Effects of Adrenocorticotropin and Cycloheximide on Adrenal Diglyceride Kinase†

Robert V. Farese,* Mohammad A. Sabir, and Ronald E. Larson

ABSTRACT: We studied the effects of adrenocorticotropin (ACTH) and cycloheximide on adrenal enzymes involved in phosphatidate synthesis. Treatment of rats in vivo with ACTH induced a rapid increase in phosphatidate synthesis from diglyceride and ATP in adrenal homogenates, and cycloheximide treatment prevented this increase if given before ACTH and rapidly reversed the increase if given after ACTH. The stimulatory effect of ACTH appeared to be largely due to an increase in diglyceride substrate, as kinase activity was not altered. The inhibitory effect of cycloheximide, on the other hand, appeared to be due to a decrease in diglyceride kinase activity. Neither ACTH nor cycloheximide treatment had any effect on the activity of glycerol-3′-phosphate acyltransferase or phosphatidate phosphatase. Our findings suggest that (a) ACTH increases the flow of phospholipid (and their levels) throughout the entire circular pathway, i.e., phosphatidate → CDP–diacylglycerol → inositides → diglycerides → phosphatidate, and (b) a labile protein may serve to allow entry into a recycling of diglyceride in this pathway. In addition, since cycloheximide blocked carbachol-induced increases in pancreatic and salivary glandular phosphatidate synthesis resulting from phosphatidylinositol hydrolysis and consequent diglyceride generation, the putative labile protein may have widespread importance.

We have recently reported that adrenocorticotropin (ACTH), via adenosine cyclic 3′,5′-phosphate (cAMP), rapidly increases adrenal concentrations of phospholipids in the phosphatidate–poly(phosphoinositide) cycle (Farese et al., 1979, 1980a,b), and polyphosphorylated phospholipids in this cycle may play a mediatory role in stimulating steroidogenesis (Farese et al., 1980a,b; Farese & Sabir, 1979, 1980). Cycloheximide and puromycin, inhibitors of protein synthesis, block ACTH-induced increases in steroidogenesis (Ferguson, 1963; Garren et al., 1965) and adrenal phospholipids (Farese et al., 1980a–c; Farese & Sabir, 1980), and a labile protein may be required in these processes. Kinetic studies (Farese et al., 1980b,c) further suggest that ACTH primarily increases de novo synthesis of phosphatidic acid by a cycloheximide-sensitive process, and the increase in phosphatidic acid concentration leads to increases in the concentrations of other phospholipids which, in turn, influence steroidogenesis. In the present study, we attempted to determine whether ACTH or cycloheximide affects the activity of several key enzymes which regulate phosphatidate synthesis, viz., glycerol-3′-phosphate acyltransferase, phosphatidate phosphatase, and diglyceride kinase. Although ACTH treatment was not found to alter the activities of these enzymes, cycloheximide treatment decreased the apparent V_{max} of diglyceride kinase. In addition, ACTH increased diglyceride levels, and this appeared to be responsible for the subsequent increase in phosphatidate synthesis via diglyceride kinase.

Experimental Procedures

Male rats (approximate weight of 250 g) were obtained from Holtzmann Co., kept in environmentally controlled quarters for 1–2 weeks before experimental use, and, where indicated, injected intraperitoneally with 2 units of short-acting $ACTH^{1-24}$ peptide (a maximally effective dose), intraperitoneally with 10 mg of cycloheximide [see Farese et al. (1980a–c) and Garren et al. (1965)], and intramuscularly with 10 units of long-acting $ACTH^{1-18}$ peptide. Blood was routinely collected from severed neck vessels after killing by guillotining. Serum and adrenal corticosterone levels, as determined by the acid-fluorescent method (Silber et al., 1958), were regularly increased 3–10-fold by ACTH treatment, and cycloheximide pretreatment (for 10 min) completely blocked these increases.

Adrenals were rapidly removed from carcasses, chilled in ice-cold physiological saline, and homogenized in 0.25 M sucrose with a motor-driven, Teflon-glass homogenizer. Nuclear, mitochondrial, microsomal, and soluble cell fractions were obtained by sequential centrifugation at 4 °C for 10 min at 400*g*, 10 min at 10000*g*, and 90 min at 105000*g*.

