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Thermal Stability of Soluble Mitochondrial H^+-ATPase

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Abstract. ATPase melting has been studied by circular dichroism and differential scanning microcalorimetry. Decomposition of the α-helix of H^+-ATPase (in which about 80% of the peptide groups of the enzyme are involved) following thermal treatment is shown to proceed gradually, beginning with room temperature. Effect of nucleotides upon melting is detected in the range of 20°–40° C. Above 40° C, the pattern of thermal decomposition of the three-dimensional structure of H^+-ATPase is independent of the nature of nucleotides present. Highly stable α-helical sites have been found in the enzyme molecule. Possible mechanism of formation of such sites is discussed, and the results obtained are compared with data on thermal stability of ATPase from thermophilic bacteria. Structural changes in the molecule following thermal treatment are compared with ATPase activity changes under similar experimental conditions.

Key words: Thermal stability – H^+-ATPase.

Introduction

The enzymatic molecule is a dynamic system whose functioning is accompanied by reversible conformational rearrangements [1, 2] Reversible changes in the protein molecule following variations in the ionic strength, pH, temperature and so on allows one to establish correlation between structural and functional properties of the enzyme. The present work is concerned with the relationship between structural and functional states of H^+-ATPase in aqueous solution under thermal treatment.

It has been established [3] that 80% peptide groups of H^+-ATPase are involved in α-helix. It was though interesting to study the pattern of thermal decomposition of the enzyme's structure, i.e., whether the unfolding occurs cooperatively according to the "all or none" principle or the polypeptide chain gradually looses its high regularity. The results were compared with the changes of enzymatic activity of ATPase under similar experimental conditions.

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Methods

Primary extract of ATPase from bovine heart mitochondria was obtained as described earlier [4]. The extract was precipitated by adding saturated ammonium sulfate to 40% concentration, centrifuged, and to the supernatant ammonium sulfate was again added to 55% saturation. The proteins that precipitate between 40% and 55% saturation contain about 90% of the enzymatic activity. This fraction was isolated by centrifugation, and the precipitate was dissolved in a small volume of medium containing 6 mM ATP and 0.5 mM EDTA, pH 7.6. The solution (protein concentration 3—5 g/l) was heated to 65° C and kept at this temperature for 3 min, then rapidly cooled to 20°—25° C and centrifuged. A large amount of enzymatically inactive proteins precipitate after this treatment while ATPase remains in the supernatant and does not change its activity. The final stage of purification consisted of gel filtration through a G-200 column equilibrated with 1 mM ATP and 0.5 mM EDTA (pH 7.5). ATPase appearing in a voil volume was collected as a single fraction. At this stage the enzyme has a specific activity of 25—35 μmol/(min · mg) and is electrophoretically homogeneous. Enzyme preparations intended for long storage were subjected to lyophilization.

The enzyme solution contained in a spherical flask was quickly (in 12—15 s) freezed in liquid nitrogen, the flask being constantly rotated to ensure spreading of the solution and freezing in a thin layer. The lyophilization procedure was carried out at 10^{-1} mm Hg until the protein was completely water-free, i.e., for 2.5—3 h. The protein powder thus obtained is very hydrophilic and must be stored sealed below O° C. The dryed preparation contains about 40% (by weight) of ATPase protein, the rest being sodium salts of ATP and EDTA. The powder is readily soluble in water, at concentrations above 4—5 mg/ml opalescence appears. Lyophilization has been shown to affect slightly if at all such ATPase properties as specific activity, optical properties (e.g., circular dichroism), electrophoretic mobility and homogeneity of the enzyme.

Before spectral measurements, the enzyme dissolved in water was passed through a Sephadex G-50 column equilibrated with 0.02 M sodium phosphate buffer (pH 7.6). ATPase activity was measured by the pH-stat method using a Radiometer-Copenhagen TTT2 pH-stat.

Circular dichroism studies were performed on a J-20 (Jasco, Japan) spectropolarimeter using thermostated cells with optical path of 1 mm. The error in measuring the temperature in the cells did not exceed 0.1° C. Protein concentration was between 0.1 and 0.3 g/l. Specific ellipticity calculated for various concentrations of the F_1 factor was similar in the range of 200 to 250 nm, the error being less than 5%. The effects observed earlier [5] take place probably when low ellipticity values are measured under conditions of significant optical density of the solution in the spectral range investigated. Protein concentration and the number of nucleotide molecules per ATPase molecule were determined spectrophotometrically [3]. UV spectra were taken on a Specord UV-Vis spectrophotometer (Karl Zeiss, GDR).

