Enzyme Catalysis in Organic Solvents with Low Water Content at High Temperatures. The Adenosinetriphosphatase of Submitochondrial Particles†

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ABSTRACT: A system composed of toluene, phospholipids, and Triton X-100 in which the ATPase activity of bovine heart submitochondrial particles can be studied at low water concentrations and high temperatures is described. In this system, ATPase activity starts to appear at 0.5% (v/v) water and increases as the concentration of water is increased. At 3.8% water, the enzyme exhibits saturation kinetics with respect to Mg-ATP with a K_m similar to that observed in an all-water system (approximately 300 μM), but the V_{max} is about 100 times lower (6 nmol min^{-1} mg^{-1}) than that in water. At concentrations of water between 0.5% and 2%, the enzyme catalyzes ATP hydrolysis at temperatures of up to 91 °C. The conditions for achieving catalysis at high temperatures are described. Even though at low water concentrations the enzyme catalyzes ATP hydrolysis at temperatures significantly higher than in totally aqueous media, the optimal temperature for hydrolysis (approximately 58 °C) is independent of the water content. The half-life of the enzyme at high temperatures is significantly higher at low water concentrations than in aqueous media. In the system described, the enzyme is located in a compartment distinct from that of the substrate and products of the reaction. Apparently, the enhancement of catalytic rates by water is due to a higher conformational mobility of the protein; the same factor causes a decrease in the thermostability of the enzyme.

Water is required for enzyme catalysis; however, Zaks and Klibanov (1985) went further by asking how much water is needed for enzyme activity. From studies in which enzymes at various levels of hydration were suspended in organic solvents, they showed that even less than one monolayer of water around the enzyme suffices to support catalysis (Zaks & Klibanov, 1988a). These findings become more interesting, since enzymes exhibit a striking thermostability when surrounded by a limiting amount of water, and placed in media in which the bulk is made up of an organic solvent. This latter property is observed with enzymes such as lipase (Zaks & Klibanov, 1984), terpene cyclase (Wheeler & Croteau, 1986), chymotrypsin (Zaks & Klibanov, 1988a), and highly complex membrane enzymes such as the mitochondrial cytochrome oxidase and the F_1-ATPase (Ayala et al., 1986; Garza-Ramos et al., 1989). These observations suggest that thermoresistance at low amounts of water is shared by many, if not all, enzymes. The explanation for this phenomenon is that the limited availability of water hinders thermal denaturation, since water, either directly or indirectly, participates in all processes that end in thermal denaturation. Accordingly, water supports catalysis, but it also decreases thermostability.

Zaks and Klibanov (1984) also observed that enzymes such as lipase could carry out catalysis at 100 °C, provided they are placed in solvents of low polarity and low water concentrations. These impressive results were obtained with substrates and products that were soluble in organic solvents. Thus, the question arises as to whether enzyme catalysis with water-soluble substrates and products can also take place in organic solvents at temperatures higher than in all-water media. A priori positive results would be of interest and would broaden the scope of studies of biocatalysis at high temperatures, since a wide variety of enzymes function with water-soluble substrates and products, and, in many, water is a direct participant of the reaction. Here, it is shown that in systems predominantly composed of an organic solvent with low amounts of water, the highly complex multisubunit mitochondrial ATPase [for a review, see Hatefi (1985) and Martins et al. (1988)] hydrolyzes ATP at temperatures as high as 91 °C.

It is also decribed that in a mixture of toluene, phospholipids, and octylphenoxypoly(ethoxyethanol) (Triton X-100),[1] which undergoes clearly defined phase changes as its water content is modified, it is possible to study enzyme behavior at high temperatures. This system was developed on the basis of previous observations on the hydrolytic activity of F_1-ATPase in toluene–phospholipid mixtures (Garza-Ramos et al., 1989), and on studies that show that detergents (Martinek et al., 1986; Luisi & Magid, 1986) and alcohols (Keiser et al., 1979; Khmelnitsky et al., 1987, 1988) can be used to place water and a wide variety of molecules in organic solvents via inverted micelles. The results also show that in this system, the enzyme may be placed in a compartment different from that of the substrates and products of the reaction, even though there is communication between them.

Materials and Methods

Chemicals. The following were obtained from the indicated sources: toluene from Merck; Triton X-100, phospholipids,

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[1] Abbreviations: DCCD, dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; TPT, mixture of 15% (v/v) Triton X-100 in toluene and 8.5 mg of phospholipid/mL; Tris, tris(hydroxymethyl)aminomethane; Triton X-100, octylphenoxypoly(ethoxyethanol).

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and ATP from Sigma Chemical Co.; [^{32}P]P_i from New England Nuclear. Phospholipids were purified according to Kagawa and Racker (1971) and [γ^{32}P]ATP was prepared as described by Glynn and Chappell (1964).

Biological Preparations. Mg-ATP submitochondrial particles were prepared as described (Beltrán et al, 1986) from bovine heart mitochondria as reported by Low and Vallin (1963). The particles suspended in 0.25 M sucrose were maintained at −60 °C in small aliquots until the time of the experiment. The concentration of protein in various preparations was between 45 and 55 mg of particle protein/mL which was determined according to Lowry et al (1951). The ATPase activity of these particles in an all-water medium was approximately 300 nmol min^{-1} mg of protein)$^{-1}$ as measured with an ATP regenerating system (Pullman et al., 1960).

Methods. In most preparations, a mixture of toluene and Triton X-100 (85:15 v/v) that contained 8.5 mg of phospholipid/mL was used. Upon addition of increasing amounts of water (from 0.5 to 7.5%, v/v), the system underwent distinct phase changes. These are illustrated in Figure 1A by the absorbance of the mixture at 520 nm.

