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Effect of Organic Solvents on the Beef Heart Mitochondrial Adenosine Triphosphatase†

Sheldon M. Schuster

ABSTRACT: The effect of organic solvents on the beef heart mitochondrial ATPase-catalyzed ATP and ITP hydrolysis was examined. It was observed that numerous organic solvents stimulated ATP hydrolysis while ITP hydrolysis was inhibited. Methanol at 20% (v/v) was found to stimulate ATP hydrolysis by over 300%, while at the same methanol concentration ITP hydrolysis was inhibited approximately 50%. In the presence of 20% methanol, ATP hydrolysis exhibited linear plots of $1/[ATP]$ vs. $1/v$, while in the absence of methanol negative cooperativity was observed. These data can be interpreted to imply that the catalytic and regulatory sites of the mitochondrial ATPase are being dissociated in 20% methanol. The effect of methanol on the hydrolysis of ATP and ITP was examined as a function of pH. It was found that, at high pH in totally aqueous solutions, the hydrolysis of ATP and ITP was inhibited, while the presence of 20% methanol either caused the hydrolytic rate to peak and remain constant above pH 8 (with ATP as substrate) or caused the rate of hydrolysis to continue to increase above pH 8 (when ITP was the substrate). These data are interpreted to indicate that an acidic group in the active site may be ionizing, limiting the ATPase-catalyzed hydrolytic rate, and, with 20% methanol, this ionization was inhibited.

The effects of organic solvent systems on the stability of beef heart mitochondrial ATPase (F_1) preparations have been explored in some detail, while the effects of these systems on F_1 catalytic activity have only been briefly described. Penefsky & Warner (1965) have shown that ethylene glycol, methanol, ethanol, and glycerol in concentrations from 2 to 20% (v/v) dramatically protected F_1 from cold inactivation. In addition, these authors found that all of the solvents except methanol caused an inhibition of F_1 activity. Methanol caused stimulations of F_1-catalyzed ATP hydrolysis activity with up to double the activity found in completely aqueous solutions. Recent work emphasizes the use of cosolvent systems such as 50% glycerol to stabilize liver and heart F_1 preparations

† From the Department of Chemistry and School of Life Sciences, The University of Nebraska, Lincoln, Nebraska 68588. *Received September 13, 1978; revised manuscript received December 29, 1978.* A portion of this work was presented in preliminary form in (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 3329. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for the support of this research.

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(Lambeth & Lardy, 1971; Rechtenwald & Hess, 1977a,b; Schuster & Wagner, 1978; Bruni et al., 1977). An important finding by Beechey and his colleagues (Beechey et al., 1975) has been the demonstration that the beef heart F_1 is removed from the membrane by a chloroform-saturated solution. This has led to a simplification of the purification of F_1 (Spitzburg & Blair, 1977) and to the knowledge that F_1 is remarkably stable to treatment by organic solvents. A report by Wang & Yang (1976) provides evidence that solvent perturbation of chloroplast membranes with cold acetone results in an enhanced rate of ATP synthesis.

Several studies on the effects of organic environments on submitochondrial particle-bound F_1 have appeared. Lenaz et al. (1975) reported that 1-butanol altered the oligomycin sensitivity of particle-bound F_1 and changed the activation energy. In additional studies, Bruni et al. (1975) and Knowles et al. (1975) have examined the effect of various acyl chains of phospholipids on the activity of an oligomycin-sensitive ATPase preparation and found that micelles of isoelectric phospholipids activate ATP hydrolytic activity. Swanljung et al. (1973) found that a similar oligomycin-sensitive ATPase is activated 18-fold by lysolecithin. Effects of both alcohols (Lenaz et al., 1971) and phospholipids (Toson et al., 1972; Bruni & Bigon, 1974) on the interactions of F_1 with the mitochondrial membrane have been reported. From these results, it appears that the functional organization of the inner mitochondrial membrane–F_1 complex is sensitive to environmental factors. Clearly, the major effect of changes in the nature of the solvent environment around this complex is evidenced by separation of the oligomycin sensitivity factors from the F_1, as well as solubilization of the ATPase complex from the membrane. None of these studies, however, are able to discriminate between effects of cosolvents directly on the F_1 or on the relation of F_1 to the normally associated proteins and lipids.