Diglyceride kinase was assayed essentially as described by Hokin & Hokin (1959). The homogenate or subcellular fraction derived from 20 mg of adrenal tissue was incubated at 37 °C in a final volume of 0.4 mL with 0.25 M sucrose, 0.05 M potassium glycylglycine buffer, pH 6.5, 2 mM $MgSO_4$, 2.5 mM sodium deoxycholate, 2 mM ATP (higher concentrations were inhibitory) containing 2 μCi of [γ-^{32}P]ATP, and other addtions, as indicated (note: exogenous diglyceride was not routinely added). After incubation, reaction mixtures were chilled, and phospholipids were extracted and purified by thin-layer chromatography in solvent systems B or C as described previously (Farese et al., 1979, 1980a,b). Radioactive phosphatidate areas (>95% of total counts per minute) were localized by radioautography and charring, scraped into vials containing Quantiflor, and counted in a liquid scintillation spectrometer. Zero-time samples contained little or no radioactivity, and the rate of reaction was linear or near-linear for 10 min. In the absence of sodium deoxycholate, incorporation of ^{32}P into phosphatidic acid was extremely poor, and the effects of ACTH and cycloheximide were not readily apparent.

The glycerol-3′-phosphate acyltransferase assay was similar to those described by Martensson & Kanfer (1968) and Lamb & Fallon (1970). Adrenal homogenate or subcellular fractions derived from 10 mg of tissue were incubated for 1 min at 37 °C in a final volume of 0.5 mL with 0.25 M sucrose, 0.05 M Tris, pH 7.5, 0.0075% (w/v) dithiothreitol, 1 mM $MgCl_2$, 4 μM ATP, 120 μM palmitoyl≈CoA (a saturating amount), and increasing amounts (6.25–150 μM) of glycerol 3′-phosphate

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Table I: Effects of ACTH and Cycloheximide on Diglyceride Kinase Activity in Particulate and Soluble Cell Fractions[a]

mitochondrial + microsomal fraction	cytosol addition: none	control	ACTH	cycloheximide + ACTH
none		5 ± 1 (4)	2 ± 1 (4)	3 ± 2 (4)
control	8 ± 2 (4)	17 ± 1 (8)	25 ± 2 (9)	18 ± 1 (9)
ACTH	15 ± 2 (4)	34 ± 4 (8)	40 ± 3 (9)	36 ± 5 (6)
cycloheximide + ACTH	6 ± 1 (4)	19 ± 4 (8)	27 ± 3 (6)	18 ± 3 (9)

[a] Shown here are the picomoles of phosphatidic acid synthesized per milligram of protein during a 5-min incubation. Other experimental conditions are as described in the Table. Values are the mean results ± SE, with the number of determinations in parentheses.

containing [U ^{14}C]glycerol 3′-phosphate (final specific activity = 8.33 μCi/μmol). After incubation, lipids were extracted as described (Farese et al., 1979, 1980a,b), and the extracts were dried and counted for radioactivity.

Phosphatidate phosphatase was assayed by the method of Hosaka et al. (1975). Diglyceride was determined by the method of Banschback et al. (1974), except that the final purified acetylated diglyceride was quantified by the method of Kabara & Chen (1976), using dipalmitin as a standard. Proteins were measured by the method of Lowry et al. (1951).

ACTH$^{1-24}$ and ACTH$^{1-18}$ were gifts from Organon. Radioactive substances were purchased from New England Nuclear. Other biological substances were purchased from Sigma.