For the assay of ATPase activity in the toluene–phospholipid–Triton (TPT) system, ATP hydrolysis was started by either of the following procedures: (1) addition of particles suspended in 0.25 M sucrose to a system that contained substrate previously transferred to the TPT mixture; (2) addition of particles previously transferred to the TPT mixture to substrate previously transferred to the TPT mixture; (3) addition of substrate in water to particles previously transferred to the TPT mixture.

The transfer of submitochondrial particles to the TPT mixture was achieved by the injection of the particles suspended in 0.25 M sucrose to the TPT mixture followed by vigorous stirring (0.5–1 min). The water volume in which the particles were added varied according to the purpose to the experiment. The transfer of substrate to the TPT mixture was achieved by the injection of a solution that contained Tris-HCl, pH 7.4, $MgCl_2$, and [γ-^{32}P]ATP to the TPT mixture followed by vigorous stirring. The concentration of the above components in the injected solution, as well as the water volume introduced, was calculated to yield the desired percentage of water and 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]ATP in the total water volume that was added to the TPT mixture. In the calculations, it was considered that the addition of particles also involved the addition of water.

The reaction was arrested by mixing 0.4 mL of the TPT reaction mixture with 0.5 mL of 6% trichloroacetic acid in water. To this mixture were added 0.5 mL of 3.3% ammonium molybdate–3.75 N H_2SO_4 and 0.2 mL of acetone. After the mixture was stirred, 1 mL of water-saturated isobutyl alcohol–benzene was added to extract the phosphomolybdate complex. After separation of the phases, an aliquot was withdrawn to measure the [^{32}P]P_i formed. In all experiments, blanks were included for every experimental point. Blanks were made exactly as the experimental samples, except that 0.25 M sucrose was added instead of the particles; the values obtained were subtracted from those detected with particles, and the difference was used to calculate ATPase activity. In all the experiments shown, not more than 30% of the [γ-^{32}P]ATP introduced was hydrolyzed. The specific activity of [γ-^{32}P]ATP was between 10 000 and 15 000 cpm/nmol.

Results and Discussion

Choosing the System. It has been reported (Garza-Ramos et al., 1989) that soluble mitochondrial F_1-ATPase transferred to a system that contained toluene and phospholipids catalyzed

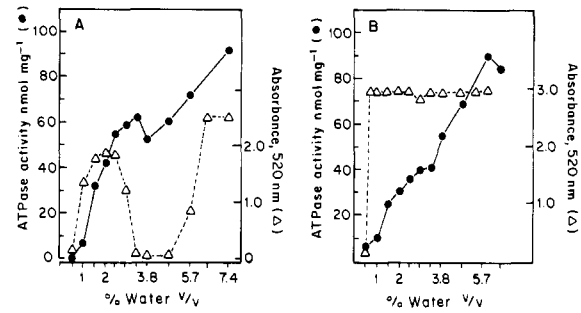

FIGURE 1: ATPase activity of submitochondrial particles in (A) toluene–phospholipid–Triton X-100 and (B) toluene–Triton mixtures and turbidity changes at various water concentrations. In (A), the basic mixture contained 15% Triton X-100 in toluene and 8.5 mg of phospholipids/mL plus the indicated concentrations of water. After the addition of 10 mM Tris-HCl, pH 7.4, to yield the indicated water concentrations, the mixture was vigorously stirred for 30 s, and its absorbance at 520 nm was measured (△). For the assay of ATPase activity (●), different aliquouts of Tris-HCl, pH 7.4, $MgCl_2$, and [γ-^{32}P]ATP were added to 1 mL of the toluene–phospholipid mixture and stirred vigorously for 30 s. ATP hydrolysis was started by the addition of 150 μg of protein suspended in 3.2 μL of 0.25 M sucrose to the latter mixture. The concentrations of Tris-HCl, $MgCl_2$, and [γ-^{32}P]ATP in the reaction mixture were such that after addition of the particles the final concentrations were 10, 3, and 3 mM, respectively, considering that the components located in the total amount of water introduced. The incubation time for measuring [γ-^{32}P]ATP hydrolysis was 10 min at a temperature of 20 °C. The experiment in (B) was identical with that in (A), except that the basic system did not contain phospholipids.

the hydrolysis of ATP at a rate that depended on the concentration of water. However, this system had a low capacity to solubilize water [see also Poon and Wells (1974)]. Thus, attempts were made to find a system in which relatively high amounts of water could be solubilized. Kumar and Balasubramanian (1979, 1980) reported that Triton X-100, in combination with long-chain alcohols, could solubilize important amounts of water in apolar solvents. In agreement with these results, and as inferred from the transparency of the system (Figure 1A), it was found that water to about 5% (v/v) could be solubilized in toluene that contained Triton X-100 and phospholipids. Most likely in these conditions, water exists in the interior of reversed micelles or in lamellar structures [see Kumar and Balasubramanian (1979, 1980)]. For the studies of ATPase activity, the use of Triton X-100 also seemed convenient, since at 20 °C in an all-water medium, Triton X-100 inhibited, but moderately, ATPase activity; i.e., 15% Triton X-100 in an all-water media inhibited ATPase activity between 30 and 35%. The ATPase activity of submitochondrial particles transferred to the TPT system at various water concentrations is shown in Figure 1A. The results showed that the particles transferred to the TPT mixture could hydrolyze ATP, provided the concentration of water was above 0.5%. As the concentration of water was raised from 0.5 to about 2.5%, the rate of hydrolysis increased; at higher water concentrations, the rates continued to increase, but with a lower slope.

