified further by DEAE-Sephacel column chromatography with a NaCl gradient in a solution of 20 mM Tris–SO_4 (pH 8) and 0.1 mM EDTA without urea. Appropriate fractions were dialyzed against distilled water and lyophilized.

ATP and ADP were purchased from Boehringer. Bes was obtained from Nakarai Chemicals, Kyoto. DEAE-Sephacel was obtained from Pharmacia Fine Chemicals.

Kinetic Measurements. The hydrogen–deuterium exchange reaction was followed by infrared absorption measurements using a Digilab FTS-14 Fourier transform infrared spectrometer system. The lyophilized protein sample was dissolved in ^2H_2O at zero time, and the spectral change caused by deuteration at time t was examined. The amount of undeuterated peptide group was taken as proportional to the ratio of the absorbance of the amide II band at $\sim$1550 cm^{-1} to that of the amide I (Blout et al., 1961; Nakanishi et al., 1973). The base line of the amide II band was taken as the absorption curve of a solution of completely deuterated protein, which was obtained by incubation in ^2H_2O at 80 °C for 4 h. The A(amide II)/A(amide I) value for completely undeuterated protein was assumed to be 0.45 (Hvidt & Nielsen, 1966; Nakanishi et al., 1973; Englander, 1975).

In this paper, we use the notation "pH" even for the deuterium ion concentration in ^2H_2O. The pH meter readings, taken with a Hitachi-Horiba pH meter, F-7SS, are given as uncorrected values. The temperature was monitored with a

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[1] Abbreviations used: TF_1, thermostable F_1 (soluble ATPase from thermophilic bacterium PS3); Bes, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; ATPase, adenosine triphosphatase; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate.

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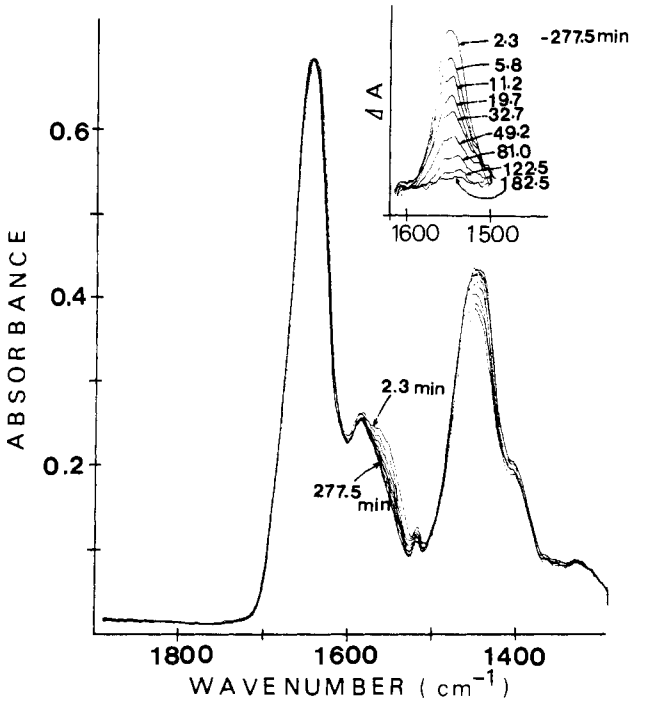

FIGURE 1: A set of recorded curves obtained in the infrared absorption study of the ^1H–^2H exchange reaction of the α subunit of TF_1–ATPase. Measurements were made in ^2H_2O solution containing Bes–Na (pH 7.0) and 5 mM $MgCl_2$ at 32 °C. The protein concentration was 40 mg/mL. The experiment was performed by using a Digilab FTS-14 Fourier transform spectrometer system. The curves given here are all photographic reproductions of the computer output displayed on a plotter; no redrawing has been done. Data acquisitions were made in the following time intervals after the protein was introduced into the ^2H_2O solution at time zero (the number of scans for data acquisition is given in parentheses): 1.3–3.3 min (80), 3.8–7.8 min (160), 8.3–14.2 min (240), 15.2–24.3 min (360), 25.4–40 min (580), 43.3–55 min (480), 75–87 min (480), 115–130 min (600), 175–190 min (600), and 175–285 min (600). Insert: A set of curves obtained by subtracting the infrared absorption curve for 277.5 min (obtained from data in the time interval 175–285 min) from each of the curves obtained at earlier times. The time shown for each curve is the midtime of data acquisition. The difference spectra indicate the amounts of peptide NH groups remaining undeuterated at each time, which were deuterated completely at 277.5 min.

