Proc. Natl. Acad. Sci. USA
Vol. 76, No. 9, pp. 4375–4379, September 1979
Biochemistry

Characterization and regulation of reductase kinase, a protein kinase that modulates the enzymic activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase

(phosphorylation/covalent modification/cholesterol/cyclic AMP/enzyme regulation)

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Communicated by Bernhard Witkop, June 20, 1979

ABSTRACT The activity of rat liver 3-hydroxy-3-methylglutaryl-coenzyme A reductase [HMG-CoA reductase; mevalonate:NADP$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34] can be modulated *in vitro* by a phosphorylation–dephosphorylation reaction sequence. A microsomal reductase kinase catalyzes the phosphorylation of HMG-CoA reductase and histones. Histone phosphorylation was enhanced 2- to 3-fold by cyclic AMP. Reductase kinase exists in interconvertible active and inactive forms. Incubation of reductase kinase with phosphoprotein phosphatase resulted in a time-dependent decrease in the ability of reductase kinase to catalyze the phosphorylation of histones and to inactivate HMG-CoA reductase. Incubation of phosphoprotein phosphatase-inactivated reductase kinase with [γ-^{32}P]ATP plus Mg^{2+} and a partially purified protein kinase designated reductase kinase kinase resulted in parallel increases in protein-bound ^{32}P radioactivity and ability to inactivate HMG-CoA reductase. Incubation of ^{32}P-labeled reductase kinase with phosphoprotein phosphatase resulted in a time-dependent loss of protein-bound ^{32}P radioactivity and a decrease in the ability to inactivate HMG-CoA reductase. Polyacrylamide gel electrophoresis of purified reductase kinase incubated with reductase kinase kinase and [γ-^{32}P]ATP plus Mg^{2+} revealed that the ^{32}P radioactivity and reductase kinase enzymic activity were located in a single electrophoretic position. Dephosphorylation of ^{32}P-labeled purified reductase kinase with phosphoprotein phosphatase was associated with significant loss of radioactivity and enzymic activity in the protein band ascribed to reductase kinase. These results provide evidence that the activity of reductase kinase, like HMG-CoA reductase, is modulated by a reversible phosphorylation–dephosphorylation reaction sequence.

Recently, we (1) demonstrated that the enzymic activity of rat liver microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase [HMG-CoA reductase; mevalonate:NADP$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34] was modulated by phosphorylation–dephosphorylation. Phosphorylation of HMG-CoA reductase was catalyzed by the microsomal enzyme reductase kinase and ATP plus Mg^{2+} (1). Ingebritsen *et al.* (2) suggested that the enzyme that inactivates HMG-CoA reductase also exists in active and inactive forms.

Reductase kinase has now been further characterized and evidence of a reductase kinase kinase has been obtained. Evidence is provided for the regulation of the activity of reductase kinase by an enzyme-mediated phosphorylation–dephosphorylation reaction sequence. Thus, two protein kinases are involved in the regulation of the catalytic activity of HMG-CoA reductase.

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MATERIALS AND METHODS

Solubilization and Purification of Microsomal Reductase Kinase. Soluble reductase kinase was prepared by extracting freshly isolated rat liver microsomes three times with 250 mM NaCl/1 mM EDTA/5 mM dithiothreitol/50 mM KH_2PO_4, pH 7.4. The combined extracts were fractionated with ammonium sulfate. The precipitate, collected between 25 and 45% saturation, was solubilized in and dialyzed against buffer A [5 mM Tris·HCl, pH 7.4/0.1 mM EDTA/5 mM dithiothreitol/10% (vol/vol) glycerol] until the conductivity was 0.65 mmho. Reductase kinase was further purified by chromatography of the ammonium sulfate precipitate (500 mg of protein) on a column (2 × 15 cm) of DE-52 with a gradient of 0.3 M NaCl in buffer A (150 ml) and buffer A (150 ml). Reductase kinase activity eluted between 1.4 and 3.8 mmho. These fractions (49 mg of protein) were pooled, dialyzed against buffer A, and chromatographed on a second DE-52 column [1.2 × 9 cm, gradient of buffer A containing 0.27 M NaCl (120 ml) and buffer A (120 ml)]. The partially purified reductase kinase used in the present studies eluted between 1.7 and 3.8 mmho; it was dialyzed against 10 mM KH_2PO_4, pH 7.4/0.1 mM EDTA/5 mM dithiothreitol and stored frozen (0.2 mg/ml). Selected preparations of partially purified reductase kinase were purified to homogeneity by utilizing phosphocellulose chromatography, gel filtration (Sepharose 6B), adsorption chromatography on alumina Cγ, and thin-layer isoelectric focusing (pH 4–6.5; pI = 5.6 ± 0.2).