Results

After 10 min of ACTH treatment in vivo, diglyceride kinase mediated phosphatidate synthesis in adrenal homogenates increased by 86%, from 28 ± 2 (n = 19) to 52 ± 2 (n = 19; $p < 0.001$) pmol of phosphatidate (mg of protein)$^{-1}$ (5 min)$^{-1}$ incubation. Cycloheximide pretreatment in vivo for 10 min completely inhibited this ACTH-induced increase (24 ± 2; n = 19), but direct addition of cycloheximide did not influence the assay. The stimulatory effect of ACTH and the inhibitory effect of cycloheximide were primarily due to changes in factors associated with the combined mitochondrial–microsomal fraction,[1] which contained the most enzyme activity (Table I). The cytosol fraction contained little enzyme activity (even with added substrate) but markedly stimulated that of the mitochondrial–microsomal fraction; however, ACTH and cycloheximide induced only relatively small changes in cytosolic stimulatory activity.[2]

The increase in ^{32}P incorporation into phosphatidic acid in ACTH-stimulated homogenates was due to increased phosphatidate synthesis rather than decreased degradation. In pulse–chase experiments in which phosphatidic acid was labeled by a 1-min exposure to [γ ^{32}P]ATP of high specific activity, and chased for 8 min by incubation with an excess of unlabeled ATP, the $T_{1/2}$ for labeled phosphatidic acid in homogenates from control, ACTH-treated, and (cycloheximide + ACTH)-treated rats was 5.2, 5.2, and 5.4 min, respectively

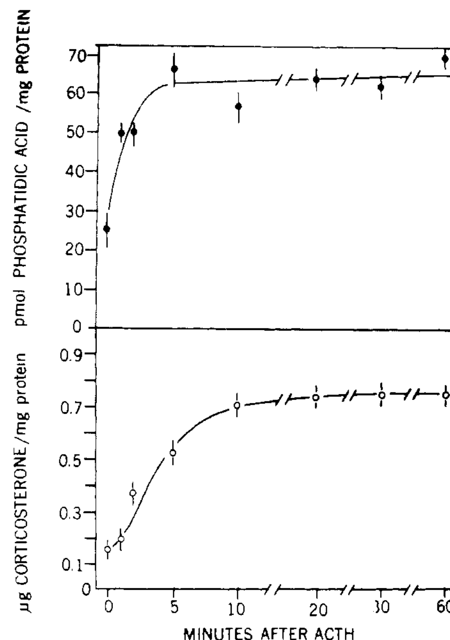

FIGURE 1: Rapid increases in adrenal steroidogenesis (lower panel) and phosphatidate synthesis from diglyceride and ATP following ACTH treatment in vivo. Rats were injected intraperitoneally with 2 units of short-acting ACTH$^{1-24}$ peptide and, if the treatment period was greater than 15 min, intramuscularly with 10 units of long-acting ACTH$^{1-18}$ peptide to provoke rapid, maximal, and sustained increases in adrenocortical secretory activity. At the designated times, the rats were killed, and adrenals were rapidly removed (within 60 s), chilled in ice-cold, physiological saline, homogenized, analyzed for corticosterone and protein content, and assayed for diglyceride kinase (incubation time = 5 min) in the absence of added substrate. Mean results ± SEM of four determinations are shown.

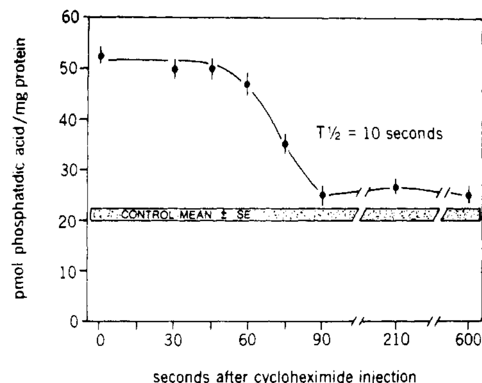

FIGURE 2: Cycloheximide-induced reversal of ACTH effects on diglyceride kinase. Rats were treated (except untreated controls) with ACTH$^{1-18}$ and ACTH$^{1-24}$ as described in Figure 1 to establish a sustained maximal effect of ACTH. After 30 min of ACTH treatment, the rats were anesthetized with 40–50 mg/kg pentobarbital. Cycloheximide (10 mg) was injected intraperitoneally, and the abdomen was opened 15 s before the designated times of adrenal removal. [This experimental protocol was virtually the same as that employed previously (Farese et al., 1980c).] The adrenals were rapidly chilled, and homogenates were assayed for diglyceride kinase mediated phosphatidate synthesis (from endogenous diglyceride) as described in Figure 1 (incubation time = 5 min). Mean results ± SEM of six to eight determinations are shown. The simultaneously determined control level of diglyceride kinase mediated phosphatidate synthesis is shown by the shaded area.