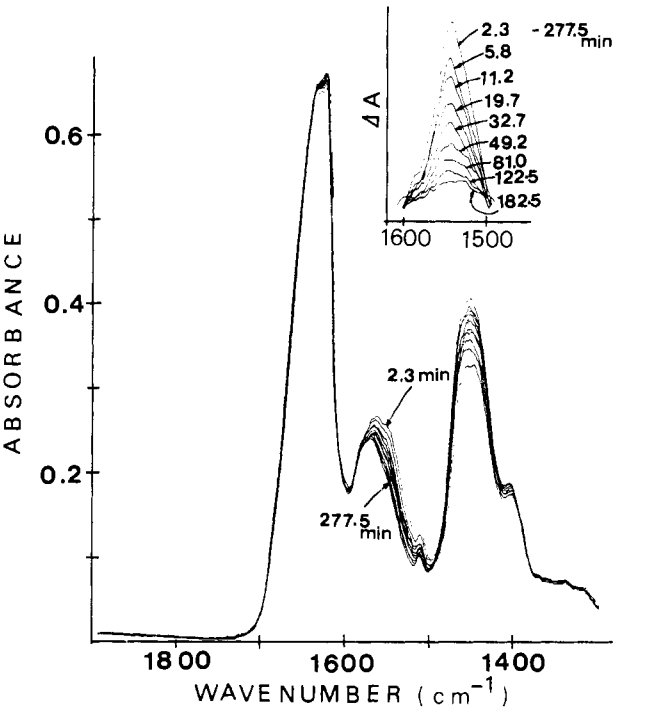

FIGURE 2: A set of recorded curves in the infrared absorption study of the ^1H–^2H exchange reaction of the β subunit of TF_1–ATPase. Experimental conditions and time schedules of data acquisition were exactly the same as in the experiment on the α subunit (see the legend of Figure 1). Insert: A set of curves obtained by computer subtraction in the same way as those for the α subunit (see the legend of Figure 1).

thermocouple and a digital voltmeter system. The infrared spectrum was always taken after passing dry air through the optical path for at least 7 h to remove water vapor.

Use of Infrared Difference Spectra. A small difference in the hydrogen–deuterium exchange rate of two given samples (A and B) was detected as follows. The lyophilized sample A was dissolved in a suitable ^2H_2O buffer (solution A) at zero time, and after 30 s the lyophilized sample B was dissolved in a ^2H_2O solution (solution B). Each solution was placed in a cell of 50-μm light path with CaF_2 windows (cells A and B). Cell A was placed in the sample beam and cell B in the reference beam of the FT-IR system. Up to this stage these procedures took 2 min. At a suitable time (t), the interferogram of the sample beam (sample A) was obtained by accumulating 20 scans. This took 30 s. Immediately afterward, the acquisition of the interferogram of the reference beam (sample B) was started. This was also obtained by accumulating 20 scans. These mutual accumulations were repeated. The interferograms were stored in disk memories in a systematic manner, and the infrared difference spectrum $A_{sample} - A_{buffer} - B_{sample} + B_{buffer}$ for any desired time (t) was obtained later by a Fourier transform. An upward (absorbance) amide II peak at 1550 cm^{-1} indicated that the deuteration of A was slower than the deuteration of B. Conversely a downward amide II band indicated that the deuteration of A was faster than the deuteration of B in the period from time 0 to t. Sometimes the difference spectrum between A at time t_1 and B at time t_2 was examined to confirm the difference in the deuteration rate (see the lowest curve in Figure 5, for example). When no peak or trough appeared at 1550 cm^{-1}, and when $t_1 > t_2$, the deuteration of A was considered to be slower than that of B.