Isolation of Inactivated Reductase Kinase. Rat liver microsomes (15 mg/ml in 50 mM imidazole, pH 7.4/250 mM NaCl/1 mM EDTA/5 mM dithiothreitol) containing endogeneous phosphoprotein phosphatase were incubated at 37° for 2 hr and then extracted three times to solubilize inactive reductase kinase. Alternatively, solubilized (6.2 mg/ml) or purified reductase kinase (0.1 mg/ml) was incubated for 30 min at 37°C with partially purified phosphoprotein phosphatase (4.5 mg). The incubation was terminated by the addition of 50 mM NaF.

Isolation of Reductase Kinase Kinase. Rat liver cytosol (100,000 × *g* supernatant) was fractionated with ammonium sulfate; the protein (5.2 g) precipitated at 60% saturation. It was dialyzed against buffer A, applied to a column of phosphocellulose (2.5 × 15 cm), and eluted with a gradient of 1 M NaCl in buffer A plus 50 μM phenylmethylsulfonyl fluoride (400 ml) and buffer A with 50 μM phenylmethylsulfonyl fluoride (400 ml). Fractions were assayed for protein kinase activity and the

Abbreviations: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; cAMP, cyclic AMP; $NaDodSO_4$, sodium dodecyl sulfate.

capacity to catalyze the phosphorylation of reductase kinase. Reductase kinase kinase activity eluted between 25 and 35 mmho. These fractions were pooled, dialyzed [5 mM 2-(*N*-morpholino)-ethanesulfonic acid, pH 6.7/5 mM dithiothreitol/10% glycerol], and stored frozen (0.73 mg/ml).

Isolation of Phosphoprotein Phosphatase. Cytosolic phosphoprotein phosphatase was partially purified from rat liver 100,000 × *g* supernatant up to the second ammonium sulfate fractionation by the method of Brandt *et al.* (3) as reported (1).

Assay of Protein Kinase Activity. The activity of protein kinase was determined by the method of Huang and Robinson (4). Incubation was performed at 37°C in 120 μl containing 40 mM β-glycerophosphate (pH 7.0), 2 mM dithiothreitol, 7 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (500–1000 dpm/pmol), and 4 mg of histone II-A, casein, or phosvitin per ml. Selected incubations also contained 25 μM cyclic AMP (cAMP).

Assay of Reductase Kinase. Microsomes that retained HMG-CoA reductase activity but no reductase kinase activity were prepared by washing microsomes five times with 50 mM potassium phosphate, pH 7.4/250 mM NaCl/1 mM EDTA/5 mM dithiothreitol. Assays contained 98 μg of washed microsomes, 50 mM β-glycerophosphate (pH 7.0), 50 mM NaF, 3 mM ATP, 12 mM $MgCl_2$, 2 mM dithiothreitol, and appropriate samples of reductase kinase in a total volume of 100 μl. After incubation for 20 min at 37°C, 40 mM EDTA was added followed by the reagents for assay of HMG-CoA reductase, and reductase activity was determined as outlined below.