Microcalorimetric studies were performed on a differential scanning microcalorimeter DASM-1M (SCB of Biological Instrument Making, Pushchino, USSR) in a 1 ml cell. Enzyme concentration was 1—5 g/l.

Light scattering measurements were performed using "Sofica" photogoniodiffusometer (France) at the angle of 90° in cells with the volume of less than 6 ml.

Results and Discussion

Studies of thermal melting of proteins require the absence of molecular aggregates in the solution since these aggregates introduce additional problems in the interpretation of the melting curves. The latter, under such circumstances, will represent superposition of two effects: intra- and intermolecular melting. The conditions ensuring no aggregation in a wide temperature range were selected using light scattering intensity at 90° as a measure of protein aggregation.

Figure 1 shows the intensity of light scattering of ATPase in water as a function of temperature, pH and ionic strength. At higher ionic strength and pH values ATPase molecules tend to aggregate. At low ionic strength and pH close to neutrality the enzyme does not aggregate in a wide temperature range. The melting of the enzyme was, therefore, studied at low ionic strength and in the pH range of 7.4—7.9. Specific activity of ATPase under these conditions was 20—30 μmol/(min · mg); the enzyme melting at lower pH values was not studied since a marked decrease of enzymatic activity was observed.

Figure 2 (1) shows ellipticity as a function of temperature at 220 nm. Apparently, the unfolding of ATPase α-helix proceeds gradually as the temperature is increased, beginning from room temperature. Microcalorimetric and spectropolarimetric data indicate that temperature-induced changes in the secondary structure of the molecule are reversible up to 60° C.

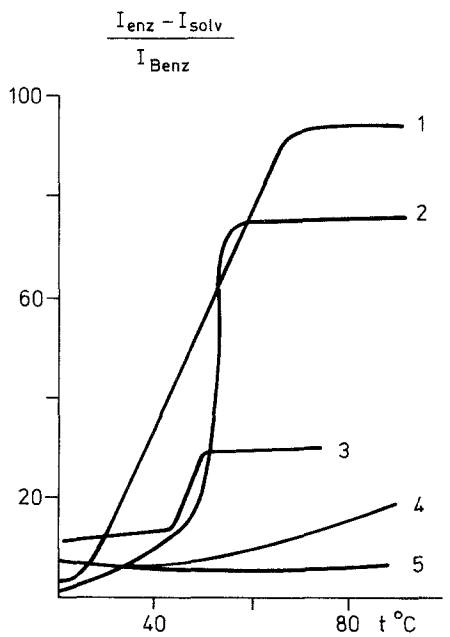

Fig. 1. Light scattering of ATPase solution as a function of temperature at different pH and ionic strength values. Enzyme concentration —0.03 g/l. The ordinate is the ratio of the difference in light scattering of the enzyme ($I_{enz.}$) and solvent ($I_{solv.}$) to the light scattering intensity of benzene (I_{benz} = 120). 1 — pH = 9.1, high ionic strength (μ = 0.6); 2 — pH = 8.62, high ionic strength; 3 — pH = 9.1, low ionic strength (μ = 0.06); 4 — pH = 8.62, low ionic strength; 5 — pH = 7.4, low ionic strength

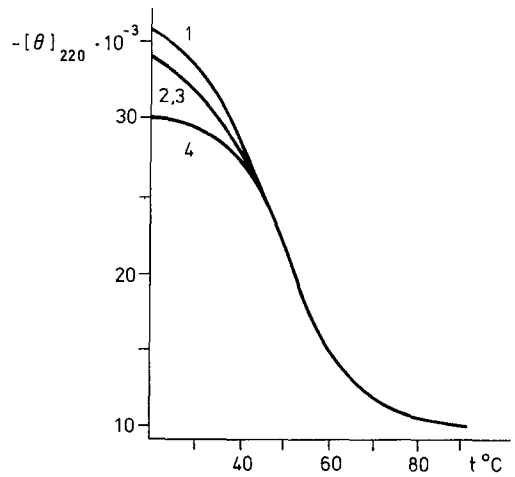

Fig. 2. The melting of H^+-ATPase in the presence of different nucleotides in the solution. Enzyme concentration 0.35 g/l, nucleotide concentration 2 mM. 1 — ATPase without nucleotides. 2,3 — ATPase with ATP and ADP, respectively. 4 — ATPase with AMP

