Catalysis and Thermostability of Mitochondrial F_1-ATPase in Toluene–Phospholipid–Low-Water Systems†

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ABSTRACT: Soluble mitochondrial F_1-ATPase from bovine heart can be transferred to systems composed of a nonpolar solvent (toluene), phospholipid, and water at concentrations between 0.02 and 0.05% (volume of water per volume toluene). In these systems, F_1 becomes resistant to cold denaturation and acquires a remarkable thermostability; i.e., its half-life at 70 °C is more than 24 h. Thermostability is due to the low content of water, since increases of water concentration bring about a progressive decrease in thermostability. At 0.04% water, the enzyme fails to catalyze a single splitting of ATP per enzyme. Gradual increases in water concentration up to 2.5% result in a progressive increase of hydrolytic activity. However, even at 2.5% water, the activity is orders of magnitude lower than in totally aqueous media. At various concentrations of water (0.1–2.5% v/v) and Mg-ATP, it was found that water affects the V_{max}, but not the K_m. The results show that, at levels of water below 0.04% (v/v), the enzyme is in a state that does not carry out catalysis and possesses high thermostability. As the water content is increased, the enzyme acquires the progressive flexibility that is required for catalysis and for undergoing rapid thermal denaturation.

Considerable work is now being done on the catalytic properties of enzymes in media that are predominantly composed of organic solvents (Martinek et al., 1986; Luisi & Magid, 1986; Klibanov, 1986). This is because in such environments enzymes exhibit characteristics that in some cases are different from those observed in totally aqueous media. It is expected that knowledge of these novel properties would provide a better understanding of enzyme structure and function, and also as to how water modulates these two parameters. Essentially, two approaches have been followed to study the properties of enzymes in organic media. One is to transfer enzymes to organic solvents via "reversed micelles" that are built with synthetic detergents with or without a cosurfactant (Wolf & Luisi, 1979; Dozou et al., 1979; Menger & Yamada, 1979; Martinek et al., 1986); in this system it is visualized that enzymes arrange themselves in the internal water space of the micelle (Grandi et al., 1981; Ramakrishnan et al., 1983; Thompson & Gierash, 1984; Chatenay et al., 1987). A second approach is to suspend an enzyme at various levels of hydration in organic solvents (Zaks & Klibanov, 1984, 1985).

In "reversed micelles", enzymes exhibit catalytic properties that in some parameters differ quantitatively from those detected in all water media (Menger & Yamada, 1979; Klyachko et al., 1984; Barbaric & Luisi, 1981). In contrast, when enzymes, such as lipases, proteases, oxidases, and dehydrogenases, are suspended in organic solvents, they display very unusual properties, i.e., a very high thermostability and catalytic reactions that are different from those observed in aqueous media (Zaks & Klibanov, 1984, 1985, 1988a,b; Wheeler & Croteau, 1986).

A variation of the first approach is the transfer of enzymes to organic media by means of phospholipids (Darszon et al., 1978; Ayala et al., 1985). In these systems, it has been shown that some membrane-bound enzymes exhibit a striking thermostability (Ayala et al., 1986); however, in these conditions their catalytic activity has not been studied. At first sight, the phospholipid system would be analogous to that in which enzymes are transferred to organic media via "reversed micelles"; however, there are no experimental data that substantiate this assumption. Here we have studied some of the basic properties of soluble F_1-ATPase of heart mitochondria in systems composed of an organic solvent (toluene), phospholipid, and water at a concentration of 0.04–2.5% (volume per volume of organic solvent). It was found that the characteristics of the enzyme are modified by the water content; at low water concentrations, the enzyme is catalytically inert and has a high thermostability, but as the water content is increased, catalysis increases and thermostability decreases.

The soluble F_1-ATPase is a complex enzyme made up of five different subunits in a defined stoichiometry (Hatefi, 1985). It has three catalytic sites that function through a concerted binding change mechanism (Boyer, 1987). Thus if an enzyme as complex as F_1 can be transferred to an organic media, it could be reasonably expected that all enzymes can be transferred and studied in such media.

Materials and Methods

The following materials were obtained from the indicated sources: toluene (Merck); soybean phospholipids [Sigma, purified according to Kagawa and Racker (1971)]; Sephadex G-50 (Pharmacia Fine Chemicals); ^3H_2O (NEN); [^{32}P]P_i (ICN), purified as described by de Meis (1984); [γ-^{32}P]ATP [prepared according to Glynn and Chappel (1964)].

