# Effect of Insulin on ATP-Citrate Lyase Phosphorylation: Regulation of Peptide A and Peptide B Phosphorylations†

Seethala Ramakrishna,* Karnam Srinivasa Murthy, and William B. Benjamin

*Diabetes Research Laboratory, Department of Physiology and Biophysics, School of Medicine, Health Sciences Center, State University of New York, Stony Brook, New York 11794*

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ABSTRACT: Insulin decreases multifunctional protein kinase (MFPK) activity in rat adipose tissue [Ramakrishna, S., & Benjamin, W. B. (1988) *J. Biol. Chem. 263*, 12677-12681]. Insulin also decreases the phosphorylation of peptide B but increases the phosphorylation of peptide A of ATP-citrate lyase (ATP-CL). The mechanism for this increase in peptide A phosphorylation was studied with purified ATP-CL from control and insulin- and isoproterenol-treated fat pads by using MFPK and the catalytic subunit of cAMP-dependent protein kinase (A-kinase). ATP-CL purified from insulin-treated fat pads is a better substrate for phosphorylation by MFPK compared to controls. This result is consistent with the hypothesis that insulin action decreases peptide B phosphorylation. To determine if the degree of phosphorylation at peptide B affects the phosphorylation rate of peptide A by A-kinase, ATP-CL was prepared with determined phosphate contents of peptides A and B. ATP-CL with a low phosphate content at peptide B is a better substrate for phosphorylation at peptide A by A-kinase than is ATP-CL with a high phosphate content at peptide B. These results suggest that the insulin-induced increase in ATP-CL phosphorylation at peptide A is due to a decrease in peptide B phosphorylation. ATP-CL prepared from isoproterenol-treated fat pads is also a better substrate for phosphorylation at peptide B by MFPK than controls. This increase in phosphorylation at peptide B by MFPK is due to positive second-site regulation by the isoproterenol-induced increase in peptide A phosphorylation.

The first demonstration that insulin action can increase rather than decrease protein phosphorylation was the finding that insulin increases the radioactivity associated with a major protein band when adipocytes are incubated with [$^{32}$P]$P_i$ and hormone (Benjamin & Singer, 1974, 1975). This result was confirmed by Avruch et al. (1976) and Forn and Greengard (1976). The protein band was soon determined to be the subunit of ATP-CL[1] found in the cytosol of hepatocytes and fat cells (Alexander et al., 1979; Ramakrishna & Benjamin 1979; Swergold et al., 1982). An insulin-induced increase in the phosphorylation of ribosomal protein S6 (Smith et al., 1980; (Smith et al., 1980; Lastick & McConkey, 1981), insulin receptor (Pertruzelli et al., 1984; White et al., 1985), acetyl-CoA carboxylase (Witters et al., 1983; Denton, 1986), and $M_r$ 23 000 protein (Belsham et al., 1980; Blackshear et al., 1983, Ramakrishna & Benjamin 1983) was soon demonstrated.

How insulin produces an increase in the phosphorylation of some proteins and a decrease in others is still not understood. The finding that both insulin and isoproterenol increase the phosphorylation of ATP-CL has added complexity to the problem (Pierce et al., 1982; Pucci et al., 1983; Ramakrishna et al., 1984; Swergold et al., 1982). The following facts about this problem are worth noting. Either insulin or isoproterenol can increase the phosphorylation of peptide A of ATP-CL, whereas insulin decreases while isoproterenol increases the phosphorylation of peptide B. In addition, the phosphorylation of peptide A enhances the ability of peptide B to be phosphorylated by MFPK (Ramakrishna et al., 1983). This latter finding is analogous to that described for glycogen synthase where the phosphorylation of site 5 enhances the ability of sites 3a-c to be phosphorylated (Picton et al., 1982; DePaoli-Roach et al., 1983; Fiol et al., 1987).

The above observations suggest that the hormone effects on ATP-CL phosphorylation are complex and could involve more than one mechanism for the control of protein phosphorylation. The mechanisms could involve both the control of the activity of a specific protein kinase by insulin and the regulation of the phosphorylation of one site by the phosphate content of a different site. These hypotheses provide the rationale for the experiments to be presented. The results show that a decrease in peptide B phosphorylation of ATP-CL leads to an increase in peptide A phosphorylation by an unchanged A-kinase by second-site regulation.