Clearly, an understanding of the hydrophobic nature of the ATP synthesis machinery is crucial to elucidation of the mechanism of ATP synthesis. The studies mentioned above are exploring the role of the hydrophobic nature of the F_1-membrane and F_1-oligomycin sensitivity conferring protein relationship. However, the effect of systems that are not totally aqueous on the catalytic properties of the purified F_1 has not been thoroughly explored. The present communication is an attempt to describe the effects of several solvent systems on the catalytic properties of isolated F_1.

Materials and Methods

Beef heart mitochondrial ATPase (F_1) was prepared by the method of Knowles & Penefsky (1972). Initial velocity experiments were performed in a total volume of 1.0 mL at 30 °C. Unless otherwise noted, the reaction mixture contained 50 mM triethanolamine chloride, pH 8.0, 200 mM sucrose, 2.5 mM free magnesium as $MgCl_2$, and nucleotides as indicated. Before use, isolated F_1 was centrifuged at 3 °C to remove the $(NH_4)_2SO_4$ and the ATP that were included in the storage medium and then resuspended in 200 mM sucrose containing 50 mM triethanolamine chloride, pH 8.0, at 30 °C. The reaction mixtures contained nucleotides whose concentrations were determined as described previously (Schuster et al., 1975a). The hydrolysis of ATP or ITP was measured exactly as previously described (Schuster et al., 1976). This assay was based upon the release of $^{32}P_i$ from [γ-^{32}P]ATP or [γ-^{32}P]ITP which was synthesized by the method of Schendel & Wells (1973). In all cases, a blank sample was treated exactly as the experimental sample, except that enzyme was replaced by buffer. All samples were done in triplicate, and

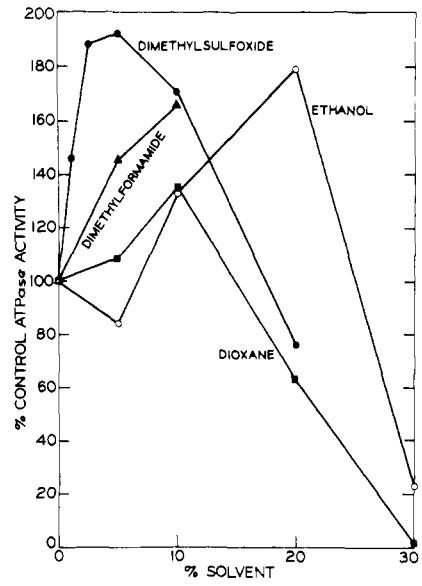

FIGURE 1: The effect of organic solvents on the rate of ATP hydrolysis catalyzed by beef heart F_1. The concentration of ATP was 1.5 mM. The procedures were as described under Materials and Methods.

the numbers presented are the averages of the multiple determinations minus the blank values. When organic solvents were used, additional blanks were run to determine a possible effect on the extraction system. When any effect was noted, that solvent was no longer used. *N,N*-Dimethylformamide at concentrations above 10% (v/v) eliminated the clear separation of the isobutyl alcohol/benzene–water interface and was used only in low concentrations (Figure 1). In all cases, percent of solvent refers to volume percent.

The pH studies (Figures 7 and 8) were performed by use of the assay system described above except that the triethanolamine was replaced by a mixture containing 50 mM each of Bes[1] (pK_a = 7.15), Hepes (pK_a = 7.5), and Bicine (pK_a = 8.35). The pH of the resultant solution was adjusted to the appropriate pH with either HCl or KOH, as appropriate. This maintained buffer capacity over the entire pH range tested. When 20% methanol was present in the buffer solutions, we adjusted the pH while compensating for the effect of organic solvents on the glass electrodes as described by Bates (1973). Since V_{max} cannot be accurately extrapolated for the F_1 hydrolysis of ATP (Ebel & Lardy, 1975; Schuster et al., 1975b), a single high concentration of ATP was chosen, i.e., 15 mM. The results were identical if 25 mM ATP was used (results not shown). When ITP was the substrate, a 20 mM concentration was used, and higher concentrations gave identical results. Therefore, with both ATP and ITP as substrates, only V_{max} information is presented.

Protein concentrations were determined by a Biuret procedure (Layne, 1957).