Mitochondria were isolated from bovine hearts (Low & Vallin, 1963), from these mitochondria F_1 was prepared as described before (Tuena de Gómez-Puyou & Gómez-Puyou, 1977). The enzyme preparation was stored at 4 °C in 2 mM ATP, 2 mM EDTA, and $(NH_4)_2SO_4$ at 50% saturation. F_1 stored at this concentration of ATP has about 3 mol of ATP bound per mole of enzyme (Garret & Penefsky, 1975). The various preparations of F_1 used in this work had ATPase

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activities that ranged between 70 and 80 μmol min^{-1} mg^{-1} as assayed by the spectrophotometric method (see below). Before use the suspension was centrifuged and the pellet dissolved in 10 mM Tris-HCl, pH 8.0, and desalted by the centrifuge column procedure using Sephadex G-50 (Kasahara & Penefsky, 1978); the column was previously equilibrated with 10 mM Tris-HCl, pH 8.0.

Transfer of Soluble F_1 to Toluene. The standard procedure for the transfer of F_1 to toluene was as follows: soybean phospholipids were dispersed by sonication at a concentration of 100 mg/mL in 10 mM Tris-HCl, pH 8.0. To 20 μL of the dispersed phospholipids placed in 1.8 × 14.8 cm test tubes was added around 100 μg of F_1 dissolved in 20 μL of 10 mM Tris-HCl, pH 8.0, and the suspension was mixed. To this mixture was added 1 mL of toluene, followed immediately by sonication for 30 s in a water bath sonicator (Bransonic 220, Plainview, NY). An emulsion was formed. Reduction in the content of water in the system was achieved by passing a N_2 current through the turbid emulsion (Darszon et al., 1988). When the volume was reduced to approximately 0.65–0.7 mL, the system became optically transparent, and its content of water ranged from 0.02 to 0.05% (v/v). If higher amounts of water were initially introduced, larger volumes had to be evaporated in order to achieve transparency.

Catalytic Properties of F_1 in Toluene Extracts. The hydrolytic properties of F_1 and its thermostability in extracts prepared by the standard procedure were studied. The detailed protocols for these experiments are described under Results, but the technique to measure ATPase activity was as follows: to the enzyme in the transparent state was added a water solution that contained 10 mM Tris-HCl, pH 8.0, [γ-^{32}P]ATP, and $MgCl_2$. In this form substrates and water are simultaneously introduced into the system; at the most 25 μL of water was added per milliliter of organic extract. Alternatively, Mg-ATP was added in toluene-transparent extracts. These were prepared by sonicating 50 μL of 10 mM Tris-HCl, pH 8.0, 200 μM [γ-^{32}P]ATP, and 1 mM $MgCl_2$ with 1 mL of toluene that contained 2 mg of phospholipid; the emulsion was exposed to a current of N_2. After partial evaporation a transparent system was obtained. The latter contained 0.02–0.05% water (v/v). The substrate in this state was added to the transparent system that contained F_1. In this form the concentration of water in the system was not significantly modified, since both the enzyme and substrate extracts contained approximately equal concentrations of water.

Independently of the state in which the substrates were added to the enzyme, the reaction was arrested by vigorously mixing 0.2 mL of the enzyme–substrate mixture with 0.5 mL of 6% trichloroacetic acid in water. To this suspension were added 0.5 mL of 3.3% ammonium molybdate/3.75 N H_2SO_4 and 0.2 mL of acetone, followed by stirring in a vortex mixer. This was followed by addition of 1 mL of water-saturated isobutyl alcohol–benzene (1:1). After being stirred in a vortex for 1 min, the mixture was centrifuged to achieve phase separation. An aliquot of the organic phase was withdrawn to assay [^{32}P]P_i; from these data ATPase activity was calculated. Blanks that contained all the ingredientes of the reaction but the enzyme were always made. Control experiments indicated that all [^{32}P]P_i or [γ-^{32}P]ATP partitioned into the water phase when a 0.2-mL aliquot of the organic extracts was mixed with 0.5 mL of trichloroacetic acid. The specific activity of the [γ-^{32}P]ATP was 90 000 cpm/nmol. It should be noted that a single hydrolytic cycle per F_1 could have been detected, since the experiments to assay ATP hydrolysis were made with an amount of enzyme that was around 100 μg (0.27 nmol).