## Materials and Methods

*Materials.* DEAE-cellulose DE-52 was purchased from Whatman. The sources of chemicals were as described (Ramakrishna & Benjamin, 1988).

*Incubation of Fat Pads with Hormones.* Epididymal fat pads from 100-125-g male Sprague-Dawley rats (Taconic Farms, Germatown, NY) were incubated in modified Krebs-Ringer Hepes buffer, pH 7.4, containing 3% BSA and 5 mM glucose at 37 °C as previously described (Benjamin & Singer, 1975). [$^{32}$P]$P_i$ was added to some flasks, and the incubations were continued for 90 min (Ramakrishna & Benjamin, 1984). Insulin (100 nM) or isoproterenol (1 $\mu$M)

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TLCK, *N*-(*p*-tosyl)-L-lysine chloromethyl ketone; SDS, Sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol); MFPK, multifunctional protein kinase; A-kinase, cAMP-dependent protein kinase; ATP-CL, ATP-citrate lyase.

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was added, and the flasks were incubated for an additional 30 min. After the incubation, the adipose tissue was quickly rinsed in Krebs–Ringer bicarbonate buffer, frozen immediately on an aluminium block (covered with Saran wrap), cooled to −70 °C, and processed as described below.

*Purification of ATP-CL.* Fat pads (5 g) were homogenized in 3 volumes of buffer A (50 mM potassium phosphate, pH 7.5, 15 mM mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 10 μM TLCK, 1 mg/L leupeptin and pepstatin A, 0.2 mM PMSF, 10 mM benzamidine, and 0.25 M sucrose). The homogenates were centrifuged at 15000*g* for 10 min, and the supernatants were centrifuged at 150000*g* for 30 min. $(NH_4)_2SO_4$ was added to the supernatants to a final concentration of 40% (25 g/100 mL), the supernatants were then cooled on ice for 1 h and centrifuged, and the precipitates were dissolved in 5 mL of buffer A. PEG ($M_r$ 8000, a 50% w/v solution) was added to the $(NH_4)_2SO_4$ fraction to give a concentration of 5% and centrifuged at 30000*g* for 15 min. The supernatant was brought to a final concentration of 15% PEG and centrifuged at 30000*g* for 15 min. The pellet was dissolved in 3 mL of buffer B (10 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 15 mM mercaptoethanol, 0.1 mM PMSF, 10 μM TLCK, 1 mg/L leupeptin and pepstatin, and 5% glycerol) and loaded on DE-52 column (0.8 × 8 cm) equilibrated in buffer B. After the columns were washed with 50 mL of buffer B, ATP-CL was eluted from the columns with buffer B containing 60 mM potassium phosphate; 2-mL fractions were collected. Fractions (2 and 3) with lyase activity were pooled, dialyzed, and concentrated with buffer C (25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.2 mM PMSF, 10 μM TLCK, 1 mg/L pepstatin and leupeptin, and 5% glycerol) by vacuum dialysis. ATP-CL is the major protein (about 75%) as assessed by Coomassie blue staining of SDS–polyacrylamide gel and specific activity measurements (7.4 units/mg of protein) in the purified fractions.

*Preparation of ATP-CL.* Rat liver ATP-CL (9.9 units/mg of protein) (2 mg/mL) was dephosphorylated with 0.4 mg/mL *Escherichia coli* alkaline phosphatase (Ramakrishna et al., 1983). The dephospho-ATP-CL was isolated by gel filtration on a Sephacryl S-200 column (1 × 55 cm). The dephospho-ATP-CL was phosphorylated at peptide A with unlabeled ATP by incubation with catalytic subunit of A-kinase at 30 °C for varying times up to 6 h. The A-site-phosphorylated lyase was isolated by gel filtration on Sephacryl S-200. In parallel assays using radioactive ATP it was determined that up to 98% of the peptide A could be phosphorylated, suggesting that the phosphatase treatment removed almost all of the phosphate on peptide A. ATP-CL prepared with defined phosphorylation content at peptide A by decreasing the incubation time with A-kinase was incubated for varying times with MFPK and 100 μM ATP to prepare ATP-CL with a low and high phosphate content at peptide B. ATP-CL thus phosphorylated was purified on a Sephacryl S-200 column and used as substrate for the kinetic analyses. Note that only 1 mol of phosphate/mol of subunit was incorporated at peptide B and in prior reports no more than 1.4 mol of phosphate/mol of subunit was incorporated at peptide B (Ramakrishna et al., 1983). These results suggest that as there are a minimum of two phosphorylation sites on peptide B, either the phosphatase treatment does not remove all the phosphate or it is difficult to fully phosphorylate both sites simultaneously.