Predeuteration of Proteins. For some experiments the subunits were predeuterated. For this, the protein (30 mg/mL) in 20% sucrose was dialyzed against 3 mL of ^2H_2O buffer consisting of 20 mM Tris–SO_4, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol. The buffer solution was changed twice and finally replaced by 5 mM Bes–Na (pH 7.0) ^2H_2O buffer. Then the preparation was centrifuged, and its protein concentration was measured spectrometrically in a cell of 1-mm light path. The protein was then lyophilized and dissolved in ^2H_2O solvent. This deuterated protein solution was added, at zero time, to an undeuterated sample, so that the deuteration kinetics of the latter (under the coexistence with the former) could be measured.

Results and Interpretations

Characterization of Isolated Subunits. The infrared absorption spectra in ^2H_2O solution of the purified α and β subunits are shown in Figures 1 and 2, respectively. The amide I peak of the α subunit is at 1648 cm^{-1}. That of the β subunit is sharper and located at a lower frequency (1640 cm^{-1}). This difference may be taken as indicating that the β subunit has a greater amount of "antiparallel β sheet structure" than the α subunit (Miyazawa & Blout, 1961).

As deuteration of the peptide NH groups proceeded, the amide II band at 1550 cm^{-1} became weaker, and the amide II′ band at 1450 cm^{-1} became stronger. The process of deu-

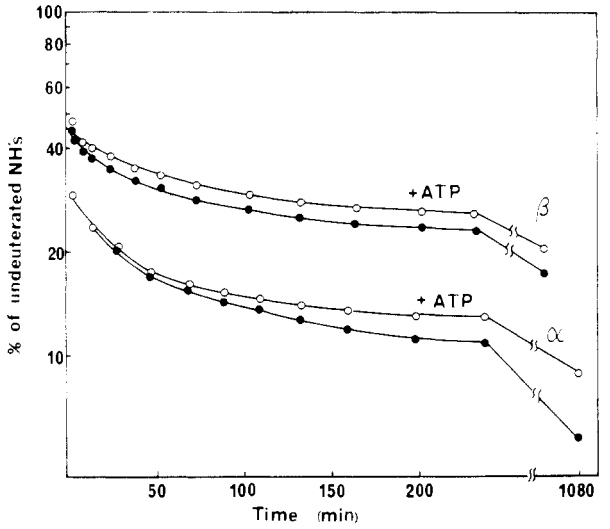

FIGURE 3: Semilogarithmic plots of deuterium exchange of α and β subunits of TF_1-ATPase from PS3. The protein concentration was 30 mg/mL in each solution. The solvents were 0.2 M Bes-Na (pH 7.0) with (○) 8 mM ATP and 10 mM $MgCl_2$ or (●) 5 mM $MgCl_2$. The temperature was 32 °C.

teration was seen more clearly by recording difference spectra (i.e., spectrum at time t minus spectrum at 277.5 min), which are shown as inserts in Figures 1 and 2. Kinetic plots obtained from these infrared absorption measurements are shown in Figure 3. As is seen here, the deuteration proceeds rather slowly, mostly in $\sim$50 min (rate constant $\approx 3 \times 10^{-4}$ s^{-1}) in the order of magnitude. One should be reminded here that the hydrogen-exchange reaction would be much faster in a completely denatured protein with all the peptide groups exposed to the solvent water; it would be $\sim$10 s^{-1} at pH 7, 32 °C (Takahashi et al., 1978). The slow deuteration of the native protein is considered to mean that many of the peptide groups are buried in the molecule or involved in strong intramolecular hydrogen bonds, so that they have only very small chances (10^{-4} or less) of being exposed to the solvent. These small chances are considered to be brought about by a structural fluctuation of the protein molecule (Tsuboi & Nakanishi, 1979). As shown in Figure 3, the hydrogen–deuterium exchange rate was much higher in the α subunit than in the β subunit. This means that the α subunit has greater structural fluctuation so that the chance of a buried peptide group being exposed to the solvent is greater than in the case of the β subunit. Namely, the β subunit seems to have a tighter structure than the α subunit.