In some cases, it was necessary to ascertain if F_1 had been inactivated or denatured either in its transfer to toluene or in toluene extracts that had been subject to various treatments. In these cases, toluene extracts were completely evaporated under a N_2 current. To the dry residue was added 200 μL of 10 mM Tris-HCl, pH 8.0, and after hydration of the lipids the tube was stirred for 2 min in order to achieve a homogeneous suspension. The ATPase activity of the aqueous suspension was assayed. This was measured either spectrophotometrically by using a coupled enzyme system, following the oxidation of NADH at 340 nm (Pullman et al., 1960), or alternatively in media that contained 30 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]ATP in a final volume of 0.2 mL; the reaction was arrested after 3 min of incubation with 6% trichloroacetic acid. Formation of [^{32}P]P_i was measured as described above.

Determination of Water Content. The amount of water in toluene extracts was determined by using ^3H_2O. In a typical experiment, phospholipids and F_1 were suspended in 10 mM Tris-HCl, pH 8.0, that contained ^3H_2O (50 000 cpm/μL). After following the steps for the transfer of the enzyme to toluene described above, an aliquot of the transparent system was removed and radioactivity determined by liquid scintillation counting. The amount of water in the resulting transparent extract was calculated; the results are expressed as percent of volume of water per volume toluene (v/v).

Protein was determined by the procedure described by Lowry et al. (1951).

Results

Transfer of F_1 to Toluene Extracts. The ATPase of whole mitochondria or submitochondrial particles can be transferred to organic solvents in a state in which the solvent–phospholipid–enzyme complex is optically transparent (Ayala et al., 1986). However, attempts to transfer soluble F_1 to organic solvents by the described methodology showed that although transparency could be achieved, only a small fraction of the activity was recovered. Accordingly, the reported methodology was systematically modified in order to establish optimal conditions for the transfer of F_1. The criterium for judging success of transfer was the degree of activity recovered after the enzyme was first taken to the transparent state and then back to an all-water system.

The transfer of F_1 to the solvent depended on the amount of phospholipids introduced; i.e., maximal transfer of an active enzyme was achieved with 2 mg of phospholipid/mL of toluene (Figure 1A). In an all-water medium the amount of phospholipid employed did not affect the activity of F_1 (not shown). It was also observed that the higher the concentration of the enzyme, the higher the activity that was recovered (Figure 1B). Also it was found that, with a fixed amount of enzyme, its transfer to the organic solvent diminished as the amount of water in the starting preparation was increased (Figure 1C). According to these results, it was decided to use as the standard extraction procedure 2 mg of phospholipid and approximately 100 μg of F_1 dissolved in 10 μL of 10 mM Tris-HCl, pH 8.0.

Stability of F_1 in Toluene Extracts. Once F_1 was extracted into the organic solvent, little loss of activity was observed after 7 h of being in the solvent; in fact, in all-water media, the decay of activity was faster (Figure 2A). In an all-water medium, F_1 is cold sensitive; this is due to dissociation of its subunits, which results from diminution of a hydrophobic effect (Penefsky & Warner, 1965). Indeed, F_1 incubated in aqueous media at 4 °C lost almost all of its activity in about 2 h, but the enzyme in the organic extract retained 70% of its activity, even after 7 h (Figure 2B) at 4 °C.