Also according to Kumar and Balasubramanian (1979, 1980), and as evidenced from the turbidity of the system, it was found that in the absence of phospholipids, only small amounts of water were solubilized in a mixture of toluene and Triton (Figure 1B). In these mixtures, particles exhibited an ATPase activity which was similar to that observed in the TPT system (compare panels A and B of Figure 1). However, all experiments were carried out in TPT mixtures, since in this system clearly defined transparent phases were detected at distinct water concentrations. A concentration of 15% Triton X-100 in toluene (and phospholipids) was chosen because,

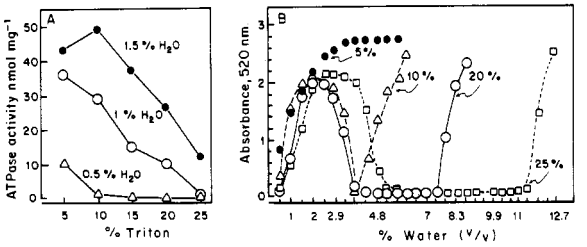

FIGURE 2: (A) ATPase activity of submitochondrial particles in a mixture of toluene–phospholipids and various concentrations of Triton X-100. (B) Turbidity of a mixture of toluene–phospholipids and various concentrations of Triton X-100 and water. In (A), the experiment was carried out as in Figure 1A, except that the basic mixture contained the indicated concentrations of Triton X-100 and water. The incubation time was 10 min, and the temperature was 20 °C. In (B), the absorbance of the mixture was measured as in Figure 1A, except that the mixture contained the indicated concentrations of Triton X-100 and water.

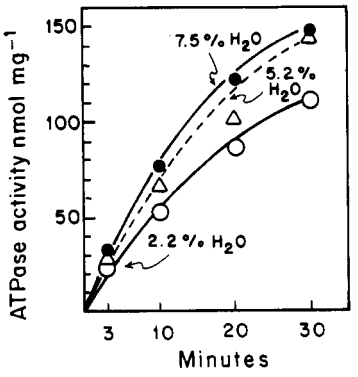

FIGURE 3: ATPase activity of submitochondrial particles in toluene–phospholipid–Triton mixtures at various incubation times. Two milliliters of TPT was mixed with a water solution that contained the substrate mixture. To this TPT mixture were introduced particles at a concentration of 150 μg/mL to start the reaction. In the final reaction system, the concentration of the components was 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]ATP in the water content that is indicated. At the indicated times, aliquots of the mixture were withdrawn to assay the amount of [γ-^{32}P]ATP hydrolyzed. The temperature was 20 °C.

although the activity with 5 and 10% Triton was higher than with 15% Triton (Figure 2A), with the former concentrations, the transparent phases were not observed in a wide range of water concentrations (Figure 2B). With 20 and 25% of Triton, the phase changes were more apparent (Figure 2B) than with 15% Triton, but the inhibition of ATPase activity was more significant (Figure 2A).

Characteristics of the ATPase Activity of Submitochondrial Particles in the Toluene–Phospholipid–Triton System. Soluble mitochondrial F_1-ATPase hydrolyzes ATP in a mixture of toluene, phospholipids, and low amounts of water (Garza-Ramos et al., 1989). In the TPT system, it was found that the ATPase activity of submitochondrial particles was higher, and relatively constant for about 20 min of incubation (Figure 3). Also, it was found that up to a concentration of about 200 μg of particle protein/mL of reaction mixture, hydrolysis was almost directly proportional to the amount of particles injected into the TPT mixture (not shown).

At a concentration of 3.8% water, the system exhibited saturation kinetics at concentrations of MgATP between 0.05 and 3.0 mM. At 20 °C, in a 5-min incubation period and a water concentration of 3.8%, the calculated K_m and V_{max} from linear Lineweaver–Burk plots were 300 μM and 30 nmol of ATP hydrolyzed/mg of protein, respectively. The K_m observed was not significantly different from that observed in aqueous systems (200 μM), but the V_{max} was about 100 times lower

Table I: Separation of Enzyme from Substrate and Products in Toluene–Phospholipid–Triton Mixtures by Centrifugation[a]

Expt A: Fractionation of the Enzyme	
fraction	activity (nmol/mL)
total system	3.4
supernatant	0.3
precipitate	2.1
Expt B: Fractionation of Substrate	
fraction	cpm/mL
total	172 000
nonsedimentable	164 590
Expt C: Fractionation of Products	
fraction	extractable [^{32}P]P_i
total	43 125
supernatant	38 590

[a] Experiment A, submitochondrial particles were transferred to the toluene–phospholipid–Triton mixture at a water concentration of 3.8% and 150 μg of protein/mL. After standing for 15 min at room temperature, an aliquot was centrifuged at 8000g for 15 min. This resulted in the formation of a pellet. After removal of the supernatant, the pellet was suspended in the standard toluene–phospholipid–Triton mixture. The ATPase activities of the original fraction, the supernatant, and the resuspended pellet were measured by adding to the mixtures the substrate reaction mixture in water. This yielded a concentration of water of 3.8% (this addition disregarded the different water concentration in the three fractions), 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]ATP. The incubation time was 5 min, at a temperature of 20 °C. In experiment B, 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]ATP were transferred to the standard toluene–phospholipid–Triton mixture at a water concentration of 3.8%. Radioactivity was determined in an aliquot of this mixture (total counts); the rest was centrifuged at 8000g for 15 min; radioactivity was determined in an aliquot of the upper portion of the tube. In experiment C, submitochondrial particles were first transferred to the standard TPT mixture. This was followed by the addition of the substrate mixture. The final water concentration was 3.8%. After 30 min of incubation, an aliquot was centrifuged at 8000g for 15 min. After centrifugation, aliquots of the supernatant and of the original mixture that had not been centrifuged were added to a water solution of 6% trichloroacetic acid. Inorganic [^{32}P]P_i was extracted as described under Materials and Methods. Total cpm indicates the radioactive extractable [^{32}P]P_i found in the mixture that was not centrifuged.