Effect of ATP Binding. Figure 3 also indicates that the addition of ATP caused a slight slowdown shift of deuteration of both the α and β subunit. This was confirmed by examining the infrared difference spectrum of (subunit + ATP) – $\text{subunit}_{\text{free}}$ – ATP_{free} as a function of time by the method described above. Results for the α subunit are shown in Figure 4. As can be seen, a positive peak develops at 1550 cm^{-1} (amide II) by 4 h and is slightly larger at 18 h. This means that the deuteration (weakening of the 1550-cm^{-1} band) is appreciably retarded by the presence of ATP. Figure 4 also indicates that ATP decreased the deuteration rate of peptide groups which are deuterated slowly and which are therefore considered to be buried deep in the α-subunit structure. Figure 5 shows the result of a similar experiment on the interaction of ATP with the β subunit. In contrast to the case of the α subunit, the 1550-cm^{-1} peak in the difference spectrum (β subunit + ATP) – β $\text{subunit}_{\text{free}}$ – ATP_{free} is already seen at 6 min, and the size of this peak does not increase with time. This means that the

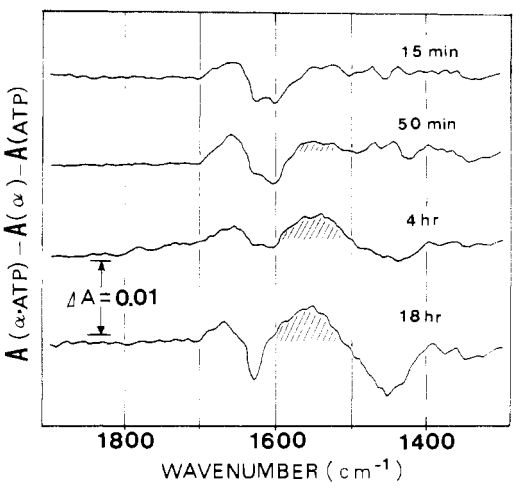

FIGURE 4: Infrared difference spectra caused by slower deuteration of the α subunit·ATP complex than of the free α subunit. The difference spectrum A–B was recorded at the indicated time (t) by the method described under Experimental Procedure (Use of Infrared Difference Spectra), where A is for the α subunit at 30 mg/mL containing 8 mM ATP and B is for the free α subunit. The spectrum of a solution of 8 mM ATP alone was also obtained, and this was subtracted from each A–B difference spectrum. The solvent was 0.2 M Bes-Na (pH 7.0), and the temperature was 32 °C. The number of scans for accumulation = 480.

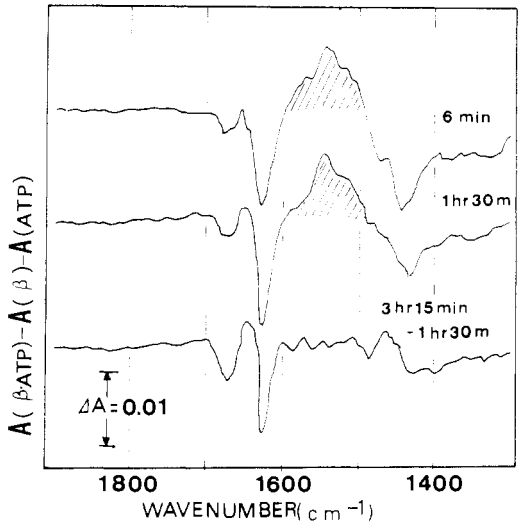

FIGURE 5: Infrared difference spectra caused by slower deuteration of the β subunit·ATP complex than of the free β subunit. Difference spectrum A–B was recorded at 6 min and also at 1 h 30 min by the method described under Experimental Procedure (Use of Infrared Difference Spectra), where A is for a β-subunit solution at 30 mg/mL containing 8 mM ATP and 10 mM $MgCl_2$ and B is for a solution at 30 mg/mL containing 5 mM $MgCl_2$. The spectrum of a solution of 8 mM ATP and 10 mM $MgCl_2$ only was obtained, and this was subtracted from each A–B difference spectrum. The lowest spectrum is the difference spectrum of A at 3 h 15 min and B at 1 h 30 min minus 8 mM ATP. The extent of deuteration of the β subunit·ATP complex at 3.25 h is shown to be nearly equal to that of the ATP-free β subunit at 1.5 h. The solvent was 0.2 M Bes-Na (pH 7.0), and the temperature was 32 °C. The number of scans for accumulation = 480.

binding of ATP induces a tightening of a flexible part (whose peptide groups are rapidly deuterated) of the subunit structure but not of an intrinsically tight portion.