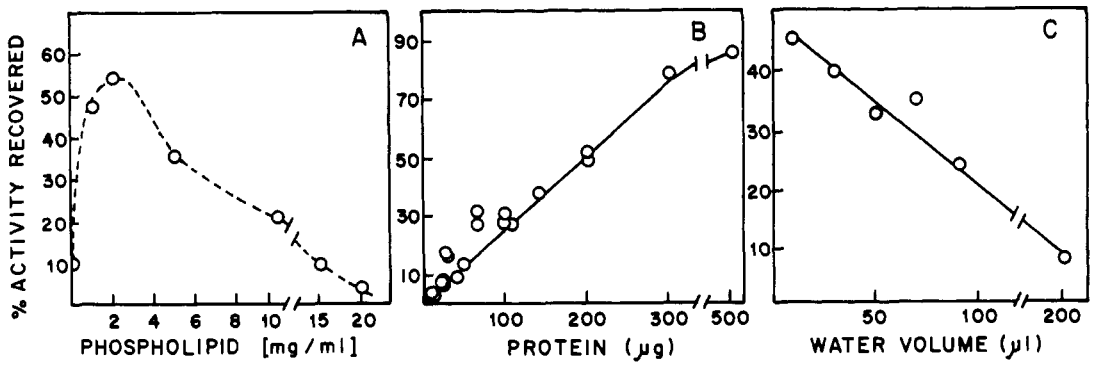

FIGURE 1: Effect of the concentration of phospholipid, protein, and water on the transfer of F_1 to toluene. The standard procedure to transfer F_1 to toluene is described under Materials and Methods, except that, in (A), the indicated amounts of phospholipids were used and the amount of F_1 used was 0.2 mg. In (B), the indicated amounts of F_1 in a volume of 50 μL were transferred; the amount of phospholipid in these experiments was 2 mg. In (C), 100 μg of F_1 was dissolved in the indicated volumes of buffer (10 mM Tris-HCl, pH 8.0) and transferred to toluene with 2 mg of phospholipids. After transfer, the extracts were evaporated under a current of N_2; the dry residue was suspended in 10 mM Tris-HCl, pH 8.0, and its ATPase activity was measured by the spectrophotometric method. The data are expressed as 100% of the activity recovered in which 100% is the activity of the starting F_1 preparation, which was 60 μmol min^{-1} mg^{-1}.

Lipases (Zaks & Klibanov, 1984), terpene cyclases (Wheeler & Croteau, 1986), proteases (Zaks & Klibanov, 1988a), and particulate mitochondrial ATPase and cytochrome oxidase (Ayala et al., 1986) in organic solvents exhibit a thermostability several orders of magnitude higher than in aqueous media. Apparently, in organic solvents, the lack of water hampers the stabilization of incorrect protein structures (Zaks & Klibanov, 1985); the breakage of H bonds by water has also been postulated to be an important factor in thermal denaturation (Klibanov, 1986). Here, it was found that soluble F_1 transferred to toluene shows a high thermostability; at a concentration of about 0.03% water (v/v) its half-life at 70 °C was more than 24 h (Figure 3A). At this temperature in an all-water medium the enzyme denatured almost instantaneously (not shown).

When toluene extracts with a relatively high amount of water are incubated at high temperatures, some of the water is lost through evaporation (Ayala et al., 1985). Although this water loss makes a precise analysis of water contribution to thermal denaturation difficult, the phenomenon may be used to ascertain if thermal denaturation in organic solvents is related to the water content of the system. Thus, transparent extracts heated to 70 °C were injected with ^3H_2O to a value of 0.18% (v/v), and the incubation at 70 °C was continued (Figure 3B). After 30 min, the activity dropped by about 70% and the water content to approximately 0.1%; after this time the activity remained stable for 8 h. Thus, values of water higher than 0.1% are needed to bring about thermal denaturation. In another experiment, the initial water concentration of toluene extracts was poised at 0.32%, and the enzyme lost almost 100% of its activity when incubated at 70 °C for 30 min (Figure 3C). In this case the water content fell during the incubation, but thermal denaturation was faster than water evaporation. Thus the data indicate that the low content of water in the transparent extracts accounts for the thermostability of F_1. Thermal denaturation of F_1 in toluene extracts supplemented with five different concentration of water was measured at 70 °C at short incubation times (Figure 4). The rate of decay of activity increased as the concentration of water was increased.

Hydrolytic Activity of F_1 in Toluene Extracts. Experiments were conducted to explore if in the transparent state the enzyme could hydrolyze ATP. Thus transparent toluene extracts of F_1 were prepared, and [γ-^{32}P]ATP hydrolysis was assayed after the addition of transparent toluene extracts that contained Mg-[γ-^{32}P]ATP (see Materials and Methods). No formation of [^{32}P]P_i was detected after 20 h of incubation of 25 °C under