(results not shown). This result, in concert with that described below, indicated that phosphatidate phosphatase was not affected by treatments.

The rapidity of the ACTH-induced increase in phosphatidate synthesis via diglyceride kinase is shown in Figure 1. As is apparent, this increase preceded the increase in steroido-

[1] We did not routinely separate mitochondria and microsomes since the yield of the latter, free of mitochondria, is very poor, and since a large fraction of microsomes (as per marker enzymes) is present in the mitochondrial fraction (see Figure 2, Farese et al., 1980a).

[2] Hepatic cytosol was as effective as adrenal cytosol in stimulating the assay, but albumin had no significant effect. The identity of the nonspecific cytosolic stimulatory is presently unknown, but it is clearly not diglyceride and is probably not influenced by ACTH or cycloheximide treatment.

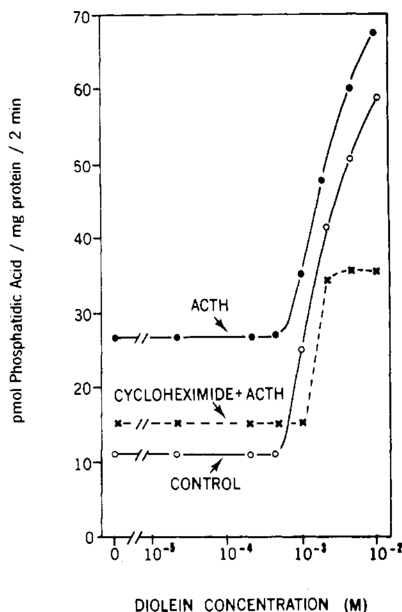

FIGURE 3: Effects of addition of increasing amounts of diolein to adrenal homogenates from control (○), ACTH-treated (●), and (cycloheximide + ACTH)-treated (×) rats. Homogenates were preincubated for 5 min at 37 °C in 0.25 M sucrose solution with 5 mM sodium deoxycholate ± diolein. ATP and other ingredients of the diglyceride kinase assay were added, and incubation was continued for 2 min. Each point is the mean of two to three determinations. Comparable results were obtained in other experiments, and in 14 comparisons in which 5 mM diolein was added to the assay, the rates of phosphatidate synthesis were 54 ± 3, 60 ± 3, and 39 ± 3 ($p < 0.001$ vs. others) pmol (mg of protein)$^{-1}$ (5 min)$^{-1}$ incubation in control, ACTH-treated, and (cycloheximide + ACTH)-treated adrenal homogenates, respectively.

genesis, and both increases persisted with continued ACTH treatment. The rapidity of reversal of on-going ACTH effects by cycloheximide is shown in Figure 2. After intraperitoneal injection of cycloheximide, there was a 45-s lag period (presumably reflecting the time required for intraadrenal accumulation of cycloheximide) followed by a return to basal levels over the next 45 s. The $T_{1/2}$ of the latter process was approximately 10 s, and this is virtually the same as the decay rate for adrenal phosphatidate levels ($T_{1/2}$ = 9 s) observed previously (Farese et al., 1980c) in similar experimental conditions.

Addition of diglyceride substrates to the diglyceride kinase assay enhanced phosphatidate synthesis substantially in control and ACTH-stimulated homogenates, but relatively poorly in homogenates from (cycloheximide + ACTH)-treated rats (Figures 3 and 4).[3] At higher levels of added substrate, the ACTH effect, relative to that of the control, was diminished or abolished; on the other hand, inhibitory effects of cycloheximide treatment remained apparent. Addition of phosphatidic acid to the assay as a trapping agent and potential substrate produced results similar to those of diolein addition (results not shown).

Diglyceride kinase could be partially solubilized by extraction of the homogenate with deoxycholate, but the substrate was severely limiting in these extracts. Interestingly, addition of 5 mM diolein markedly stimulated phosphatidate synthesis in these extracts, and induced effects of ACTH and cycloheximide were no longer apparent (results not shown).