(in aqueous media, it is about 600 nmol min^{-1} mg^{-1}). It is to be noted that some enzymes when placed in reversed micelles composed of synthetic detergents have kinetic parameters similar to those observed in aqueous media [see Table 7 of Luisi and Magid (1986)]; however, it has been reported that in reverse micelles, certain enzymes exhibit changes of their K_m, but not in their V_{max} (Samama et al., 1987; Fletcher et al., 1985), and vice versa (Sanchez-Ferrer et al., 1988).

In the characterization of the system, it was considered necessary to establish if the enzyme and substrates and products of the reaction were located in the same or in different and separable compartments. In systems in which enzymes were studied as a suspension in organic solvents, the substrates and products were hydrophobic, and thus they were located in the organic phase (Zaks & Klibanov, 1984, 1985, 1988a,b; Wheeler & Croteau, 1986). In the reverse micelle systems, the location of the protein depends on its characteristics [for discussion, see Bru et al. (1989)], and it may locate in the water space of the micelle (Grandi et al., 1986; Thompson & Gierash, 1984; Chatenay et al., 1987), in the interphase, or in both the water and organic phases (Ramakrishnan et al., 1983; Nicot et al., 1985). In the reverse micelle systems, the substrates and products distribute according to their solubility properties. In the experiment of Table I, submitochondrial particles were transferred to the standard TPT system at a water concentration of 3.8% (v/v) which yielded a transparent system. Upon centrifugation, a pellet was formed. The ATPase activities of the supernatant and the resuspended pellet

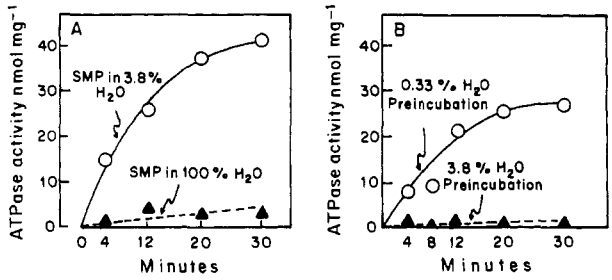

FIGURE 4: Effect of water on the ATPase activity of submitochondrial particles at 73 °C. In (A), the standard toluene–phospholipid–15% Triton mixture that contained Tris-HCl, $MgCl_2$, and [γ-^{32}P]ATP was preincubated for 10 min at 73 °C. At this time, particles suspended in 0.25 M sucrose were added to start the reaction [this is depicted as SMP in 100% H_2O (▲)]. The reaction was also started by the addition of particles previously transferred to the toluene–phospholipid mixture at room temperature, at a water concentration of 3.8% [this is depicted as SMP in 3.8% H_2O (○)]. After the reaction was initiated, in both sets of tubes the concentration of water was 3.8%, and 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]ATP in the total water content. One milliliter of the reaction mixture contained 150 μg of particle protein. At the indicated times of incubation at 73 °C, the reaction was arrested. In (B), submitochondrial particles were first transferred to the toluene–phospholipid–Triton mixture at room temperature at 0.33% (○) and 3.8% (▲) water. The tubes were placed in a bath at 73 °C for 10 min to achieve thermoequilibration. At this time, the components of the hydrolytic reaction were added to start the reaction at 73 °C. The addition of substrate to the enzyme was made in an all-water solution, or in the TPT mixture so as to yield 3.8% water with 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 3 mM[γ-^{32}P]ATP in the reaction media. The concentration of the particles was 150 μg of protein/mL of reaction mixture.

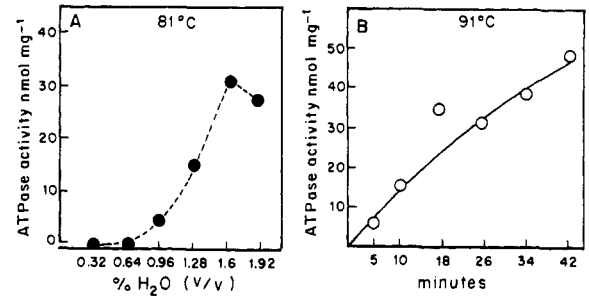

FIGURE 5: ATP hydrolysis by submitochondrial particles in the standard TPT mixture at 81 °C (A) and 91 °C (B). In (A), the particles were transferred to the TPT mixture at room temperature at a water concentration of 0.32%. Aliquots of this mixture were placed in a bath at 81 °C for 10 min to achieve thermoequilibration. At this time, a water solution that contained the components of the hydrolytic reaction was added. The latter addition was made so as to yield the indicated concentrations of water and 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]ATP in the total water content. The concentration of the particles was 150 μg of protein/mL of reaction mixture. After 20 min of reaction time at 81 °C, the reaction was arrested. In (B), the same protocol was followed except that the experiment was carried out at 91 °C. To start the hydrolytic reaction, to the particles preincubated for 10 min at 91 °C at a water concentration of 0.32% was added substrate in water to yield 1.28% water. The incubation was continued at 91 °C for the times shown. During the handling of the tubes for the addition of the substrate, the temperature fell in 3–5 °C.

were measured. Most of the activity was found in the pellet. The same procedure was used to ascertain the distribution of the substrates and products of the reaction; it was found that both failed to sediment at centrifugal forces that pelleted the particles (Table I). Therefore, it appears that in the standard TPT system, the enzyme is in a compartment with sedimentation properties different from that of the substrates and products, even though there is communication between them.