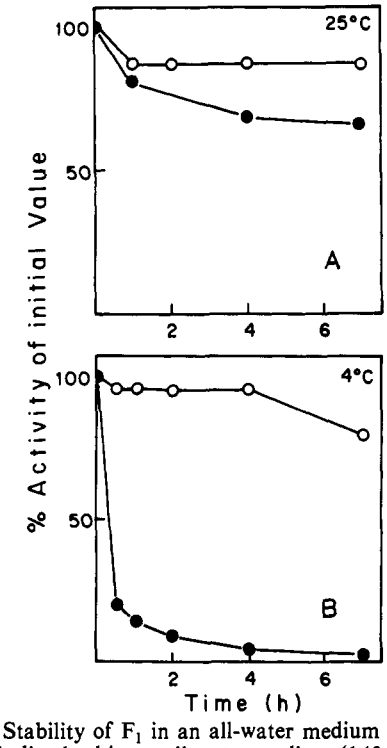

FIGURE 2: Stability of F_1 in an all-water medium and in toluene extracts. F_1 dissolved in an-all water medium (140 μg in 10 μL of 10 mM Tris-HCl, pH 8.0) was transferred to toluene by the standard procedure. The extract was incubated at 25 °C (A) and 4 °C (B). Aliquots were withdrawn at the indicated times, and their ATPase activity was measured after evaporation of the solvent as in Figure 1 (○). The traces indicated by (●) show the ATPase activity of F_1 incubated in an all-water system. The ATPase activity of the starting F_1 preparation was 70 μmol min^{-1} mg^{-1} in an all-water system, whereas the initial activity of the enzyme after transfer to toluene and back to water was 28 μmol min^{-1} mg^{-1}.

vigorous mixing. The lack of activity could be due to inaccessibility of the substrate to the enzyme; thus F_1 was sonicated with phospholipids and toluene as in the standard procedure. After 15 s of sonication 50 μL of a water solution that contained Mg-[γ-^{32}P]ATP was added, and sonication was continued for another 15 s. The emulsion was thereafter taken to the transparent state, and an aliquot was withdrawn to assay the amount of [^{32}P]P_i. This was about 20% of the total [γ-^{32}P]ATP introduced. Thereafter, aliquots were withdrawn for as long as 20 h to determine if hydrolysis took place in the transparent system. According to the protocol, it was expected that in the transparent system both [γ-^{32}P]ATP and the en-

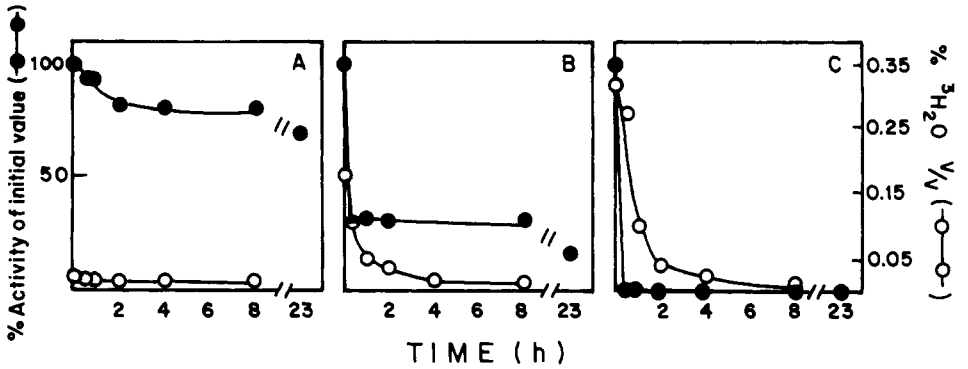

FIGURE 3: Relation between the content of water in toluene extracts and thermal inactivation of F_1. In (A), transparent toluene extracts were prepared by the standard procedure except that the phospholipids and F_1 were suspended in 10 mM Tris-HCl, pH 8.0, that contained ^3H_2O. This had the amount of ^3H_2O shown in the right ordinate at time zero (v/v). Therafter, a 2-mL aliquot of the extract was incubated in tightly stopped 1.0 × 8.5 cm tubes at 70 °C. At the indicated times, aliquots were withdrawn to measure radioactivity (○) and ATPase activity (●). In (B) and (C), the transparent extracts were injected with 10 mM Tris-HCl, pH 8.0, made in ^3H_2O to yield the amount of ^3H_2O shown in the ordinates. After injection, the tubes were incubated at 70 °C, and aliquots were withdrawn at the indicated times to assay ATPase activity (●) and radioactivity (○). ATPase activity was measured after evaporation of the extract as in Figure 1. One hundred percent ATPase activity corresponds to the activity of the extract before it was incubated at 70 °C (30 μmol min^{-1} mg^{-1}).