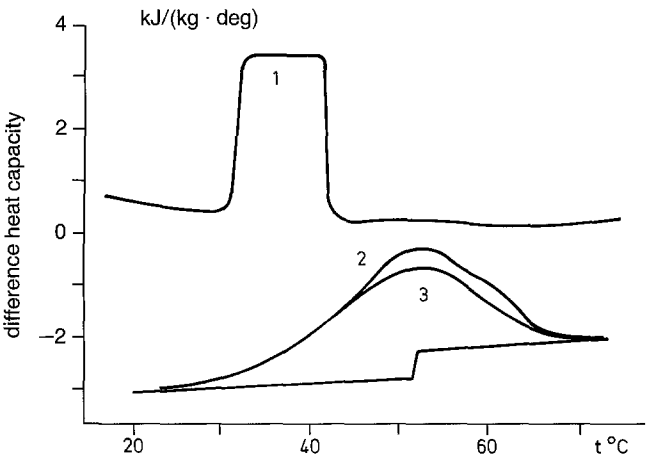

Fig. 3. Heat-absorption of 0.14% ATPase solution in 20 mM sodium phosphate buffer, pH 7.4. Cell volume about 1 ml, rate of solution heating 1 degree/min. 1 — reference point, S = $7.28 \cdot 10^{-3}$ cal. 2 — heat-absorption of H^+-ATPase. 3 — heat-absorption of H^+-ATPase 15 h after taking curve 2

Melting curve analysis performed according to Lamry et al. [6] shows that ATPase undergoes no cooperative transition from one state to the other, as is the case with such proteins as myoglobin and cytochrom C. Since ATPase melting involves several intermediate states and the division of the overall process into individual transitions appears questionable, the transition enthalpy value can be obtained only through microcalorimetric measurements.

Figure 3 presents melting curves obtained by the latter method. Melting of the enzyme appears to be reversible when it is highly purified from ATP (less than 4–5 ATP molecules per enzyme molecule). When more than 8–10 ATP molecules per protein molecule are present, the enzyme irreversible aggregates at 74° C. Figure 3 shows that ATPase melting is a composite process representing superposition of individual, more simple thermal transitions in the enzyme molecule. Heat-absorption maximum on the melting curves is located near the 52° C point. At least two peaks can be seen in the region of maximum heat-absorption. Transition enthalpy, as calculated from the microcalorimetry data, equals 1860 kcal/mol, transition temperature

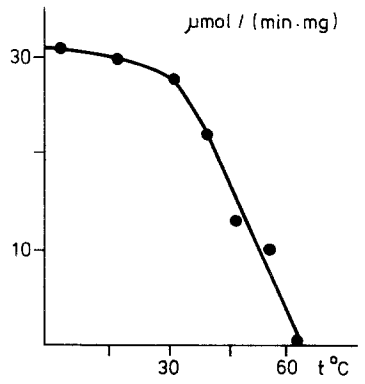

Fig. 4. Specific activity of ATPase as a function of temperature at which the solution was pre-heated for 10 min. ATPase activity was measured in medium containing 0.1 M sucrose, 0.1 M KCl, 2 mM tris-buffer (pH 7.9) at 20° C. Cell volume 2—3 ml. Enzyme concentration in the cell $2 \cdot 10^{-3}$ g/l

is about 52° C. Marked heat-absorption can be observed near 62° C. As follows from Figure 2 (1), the changes due to destruction of α-helix are essentially completed at this temperature, and the heat-absorption maximum results from disruption of a certain conformation which does not manifest itself spectropolarimetrically, i.e., is optically inactive. Noteworthy, is the fact that α-helix unfolding under thermal treatment is incomplete. About one third of α-helical regions in the ATPase polypeptide chain are highly thermostable. We suppose that the complex pattern of thermal destruction of ATPase is due to different stability of α-helical sites in different enzyme subunits. Light scattering data indicate that the enzyme does not dissociate into the subunits in the temperature range studied. Heat effect resulting from aggregation of enzyme molecules appears to contribute very little to the melting pattern observed. It is also possible that the highly thermostable portion of α-helical sites in different polypeptide chains form a compact structure, which is stabilized either chemically, e.g., by disulfide bonds, or by dissociated α-helical sites which form an intramolecular matrix that possesses certain rigidity (at 70° C) and stabilizes some α-helical regions. Such a matrix is composed of polypeptide regions with sufficient number of degrees of freedom to damp the impacts of exogenous molecules, whose mobility is a function of temperature. Polypeptide regions that have acquired irregular secondary structure but are still bound to each other in some way can thus function as a thermostate for those regions that retain α-helical configuration. Melting properties of ATPase preparations with low and relatively high content of bound ATP molecules are somewhat different. The difference, essentially, is that the latter preparations exhibit greater tendency to aggregation: it has been noted that irreversible aggregation in this case is observed beginning with 74° C.