*Enzyme Assays.* ATP-CL was assayed by the malate dehydrogenase coupled procedure (Singh et al., 1976). One unit of ATP-CL is defined as the amount of enzyme necessary to catalyze the oxidation of 1 μmol of NADH/min at 37 °C.

Table I: Phosphorylation of ATP-CL Isolated from Hormone-Treated Fat Pads[a]

| treatment | MFPK[b] | A-kinase[b] | A-kinase, then MFPK[b] |
|---|---|---|---|
| control | 0.20 ± 0.02 | 0.63 ± 0.03 | 0.74 ± 0.09 |
| insulin (100 nM) | 0.59 ± 0.02[c] | 0.48 ± 0.04[d] | 0.76 ± 0.05 |
| isoproterenol (1 μM) | 0.53 ± 0.03[c] | 0.47 ± 0.03[c] | 0.65 ± 0.07 |

[a] ATP-CL (0.1–0.5 mg/mL) purified as described was phosphorylated either by MFPK (10 μg/mL) or by A-kinase (5 μg/mL). The amount of ATP-CL phosphorylation was quantitated by counting the $^{32}P$-labeled ATP-CL band after SDS–PAGE and acid treatment. When ATP-CL was first phosphorylated by A-kinase, the reaction was stopped by the addition of protein kinase inhibitor. MFPK reaction was initiated by the addition of MFPK and [$^{32}P$]ATP and incubated for 40 min. The values are the mean ± SEM. $n$ = 13 for insulin, 15 for isoproterenol, and 19 for the control. [b] In units of mol of phosphate/mol of subunit. [c] The statistical significance of the difference between control and hormone treatment was calculated by Student's $t$ test and $p < 0.001$. [d] The statistical significance of the difference between control and hormone treatment was calculated by Student's $t$ test and $p < 0.002$.

ATP-CL phosphorylation by MFPK or A-kinase was as described previously (Ramakrishna & Benjamin, 1985). The reaction mixture contained 50 mM Hepes, pH 7.0, 2 mM DTT, 8 mM magnesium acetate, 0.3 mM EGTA, 0.5 mM EDTA, 0.1 mM [$\gamma^{32}P$]ATP, 1 mg/mL ATP-CL, and the appropriate kinase. Samples were incubated for 5 min–6 h at 30 °C. The reaction was terminated by adding sample buffer. Samples were subjected to SDS–polyacrylamide gel electrophoresis, and the phosphate incorporated into peptide A or peptide B of ATP-CL was measured as described previously (Ramakrishna & Benjamin, 1985).

## Results and Discussion

*In Vitro*[2] *Phosphorylation of ATP-CL Purified from Hormone-Treated Fat Pads.* Because previous studies had shown that insulin changes the phosphate content of specific sites on glycogen synthase (Parker et al., 1983; Sheorain et al., 1984, 1985), we hypothesized that insulin should rapidly change the phosphorylation state of ATP-CL. ATP-CL purified from fat pads incubated with insulin should contain less phosphate at peptide B and more phosphate at peptide A compared to controls (Ramakrishna et al., 1984). The protein should reflect these changes when used as substrate in phosphorylation studies. Fat pads were incubated with hormone, and in each group some tissue was also incubated with [$^{32}P$]$P_i$. ATP-CL from fat pads incubated with [$^{32}P$]$P_i$ was immunoprecipitated as described previously (Pucci et al., 1983). The immunoprecipitated ATP-CL was resolved on SDS gels, and the specific radioactivity of the ATP-CL band was calculated. In each experiment it was demonstrated that insulin (nine experiments) and isoproterenol (six experiments) increased the specific radioactivity of ATP-CL by 115% and 85%, respectively. ATP-CL rapidly purified from fat pads incubated in parallel experiments but without [$^{32}P$]$P_i$ was used as substrate for in vitro phosphorylation by MFPK and A-kinase. As shown in Table I, ATP-CL from insulin-treated fat pads was phosphorylated to 0.59 mol/mol of subunit at peptide B by MFPK compared to 0.20 mol/mol of subunit in controls. A small but significant decrease (0.15 mol/mol of subunit) in peptide A phosphorylation by A-kinase was also found with ATP-CL from insulin-treated adipose tissue compared to control. Thus, insulin in vivo makes peptide B a better sub-

[2] In this report the term "in vivo" refers to intact tissue or cells, and "in vitro" refers to cell-free systems.