Assay of HMG-CoA Reductase. Microsomes from rat liver were isolated as described (1). Assays for HMG-CoA reductase contained 50 mM KH_2PO_4 (pH 7.2), 10 mM dithiothreitol, 4 mM NADPH, 0.1 mM *dl*-[3-^{14}C]HMG-CoA (7000 cpm/nmol), and enzyme in a total volume of 200 μl. The reaction was terminated by addition of 50 μl of 10 M HCl, and [^{14}C]mevalonolactone was separated by either thin-layer chromatography (5) or column chromatography (6).

Additional Materials and Methods. Aqueous and sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis was performed as reported (6). Protein was quantified by the method of Bradford (7). [γ-^{32}P]ATP and *dl*-[3-^{14}C]HMG-CoA were purchased from New England Nuclear; HMG-CoA and agarose-hexane-cAMP were from PL Biochemicals; casein, histone II-A, phosvitin, phenylmethylsulfonyl fluoride, and alumina C_γ were from Sigma; DE-52 and phosphocellulose were from Whatman.

RESULTS

Inactivation of Microsomal HMG-CoA Reductase Activity by Reductase Kinase and ATP plus Mg^{2+}. Microsomal HMG-CoA reductase was inactivated by partially purified reductase kinase and ATP plus Mg^{2+} in a time-dependent manner (Fig. 1). Inactivated reductase kinase with or without ATP plus Mg^{2+} failed to decrease the enzymic activity of microsomal HMG-CoA reductase. Incubation of inactive reductase kinase with ATP plus Mg^{2+} and partially purified reductase kinase kinase restored the ability of reductase kinase to inhibit the enzymic activity of HMG-CoA reductase. These results are consistent with the proposal that reductase kinase exists in interconvertible active and inactive forms.

Characterization of Reductase Kinase. Microsomal reductase kinase required ATP plus Mg^{2+} for phosphorylation and inhibition of the enzymic activity of HMG-CoA reductase (1), (Fig. 1). Partially purified reductase kinase catalyzed the phosphorylation of histone 2A (Fig. 2A) but had little capacity to phosphorylate casein (20% > control). Phosphorylation of histone 2A by reductase kinase was enhanced 2- to 4-fold by

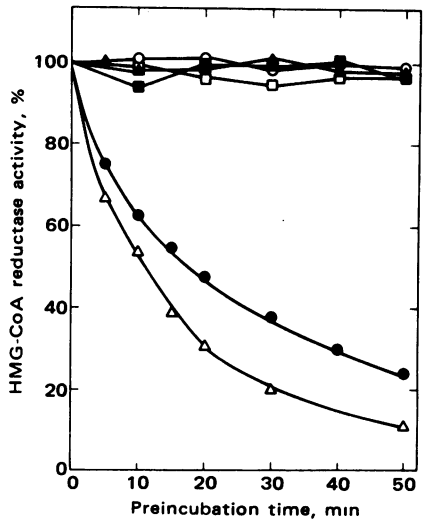

FIG. 1. Inactivation of HMG-CoA reductase by reductase kinase and ATP plus Mg^{2+}. Samples of reductase kinase were incubated for the indicated times at 37°C in 100 μl containing 50 mM β-glycerophosphate (pH 7.0), 50 mM NaF, and 2 mM dithiothreitol with or without 3 mM ATP plus 12 mM $MgCl_2$. After incubation, 40 mM EDTA was added and the enzymic activity of microsomal HMG-CoA reductase was determined: solubilized reductase kinase (175 μg) with (△) or without (▲) ATP plus Mg^{2+}; inactivated reductase kinase (122 μg) with (■) or without (□) ATP plus Mg^{2+}; inactivated reductase kinase (122 μg) plus reductase kinase kinase (23 μg) with (●) or without (○) ATP plus Mg^{2+}.

cAMP. The enhancement of phosphorylation of histone by cAMP was not observed after fractionation of solubilized reductase kinase on an agarose-cAMP affinity column, consistent with the binding of the regulatory subunit of the holoenzyme by cAMP and the dissociation of the catalytic subunit, resulting in a loss of cAMP activation of the enzyme.

A cytosolic reductase kinase has also been characterized, and its properties appear to be similar to those of the microsomal enzyme.