In previous work on intact TF_1 (Ohta et al., 1978) we found that nucleotide binding slows down the deuteration of TF_1 and that this slowdown occurs within a few minutes of the beginning of deuteration. Therefore, the conformational change induced by nucleotide binding seems to occur on the β moiety

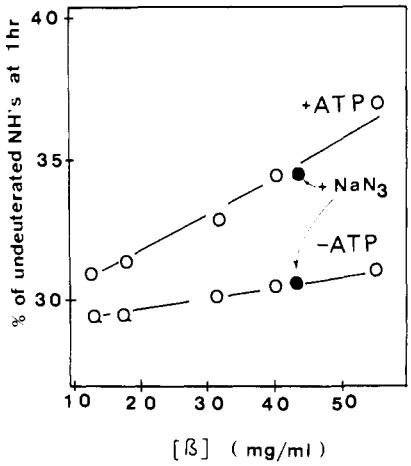

FIGURE 6: Dependence of the rate of deuteration of the β subunit upon the β-subunit concentration. The amount of NH groups remaining undeuterated in the β subunit at 1 h with various concentrations of the β subunit is given along the ordinate. The solvents were 0.2 M Bes–Na (pH 7.0) containing 8 mM ATP and 10 mM $MgCl_2$ (+ATP) or 5 mM $MgCl_2$ only (–ATP) at 32 °C. (●) The solutions contained 5 mM NaN_3.

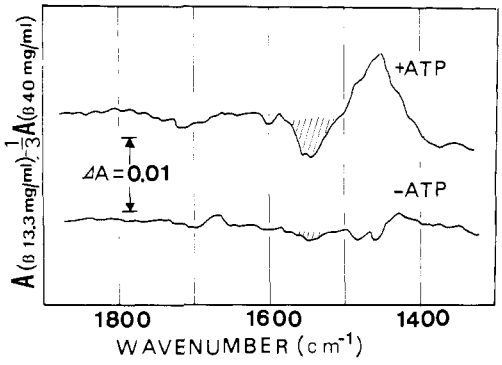

FIGURE 7: Infrared difference spectra of two different concentrations of the β subunit. The spectra were obtained by subtracting the curve of 40 mg/mL β subunit × 1/3 from that of 13.3 mg/mL β subunit at 1 h. The solvents were 0.2 M Bes–Na (pH 7.0) containing 8 mM ATP and 10 mM $MgCl_2$ (upper) or 5 mM $MgCl_2$ (lower) at 32 °C. The number of scans for accumulation = 480.

of TF_1. With intact TF_1, ATP had a greater effect than ADP. With the isolated β subunit, however, ATP and ADP had similar effects on the deuteration kinetics (data not shown).

Interaction of β Subunits. The effect of ATP on the deuteration kinetics of the β subunit was more pronounced at a higher concentration of β subunits (Figure 6); when the β concentration was only 13.3 mg/mL, 8 mM ATP caused only 1.4% retardation of the deuteration of peptide groups at 1 h, whereas when the β concentration was 40 mg/mL, it caused 4% retardation at 1 h. This finding is also shown clearly by the difference spectra between solutions of high and low concentrations of β subunits (see Figure 7). In the absence of ATP, the deuteration kinetics did not depend on the β-subunit concentration, whereas in the presence of ATP, deuteration was slower at high concentrations of β subunits. With intact TF_1, the rate of deuterium exchange was independent of the concentration at concentrations of 20–50 mg/mL, with or without ATP (data not shown). Studies by gel filtration showed that the β subunit formed a dimer and/or a trimer in solutions of low ionic strength. Analytical ultracentrifugation studies supported this finding (data not shown). Studies on circular dichroism, on the other hand, showed that the amount of ATP binding is independent of the β concentration (Ohta et al., 1980). It is, therefore, supposed that the β subunit

