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Structural dynamics in F_1ATPase during the first reaction cycle of ATP hydrolysis

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The velocity of ATP hydrolysis, catalyzed by purified F_1ATPase from *Micrococcus luteus*, was decelerated on decreasing the temperature. At 13°C one reaction cycle is completed after 20 s. Hydrolysis was triggered upon rapid mixing of the enzyme with ATP. During the first reaction cycle, succeeding structural alterations of the F_1ATPase were traced by time resolved X-ray scattering. The scattering spectra obtained from consecutive intervals of 1 s, revealed the F_1ATPase to pass a conformational state exhibiting an expanded (6%) molecular shape. The expanded state was observed between 45% and 65% of the time required to complete the reaction cycle. This points out a conformational pulsation during ATP hydrolysis.

F_1ATPase; Reaction cycle; Dynamic structure transition; Time resolved X-ray scattering

1. INTRODUCTION

F_1ATPase (EC 3.6.1.34) of the aerobic bacterium *Micrococcus luteus* is a typical representative of the catalytic active moiety of bacterial F-type ATP-synthases. The complete enzyme (δ subunit associated) hydrolyzes CaATP with a specific activity of 12 μmol phosphate · mg^{-1} · min^{-1} at 37°C [1]. The present knowledge about substates in the catalytic process of F_1ATPases converting ATP to ADP and phosphate is fragmentary. Most models describing the pathway of ATP hydrolysis postulate dynamic conformational changes to be involved [2–4]. The detection of such structural transitions requires a major fraction of the molecules acting synchronously. All molecules of this fraction then pass the succeeding conformational states simultaneously. Thus, the time course of any appropriate molecular parameter of a single molecule now become observable and because of all the synchronized molecules they interfere only in a constructive manner.

We synchronized molecules of F_1ATPase upon rapid mixing of enzyme and substrate solutions. By small angle X-ray scattering we traced the shape of the F_1ATPase during the first enzymatic reaction cycle, looking for changes indicating intermediate conformational states.

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2. EXPERIMENTAL

2.1. *F_1ATPase*

F_1ATPase from *Micrococcus luteus* bacteria (ATCC4698) was purified as described previously [5,6]. Purity was >98% as roughly estimated by densitometry of Coomassie blue stained SDS-PAGE gels. A relative molecular mass of 360 000 [7] was used for the computations of the time required for one reaction cycle. Protein concentration was determined by the method of Lowry [8] using bovine serum albumin as standard. Dissociation of the δ subunit was prevented, maintaining the enzyme concentration in Tris-HCl buffer (100 mM, pH 8.0) above 2 g/l [9].

2.2. *ATPase assay*

The hydrolytic activity (in 100 mM Tris-HCl, pH 8.0) was determined estimating the phosphate released from CaATP photometrically. The colorimetric assay with Malachite green was used [10]. Temperature dependence of the Michaelis-Menten constants V_{max} and K_m between 47°C and 15°C was obtained from Hanes plots with substrate (CaATP) varied from 0.1 to 4.0 mM.

2.3. *Mixing device*

Enzyme/substrate mixtures for the scattering experiments were generated with a double-syringe (1 ml) device, using linear ball-bearing mechanics from ISERT (Eiterfeld, Germany). Syringes were actuated by a PC-controlled stepping motor drive mixing 80 μl enzyme (10 g/l F_1ATPase) with an equal volume of substrate (2 mM ATP, 10 mM $CaCl_2$) within 0.22 s. The flux of the reagents transferred the hydrolyzing F_1ATPase into the quartz capillary (Hilgenberg, Malsfeld, Germany) of the thermostated X-ray sample guide. Only 2 μl of the mixture was irradiated by the X-ray beam before being replaced by the next one of the succeeding experiment. Prior to mixing, each of the constituent solutions passed through of 150 cm thermostated stainless steel HPLC capillary (0.8 mm inner diameter) to attain the desired temperature (13°C).