The ATPase of submitochondrial particles is made up of a membrane moiety, F_0, and a detectable, soluble catalytic factor, F_1 (Hatefi, 1985; Martins et al., 1988). To ascertain if F_1 remained attached to F_0 in TPT mixtures, particles were incubated for 24 h with DCCD (0.9 nmol/mg); the ATPase activity was inhibited 80%, presumably due to the covalent binding of DCCD to F_0. The inhibited particles were transferred to the TPT system, and their ATPase activity was assayed in 3.8% water. The DCCD-treated and the control particles hydrolyzed ATP at equal rates. This indicates that during the transfer of particles to the TPT mixture or in the system, F_1 is detached or loosely bound to F_0.

Enzyme Activity at High Temperatures. Enzymes suspended in organic solvents can carry out catalysis at temperatures as high as 100 °C (Zaks & Klibanov, 1984). Few studies have been carried out on the effect of temperature on enzyme catalysis in reverse micelles. For instance, alcohol dehydrogenase (Samama et al., 1987; Lee & Bielmann, 1986) and cholesterol oxidase (Khmelnitsky et al., 1988; Lee & Bielmann, 1986), exhibited significant inactivation at temperatures around 30 °C, whereas polyphenol oxidase inactivated at 45 °C (Sanchez-Ferrer et al., 1988). In contrast, soluble mitochondrial F_1-ATPase exhibited a remarkable thermostability at temperatures of 70 °C in a system of toluene–phospholipids (Garza-Ramos et al., 1989); however, catalysis by this enzyme in the high-temperature range was not studied. Thus, the ATPase activity of submitochondrial particles in TPT mixtures was studied at high temperatures.

ATP hydrolysis was detected at 72 °C, but it was found that the preservation of catalytic activity depended on the water content of the enzyme at the time of its introduction to the reaction mixture (Figure 4). Particles suspended in an all-water medium, or previously transferred to the apolar media in 0.32% water at room temperature, were added to a TPT system that contained ATP, and which had been previously taken to 72 °C. In the first case, after the addition of particles, little hydrolysis was detected, while in the latter, significant activity was observed (Figure 4A). This suggested that the enzyme added in 100% water was denatured before it reached a space of limited water content whereas the enzyme previously located in a compartment with low water was protected against thermal denaturation, and thus could carry out ATP hydrolysis.

In another set of experiments, submitochondrial particles were first transferred to the TPT mixture at 0.32 and 3.8% water and preincubated at 73 °C for 30 min. At this time, substrate was added in an all-water solution, or in the TPT mixture. These additions were made so as to yield in the reaction mixture a final water concentration of 3.8%; after the addition, the amount of ATP hydrolyzed was measured throughout time (Figure 4B). ATP hydrolysis was observed when the water concentration in the preincubation was 0.32%, but not when it was 3.8%. Experiments similar to those of Figure 4B, in all-water media, indicated that at this temperature the enzyme failed to hydrolyze ATP.

Since ATPase activity was detected in TPT mixtures at temperatures where in all-water media the enzyme was inert, it was decided to determine the maximal temperature at which the mitochondrial ATPase could exert catalytic activity. Submitochondrial particles were first transferred to the standard TPT system at a water concentration of 0.32% and preincubated for 15 min at 81 °C. At this time, substrate in various water volumes was added to initiate the reaction, and the incubation was continued at 81 °C (Figure 5A). At water concentrations below 0.96%, no significant hydrolysis was detected, but at higher water concentrations, substantial hydrolysis took place. It was also observed that particles transferred to the TPT mixture containing 0.32% water and

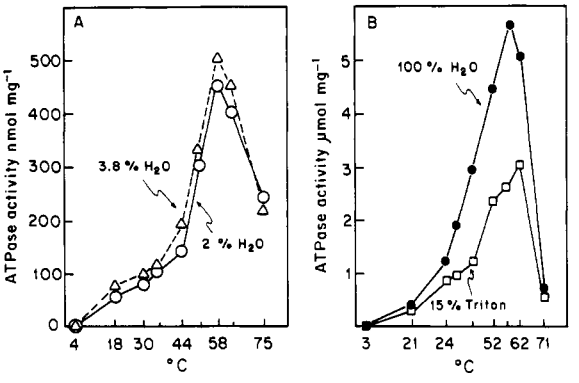

FIGURE 6: Optimal temperature for ATP hydrolysis in (A) toluene–phospholipid–15% Triton mixtures and (B) 100% water (●—●) and 15% Triton in water (□—□). In (A), 0.8 mL of a mixture that contained the substrate reaction mixture in the standard toluene–phospholipid–Triton mixture was incubated at the indicated temperature for 5 min. At this time, 0.2 mL of a toluene–phospholipid–Triton mixture that contained 150 μg of particle protein was added to start the reaction. In the reaction mixture, water was at a concentration of 2.0 (○—○) and 3.8% (△---△); other final concentrations were 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]ATP in the total water content. After 10-min incubation at the indicated temperatures, the reaction was stopped. In (B), the same protocol was followed, except that it was carried out in an all-aqueous media, or in 15% Triton X-100 in water (v/v). The reaction media contained 30 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 3 mM [γ-^{32}P]ATP, and 1 μM FCCP. The concentration of the particles was 200 μg/mL. The incubation time was 3 min.

preincubated for 15 min at 91 °C exhibited ATPase activity at this temperature when the substrate was introduced with 1.28% water (Figure 5B). The activity at high temperatures declined with the time of incubation (Figures 4 and 5). This suggests that during catalysis the enzyme underwent thermal denaturation. This may imply that in the conditions of the experiment, the introduction of water (with the substrate) allowed the enzyme to acquire the conformational mobility needed for catalysis, but at the same time decreased its thermostability.