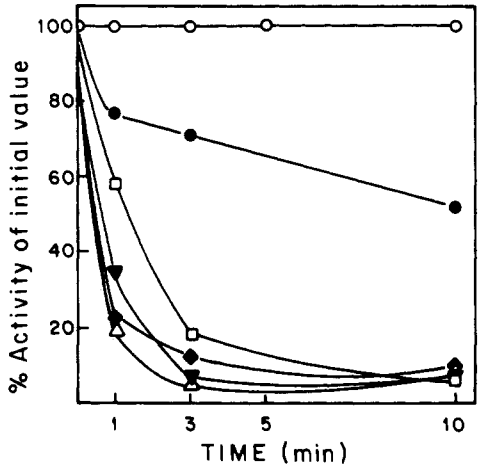

FIGURE 4: Time course of thermal inactivation of F_1 at 70 °C in toluene extracts supplemented with various amounts of water. Transparent toluene extracts were prepared by the standard procedure. One-milliliter aliquots of these extracts were incubated at 70 °C. After 2 min (the milliliter inside of the tube reached a temperature of 70 °C in less than 15 s), an aliquot was withdrawn for assay of ATPase activity (see below) and different volumes of 10 mM Tris-HCl, pH 8.0, were injected and stirred vigorously. In the time used for this procedure, the temperature dropped by 1–2 °C. The amount of water injected yielded the following final concentrations (v/v): (●) 0.15%; (□) 0.3%; (▼) 0.45%; (♦) 1.35%; (△) 2.5%. In (○) no water was added. Aliquots of 0.2 mL were withdrawn at the indicated times to determine maximal ATPase activity; this was done after evaporation of the solvent as described in Figure 1, except that ATPase activity was determined by using [γ-^{32}P]ATP (see Materials and Methods).

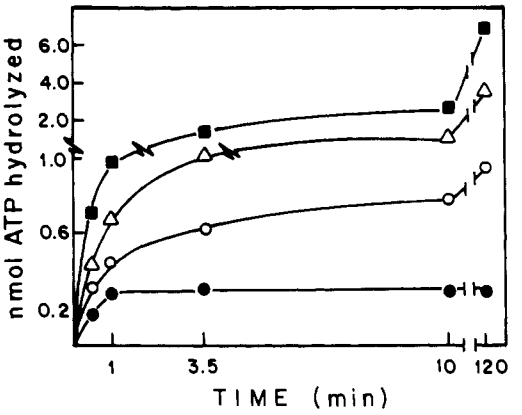

FIGURE 5: Time course of ATP hydrolysis by F_1 in toluene extracts that contained different amounts of water. F_1 (0.11 mg) was transferred by the standard procedure. Several extracts were prepared, pooled, and divided in aliquots of 2.0 mL. Into the 2-mL aliquots were injected different volumes of substrate solution that contained 10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, and 200 μM [γ-^{32}P]ATP. After the injection, the mixture was vigorously stirred, and 0.2-mL aliquots were withdrawn and added to 0.5 mL of 6% trichloroacetic acid. The amount of [^{32}P]P_i formed was determined. The amount of water in the ternary system (v/v) was as follows: (●) 0.25%; (○) 0.5%; (△) 1.0%; (■) 2.5%. The results indicate the amount of ATP hydrolyzed in the 2-mL system.

zyme would be in the same compartment. The results showed that no hydrolysis of [γ-^{32}P]ATP took place, even though the specific activity of the substrate and the amount of enzyme were such that a single ATP splitting per enzyme could have been detected.

Klibanov and co-workers (Zaks & Klibanov, 1984, 1985, 1988a,b) reported that enzymes suspended in organic solvents exhibit catalysis; however, their experiments indicate that water is needed for catalysis, even though the amount could be as low as that required to form a monolayer around the protein. It was explored if the injection of low amounts of water into the F_1–phospholipid–toluene system could induce catalysis. This proved to be the case. In the experiment of Figure 5, various volumes of water that contained Mg-[γ-^{32}P]ATP were added to F_1 in the transparent state; at various times formation of [^{32}P]P_i was assayed. At water concentrations of 0.15% (v/v), hydrolysis was detected and increased progressively up to 2.5% water; the rate of ATP hydrolysis was high in the first minute after the addition of substrate and then decreased. The cause of this biphasic pattern is not clear, but it is not due to denaturation of the enzyme during catalysis, since the enzyme transferred back to an all-water system showed catalytic activity. It is noteworthy that, in a 2-h incubation, not all the ATP was hydrolyzed. In this respect, it is interesting that Han et al. (1987) reported that the extent of lipid hydrolysis by lipase in organic solvents depended on the amount of water in the system. Also it is pointed out that, even at the highest amount of water injected into the system, the ATPase activity was orders of magnitude lower than that detected in totally aqueous media.