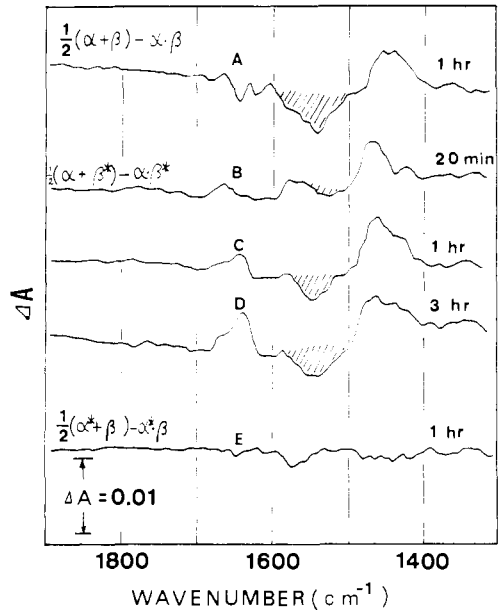

FIGURE 8: Infrared difference spectra caused by slower deuteration of a mixture of the α and β subunits than of the isolated α and β subunits. Protein concentration: (A) 30, (B) 40, (C) 40, (D) 40, and (E) 30 mg/mL. α^* and β^* mean predeuterated α and β subunits, respectively. The spectra were obtained at indicated times after the start of deuteration, by accumulating 480 scans. The solvent was 0.2 M Bes–Na (pH 7.0) and 5 mM $MgCl_2$ at 32 °C.

itself can bind to ATP but that only the dimer or trimer of the β subunit undergoes an ATP-induced conformational change.

It should be mentioned that 5 mM NaN_3, which inhibits ATPase, had no effect on the deuteration kinetics of the β subunit (filled circles in Figure 6).

Interaction of α and β Subunits. Figure 8 shows the infrared difference spectra, α $subunit_{free}$ + β $subunit_{free}$ – α and β $subunit_{mixture}$, which had a negative peak at 1550 cm^{-1} at 1 h after the start of deuteration. Thus, the coexistence of the α and β subunits must cause retardation of peptide deuteration even in the absence of ATP. This raises the question of whether retardation takes place on the α subunit or the β subunit or both. This question was examined by using predeuterated α and β subunits (see Experimental Procedure). As seen in curves B–E of Figure 8, the 1550-cm^{-1} trough appears only when the α subunit is subjected to deuteration. Therefore, the ATP-free α and β interaction is concluded to cause a tightening of the structure of the α-subunit moiety but not of the β moiety.

When ATP is involved in the system, however, an appreciable tightening of the structure of the β subunit is induced by the α–β subunit interaction. As shown above, peptide deuteration of the purified subunit is retarded in the presence of ATP. The extent of retardation is, however, only 2% at 1 h at 18 mg/mL of the β subunit. As shown in Figures 9 and 10, the extent of retardation is greatly enhanced on adding deuterated α subunit. Since the α subunit is already deuterated, the difference between values with and without ATP was entirely attributable to the β subunit. When the ratio of the α and β subunits was 1, retardation amounted to 7% of the total peptide groups of the β subunit. This effect was specific, because the predeuterated albumin had no effect (filled circles in Figure 9 and the lowest curve in Figure 10).