Characterization of Reductase Kinase Kinase. Reductase kinase kinase activity was found in both liver microsomes and cytosol. The enzyme used in these studies was partially purified from cytosol. Reductase kinase kinase catalyzed the phosphorylation of casein and, to a lesser degree, of phosvitin in a

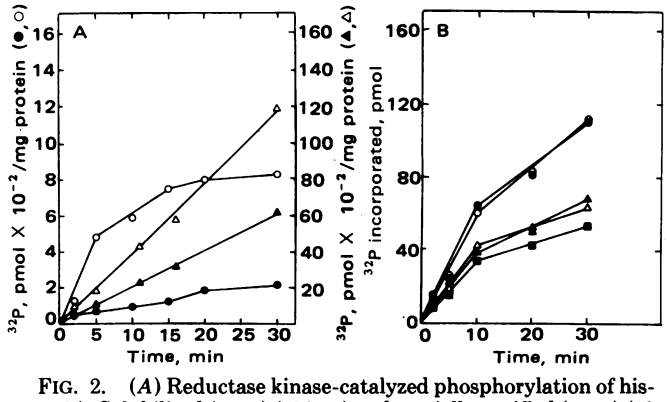

FIG. 2. (*A*) Reductase kinase-catalyzed phosphorylation of histone 2A. Solubilized (●, ○) (450 μg) and partially purified (▲, △) (19 μg) reductase kinase activities were determined in the absence (●, ▲) and presence (○, △) of 25 μM cAMP. (*B*) Reductase kinase kinase-catalyzed phosphorylation of casein and phosvitin. Partially purified reductase kinase kinase (58 μg) was assayed with casein (●), casein plus 25 μM cAMP (○), casein plus 25 μM cAMP plus 50 μg polyarginine (■), phosvitin (▲), or phosvitin plus 25 μM cAMP (△).

cAMP-independent manner (Fig. 2*B*); it had little activity with histone 2A as substrate (23% > control). Polyarginine significantly decreased the phosphorylation of casein by reductase kinase kinase (Fig. 2*B*).

Modulation of Reductase Kinase Activity. The inhibition of the enzymic activity of HMG-CoA reductase by partially purified reductase kinase and ATP plus Mg^{2+} was enhanced by cAMP (Table 1). Incubation of reductase kinase with partially purified reductase kinase kinase and ATP plus Mg^{2+} increased the inhibition of the enzymic activity of HMG-CoA reductase. Reductase kinase inactivated by phosphoprotein phosphatase had little effect on the activity of HMG-CoA reductase. Incubation of inactivated reductase kinase with reductase kinase kinase and ATP plus Mg^{2+} was associated with an increase in the reductase kinase inhibition of HMG-CoA reductase activity. Reductase kinase kinase alone had no effect on the enzymic activity of HMG-CoA reductase. The enzymic modulation of the catalytic activity of reductase kinase on the activity of HMG-CoA reductase was also confirmed by analysis of the phosphorylation of histone 2A (Fig. 3). These combined results are interpreted as indicating that reductase kinase as isolated in the present studies contains both active and inactive forms. The active and inactive forms of the enzyme can be interconverted by a phosphoprotein phosphatase and a protein kinase, reductase kinase kinase.

The mechanism of the activation and inactivation of reductase kinase was investigated further with [γ-^{32}P]ATP plus Mg^{2+}. Incubation of inactivated reductase kinase with reductase kinase kinase and [γ-^{32}P]ATP plus Mg^{2+} was associated with a time-dependent increase in protein-bound ^{32}P radioactivity (Fig. 4) and a 47 and 52% increase in the capacity of reductase kinase to catalyze the phosphorylation of histones and to inactivate HMG-CoA reductase, respectively. Incubation of ^{32}P-labeled reductase kinase with a phosphoprotein phosphatase resulted in a time-dependent release of protein-bound ^{32}P radioactivity and decrease in the ability of reductase kinase to inactivate HMG-CoA reductase (2% > control).