Results

The results presented in Figure 1 show the effects of varying concentrations of several organic solvents on the ATP hydrolysis rate of F_1. Some of the solvents used cause nearly twofold stimulations of the ATP hydrolysis rate catalyzed by F_1. The most effective solvent for this rate enhancement is methanol (Figure 2). At a concentration of 20% (v/v)

[1] Abbreviations used: Bes, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

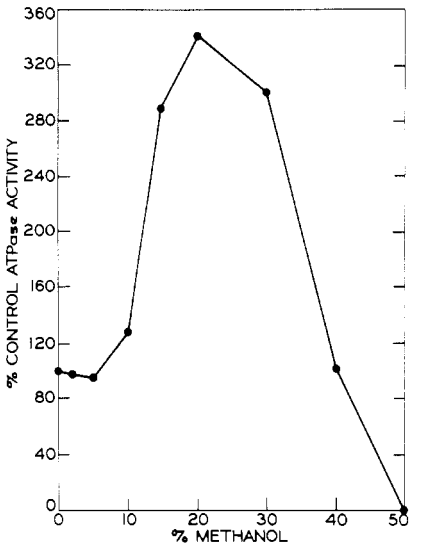

FIGURE 2: The effect of methanol on the rate of ATP hydrolysis catalyzed by beef heart F_1. The concentration of ATP was 1.5 mM. The procedures were as described under Materials and Methods.

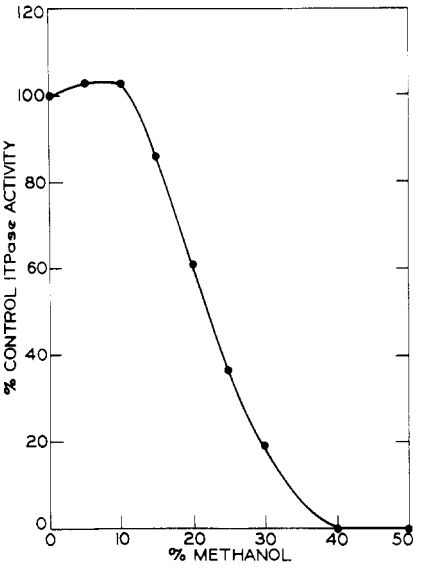

FIGURE 3: The effect of methanol on the rate of ITP hydrolysis catalyzed by beef heart F_1. The concentration of ITP was 2.0 mM. The procedures were as described under Materials and Methods.

methanol, a more than threefold stimulation of the F_1-catalyzed ATP hydrolysis rate is seen. There is virtually no effect by methanol on the ATP hydrolysis rate if less than 10% organic solvent is added. A somewhat surprising finding is that even at a concentration as high as 40% methanol, control levels of ATPase activity are evident (Figure 2).

Our previous work and the results of others have indicated that the enzymatic properties of F_1 observed with ATP as substrate are not necessarily the same as those found when ITP is the substrate (Ebel & Lardy, 1975; Schuster et al., 1975a,b, 1976; Pederson, 1976). Therefore, the effect of methanol on the F_1-catalyzed hydrolysis of ITP was monitored (Figure 3). At none of the concentrations tested did methanol increase the rate of F_1-catalyzed ITP hydrolysis. When the methanol concentration was 20%, the ITP hydrolysis rate was decreased to nearly one-half the control rate (Figure 3). Although the data are not shown, several other organic solvents were tested (dioxane, ethanol, and dimethyl sulfoxide), and all caused a decrease in activity, similar to that seen in Figure 3.

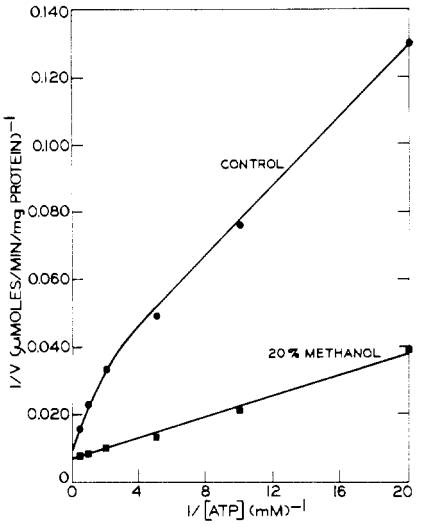

FIGURE 4: A double-reciprocal plot of initial velocity of ATP hydrolysis vs. ATP concentration in either aqueous (control) or 20% methanol assay medium. The procedures were as described under Materials and Methods.