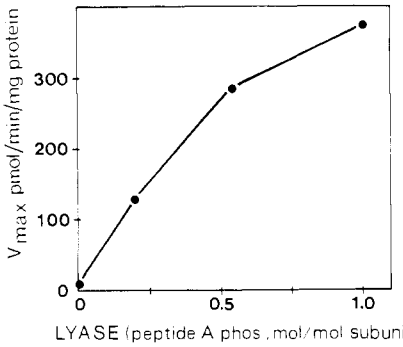


FIGURE 1: Effect of peptide A phosphate content of ATP-CL on MFPK activity. Dephospho-ATP-CL was phosphorylated on peptide A to 0.22, 0.54, and 0.98 mol/mol by A-kinase and purified. The $V_{max}$ and apparent $K_m$ values were determined from the Lineweaver–Burk plot of the relationship between the lyase concentration and MFPK activity. The relationship between maximal velocity and different ATP-CL peptide A phosphate contents is plotted. These assays were repeated twice with similar results.

strate for in vitro phosphorylation by MFPK by either decreasing peptide B phosphorylation and/or increasing peptide A phosphorylation. ATP-CL purified from isoproterenol-treated fat pads was phosphorylated at peptide A by A-kinase to 0.47 mol/mol of subunit compared to 0.63 mol/mol of subunit in samples from control. These observations are consistent with peptide A being more highly phosphorylated due to the in vivo activation of A-kinase by isoproterenol. The increase in the phosphorylation of peptide B by MFPK from ATP-CL purified from isoproterenol can be explained by the $\beta$-agonist causing in vivo an increase in phosphorylation at peptide A and a small increase in the phosphorylation of peptide B, still rendering peptide B a better substrate for in vitro added MFPK by positive second-site regulation. When ATP-CL from control and insulin- and isoproterenol-treated fat pads is first phosphorylated by A-kinase, peptide B phosphorylation in vitro by MFPK increases, demonstrating "positive second-site regulation" of peptide B by peptide A. Insulin decreases peptide B phosphorylation by decreasing MFPK activity (Ramakrishna & Benjamin, 1988). How this decrease in peptide B phosphorylation leads to an increase in peptide A phosphorylation is not explained by these experiments. Insulin could activate a "new protein kinase" that phosphorylates peptide A or by second-site regulation could increase the availability of peptide A for phosphorylation by an unchanged A-kinase. Our prediction is that an insulin-induced decrease in peptide B phosphorylation leads to an increase in peptide A phosphorylation. Experiments were undertaken to test this hypothesis.

*Phosphorylation of ATP-CL with Defined Phosphate Contents of Peptides A and B.* To determine the kinetic parameters for the phosphorylation of peptide B as a function of peptide A phosphorylation, dephospho-ATP-CL was first phosphorylated by A-kinase to 0.22 and 0.54 mol/mol of subunit. These preparations were used to study the phosphorylation of peptide B by MFPK. The dephospho-ATP-CL was not a good substrate for MFPK, and little phosphorylation was observed. As shown in Figure 1, the $V_{max}$ for ATP-CL markedly increased with an increase in peptide A phosphorylation, confirming our initial observation of a positive second-site regulation (Ramakrishna et al., 1983). The $K_m$ for ATP-CL also increased with increasing $V_{max}$ (data not shown).