Discussion

Method. The new method described here is very useful for studies on intersubunit (A and B) interaction. The method

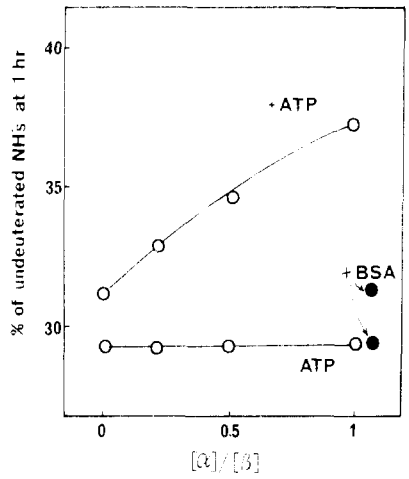

FIGURE 9: Effect of the α subunit on the deuteration rate of the β subunit. The amount of NH groups remaining undeuterated in the β subunit (18 mg/mL) at 1 h with various concentrations of the predeuterated α subunit is shown on the ordinate. The solvents were 0.2 M Bes-Na (pH 7.0) containing 8 mM ATP and 10 mM $MgCl_2$ (+ATP) or 5 mM $MgCl_2$ only (-ATP) at 32 °C. (●) Values obtained by adding 18 mg/mL predeuterated bovine serum albumin instead of the α-subunit to the β-subunit solution.

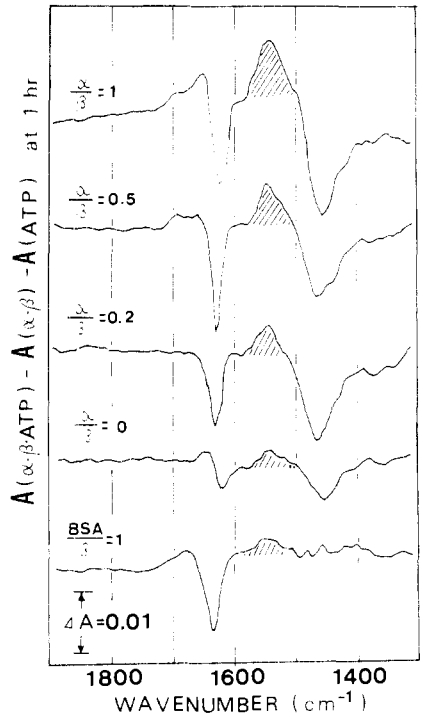

FIGURE 10: Infrared difference spectra caused by slower deuteration of the β subunit with various amounts of the α subunit in the presence of ATP than in the absence of ATP. Each spectrum was obtained at 1 h after the start of deuteration, by accumulating 480 scans. The effect of the α subunit was examined by adding various amounts of predeuterated α subunit to lyophilized β subunit. The lowest spectrum was obtained by adding 18 mg/mL predeuterated bovine serum albumin instead of the predeuterated α subunit. The solvent was 0.2 M Bes-Na (pH 7.0) with 8 mM ATP and 10 mM $MgCl_2$ (+ATP) or with 5 mM $MgCl_2$ only (-ATP) at 32 °C.

consists of measurement of the difference spectrum of A-$B_{mixture}$ - A_{free} - B_{free} as a function of time. Measurements are made from the start of deuteration of the three protein samples, A, B, and A-B mixture. By this method a difference in the deuteration rates of 1% of the total peptide groups at a particular time can be detected. Moreover, it is possible to determine which moiety (A or B) is directly responsible for the retardation (or acceleration) of deuteration by using a predeuterated sample of A or B. This method should be generally applicable to any subunit structure of a protein.

Conformation of the α Subunit. We found that in a mixture of the α and β subunits of proton-translocating ATPase, the flexibility of the α subunit is appreciably reduced, while the structure of the β subunit remains apparently unchanged in the absence of ATP (Figure 8). This seems understandable, because the α subunit has a much more flexible structure than the β subunit. As reported previously, the binding of nucleotide to the isolated α subunit was much looser and more rapid than its binding to the subunit in intact TF_1 (Ohta et al., 1980). These findings suggest that both the slow release of nucleotide from the α subunit in TF_1 and the conformational change of the α subunit are caused by the interaction with the β subunit. Several authors have demonstrated the rapid release of nucleotide from chloroplast F_1-ATPase caused by illumination or application of an external electric field (Harris & Slater, 1975; Magnusson & McCarty, 1976; Gräber et al., 1977). Thus, it is speculated that a flow of protons through ATPase affects the α-subunit-β-subunit interaction, so that the conformation of the α subunit changes, resulting in release of nucleotide. The α conformational change induced by the binding of nucleotide and/or by the binding of the β subunit is considered to be small, because the amide I band in the difference spectra was not large (see Figures 4 and 8). However, this small conformational change seems to play an important role.