Experiments were done to determine if ATP hydrolysis by F_1 in the conditions of Figure 5 depended on Mg-ATP concentrations. Fixed volumes of water that contained different concentrations of Mg-[γ-^{32}P]ATP were injected to transparent extracts that were prepared with 100 μg of F_1. The amount of substrate hydrolyzed in 1 min was determined. The volumes of water injected yielded the following final concentrations (v/v): 0.15%, 0.3%, 0.45%, 1.35%, and 2.5%. Each water

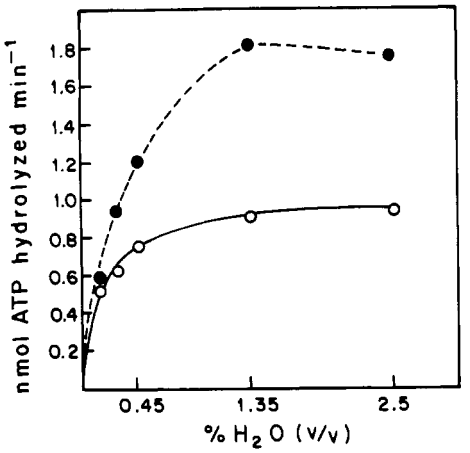

FIGURE 6: Dependence of enzymatic activity on the concentration of water in toluene extracts. Into 1 mL of transparent toluene extracts were injected different volumes of water; this yielded the indicated water concentrations (v/v). The different volumes of water added contained 1.5 (○) and 3.0 (●) nmol of Mg-[γ-^{32}P]ATP. After 1 min of incubation, the reaction was arrested and the amount of [^{32}P]P_i formed was measured.

concentration had various Mg-[γ-^{32}P]ATP concentrations that ranged from 0.1 to 2 mM. The activity was higher as the amount of water was raised, and also it was observed that, at all concentrations of water, hydrolysis exhibited saturation kinetics and that double-reciprocal plots yielded straight lines. In the latter, the lines intersected the abscissas at nearly the same point; the apparent K_m ranged from 0.8 to 1 mM. Thus the effect of water was mainly on the V_{max}, i.e., 1.0, 1.8, 2.9, 10.2, and 16.4 nmol min^{-1} [mL of extract (containing the respective aforementioned water concentrations)]$^{-1}$. These data indicated that when increasing volumes of water of equal substrate concentrations were injected, the activity increased. This could result from an increase in the amount of water or from an increase in the amount of substrate in the whole system. Thus from the data of the preceding experiments, enzyme activity at a *fixed amount of substrate* in the system was plotted against water concentration (Figure 6). The activity increased with water concentration, notwithstanding that the concentration of ATP in the water injected decreased as the amount of water increased. However, the extent to which water participates as substrate in the hydrolytic reaction could not be evaluated from the present data.

Discussion

Catalysis starts to appear in lysozyme when the amount of water is about 0.2 g/g of enzyme (Careri et al., 1980, 1986; Rupley et al., 1983; Shinkel et al., 1985); at this hydration level, lysozyme exhibits motional properties in its interior and surface. As hydration is increased, the activity of lysozyme continues to increase (Careri et al., 1980; Rupley et al., 1983). F_1, transferred to a system that contains 0.04% water, fails to hydrolyze ATP and has a high thermostability. This suggests that, in this state, the enzyme flexibility required for catalysis and thermal denaturation is hindered. As the water content of the system is increased, the enzyme exhibits an increasingly higher catalytic activity and a decrease in thermostability (Figure 7 illustrates the relation). Some of the data of Zaks and Klibanov [Figures 1 and 2B of Zaks and Klibanov (1984)] also show that catalysis by lipase in organic solvents appears at concentrations of water at which thermostability decreases. Thus it is likely that, in all enzymes, there may be an inverse relation between catalysis and thermostability that may be controlled by the amount of water surrounding the enzyme.