To study effect of different nucleotides on the enzyme in more detail, we measured ellipticity at 220 nm as a function of temperature in the presence of ADP, ATP and AMP. Figure 2 shows circular dichroism of ATPase with a minimum content of nucleotides (4—5 nucleotide molecules per 1 enzyme molecule, see [3]) as compared to that in the presence of high concentration of nucleotides. Binding of additional nucleotides appears to result in a slight deformation of the α-helix which, in turn, leads to a decrease of ellipticity near 220 nm. The thermal stability of F_1 factor, however, is independent of the nature of bound nucleotide, and moreover, is independent of whether the nucleotides are present in the solution or not, at least for

the melting stage that begins with 40° C (Fig. 2). Between 20° C and 40° C the melting profiles change in the presence of nucleotides, the effects of ATP and ADP being practically the same. The higher liability of ATPase to aggregation in the presence of nucleotides seems to be due to the screening of charged groups in the enzyme molecule.

As is shown above, the melting of a-helix is reversible within a wide temperature range. In the following experiments we studied the extent to which the structural reversibility correlates with enzymatic reversibility. Figure 4 shows specific activity of F_1 as a function of temperature at which the solution of ATPase was kept for a certain period of time. The experiment was run as follows. Aqueous solution of F_1 was kept for 10 min at the various indicated temperatures, quickly cooled to 20° C, and assayed for enzymatic activity under similar conditions for all experimental points. The curve on Figure 4 thus presents ATPase activity as a function of incubation temperature. The comparison of structural and enzymatic data indicates that structural changes are completely reversible up to the temperature of 40° C. At higher incubation temperature, the enzymatic activity becomes irreversibly destroyed. This effect is probably due to either rearrangements on the quaternary structure level or irreversible alterations in the vicinity of the active site, since the changes of secondary structure are completely reversible up to 60° C. Incubation of F_1 solution at 60° C for 10 min results in irreversible disappearance of ATPase activity. At this temperature the structure of ATPase undergoes irreversible changes.

In view of partial thermostability of α-helix in the F_1 molecule, it is interesting to compare functional and structural properties of membrane-bound ATPases isolated from organisms with different habitat temperature. Temperature optimum of H^+-ATPase (factor TF_1) isolated from thermophilic bacteria is 70° C, i.e., the temperature at which the bacteria grow in nature [7]. The ATPase activity was detected by the authors up to 80° C [7], the absolute ellipticity value growing only slightly in the 200—250 nm range, and the shape of the spectrum remained the same. Essentially no structural changes of TF_1 molecule were observed in the range of 0°—80° C. Evolution of the primary structure resulted in a higher thermostability of TF_1 as compared to F_1. When TF_1 is heated to 86° C, an $\alpha \rightarrow \beta$ transition probably occurs in the molecule, and this could be confirmed by infrared spectra analysis.

Comparison of circular dichroism spectra of F_1 and TF_1 as a function of temperature reveals certain features of the transition from α-helix to another conformation following thermal treatment. As a rule, the α-helix in water is destroyed in the range of 40° C to 80° C resulting in formation of irregular structure. The heat-induced destruction occurs either gradually (e.g., in the case of tropomyosin and ATPase) or cooperatively, within a narrow (2°—3° C) range. The mechanism of α-helix melting is markedly different in TF_1 and F_1. The results of earlier studies [2] as well as circular dichroism spectra of TF_1 in the 85°—95° C interval indicate that a transition from α-helix to β-structure takes place, the latter being evidently the most thermodynamically advantageous conformation. In the course of evolution, the requirement for higher thermostability of the enzyme governed selection, in the TF_1 molecule, of polypeptide sites with high thermostability, which resulted in different mechanisms of α-helix melting in TF_1 and F_1.

The phenomenon of $\alpha \rightarrow \beta$ transition in films of certain proteins has been observed earlier [2]. Significant regions of the polypeptide chain are involved in this

process at 90°–120° C. These studies suggest that above 85°–90° C the α-helix becomes unstable. Upon heating, the α-helical regions of the polypeptide undergo transition to a more stable organized state (often to the β-structure) via an intermediate state in which hydrogen of the peptide group becomes accessible for contact with small molecules of the environment, e.g., water molecules. In the more stable state the peptide group hydrogen becomes again inaccessible to small molecules. At a lower temperature, the α-helix is usually transformed into an irregular conformation. A more detailed comparative study of thermodynamic properties of F_1 and TF_1 in one laboratory will, undoubtly, bring about better understanding of the mechanism of $\alpha \rightarrow \beta$ transition, as well as the transition from α-helix to another conformation.

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