Optimal Temperature for ATP Hydrolysis. There are few reports on the optimal temperature for catalysis by enzymes suspended in organic solvents or placed in reverse micelles (Sanchez-Ferrer et al., 1988; Khmelnitsky et al., 1988). Thus, the optimal temperature of the mitochondrial ATPase in all-water media, and in a space of limited water availability, was determined. At 2 and 3.8% water in the standard toluene–phospholipid–Triton mixture, the optimal temperature for ATP hydrolysis was around 58 °C (Figure 6A). In all-water media, and in water with 15% Triton, the optimal temperature was in the same range (Figure 6B); similar results were obtained by Adade et al. (1987). Although these studies indicated that the concentration of water did not affect the optimal temperature of the enzyme, it was noted that the decay of enzyme activity that appeared at temperatures higher than 58 °C was lower at low water concentrations (Figure 6, compare panels A and B). These latter findings (and those of Figures 4 and 5) indicated that in a low-water environment, the preservation of enzyme activity, at temperatures above the optimal temperature, was higher than in all-water media. A comparison of the thermophilic and mesophilic ATPases revealed that thermostability of the former is related to a higher number of ioinic bonds, which apparently could intensify subunit–subunit interactions (saishu et al., 1986). A similar phenomenon may take place when particulate F_1 is placed in a limited water space.

Stability of the Mitochondrial ATPase at High Temperatures. It has been shown that particulate and soluble mitochondrial ATPases have a high thermostability in systems that contain toluene, phospholipids, and a low water content (Ayala et al., 1986; Garza-Ramos et al., 1989). The same phenomenon was observed in the TPT mixture; Table II shows the half-life of the enzyme at various temperatures and water concentrations. From the data, it is apparent that between 0.32 and 0.9% water, there was a marked decrease in thermostability. These are the levels of water at which catalysis became apparent. In addition, it is important to note that in the experiment of Figure 5, ATPase activity was started by the addition of substrate to enzymes preincubated at 81 or 91 °C. Since during the preincubation a substantial number of enzymes were inactivated (Table II), the activity detected (Figure 5) was due to the remaining active enzymes. This suggests that at high temperatures the activity would be significantly higher if expressed per number of remaining active enzymes.

Table II: Half-Lives of ATPase Activity of Various Temperatures and Concentrations of Water[a]

	half-lives (min), % H_2O (v/v)			
temp (°C)	0.32	0.96	1.28	100
58	74	45	21	2
72	15	4	4	1
81	8	4	3	

[a] Particles were transferred at room temperature to the standard toluene–phospholipid–15% Triton mixtures at the indicated water concentrations. The concentration of the particles was 150 μg/mL of the mixture. The tubes were stoppered and incubated at the indicated temperatures. At different times, aliquots were withdrawn and placed in ice. Afterward, the ATPase activity of the samples was measured by adding the reaction mixture in water so as to yield 5% water and 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]ATP in the total water content; the incubation time was 10 min at a temperature of 20 °C. In 100% water, submitochondrial particles were incubated in 0.25 M sucrose at a concentration of 1 mg/mL. In this case, ATPase activity was measured in 30 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 3 mM [γ-^{32}P]ATP, 1 μM FCCP, and 100 μg of particle protein/mL, in a 5-min incubation time at a temperature of 20 °C.

Question of the Low ATPase Activity of Submitochondrial Particles in the Toluene–Phospholipid–Triton System. In a totally aqueous system, at 20 °C the V_{max} of ATPase activity of the particles employed was around 600 nmol min^{-1} mg^{-1}. In the TPT system, it was on the order of 6 nmol min^{-1} (mg of particles)$^{-1}$. There may be several explanations for this low activity. One is that the enzyme denatures when transferred to the TPT system. This possibility was explored by transferring particles to the TPT system followed by centrifugation of the mixture (as in Table I). The resulting pellet was suspended in 20 mM Tris-HCl, pH 7.4, and its ATPase activity was assayed in an all-water system; the total activity of the pellet was around 80% of that of the starting particles. Very similar data were obtained after the particles were tranferred to TPT mixtures in 0.32, 0.64, 2.32, 3.72, 4.02, 4.8, and 5.8% water. Thus, irreversible denaturation of the enzyme does not seem to be the cause of the low activity in TPT mixtures. Alternatively, the low ATPase activity could be due to a limitation in the transfer of substrate to the enzyme, particularly if it is considered that the substrate is in a compartment different from that of the enzyme (Table I); also, it is possible that in the TPT system, only a fraction of the enzymes are active. The former possibility was not tested, but it is known that there is fast communication between reverse micelles (Fendler, 1982). With respect to the number of active enzymes, it was reported that about 80% of cytochrome oxidase transferred to a system of toluene and phospholipids could be reduced by ascorbate (Escamilla et al., 1989). Although these experiments were made in the absence of Triton, the data

suggest that in the system, at least 80% of the enzyme population was accessible to substrate.

Finally, there is the possibility that in a low-water environment, the low ATPase activity is due to a hindrance of the conformational changes that the enzyme undergoes during the catalytic cycle. In fact, Rupley et al. (1983), Schinkel et al. (1985), and Careri et al. (1980, 1986) observed that as the hydration shell of lysozyme increased from 0.25 to 0.5 g of water/g of enzyme, there was an increase in catalytic rates. Moreover, Escamilla et al. (1989) found that in a low-water environment, the catalytic cycle of cytochrome oxidase was arrested after reduction of cytochrome *a*; apparently, higher amounts of water were required for the operation of a full catalytic cycle. Therefore, all these findings are consistent with the idea that enzymes placed in a low-water environment lack the freedom to undergo the conformational changes involved in catalysis, and thus the overall rates of catalysis would be diminished.