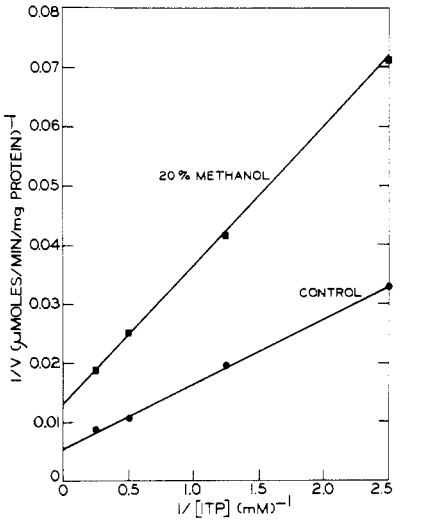

FIGURE 5: A double-reciprocal plot of initial velocity of ITP hydrolysis vs. ITP concentration in either aqueous (control) or 20% methanol assay medium. The procedures were as described under Materials and Methods.

A possible explanation for the differences in the methanol effect on the hydrolytic rates with ATP as opposed to ITP as a substrate could lie with the relationship between the regulatory and catalytic sites of F_1 (Schuster et al., 1975a,b; Lardy et al., 1975). For example, if methanol were interfering with the relationship of these sites, ATP hydrolysis might not be subject to control from the regulatory site. At the same time, ITP hydrolysis rates could be unaffected until the solvent concentrations get to a point where they interfere with the catalytic process. To explore such possibilities, the kinetics of F_1-catalyzed ATP hydrolysis were compared in completely aqueous (control) and 20% methanolic solutions (Figure 4). In the aqueous solution, the double-reciprocal plot of $[ATP]^{-1}$ vs. v^{-1} shows marked curvature indicative of negative cooperativity (Figure 4). In the presence of 20% methanol, this curvature disappears (Figure 4). When ITP is the F_1 substrate, neither the aqueous (control) nor the 20% methanolic medium causes double-reciprocal plots to deviate from linearity (Figure 5). It appears from the data of Figure 5 that the K_m for ITP (1.5 mM) does not change appreciably in 20% methanol, while V_{max} is decreased about 50%.

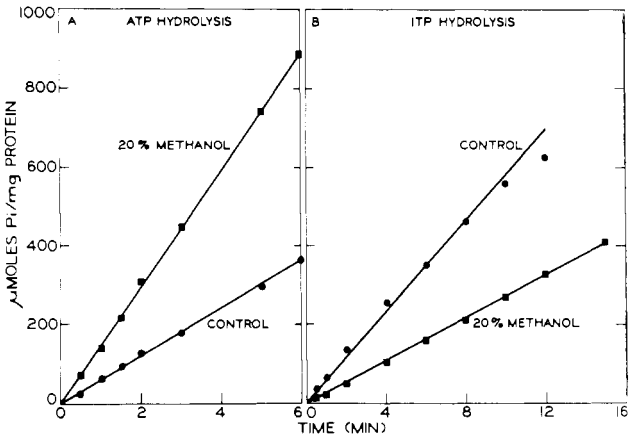

FIGURE 6: Beef heart F_1 hydrolysis of ATP (panel A) and ITP (panel B) as a function of time in either aqueous (control) or 20% methanol solutions. The procedures were as described under Materials and Methods.

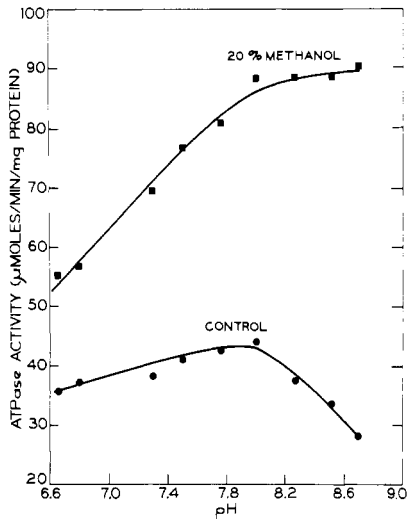

FIGURE 7: The pH dependence of the V_{max} of beef heart F_1 hydrolysis of ATP in either aqueous (control) or 20% methanol solutions. The procedures were as described under Materials and Methods.