[3] The failure to observe further increases in phosphatidate synthesis with concentrations of dipalmitin greater than 0.2 μM is probably due to the inability to maintain uniformly dispersed suspensions of this diglyceride at the higher concentrations.

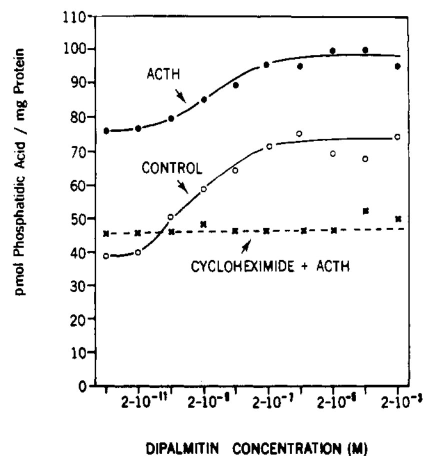

FIGURE 4: Effects of addition of increasing amounts of dipalmitin to adrenal homogenates from control (○), ACTH-treated (●), and (cycloheximide + ACTH)-treated (×) rats. Homogenates were preincubated for 5 min at 37 °C in 0.25 M sucrose solution with 5 mM sodium deoxycholate ± dipalmitin. ATP and other ingredients of the diglyceride kinase assay were added, and incubation was continued for 5 min. Each point is the mean of three determinations.

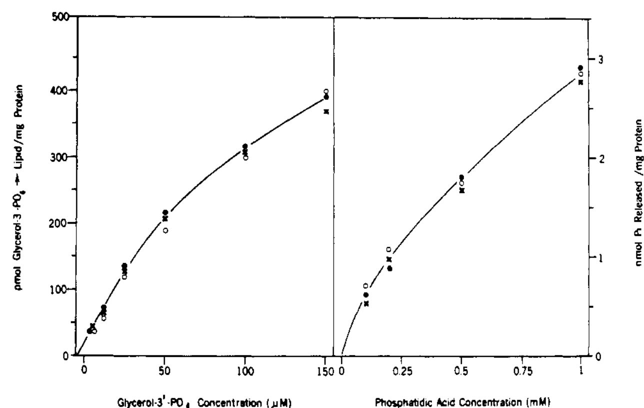

FIGURE 5: Lack of effect of ACTH (●) and cycloheximide (×) on activity of glycerol-3'-phosphate acyltransferase (left) and phosphatidate phosphatase (right) in adrenal homogenates. The time of assay was 1 and 20 min, respectively, for glycerol-3'-phosphate acyltransferase and phosphatidate phosphatase. Controls are shown by open circles.

Acute 10-min ACTH treatment in vivo increased adrenal diglyceride levels severalfold, from 143 ± 13 (n = 4) to 596 ± 61 (n = 4) ng (mg of protein)$^{-1}$ ($p < 0.001$). This effect was evident despite pretreatment with cycloheximide for 10 min [428 ± 16 (n = 4) ng of diglyceride (mg of protein)$^{-1}$, $p < 0.001$, vs. control].

There were no appreciable effects of ACTH or cycloheximide on glycerol-3'-phosphate acyltransferase mediated phosphatidate synthesis[4] or on phosphatidate phosphatase (Figure 5). Because of the apparent importance of de novo synthesis of phosphatidic acid in ACTH action (Farese et al., 1980b,c), time-course (1–20 min) glycerol-3'-phosphate acyltransferase assays were also conducted in the presence of severely limiting (3 μM) or excessive (3 mM) concentrations of glycerol 3'-phosphate; in both cases, there were no apparent effects of ACTH or cycloheximide treatment.

Since the above results suggested that cycloheximide inhibited diglyceride kinase activity, it was of interest to determine whether cycloheximide inhibits the increase in

[4] There also were no observed effects of ACTH or cycloheximide during incubations of isolated microsomes or mitochondria in this assay; results of whole homogenates are shown since cytosol (inactive alone) stimulated the activity of microsomes and mitochondria.