To study the effect of peptide B phosphorylation on peptide A phosphorylation by A-kinase, ATP-CL phosphorylated at peptide A to 0.22 and 0.54 mol/mol of subunit was used to phosphorylate peptide B by MFPK to different phosphate

Table II: Effect of ATP-CL Peptide B on Peptide A Phosphorylation by A-Kinase[a]

| ATP-citrate lyase | | | |
|---|---|---|---|
| peptide A (mol of phosphate/mol of subunit) | peptide B (mol of phosphate/mol of subunit) | $V_{max}$ [pmol/(min·mg of protein)] | $K_m$ ($\mu$M) |
| 0 | 0 | 5007 | 20.0 |
| 0.22 | 0 | 1429 | 5.9 |
| 0.22 | 0.50 | 667 | 2.0 |
| 0.22 | 0.68 | 400 | 1.3 |
| 0.54 | 0 | 800 | 3.6 |
| 0.54 | 0.77 | 625 | 1.5 |
| 0.54 | 1.01 | 370 | 1.2 |

[a] ATP-CL was dephosphorylated with alkaline phosphatase and then phosphorylated at peptide A with A-kinase to 0.22 and 0.54 mol/mol of subunit. These lyase samples were further phosphorylated at peptide B with MFPK. Note that after each dephosphorylation or phosphorylation step the lyase was purified by gel filtration on Sephacryl S-200. The reaction velocities for the catalytic subunit of A-kinase were determined for various concentrations of these prepared lyases. The data were plotted as double-reciprocal plots, and lines were fitted by linear regression. The $V_{max}$ and $K_m$ values were obtained from the Lineweaver–Burk plots. The values are averages of duplicate samples. These assays were repeated two times with different amounts of phosphate at peptide A and peptide B, and similar results were obtained.

contents. As shown in Table II, ATP-CL containing a large amount of phosphate at peptide B is not as good a substrate for further phosphorylation at peptide A by A-kinase as ATP-CL containing a lesser amount of phosphate at peptide B. Kinetic studies demonstrate that the $V_{max}$ for peptide A phosphorylation decreases as a function of peptide B content. The decrease over a possible physiologic range of phosphate contents for peptides B and A was 40% for the two instances tested. Note that the $K_m$ for ATP-CL decreases with an increase in peptide B phosphorylation. Because the molar concentration of ATP-CL is likely to be less than 10 $\mu$M in normal (uninduced) rat liver or adipose tissue (Guy et al., 1980), it is possible that the fall in the $K_m$ from 5.9 $\mu$M to 1.2 $\mu$M with peptide B phosphorylation could be of physiological significance.

This paper presents evidence that a dephosphorylation at one site renders another site more able to be phosphorylated. Therefore, a decrease in the activity of a specific kinase or an increase in the activity of a specific phosphatase could at steady state increase the phosphate content of a distant site phosphorylated by an unchanged other kinase. Note that these observations are different from the findings that increased phosphorylation at peptide A increases the ability of peptide B to be phosphorylated (Ramakrishna et al., 1983) and that increased phosphorylation at site 5 on glycogen synthase by casein kinase 2 increases the phosphorylation at sites 3a–c by glycogen synthase kinase 3 (Picton et al., 1982; DePaoli-Roach et al., 1983) because in the results described in this paper a decrease in the phosphorylation of peptide B increases the ability of peptide A to be phosphorylated.

A general scheme for the effect of insulin on ATP-CL phosphorylation is shown in Figure 2. Insulin action decreases peptide B phosphorylation by decreasing the activity of MFPK (Ramakrishna & Benjamin 1988) (step i) and possibly by increasing the activity of a broad-spectrum protein phosphatase (Cohen, 1982). This decrease in peptide B phosphorylation makes peptide A a better substrate for phosphorylation by A-kinase (step ii). The increase in peptide A phosphorylation now renders peptide B a better substrate for phosphorylation by MFPK (step iii). Thus a further fall in peptide B phosphorylation produced by a decrease in MFPK activity is limited by this positive feedback system. Isoproterenol increases