A possible effect of methanol and the other organic solvents tested was via a slow, time-dependent activation (with 20% methanol in the medium) or inactivation (when ITP is the substrate). The data of Figure 6 demonstrate that this is clearly not the case. Panel A of Figure 6 shows that the hydrolysis of ATP proceeded linearly with respect to time with or without methanol present. When ITP was the substrate, the rates were also constant (panel B, Figure 6). Although the data are not shown, these changes in rate were immediately reversible if the methanol was diluted with aqueous buffer. It seems apparent, then, that the change in rate caused by methanol to ATP and ITP hydrolysis is immediate (within the time frame of the techniques employed) and reversible.

In order to more fully appreciate the role of the organic solvents used on the F_1 molecule, pH profiles of the various activities were examined. The results shown in Figure 7 demonstrate that in the aqueous medium used (control, Figure 7), a pH optimum of about 8 is evident. Above this pH, the activity rapidly decreases. In the presence of 20% methanol (Figure 7), the activity is optimal from pH 8 to the highest tested, pH 8.7. Although, within the entire pH range tested, methanol stimulated the rate of F_1-catalyzed ATP hydrolysis, the differences of rate in various media are more pronounced at high pH. An analogous series of results is obtained when ITP hydrolysis is measured as a function of pH (Figure 8).

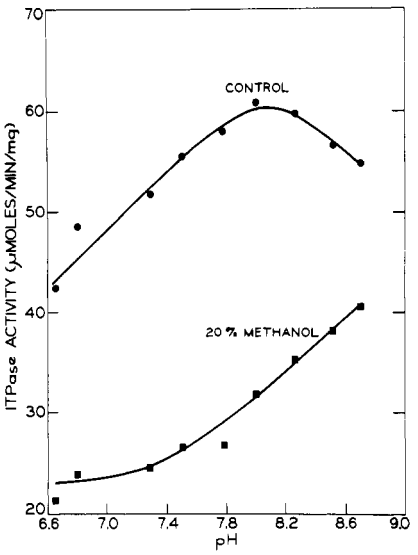

FIGURE 8: The pH dependence of the V_{max} of beef heart F_1 hydrolysis of ITP in either aqueous (control) or 20% methanol solutions. The procedures were as described under Materials and Methods.

Although the hydrolysis rates are lower in the 20% methanolic medium at all pH values tested, the high pH range caused the control (aqueous) and the methanolic rates to converge. The methanolic F_1-catalyzed ITP hydrolysis rates were consistently increased in more basic medium, while above pH 8 the aqueous solution inhibited F_1-catalyzed ITP hydrolysis.

Discussion

In an attempt to examine the role of a nonaqueous area in the vicinity of the F_1, various organic solvents were included in an ATPase reaction medium (Figures 1 and 2). It is apparent from the data presented in Figure 1 that most of the common organic solvents employed caused an increase in the rate of F_1-catalyzed ATP hydrolysis at some solution percentage. At high solvent concentrations, all the reagents tested inhibited the F_1-catalyzed ATP hydrolysis rate, but stimulation of the hydrolysis was noted at lower concentrations. It is somewhat surprising that 20% methanolic solutions were so stimulatory to the ATPase rates (Figure 2). Even with 40% methanol, the F_1-catalyzed ATP hydrolysis rate was at the same level as the totally aqueous control. Even though ATP hydrolysis was more than three times faster in 20% methanol than in the control situation, the results with ITP as a substrate are quite different. Figure 3 shows that methanol (or any other solvent tested) not only fails to cause a stimulation of the F_1-catalyzed ITP hydrolysis rate but also instead causes inhibition. In 20% methanol, while ITP hydrolysis was 50% inhibited, ATP hydrolysis was more than three times faster than in the aqueous control. It is possible that organic environments enhance the specificity of F_1 for adenine nucleotides over inosine or guanosine nucleotides. If the membranous environment of F_1 can be extrapolated from these findings, a sevenfold increase in specificity is apparent for the intact system. Such specificity is, in fact, seen when various nucleotides are used as substrates for nucleoside triphosphate synthesis in submitochondrial systems (Hohnadel & Cooper, 1975; Schuster et al., 1977; Pederson, 1976).