2.4. *Time resolved X-ray scattering*

X-Ray scattering was performed at DESY/HASYLAB (Hamburg, Germany) using the X-ray scattering camera at the synchrotron beam line A1 of the DORIS storage ring [11]. Scattered X-ray photons (0.13

nm wavelength) from a beam focussed by bent mirrors were counted by a position-sensitive Ar/CO_2 detector at 1.9 m distance to the sample. So a Q range between 0.18 and 6.2 nm^{-1} was covered ($Q = 4\pi\sin(\vartheta/\lambda)$ and 2ϑ is the scattering angle). Data aquisition was started after complete replacement of the preceding enzyme/substrate mixture in the sample guide. Then scattering spectra during consecutive intervals of one second were recorded. Accumulation of the corresponding data sets from 5 experiments improved the statistical error. Data reduction and radial averaging was performed by the MADMAN software on a VAX 780 computer. Radii of gyration were evaluated from Guinier plots [12] of the buffer solvent subtracted scattering spectra up to $Q = 0.6$ nm^{-1}.

3. RESULTS AND DISCUSSION

The study of intermediate states during the reaction cycle of F_1ATPase by time-resolved X-ray scattering requires a solution containing enzyme molecules hydrolyzing the ATP synchronously. Utilizing jump methods [13], e.g. a sudden increase in the concentration of substrate, a major fraction of enzyme molecules enters the reaction cycle in a short time. The time needed to obtain a single scattering spectrum with an appropriate statistical error determines the extent to which a dynamically appearing structural change may be resolved (time resolution). An experimental compromise must be found between the number of data sets taken during one reaction cycle and the significance of the structural parameter calculated from the scattering spectra. As experimental expense increases with time

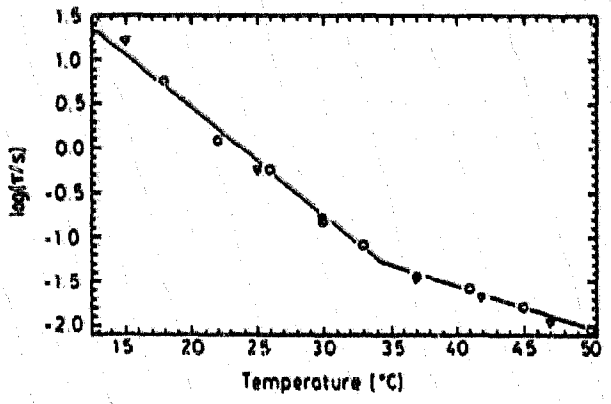

Fig. 1. Temperature dependence of the time (τ) required to release 2 mol of phosphate from 1 mol of F_1ATPase, calculated from the ATP hydrolyzing activity. (circles) Enzymatic activity obtained from an assay using diluted enzyme and a 5-fold K_m concentration of substrate (1 mM ATP, 5 mM $CaCl_2$) [15]; (triangles) τ computed from enzymatic activity of F_1ATPase being entirely saturated with substrate (V_{max}).

resolution on the millisecond scale, we prolonged the reaction cycle of the F_1ATPase decreasing the temperature [14]. Due to its high activation energy the hydrolyzing activity of the F_1ATP from *Micrococcus luteus* depends strongly on the temperature [15]. The time to complete one reaction cycle was calculated from the enzymatic activity. It depends on the number of