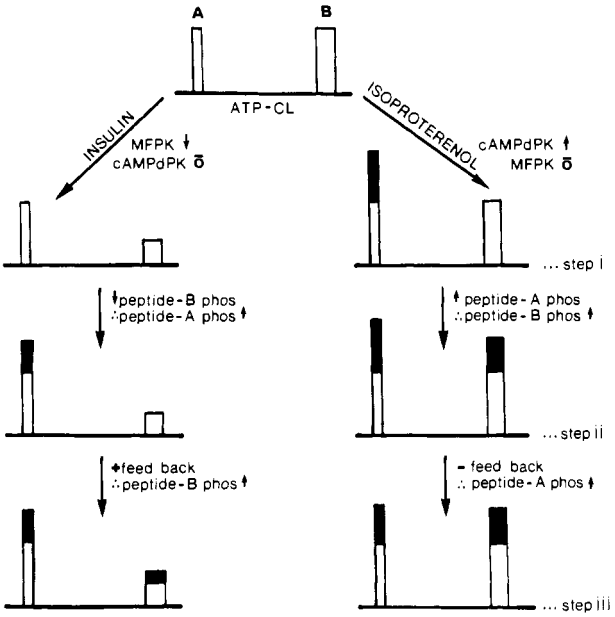


FIGURE 2: Schema for the regulation of ATP-CL phosphorylation by insulin and isoproterenol. The shaded area represents increased phosphorylation. ATP-CL contains at least three phosphorylation sites on the two peptides A and B. Peptide A contains the A-kinase site containing phosphoserine. Peptide B contains the MFPK site containing both phosphoserine and phosphothreonine. The hormone effect is shown in three steps. Insulin decreases MFPK activity (Ramakrishna & Benjamin, 1988) without affecting A-kinase activity, resulting in a decrease in peptide B phosphorylation. This decrease in peptide B phosphorylation allows more peptide A phosphorylation by an unchanged A-kinase (step ii). This increase in peptide A phosphorylation increases peptide B phosphorylation by second-site regulation (step iii). Isoproterenol increases A-kinase activity and hence peptide A phosphorylation (step i). This increase in peptide A phosphorylation by positive feedback action increases peptide B phosphorylation by an unchanged MFPK (step ii). This increase in peptide B phosphorylation by negative feedback reduces peptide A phosphorylation by the elevated A-kinase (step iii).

peptide A phosphorylation by increasing A-kinase activity (step i). This increase in peptide A phosphorylation makes peptide B a better substrate for subsequent phosphorylation by MFPK (step II). This increase in peptide B phosphorylation, by negative feedback, limits the further phosphorylation of peptide A by the activated A-kinase (step iii). Thus, in this schema insulin and its counterregulatory hormones do not generate the same phosphorylation state in ATP-CL. Insulin generates an ATP-CL molecule with a low peptide B phosphate content and a minimally increased peptide A phosphate content whereas a $\beta$-agonist leads to a maximally increased peptide A phosphate content and in increased peptide B content. It should be noted that this schema represents the predicted changes in protein phosphorylation as taking place on a single peptide chain. Because ATP-CL is composed of four identical subunits (Singh et al., 1976), it is possible that changes in protein phosphorylation at one site could affect the phosphorylations at sites on the same subunit and/or other subunits of the holoenzyme.

It is likely that other enzymes whose activities are varied by phosphorylation/dephosphorylation reactions and are phosphorylated at multiple sites are also regulated by similar complex interrelations between the degree of phosphorylation at specific sites and changes in kinase activities as described for ATP-CL. The relationship of the recently described insulin-induced increases in protein kinase activities [ribosomal protein S6 kinase (Tabarini et al., 1985; Cobb, 1986), microtubule-associated protein 2 kinase (Ray & Sturgill, 1987), Kemptide kinase (Yu et al., 1987), and casein kinase II (Sommercorn et al., 1987)] to the insulin-induced decrease in MFPK activity (Ramakrishna & Benjamin, 1988) is unknown. It should be noted that results that demonstrate a decrease in MFPK activity by insulin are consistent with observations that show an insulin-induced net dephosphorylation of specific sites that correlates with changes in enzyme activities (Parker et al., 1983; Sheorain et al., 1984; Lawrence et al., 1986).