Table II: Effects of Cycloheximide on Phosphatidate Synthesis Resulting from Secretagogue-Induced Phosphatidylinositol Hydrolysis in Rat Pancreas and Salivary Gland[a]

addition	ng of phosphatidylinositol–P/mg of protein		ng of phosphatidic acid–P/mg of protein	
	pancreas	salivary gland	pancreas	salivary gland
(A) none (control)	684 ± 16	351 ± 9	31 ± 2	83 ± 9
(B) 0.1 mM cycloheximide	728 ± 23	336 ± 2	30 ± 3	68 ± 9
(C) 10 μM carbachol	444 ± 14 [b]	241 ± 9 [b]	49 ± 3 [e]	133 ± 9 [f]
(D) 0.1 mM cycloheximide + 10 μM carbachol	430 ± 8 [c]	268 ± 16 [d]	34 ± 2	64 ± 6

[a] Rat pancreas and salivary glands were minced and dispersed with collagenase as described previously (Farese et al., 1980d). Tissue fragment suspensions (approximately 100 mg) were preincubated under 95% O_2 + 5% CO_2 for 60 min at 37 °C in 0.5 mL of Krebs Ringer bicarbonate buffer containing 5 mM glucose and 0.5% albumin. Where indicated, cycloheximide was added during the last 30 min of preincubation. Carbachol was then added, as indicated, and incubation was continued for 60 min. Tissue phospholipids were extracted, purified, and quantified by phosphorus (P) determination as described previously (Farese et al., 1980d). Mean values ± SEM of four determinations are shown. Similar results were obtained in repeat experiments with other secretagogues. The dose of carbachol employed in these experiments produced maximal effects, and carbachol-induced changes in phospholipid concentrations reached a steady state at about 30 min of incubation. P was determined by standard t test. [b] P vs. (A) < 0.001. [c] P vs. (B) < 0.001. [d] P vs. (B) < 0.05. [e] P vs. (A) < 0.005. [f] P vs. (A) < 0.01.

phosphatidic acid which occurs with secretagogue-induced phosphatidylinositol breakdown and the subsequent increase in diglyceride levels (Banschback et al., 1974). For this purpose, we incubated collagenase-dispersed fragments of rat pancreas and salivary gland (Table II) with carbachol: in both tissues, cycloheximide did not block carbachol-induced phosphatidylinositol breakdown but did block the expected increase in phosphatidic acid.

Discussion

The present results show that ACTH acutely increases phosphatidate synthesis from endogenous diglyceride and ATP. Since substrate limits this synthesis, and since ACTH acutely increases adrenal diglyceride levels, the observed ACTH effect may largely be due to an increase in substrate concentration. Support for the latter is derived from the observed effects of substrate addition to the diglyceride kinase assay: the latter results, although not fully interpretable by a classical enzyme kinetic analysis (because of membrane-bound enzymes and poorly dissolved substrate), did not suggest that ACTH alters the K_m or V_{max} of diglyceride kinase.

The ACTH-induced increase in diglyceride concentration may be derived from (a) increased de novo phosphatidate synthesis from glycerol 3′-phosphate and fatty acyl∼CoA (Farese et al., 1979, 1980a–c) and/or (b) increased conversion of triglyceride to diglyceride. Both possibilities seem plausible since ACTH promotes glycogenolysis (Haynes & Berthet, 1957), glycolysis (Bell et al., 1970), and possibly lipolysis (Butcher et al., 1968). Further studies are obviously needed to determine which of these possibilities is (are) more important in ACTH action.

The fact that ACTH increased adrenal diglyceride concentrations and the overall amount of phosphatidate synthesis from diglyceride and ATP is of interest in that it is now clear that all measurable substances in the entire circular phospholipid pathway, i.e., phosphatidate → CDP–diacylglycerol → inositides → diglycerides → phosphatidate, are increased severalfold by acute ACTH treatment. Thus, previously noted increases in individual phospholipids in this pathway cannot be explained by a decrease in their turnover. Furthermore, the increased resynthesis of phosphatidate from diglyceride probably serves to maintain the highest level of phospholipids in this pathway for any given degree of enhancement of de novo synthesis of phosphatidic acid, and this may be important for steroidogenesis (Farese et al., 1979, 1980a–c; Farese & Sabir, 1979, 1980).