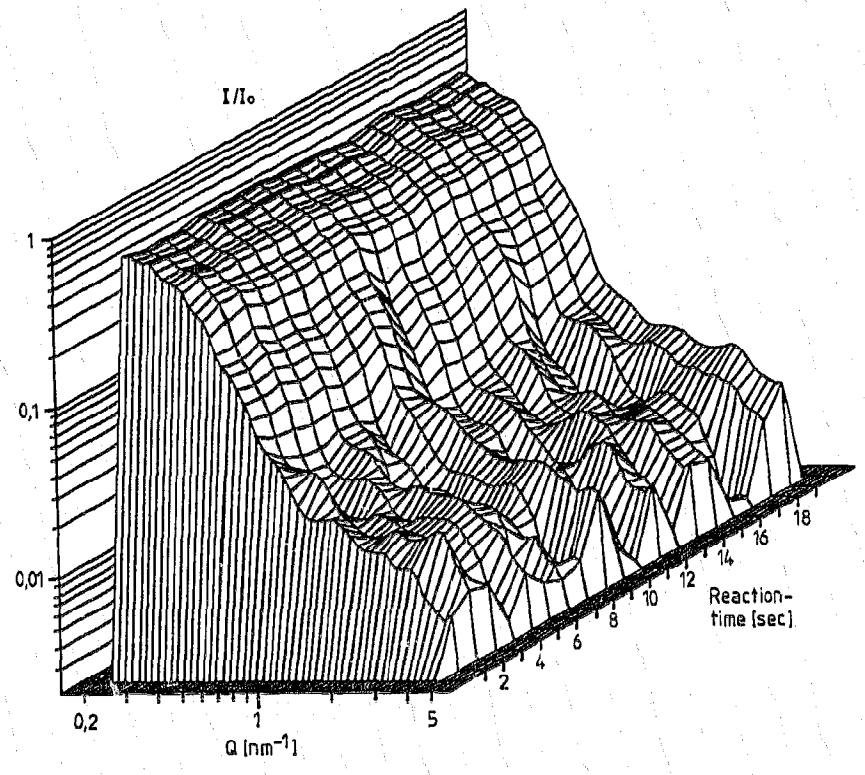

Fig. 2. Isometric drawing of the time resolved X-ray scattering spectra of ATP hydrolyzing F_1ATPase at 13°C obtained during the first reaction cycle. Spectra on the double logarithmic scale were normalized to zero angle scattering ($I_0 = 1$), computed from the corresponding Guinier plots.

functional catalytic sites on the F_1ATPase. It is not clear whether there are two or three sites contributing mainly to the hydrolytic reaction [17]. Previous results from our laboratory [16] favour the bi-site model. The temperature dependence of the time (τ), whilst two moles phosphate become released by one mole F_1ATPase is shown in Fig. 1. From substrate variation studies (0.1–4 mM CaATP), carried out with concentrated enzyme (2 g/l) between 47°C and 15°C, the Michaelis constant K_m was evaluated. Over the whole temperature range no significant change could be detected ($K_m = 200 \pm 50\ \mu M$), thus excluding an altered affinity in substrate binding. The cycle time τ, computed from the extrapolated enzymatic activities (V_{max}) under total saturation with substrate (triangles in Fig. 1), is comparable to the time calculated from enzymatic activities obtained with a 5-fold K_m concentration of substrate in an assay with diluted enzyme (circles in Fig. 1). Hence, at 37°C the F_1ATPase in the high temperature conformation would complete one reaction cycle after 28 ms. Lowering the temperature to 35°C switches the F_1ATPase to the low temperature conformation, related with an enlarged activation energy (220 ± 10 kJ/mol) as derived from the Arrhenius plot [15]. This causes the time calculated for one reaction cycle at 37°C to rise to 20 s at 13°C. A cycle time of 20 s during which two mol ATP are cleaved by 1 mol F_1ATPase is in agreement with the previous observed kinetics of the phosphate release [16]. While using synchrotron radiation and a concentrated protein solution, this velocity is suitable for X-ray data collection in time intervals of one second. The time resolved X-ray scattering of F_1ATPase during synchronized ATP hydrolysis is depicted in Fig. 2. Scattering at low angles depends on the expansion of the scattering molecule characterized by the radius of gyration (R_g) [12]. The radius of gyration of a molecule is defined as the root mean square distance of its electrons from the center of charge. This structure parameter was evaluated from Guinier plots of the innermost part of the scattering profiles. The time course of the radius of gyration during ATP hydrolysis, given in Fig. 3, reveals an enormous flexibility in the F_1ATPase molecule: after triggering the enzymatic reaction, the radius of gyration appeared to be invariable during the first 9 s of the reaction cycle. The initial value of $R_g = 4.38 \pm 0.11$ nm (arithmetic mean between 1 and 9 s) corresponds to that elicited by small angle neutron scattering for the low temperature conformation of the F_1ATPase in the absence of substrate ($R_g = 4.26 \pm 0.05$ nm at 22°C) [15]. Then a sudden increase to $R_g = 4.66 \pm 0.07$ nm (arithmetic mean between 10 and 13 s) occurred. After 4 s in an expanded state, the F_1ATPase proceeded to a spatial form where the radius of gyration ($R_g = 4.39 \pm 1.3$ nm, arithmetic mean between 14 and 19 s) resembled that during the early period of the reaction cycle.

The radius of gyration of the short expanded state is comparable to that found by small angle neutron scattering studies of the high temperature form ($R_g = 4.69 \pm 0.1$ nm at 50°C) [15]. Although we do not yet know whether the two static conformational states detected by neutron scattering and enzyme kinetics are similar to the two well-distinguishable dynamically occurring conformations, the temperature-induced static changes demonstrate the inherent flexibility of the ATPase molecule observed during enzymatic action.