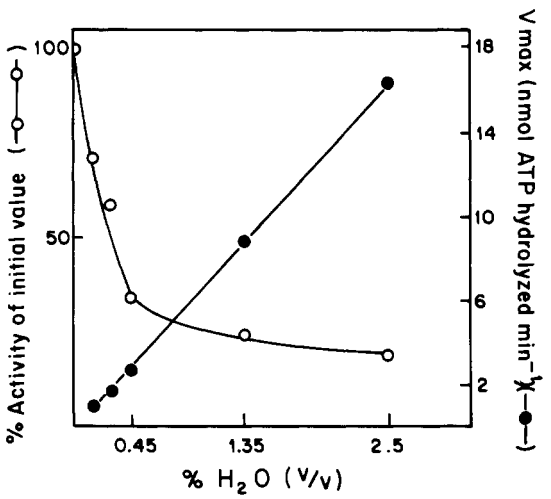

FIGURE 7: V_{max} and thermal inactivation of F_1 in toluene extracts with different amounts of water. The values of V_{max} (●) were calculated from Lineweaver–Burk plots at various concentrations of Mg-[γ-^{32}P]ATP at the indicated concentrations of injected water (see text under Results). The values for thermal inactivation were taken from the experiment in Figure 4. The percent of activity remaining after 1 min of incubation at 70 °C with the indicated amounts of water (○) is plotted.

In systems composed of organic solvents and synthetic detergents, catalysis by numerous enzymes has been observed at molecular water to detergent ratios of 10–50 without loss of transparency (Martinek et al., 1986). Here at water to phospholipid ratios of about 10 (assuming an average molecular weight of 800 for phospholipids), F_1 is catalytically inert, and if water is added, the system becomes turbid. Thus there are differences in the two systems. Poon and Wells (1974) reported that phosphatidylcholine in ethereal solutions has a first hydration shell of 7 water molecules per lipid molecules. Thus in the transparent state the ratio of water molecules per phospholipid molecule would roughly correspond to the first hydration shell of phospholipids; this suggests that, in this state, F_1 is surrounded by water molecules that are in contact with the lipid. It is of relevance that Poon and Wells (1974) observed that phospholipase failed to hydrolyze phosphatidylcholine when it was in its first hydration state.

The formation of reversed micelles in organic solvents is a complex phenomenon (Wells, 1974; Maitra, 1984). Here it was found that, from 2 to 20 mg of phospholipid/mL of toluene, the amount of water in the solvent increased almost directly with the amount of phospholipid and that this was not affected by the inclusion of 100 or 300 μg of F_1. This suggests that phospholipids affect the number, but not the size, of the micelles and also that the protein could arrange itself in the water space limited by the lipids. If this is the case, in the low water volume of the transparent system, 100 μg of F_1 with its bound nucleotides would reach concentrations of about 250 mg/mL of water and adenine nucleotides millimolar concentrations. The concentration of enzyme and adenine nucleotides during the preparation of extracts probably explains why F_1 transfers better at initial low water values or high protein concentrations (Figure 1). Also it is likely that the high concentration of enzyme and nucleotide in the available water of the extracts contributes to the thermoresistance of the enzyme. Indeed, ATP exerts some protection against thermal denaturation (Penefsky et al., 1960).

A point that deserves comment is whether catalysis that appears when water is injected into the transparent system is due to a fraction of the enzyme that is transferrred to this water compartment or alternatively to all enzymes catalyzing

at limited rates. The observation that the water content of the system could not be increased without loss of transparency suggests that injected water results in formation of "water drops" where the enzyme may partition and exhibit catalytic activity. In fact, increasing volumes of injected water brought about a proportional increase in the V_{max} of the enzyme (Figure 7).

In conclusion, we think that the present data indicate that, in the transparent state, the enzyme is in an environment in which the low water content hinders catalysis and thermal denaturation. Thus the phospholipid–organic solvent system appears to be potentially useful for the study of enzymes in a state of limited interaction with water, particularly if it is considered that highly complex enzymes can be placed in such conditions.

Registry No. ATPase, 9000-83-3; ATP, 56-65-5; Mg–ATP, 1476-84-2; toluene, 108-88-3.

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