A-kinase and MFPK phosphorylate stoichiometrically ATP-CL, acetyl-CoA carboxylase, and glycogen synthase. Though the physiological effect of ATP-CL phosphorylation has not been determined, these kinases via phosphorylation do inhibit the activities of acetyl-CoA carboxylase[3] and glycogen synthase (Sheorain et al., 1985; Kim, 1979). The evidence presented in this paper suggests that insulin could decrease the phosphorylation of specific sites on acetyl-CoA carboxylase and glycogen synthase and thus increase their enzyme activities. As described for ATP-CL phosphorylation, the decrease in MFPK activity by insulin (Ramakrishna & Benjamin, 1988) not only could decrease phosphorylation at MFPK-directed sites on enzymes of metabolism but could also increase phosphorylation at other regulatory sites, limiting wide swings in the phosphorylation state and enzyme activities. Indeed, an insulin-induced decrease in phosphorylation of an MFPK-sensitive site of acetyl-CoA carboxylase could regulate the phosphorylation of an A-kinase or casein kinase II phosphorylated site.

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**Registry No.** MFPK, 9026-43-1; ATP-CL, 9027-95-6; insulin, 9004-10-8; isoproterenol, 7683-59-2.

## REFERENCES

Alexander, M. C., Kowaloff, E. M., Witters, L. A., Dennihy, D. T., & Avruch, J. (1979) *J. Biol. Chem. 254*, 8052–8056.

Avruch, J., Leone, G. R., & Martin, D. B. (1976) *J. Biol. Chem. 251*, 1511–1515.

Belsham, G. S., Brownsey, R. W., & Denton, R. M. (1982) *Biochem. J. 204*, 345–352.

Benjamin, W. B., & Singer, I. (1974) *Biochim. Biophys. Acta 351*, 28–41.

Benjamin, W. B., & Singer, I. (1975) *Biochemistry 14*, 3301–3309.

Blackshear, P. J., Nemenoff, R. A., & Avruch, J. (1983) *Biochem. J. 214*, 11–19.

Cobb, M. H. (1986) *J. Biol. Chem. 261*, 12994–12999.

Cohen, P. (1982) *Nature 296*, 613–620.

Denton, R. M. (1986) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 20*, 293–341.

DePaoli-Roach, A. A., Ahamad, Z., Camici, M., Lawrence, J. C., Jr., & Roach, P. J. (1983) *J. Biol. Chem. 258*, 10702–10709.

Fiol, C. J., Mahrenholz, A. M., Wang, Y., Roeske, R. W., & Roach, P. J. (1987) *J. Biol. Chem. 262*, 14042–14048.

Forn, J., & Greengard, P. (1976) *Arch. Biochem. Biophys. 176*, 721–733.

Kim, K.-H. (1979) *Mol. Cell. Biochem. 28*, 27–43.

Lastick, S. M., & McConkey, E. H. (1981) *J. Biol. Chem. 256*, 583–585.

[3] S. Ramakrishna and W. B. Benjamin, unpublished results.

Lawrence, J. C., Jr., James, C., & Hiken, J. F. (1986) *J. Biol. Chem. 261*, 669–677.
Parker, P. J., Caudwell, F. B., & Cohen, P. (1983) *Eur. J. Biochem. 130*, 227–234.
Pertruzelli, L., Herrera, R., & Rosen, O. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3327–3331.
Picton, C., Woodgett, J., Hemmings, B., & Cohen, P. (1982) *FEBS Lett. 150*, 191–196.
Pierce, M. W., Palmer, J. L., Keutman, H. T., Hall, T. A., & Avruch, J. (1982) *J. Biol. Chem. 257*, 10681–10686.
Pucci, D. L., Ramakrishna, S., & Benjamin, W. B. (1983) *J. Biol. Chem. 258*, 12907–12911.
Ramakrishna, S., & Benjamin, W. B. (1979) *J. Biol. Chem. 254*, 9232–9236.
Ramakrishna, S., & Benjamin, W. B. (1983) *Biochem. Biophys. Res. Commun. 117*, 758–764.
Ramakrishna, S., & Benjamin, W. B. (1988) *J. Biol. Chem. 263*, 12677–12681.
Ramakrishna, S., Pucci, D. L., & Benjamin, W. B. (1983) *J. Biol. Chem. 258*, 4950–4956.
Ramakrishna, S., Pucci, D. L., & Benjamin, W. B. (1984) *Biochem. Biophys. Res. Commun. 122*, 1047–1056.
Ray, L. B., & Sturgill, T. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1502–1506.
Sheorain, V. S., Juhl, H., Bass, M., & Soderling, T. R. (1984) *J. Biol. Chem. 259*, 7024–7030.
Sheorain, V. S., Ramakrishna, S., Benjamin, W. B., & Soderling, T. R. (1985) *J. Biol. Chem. 260*, 12287–12292.
Singh, M., Richards, E. G., Mukherjee, A., & Srere, P. A. (1976) *J. Biol. Chem. 251*, 5242–5250.
Smith, C. J., Rubin, C. S., & Rosen, O. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2641–2645.
Sommercorn, J., Mulligan, J. A., Lozeman, F. J., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8834–8838.
Swergold, G. D., Rosen, O. M., & Rubin, C. S. (1982) *J. Biol. Chem. 257*, 4207–4215.
Tabarini, D., Heinrich, J., & Rosen, O. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4369–4373.
White, M. F., Takayama, S., & Kahn, C. R. (1985) *J. Biol. Chem. 260*, 9470–9478.
Witters, L. A., Tipper, J. P., & Bacon, G. W. (1983) *J. Biol. Chem. 258*, 5643–5648.
Yu, K.-T., Khalaf, N., & Czech, M. P. (1987) *J. Biol. Chem. 262*, 16677–16685.