Shrinking of F_1ATPases upon ligation with nucleotides (2–3% with ADP [18], 0–0.6% with ATP [19]), as detected by static small angle scattering, is not in contradiction to the observed dynamic expansion (6%) in the presence of ATP. The average radius of gyration for a whole reaction cycle exceeds that from the beginning by only 1%. In general, from static small

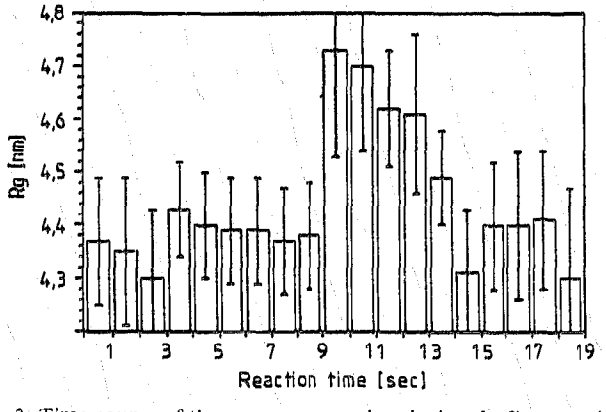

Fig. 3. Time course of the enzyme expansion during the first reaction cycle at 13°C. The radii of gyration were calculated from Guinier plots. Upon hydrolyzing CaATP the F_1ATP passed an expanded state ($R_g = 4.66 \pm 0.07$ nm, averaged from 10 to 13 s). No significant change could be detected during 0–9 and 14–19 s with an average radius of gyration of $R_g = 4.38 \pm 0.05$ nm.

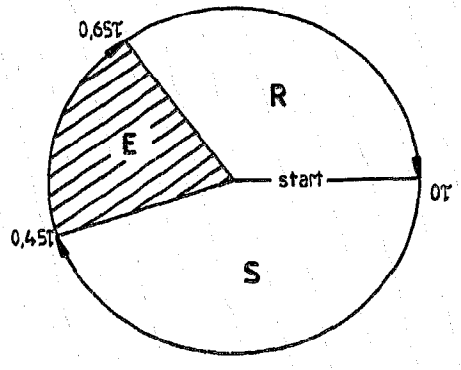

Fig. 4. Referring to the structural transitions, the reaction cycle of F_1ATPase tentatively consists of at least 3 phases: after substrate binding in the S-phase an expanded molecule occurs in the E-phase. The cycle is completed after refolding of the enzyme in the R-state.

angle scattering of a solution containing hydrolyzing F_1ATPase, just the time-averaged radius of gyration can be derived. Hence, a static shrinking of the F_1ATPase in the percent range, induced by nucleotide binding, would superimpose the averaged dynamical expansion during ATP hydrolysis. This would lead to an apparent static nucleotide effect on the radius of gyration from the magnitude cited above.

The double logarithmic representation of the scattering curves (Fig. 2) emphasizes the subsidiary maxima and minima at larger scattering angles. This wide-angle region gives more molecular shape information [20]. In the case of F_1ATPase the maximum around 1.0 nm^{-1} depends on the arrangement of the compository subunits [21,22]. Although the increasing statistical error limits the significant interpretation of the scattering curves at larger angles, the alterations of the maximum at 1.0 nm^{-1} during ATP hydrolysis suggest a dynamic rearrangement of the subunits even in phases of the reaction cycle, where the radius of gyration remained unaffected. For the description of dynamic events during the reaction cycle of F_1ATPase we use a clock-like diagram, shown in Fig. 4. This type of diagram has been established well for the description of the reaction cycle of other enzymes, e.g. bacteriorhodopsin [23]. Data presented in this paper propose the reaction cycle of F_1ATPase to be divided into at least three phases: the reaction cycle is entered by binding the substrate to a compact enzyme conformation (S-state). After passage through the expanded E-state, a further compact conformation of the F_1ATPase occurs in the R-state, indicating a refolding of the molecule. However, the accuracy of the scattering data is insufficient to distinguish between the structural parameters of the enzyme in the S- and the R-state. Supposing that the sequence of structural transitions will be repeated only after the completion of a whole reaction cycle, the S- and R-phases differ.

With an extended version of the mixing device we intend to trace the phosphate release during one turnover and relate enzymatic function to the structural changes.

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