Table 1. Reductase kinase-catalyzed inactivation of enzymic activity of HMG-CoA reductase

Enzyme fraction*	Reductase kinase assay† ATP	Reductase kinase assay† cAMP	HMG-CoA reductase activity, % of control
Reductase kinase	−	−	100 (100)‡
	+	−	47 (73)‡
	+	+	33 (56)‡
Reductase kinase plus reductase kinase kinase	−	−	100
	+	−	29
Inactivated reductase kinase	−	−	100
	+	−	106
Inactivated reductase kinase plus reductase kinase kinase	−	−	100
	+	−	69
Reductase kinase kinase	−	−	100
	+	−	105
	+	+	104

* Solubilized reductase kinase (0.3 mg), phosphoprotein phosphatase-inactivated reductase kinase (0.6 mg), or reductase kinase kinase (0.14 mg) was preincubated at 37°C for 1 hr in 500 μl containing 50 mM β-glycerophosphate (pH 7.0), 0.2 mM ATP, and 3 mM $MgCl_2$. After incubation, 50-μl aliquots were taken for assay of reductase kinase activity. Additional 100 μl samples were taken for assay of protein kinase activity with histone 2A as substrate (see Fig. 3).

† When indicated: 3 mM ATP plus 12 mM $MgCl_2$; 25 μM cAMP.

‡ In separate experiments, highly purified HMG-CoA reductase (10 μg) was inactivated with reductase kinase (30 μg) and 3 mM ATP plus 12 mM $MgCl_2$ with or without 1 μM cAMP.

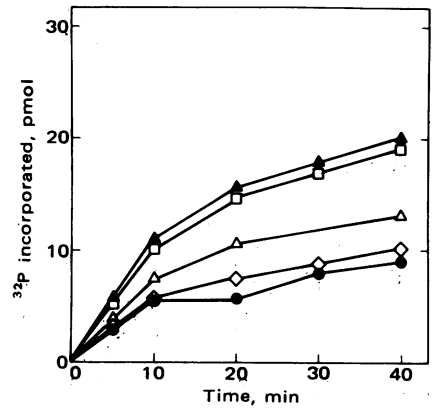

FIG. 3. Modulation of the reductase kinase-catalyzed phosphorylation of histone 2A. The effect of cAMP on the reductase kinase-catalyzed inactivation of HMG-CoA reductase (see Table 1 for details of assay conditions and inhibition of enzymic activity of HMG-CoA reductase) and phosphorylation of histone 2A was determined after incubation with ATP plus Mg^{2+} and the following enzyme preparations: reductase kinase and reductase kinase kinase (□); inactivated reductase kinase and reductase kinase kinase (▲); reductase kinase (△); reductase kinase kinase (◇); inactivated reductase kinase (●).

To establish definitively that activation–inactivation of reductase kinase was due to phosphorylation–dephosphorylation, studies were performed on the purified kinase. Reductase kinase purified by isoelectric focusing had a pI of 5.6 ± 0.2 and was a single band on polyacrylamide electrophoresis (Fig. 5 *inset*). Assay of gel slices demonstrated that the kinase activity (>95%) was localized to the single electrophoretic band, and incubation of gel slices containing reductase kinase resulted in the inactivation of HMG-CoA reductase activity (47%) and increase in the ability to catalyze the phosphorylation of reductase. The