Localization of the inhibitory effect of cycloheximide primarily to the particulate fraction suggested, first, that the cycloheximide-sensitive factor is present therein and, second, that changes in water-soluble substances, e.g., ppGpp, a metabolite which accumulates with protein synthesis inhibition and may serve as a possible repressor for phospholipid synthesis (Merlie & Pizer, 1973), cannot explain the inhibitory effect of cycloheximide.

The observed inhibitory effect of cycloheximide on diglyceride kinase activity was not due to decreased substrate availability since (a) diglyceride levels were increased in (cycloheximide + ACTH)-treated adrenals, (b) diglyceride addition to the diglyceride kinase assay provoked a lesser increase in phosphatidate synthesis after cycloheximide treatment, and (c) in the rat pancreas and salivary gland, cycloheximide blocked the expected increases in phosphatidic acid which result from secretagogue-induced phosphatidylinositol hydrolysis and diglyceride generation (Banschback et al., 1974).

Whether or not the inhibition of diglyceride kinase by cycloheximide is due to deficiency of a labile protein is presently not certain. Nevertheless, cycloheximide inhibits the increases in phospholipids which occur during the action of agents which enhance phosphatidate synthesis primarily (i.e., de novo), e.g., ACTH, parathyroid hormone, and angiotensin (Farese et al., 1980b,c,e,f), or secondarily, as with carbachol-induced phosphatidylinositol hydrolysis (Hokin & Hokin, 1958; Michell, 1975). Knowledge of this inhibitory effect of cycloheximide should be important to many investigators, since cycloheximide (or puromycin) sensitivity may signify involvement of phospholipids, as well as proteins, in observed responses in target tissues.

References

Banschback, M. W., Geison, R. L., & Hokin-Neaverson, M. (1974) *Biochem. Biophys. Res. Commun. 58*, 714.

Bell, J., Brooker, G., & Harding, B. W. (1970) *Biochem. Biophys. Res. Commun. 4*, 938.

Butcher, R. W., Baird, C. E., & Sutherland, E. W. (1968) *J. Biol. Chem. 243*, 1705.

Farese, R. V., & Sabir, M. A. (1979) *Biochim. Biophys. Acta 575*, 299.

Farese, R. V., & Sabir, M. A. (1980) *Endocrinology (Baltimore) 106*, 1869.

Farese, R. V., Sabir, M. A., & Vandor, S. L. (1979) *J. Biol. Chem. 254*, 6842.

Farese, R. V., Sabir, M. A., Vandor, S. L., & Larson, R. E. (1980a) *J. Biol. Chem. 255*, 5728.

Farese, R. V., Sabir, M. A., & Larson, R. E. (1980b) *J. Biol. Chem. 255*, 7232.

Farese, R. V., Sabir, M. A., & Larson, R. E. (1980c) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7189.
Farese, R. V., Larson, R. E., & Sabir, M. A. (1980d) *Biochim. Biophys. Acta 633*, 479.
Farese, R. V., Bidot-Lopez, P., Sabir, M. A., Smith, J., Schinbeckler, B., & Larson, R. (1980e) *J. Clin. Invest. 65*, 1523.
Farese, R. V., Sabir, M. A., & Larson, R. E. (1980f) *J. Clin. Invest. 66*, 1428.
Ferguson, J. J., Jr. (1963) *J. Biol. Chem. 238*, 2754.
Garren, L. E., Ney, R. L., & Davis, W. W. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 1443.
Haynes, R. C., & Berthet, L. (1957) *J. Biol. Chem. 225*, 115.
Hokin, L. E., & Hokin, M. R. (1958) *J. Biol. Chem. 233*, 805.
Hokin, M. R., & Hokin, L. E. (1959) *J. Biol. Chem. 234*, 1381.
Hosaka, K., Yamashita, S., & Numa, S. (1975) *J. Biochem. (Tokyo) 77*, 501.
Kabara, J. J., & Chen, J. S. (1976) *Anal. Chem. 48*, 814.
Lamb, R. E., & Fallon, H. J. (1970) *J. Biol. Chem. 254*, 3075.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, J. (1951) *J. Biol. Chem. 193*, 265.
Martensson, E., & Kanfer, J. (1968) *J. Biol. Chem. 243*, 497.
Merlie, J. P., & Pizer, L. I. (1973) *J. Bacteriol. 116*, 355.
Michell, R. H. (1975) *Biochim. Biophys. Acta 414*, 81.
Silber, R. H., Busch, R., & Oslapas, R. (1958) *Clin. Chem. (Winston-Salem, N.C.) 4*, 278.