This suggested the relative rigidity of the enzyme in a low-water environment would be in consonance with the high thermostability of the enzyme in such a condition, particularly because thermal denaturation also involves water-dependent structural transitions of the protein (Ahern & Klibanov, 1985; Klibanov, 1983; Zale & Klibanov, 1986; Ooi & Oobatake, 1988). Thus, taken together, the overall data suggest that the amount of water that surrounds the enzyme effects its catalytic rates, as well as its sensitivity to thermal denaturation. That is, at increasing amounts of water around the enzyme, higher catalytic rates are attained, but at the cost of an increased sensitivity to thermodenaturation.

In conclusion, the mitochondrial ATPase when placed in a low-water environment exhibited a marked thermostability and carried out catalysis with water-soluble substrates and products at temperatures as high as 91 °C. These findings agree with those of Zaks and Klibanov (1984), who found that enzyme activity with hydrophobic substrates and products could take place at temperatures of 100 °C with enzymes suspended in organic solvents. However, with an enzyme that deals with water-soluble substrates and products, water, in addition to being required for enzyme conformational changes, is also needed for solubilization of substrates and products. Thus, it is possible that, with water-soluble substrates and products, catalysis would not be observed and/or preserved for significant lengths of time at the highest temperature at which the enzyme could work, since water that necessarily accompanies the substrates and products would favor denaturation of the enzyme. In this respect, it is interesting that in the system described here, the enzyme seems to localize to a compartment distinct from that of the substrates and products of the reaction, even though there is communication between the two compartments. This suggests that it may be technically feasible to separate water that surrounds the substrates and products of the reaction from that which surrounds the enzyme, thus allowing it to work at higher temperatures.

Registry No. Triton X-100, 9002-93-1; ATPase, 9000-83-3; Mg-ATP, 1476-84-2; toluene, 108-88-3.

References

Adade, A. B., O'Brien, K. L., & Vanderkooi, G. (1987) *Biochemistry 26*, 7297–7303.

Ahern, T. J., & Klibanov, A. M. (1985) *Science (Washington, D.C.) 228*, 1280–1284.

Ayala, G., Tuena de Gómez-Puyou, M., Gómez-Puyou, A., & Darszon, A. (1986) *FEBS Lett. 203*, 41–43.

Beltrán, C., Tuena de Gómez-Puyou, M., Darszon, A., & Gómez-Puyou, A. (1986) *Eur. J. Biochem. 160*, 163–168.

Bru, R., Sanchez-Ferrer, A., & Garcia-Carmona, F. (1989) *Biochem. J. 259*, 355–361.

Careri, G., Gratton, E., Yong, P. H., & Rupley, J. A. (1980) *Nature 284*, 572–573.

Careri, G., Giansenti, A., & Rupley, J. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6810–6814.

Chatenay, D., Urbach, W., Nicot, C., Vacher, M., & Waks, M. (1987) *J. Phys. Chem. 91*, 2198–2201.

Escamilla, E., Ayala, G., Tuena de Gómez-Puyou, M., Gómez-Puyou, A., Millán, L., & Darszon, A. (1989) *Arch. Biochem. Biophys. 272*, 332–343.

Fendler, J. H. (1982) in *Membrane Mimetic Chemistry* (Fendler, J. H., Ed.) pp 48–77, Wiley, New York.

Fletcher, P. D. I., Rees, G. D., Robinson, B. H., & Freedman, R. B. (1985) *Biochim. Biophys. Acta 832*, 204–214.

Garza-Ramos, G., Darszon, A., Tuena de Gómez-Puyou, M., & Gómez-Puyou, A. (1989) *Biochemistry 28*, 3177–3182.

Glynn, I. M., & Chappell, J. B. (1964) *Biochem. J. 90*, 147–149.

Grandi, C., Smith, R. E., & Luisi, P. L. (1981) *J. Biol. Chem. 256*, 837–843.

Hatefi, Y. (1985) *Annu. Rev. Biochem. 54*, 1015–1069.

Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Keiser, B. A., Varie, D., Barden, R. E., & Holt, S. L. (1979) *J. Phys. Chem. 83*, 1276–1280.

Khmelnitsky, Y. L., Zharinova, I. N., Berezin, I. V., Levashov, A. V., & Martinek, K. (1987) *Ann. N.Y. Acad. Sci. 501*, 161–164.

Khmelnitsky, Y. L., Hilhorst, R., & Veeger C. (1988) *Eur. J. Biochem. 176*, 265–271.

Klibanov, A. M. (1983) *Ad. Appl. Microbiol. 29*, 1–28.

Kumar, C., & Balasubramanian, D. C. (1979) *J. Colloid Interface Sci. 69*, 271–279.

Kumar, C., & Balasubramanian, D. C. (1980) *J. Colloid Interface Sci. 74*, 64–70.

Lee, K. M., & Bielman, J. F. (1986) *Bioorg. Chem. 14*, 262–273.

Low, H., & Vallin, I. (1963) *Biochim. Biophys. Acta 69*, 361–374.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Luisi, P. L., & Magid, L. J. (1986) *CRC Crit. Rev. Biochem. 20*, 409–474.

Martinek, K., Levashov, A. V., Klyacko, N., Khmelnitsky, Y. L., & Berezin, I. V. (1986) *Eur. J. Biochem. 155*, 453–468.

Martins, O. B., Gómez-Puyou, A., & Tuena de Gómez-Puyou, M. (1988) *Biophys. Chem. 29*, 111–117.

Nicot, C., Vacher, M., Vincent, M., Gallay, J., & Waks, M. (1985) *Biochemistry 24*, 7024–7032.

Ooi, T., & Oobatake, M. (1988) *J. Biochem. 103*, 114–120.