# Biosynthesis of the 7-Mercaptoheptanoic Acid Subunit of Component B [(7-Mercaptoheptanoyl)threonine Phosphate] of Methanogenic Bacteria†

Robert H. White

*Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061*

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ABSTRACT: $^{2}H$- and $^{13}C$-labeled precursors were used to establish the pathway for the biosynthesis of the 7-mercaptoheptanoic acid moiety of component B in methanogenic bacteria. The extent and position of the label incorporated into 7-mercaptoheptanoic acid were measured from the molecular and fragment ions in the mass spectrum of the methyl ester methylthiol derivative of the 7-mercaptoheptanoic acid. Deuterium from [2,2,2-$^{2}H_3$]acetate was found to be incorporated into four separate positions of 7-mercaptoheptanoic acid. One deuterium was equally distributed between the C-2 and the C-3 of the 7-mercaptoheptanoic acid, and the remaining three were at carbons 4–6. The extent of incorporation at the C-2 and C-3 positions was the same as that observed for the incorporation of [2,2,2-$^{2}H_3$]acetate into the $\alpha$-ketoglutarate produced by the cells. [1,2-$^{13}C_2$]Acetate was incorporated into four separate sites of the 7-mercaptoheptanoic acid molecule. An intact acetate unit was incorporated at C-2 and C-3, and *single* carbons of the acetate were incorporated at C-5, C-6, and C-7. [2,2,3,3-$^{2}H_4$]Succinate was incorporated with the retention of all four deuteriums, and it supplied carbons 1–4. On the basis of this and additional information, it is concluded that 7-mercaptoheptanoic acid is biosynthesized from $\alpha$-ketosuberate, which arises from $\alpha$-ketoglutarate by repeated $\alpha$-keto acid chain elongation. The mechanism for the conversion of $\alpha$-ketosuberate to a thiol appears to be analogous to that for the conversion of sulfopyruvate to coenzyme M (2-mercaptoethanesulfonic acid).

Component B [(7-mercaptoheptanoyl)threonine phosphate] is a small molecular weight cofactor required for the reduction of methyl coenzyme M to methane in methanogenic bacteria (Noll et al., 1986). The functional portion of the molecule appears to be the thiol group of the 7-mercaptoheptanoic acid, which is known to form a heterodisulfide bond with coenzyme M when methane is produced from methyl coenzyme M in the methylreductase reaction (Bobik et al., 1987). The presence of the 7-mercaptoheptanoic acid portion of component B in archaebacteria is unusual, since, by first inspection, it appears to be a derivative of a fatty acid, a class of compounds that only occurs in very small amounts in methanogenic bacteria (Langworthy, 1985). 7-Mercaptoheptanoic acid is also a homologue of 8-mercaptooctanoic acid, a known intermediate in the biosynthesis of lipoic acid (White, 1980a), a coenzyme known to occur in much lower amounts in archaebacteria than in other cells (Noll & Barber, 1988).

In order to determine if a connection exists between the biosynthesis of lipoic acid and the 7-mercaptoheptanoic acid moiety of component B, the following work was undertaken. The results of this work show that 7-mercaptoheptanoic acid is generated by a biosynthetic pathway completely different from that of lipoic acid. This pathway is novel in that it

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