Organic solvent-dependent enhancement of substrate specificity has been reported in other systems (Tan & Lovrien, 1972), but the mechanisms are unclear (Pesheck & Lovrien, 1977). In fact, dramatic rate enhancements with organic cosolvent systems are well-known. Sanwall et al. (1966) found cosolvent systems that stimulated bacterial phosphoenopy-

ruvate carboxylase nearly 30-fold, and others have found similar results with biotin carboxylase (Dimroth et al., 1970). The explanation for these phenomena seems to lie only in V_{max} changes on the processing of the various substrates. However, such explanations appear rather speculative. Recently, Pesheck & Lovrien (1977) have suggested that, at least in the case of cosolvent stimulation of β-glucuronidase, binding properties are affected to the same extent as V_{max}.

The mechanism by which 20% methanol can stimulate F_1-catalyzed ATP hydrolysis yet inhibit ITP hydrolysis is probably rather complex. Neither a simple inactivation mechanism nor a general stimulation explanation can be convincingly defended. More likely, there is an element of both phenomena occurring simultaneously. It has been shown that F_1-catalyzed ATP hydrolysis exhibits negative cooperativity [Schuster et al. (1975a,b), Ebel & Lardy (1975), Pederson (1976)]. This and other data have been interpreted to indicate the existence of catalytic and regulatory sites on F_1. It was concluded from this work that the nucleotide binding at the regulatory site caused a conformational change in the catalytic site, making hydrolysis slower. It is therefore possible that, in 20% methanol, the catalytic and regulatory sites are separated purely by a solvation of the F_1 subunits. This would account for the methanolic stimulation of F_1-catalyzed ATP hydrolysis. The observation that in 20% methanol the plots of $1/[ATP]$ vs. $1/v$ became linear (see Figure 4) is consistent with such an explanation.

While the 20% methanol may be dissociating the catalytic from the regulatory sites of F_1 (and thereby increasing the rate of ATP hydrolysis), there may be an effect at the active site causing a slowdown of ITP hydrolysis. Since the effect of 20% methanol on ITP hydrolysis is confined to a decrease in V_{max}, but not K_m (see Figure 5), such an explanation is reasonable. It is probable, therefore, that the ATP "stimulation" might be more a release of inhibition by ATP binding to the regulatory site, while ITP hydrolysis is being affected by an action at the active site. This same methanol action at the catalytic site must certainly be operative when ATP is the F_1 substrate, so the increase in activity seen by us is probably the sum of the stimulation (by dissociating catalytic and regulatory sites) and the inhibition of some function at the catalytic site.

Whenever hydrolytic enzymes are being examined in the presence of alcohols as cosolvents, the possibility of alcoholysis of intermediates must be considered. Greenzaid & Jencks (1971) found, for example, that numerous short-chain alcohols act as nucleophiles during esterase catalysis of phenyl acetate hydrolysis. It seems unlikely, however, that alcoholysis is responsible for the stimulation of F_1-catalyzed ATP hydrolysis reported in this communication. First, there is no evidence that any intermediate exists that would be nearly so labile to alcoholysis as the aryl intermediates of other hydrolases (Tan & Lovrien, 1972). Second, dioxane has been shown to exert the same effect as alcohols (Figure 1), and this compound cannot be involved in alcoholysis.

The effects of 20% methanol on the V_{max} of both ATP and ITP hydrolysis give a clue as to the nature of the limiting processes of nucleotide hydrolysis. When pH is varied, it is clear that, in aqueous solutions, both ATP and ITP hydrolysis exhibit optima at pH 8 (see Figures 7 and 8). When 20% methanol is included in the reaction medium, the decrease in V_{max} at pH >8 is no longer seen (Figure 7 and 8). These data imply that, in completely aqueous solutions, the deprotonation of a susceptible group causes a decreased V_{max}. When the medium contains methanol, this deprotonation no longer occurs and the V_{max} either increases (with ITP as substrate, Figure 8) or no longer decreases (with ATP as substrate, Figure 7). Although a guanido group is known to be present in the F_1 active site (Marcus et al., 1976), it is unlikely that the ionization of this group is responsible for the pH-dependent rate changes seen by us in Figures 7 and 8. It is known that deprotonation of a basic molecule is enhanced as the solvent dielectric constant decreases (Bates, 1973). This would suggest that deprotonation of the guanido group is favored in methanolic solutions, making a carboxyl or hydroxyl group a more likely choice. One such candidate whose ionization could be affected by methanol in the pH range studied is a tyrosine. Indeed, a tyrosine group has been implicated by Ferguson et al. (1975a,b) as possibly existing in the F_1 active site. Although it may be that the protonation of the tyrosinate ion is necessary for rapid ATP hydrolysis by F_1, much more needs to be done to establish such a hypothesis.