Conformation of the β Subunit. When the β subunit combined with ATP, its flexible portion became tighter, and this tightening was further enhanced by the interaction of the β subunit with the α subunit. This tightening was also enhanced by β-subunit-β-subunit interaction. In other words, ATP binding causes a marked structural change in the β subunit only when the β subunit is bound to another β subunit or to the α subunit. As α-β-subunit interaction caused a greater conformational change than β-β-subunit interaction, α-β-subunit interaction may be essential in TF_1-ATPase. This finding implies that the conformation of the β subunit may be changed indirectly by an effector. For example, an effector attached to the α subunit could induce a conformational change of the β subunit.

In our previous paper (Ohta et al., 1980) we suggested that the catalytic sites are located in the β subunits and that nucleotide binding to the β subunit is regulated by the α subunit. Here, we discussed the interactions at the conformational level. The coupling of nucleotide binding to the catalytic site and the flow of protons may be explained by supposing that transport of protons is coupled to subunit-subunit interaction.

Acknowledgments

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Purification and Properties of Chick Renal Mitochondrial Ferredoxin†

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ABSTRACT: Chick renal mitochondrial ferredoxin, as detected by its adrenal flavoprotein dependent NADPH–cytochrome *c* reductase activity, has been isolated from vitamin D_3 replete chicks. This ferredoxin, active in the reconstituted 1α-hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3), was purified about 4000-fold to homogeneity, in 2% yield, using ion exchange chromatography on DEAE-cellulose and discontinuous preparative, as well as polyacrylamide disc gel, electrophoresis. The purified protein has an estimated molecular weight of 11 900 based on migration on gel electrophoresis in the presence of sodium dodecyl sulfate and chromatography on Sephadex G-100. The oxidized form exhibits optical absorbances with λ_{max} at 412 and 454 nm, which are diminished upon reduction. Enzymatically active renal ferredoxin from mitochondrial extracts of chicks raised on vitamin D_3 deficient and vitamin D_3 replete diets showed no significant difference in total units recovered from crude mitochondrial sonic extracts. The renal ferredoxin from vitamin D_3 supplemented animals was fully functional in the reconstituted 25-hydroxyvitamin D_3–1-hydroxylase system. A soluble *b*-type cytochrome, a major contaminant in previous preparations, was separated from the renal ferredoxin, and was found to be inactive in the reconstituted 1α-hydroxylase system. Since this soluble cytochrome, which was isolated from the same mitochondrial extracts, does not support 1α-hydroxylation, nor does it affect the rate of the ferredoxin-catalyzed reaction, the iron–sulfur component, and not the *b*-type hemoprotein, is the specific electron carrier between NADPH-reduced flavoprotein and cytochrome P-450 during 1α-hydroxylation of 25-hydroxyvitamin D_3.

The metabolic activation of vitamin D_3 to the dihydroxylated hormone, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$),[1] is known to involve oxidation at the C-25 position in the liver (Blunt et al., 1968; Horsting & DeLuca, 1969) and C-1 hydroxylation in the kidney (Fraser & Kodicek, 1970; Holick et al., 1971). Whether renal hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3) occurs at the C-1 or C-24 positions appears to be regulated by serum calcium concentration (Boyle et al., 1972; DeLuca, 1974), parathyroid hormone (Garabedian et al., 1972; Fraser & Kodicek, 1973), serum phosphorus concentration (Tanaka & DeLuca, 1973), sex hormones (Tanaka et al., 1976; Castillo et al., 1977), and 1,25-$(OH)_2D_3$ itself (Larkins et al., 1974; Tanaka & DeLuca, 1974). Though the mechanisms of regulation are still unknown, the chick renal

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[1] Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; KP_i, potassium phosphate buffer; DTT, 1,4-dithiothreitol; high-pressure LC, high-pressure liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; EPR, electron paramagnetic resonance; Mops, 4-morpholinepropanesulfonic acid.

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