Divalent Metal Ion, Inorganic Phosphate, and Inorganic Phosphate Analogue Binding to Yeast Inorganic Pyrophosphatase†

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ABSTRACT: Four different techniques, equilibrium dialysis, protection of enzymatic activity against chemical inactivation, ^{31}P relaxation rates, and water proton relaxation rates, are used to study divalent metal ion, inorganic phosphate, and inorganic phosphate analogue binding to yeast inorganic pyrophosphatase, EC 3.6.1.1. A major new finding is that the binding of a third divalent metal ion per subunit, which has elsewhere been implicated as being necessary for enzymatic activity [Springs, B., Welsh, K. M., & Cooperman, B. S. (1981) *Biochemistry* (in press)], only becomes evident in the presence of added inorganic phosphate and that, reciprocally, inorganic phosphate binding to both its high- and low-affinity sites on the enzyme is markedly enhanced in the presence of divalent metal ions, with Mn^{2+} causing an especially large increase in affinity. The results obtained allow evaluation of all of the relevant equilibrium constants for the binding of Mn^{2+} and inorganic phosphate or of Co^{2+} and inorganic phosphate to the enzyme and show that the high-affinity site has greater specificity for inorganic phosphate than the low-affinity site. In addition, they provide evidence against divalent metal ion inner sphere binding to phosphate for enzyme subunits having one or two divalent metal ions bound per subunit and evidence for a conformational change restricting active-site accessibility to solvent on the binding of a third divalent metal ion per subunit.

Work in this laboratory over the last few years has been devoted to elucidating the mechanism of action of the dimeric enzyme yeast inorganic pyrophosphatase, EC 3.6.1.1 (PPase).[1] This enzyme catalyzes both pyrophosphate (PP_i) hydrolysis and water–phosphate (P_i) oxygen exchange. In recent papers (Hamm & Cooperman, 1978; Springs et al., 1981) we have presented a unified scheme for PPase action which accounts quantitatively for the observed overall rate constants for both processes and, in addition, presented evidence that three Mg^{2+} per active subunit are required for enzymatic activity and that there are two P_i sites per subunit, one of high and one of low affinity. We will henceforth denote these sites as site 1 and site 2, respectively.

Although Mg^{2+} confers maximal activity on PPase, appreciable activity is also found in the presence of Zn^{2+}, Co^{2+}, and Mn^{2+} (Butler & Sperow, 1977; Janson et al., 1979). In this paper we use four different techniques, equilibrium dialysis, protection of enzymatic activity against chemical modification, ^{31}P relaxation rates, and water proton relaxation rates, to investigate divalent metal ion, P_i, and P_i analogue (methylphosphonate, thiophosphate, and phosphoramidate) binding to PPase. The binding of P_i in the presence of either Mn^{2+} or Co^{2+} has been investigated in the most detail, because the paramagnetism of these two metal ions will make them extremely useful in future NMR and ESR studies.

Our major findings and conclusions are as follows. (1) In addition to the two divalent metal ions bound with approximately equal affinity per PPase subunit in the absence of P_i, a third divalent metal ion is bound with approximately equal affinity in the presence of P_i. (2) Reciprocally, the affinity of P_i for both of its sites on PPase is markedly increased in the presence of divalent metal ion. In the presence of either Mn^{2+} or Co^{2+}, the binding to site 1 is strong enough to permit direct stoichiometric titration. (3) The equilibrium dialysis and ^{31}P relaxation rate studies permit evaluation of all of the relevant equilibrium constants for the binding of three Mn^{2+}

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[1] Abbreviations used: BSA, bovine serum albumin; PEI, poly(ethylenimine); EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; PhGx, phenylglyoxal; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; PPase, yeast inorganic pyrophosphatase; PRR, proton relaxation rate; Cl_3CCOOH, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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