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Phospholipid Composition of Substrate Adhesion Sites of Normal, Virus-Transformed, and Revertant Murine Cells†

Martha K. Cathcart and Lloyd A. Culp*

ABSTRACT: The phospholipid composition of cell–substratum adhesion sites, obtained after EGTA-mediated detachment of cells from the tissue-culture substratum, was determined for [^{32}P]orthophosphate radiolabeled Balb/c 3T3, SV40-transformed (SVT2), and concanavalin A selected revertant variant cell lines. All of the major phospholipid classes were found in the substrate-attached material, but there was an enrichment for specific phospholipid species in this adhesive material as compared to whole-cell and surface-enriched membranes. The phospholipid composition was remarkably similar for the whole-cell and surface-enriched membrane fractions from the three cell lines. However, pronounced differences in the phospholipid composition of the adhesion sites were observed as a result of viral transformation—SVT2 sites were clearly enriched in phosphatidylethanolamine and depleted in phosphatidylcholine when compared to 3T3 sites. This alteration in adhesion site phospholipids of transformed cells reverted to 3T3-like values in the adhesive material of revertant cells. The composition of adhesive material of newly attaching cells was also examined to differentiate compositional differences between "footpad" adhesion sites and "footprints", adhesive material pinched off from the posterior of cells as they move across the substratum. Pulse and pulse-chase analyses of the [^{32}P]phospholipids revealed some differences in synthesis and turnover rates in the three cell lines; in addition, altered rates of deposition of newly synthesized material into adhesion sites of transformed cells were observed. These data afford further evidence that the cell–substratum adhesion sites are highly specialized areas of the cell surface enriched in components which are intricately involved in the adhesive process. The transformation-dependent changes in adhesion site phospholipids may help to determine the basis for the altered adhesive properties of transformed cells.

Normal cells and oncogenic virus-transformed cells exhibit considerably different growth, morphologic, and motility properties in vitro. One basic alteration in transformed cells is that they are less adherent to their tissue-culture substratum than normal cells (Gail & Boone, 1972; Sanford et al., 1967; Shields & Pollock, 1974; Weber et al., 1977), a characteristic which may mimic their behavior during metastasis in vivo. The adhesive interaction between a cell and its tissue-culture substratum has been determined to involve discrete attachment points on the undersurface of the cell (Brunk et al., 1971; Abercrombie et al., 1971; Harris, 1973; Revel et al., 1974; Culp, 1975; Rosen & Culp, 1977). The interaction is mediated by one or more serum components absorbed to the substrate (Revel & Wolken, 1973; Grinnell, 1974; Culp & Buniel, 1976; Stamatoglou, 1977), but the serum components as well as the cellular components which participate in this adhesive interaction at present remain undefined. The determination of the molecular mechanism of the interaction is of great interest not only for understanding growth and movement of cells in culture but also for understanding the alterations which accompany malignant conversion.

Treatment of normal or SV40[1]-transformed murine cells, attached to their serum-coated tissue-culture substrates, with the Ca^{2+}-specific chelator EGTA results in cell rounding and detachment (with the aid of rotary movement and gentle pipetting) (Culp & Black, 1972b). Scanning electron microscopic studies have revealed that, as the cell rounds up and detaches under these conditions, it pinches off and leaves behind firmly adherent footpads which had mediated the cell–substratum adhesion (Rosen & Culp, 1977). Pinched-off

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[1] Abbreviations used: CL, cardiolipin; DiPI, diphosphatidylinositol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; $NaDodSO_4$, sodium dodecyl sulfate; PA, PC, PE, PG, PI, and PS represent respectively phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylserine; SM, sphingomyelin; SV40, Simian virus 40; SAM, substrate-attached material; REV, revertant cells; PBS, phosphate-buffered saline; PBS II, phosphate-buffered saline containing 100 mg/L each of $MgCl_2$ and $CaCl_2$.

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