Poon, P. H., & Wells, M. A. (1974) *Biochemistry 13*, 4928–4936.

Pullman, M. E., Penefsky, H. S., Datta, A., & Racker, E. (1960) *J. Biol. Chem. 235*, 3322–3329.

Ramakrishnan, V., Darszon, A., & Montal, M. (1983) *J. Biol. Chem. 258*, 4857–4860.

Rupley, J. A., Gratton, E., & Careri, G. (1983) *Trends Biochem. Sci. (Pers. Ed.) 8*, 18–22.

Saishu, T., Nojima, H., & Kagawa, Y. (1986) *Biochim. Biophys. Acta 867*, 97–106.
Samama, J. P., Lee, K. M., & Biellmann, J. F. (1987) *Eur. J. Biochem. 163*, 609–617.
Sanchez-Ferrer, A., Bru, R., & Garcia-Carmona, F. (1988) *FEBS Lett. 233*, 363–366.
Schinkel, J. E., Downer, N. W., & Rupley, J. A. (1985) *Biochemistry 24*, 352–366.
Thompson, K. F., & Gierasch, L. M. (1984) *J. Am. Chem. Soc. 106*, 3648–3652.
Wheeler, C. J., & Croteau, R. (1986) *Arch. Biochem. Biophys. 248*, 429–434.
Zaks, A., & Klibanov, A. M. (1984) *Science 224*, 1249–1251.
Zaks, A., & Klibanov, A. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3194–3196.
Zaks, A., & Klibanov, A. M. (1988a) *J. Biol. Chem. 263*, 3194–3201.
Zaks, A., & Klibanov, A. M. (1988b) *J. Biol. Chem. 263*, 8017–8021.
Zale, S. E., & Klibanov, A. M. (1986) *Biochemistry 25*, 5432–5444.

Binding Modes of Inhibitors of Ribonuclease T_1 As Elucidated by Analysis of Two-Dimensional NMR

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ABSTRACT: Aromatic proton and high field shifted methyl proton resonances of RNase T_1 complexed with Guo, 2′GMP, 3′GMP, or 5′GMP were assigned to specific amino acid residues by analyses of the two-dimensional NMR spectra in comparison with the crystal structure of the RNase T_1–2′GMP complex. These assignments were subsequently correlated to those of free RNase T_1 [Hoffmann & Rüterjans (1988) *Eur. J. Biochem. 177*, 539–560]. The spatial proximities of amino acid residues as elucidated by NOESY spectra were found to be quite similar among free RNase T_1 and the inhibitor complexes, showing that large conformational changes did not occur upon complex formation. However, small but appreciable conformational changes were induced, which were reflected by the systematic chemical shift changes of some amino acid residues in the active site. Furthermore, we confirmed that RNase T_1 contains two specific binding sites, one for the guanine base and the other for the phosphate moiety. The inhibitors are forced to adapt their conformations to fit the guanine base and the phosphate moiety to each binding site on the enzyme. This is consistent with our previous studies that 2′GMP and 3′GMP take the syn form as a bound conformation, while 5′GMP takes the anti conformation around glycosidic bonds [Inagaki et al. (1985) *Biochemistry 24*, 1013–1020]. The slow-exchange process between free and bound forms involving Tyr42 and Tyr45 was found to be specific to the recognition of the guanine base.

Ribonuclease T_1 (RNase T_1)[1] (EC 3.1.27.3) is an acidic protein (104 amino acid residues) isolated from Takadiastase, a commercial product of *Aspergillus oryzae* (Sato & Egami, 1957). RNase T_1 specifically cleaves ribonucleic acid (RNA) chains at guanylic acid residues. Rigorous recognition of a guanine base by RNase T_1 is a typical example of an RNA–protein interaction. A number of studies have been made to elucidate the specific interaction and the enzymatic mechanism of RNase T_1. From chemical and kinetic studies, amino acid residues located in the active site have been suggested: His40, His92, Glu58, and Arg77 (Takahashi & Moore, 1982; Osterman & Walz, 1979). Nuclear magnetic resonance (NMR) analyses also have given valuable information on this point (Rüterjans & Pongs, 1971; Arata et al., 1979; Inagaki et al., 1981, 1985; Kyogoku et al., 1982; Hoffmann & Rüterjans, 1988). Recently, the crystal and molecular structures of RNase T_1–2′GMP (Heinemann & Saenger, 1982; Sugio et al., 1985a, 1988; Arni et al., 1988) and RNase T_1–3′GMP (Sugio et al., 1985b) have been elucidated by X-ray analysis. In the present study, we report the analysis of the NMR spectra of RNase T_1 complexed with inhibitors by the 2D NMR method. By comparing the results of the NOE experiments with the molecular structure obtained by the X-ray analysis, the aromatic proton and the high field shifted methyl proton resonances in the RNase T_1–inhibitor complexes were assigned to the specific amino acid residues. These assignments were subsequently compared with those obtained by the analysis of free RNase T_1 (Hoffmann & Rüterjans, 1988). On the basis of the present assignments and the conformational properties of the inhibitors, the binding modes of the inhibitors to RNase T_1 were discussed.

Experimental Procedures

RNase T_1 was prepared from Takadiastase by affinity chromatography (Kanaya & Uchida, 1981). The enzymatic

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[1] Abbreviations: RNase T_1, ribonuclease T_1; Guo, guanosine; 2′GMP, guanosine 2′-monophosphate; 3′GMP, guanosine 3′-monophosphate; 5′GMP, guanosine 5′-monophosphate; DQF-COSY, double quantum filtered correlated spectroscopy; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; 2D NMR, two-dimensional NMR; CIDNP, chemically induced dynamic nuclear polarization; 1D NMR, one-dimensional NMR.

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