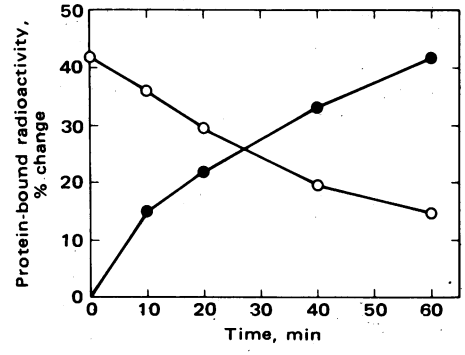

FIG. 4. Phosphorylation and dephosphorylation of reductase kinase. Phosphorylation (●): Inactivated reductase kinase (1.5 mg) was incubated at 37°C with reductase kinase kinase (0.37 mg) in 1.0 ml containing 0.1 mM [γ-^{32}P]ATP, 3 mM $MgCl_2$, 2 mM dithiothreitol, 50 mM NaF, and 50 mM β-glycerophosphate (pH 7.0). At the indicated intervals 10-μl aliquots were removed in duplicate and assayed for protein-bound ^{32}P radioactivity. Dephosphorylation (○): At the end of the 60-min incubation, 25 mM EDTA was added and reductase kinase activity was determined. Reductase kinase inhibited HMG-CoA reductase activity by 52% (±5%). A 0.5-ml sample of the incubation mixture was then removed, 2 mg of phosphoprotein was added, and the incubation was continued at 37°C. Aliquots were taken at the indicated times and the protein-bound ^{32}P radioactivity was determined. At the end of the second 60-min incubation, 0.1 ml was removed, 50 mM NaF was added, and reductase kinase activity was determined. Reductase kinase inhibited only 2% (±5%) of the enzymic activity of microsomal HMG-CoA reductase.

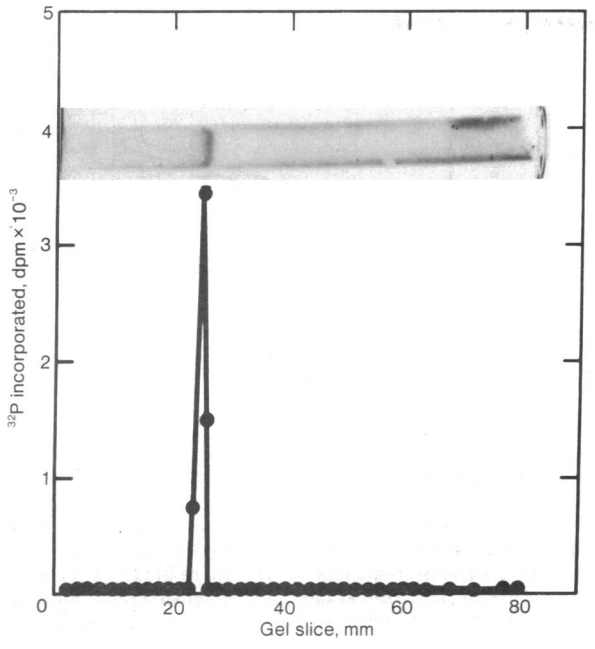

FIG. 5. Polyacrylamide gel electrophoresis of ^{32}P-labeled reductase kinase. Purified reductase kinase (0.09 mg/ml) was incubated in a reaction mixture (100 μl) containing 50 mM β-glycerophosphate (pH 7.0), 2 mM dithiothreitol, 1 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (6 μCi), and reductase kinase kinase (0.06 mg/ml) at 37°C for 1 hr. The control incubation contained all the components plus reductase kinase or reductase kinase kinase. The reaction mixture was subjected to polyacrylamide gel electrophoresis (13%; ref. 6). The gels were cut into 1-mm slices, and radioactivity and reductase kinase activity were determined. Radioactivity, data are corrected by subtraction of both control values. Reductase kinase activity from the gel slices was determined by measuring the inactivation of HMG-CoA reductase and histone phosphorylation. A duplicate gel of reductase kinase was stained with Coomassie blue for protein. Reductase kinase activity was detected only in the gel area coincident with the single electrophoretic band (inactivation of HMG-CoA reductase, 47%). After 1 hr of phosphorylation, selected samples were dephosphorylated by addition of EDTA and phosphatase. These samples obtained by gel electrophoresis showed significant loss (70%) of ^{32}P in the area corresponding to the electrophoretic position of reductase kinase.

monomeric molecular weight by $NaDodSO_4$ gel electrophoresis and the oligomeric molecular weight by column chromatography (Sepharose 6B) were 58,000 (±5000) and 380,000, respectively. In contrast to solubilized or partially purified reductase kinase, no enhancement of inactivation of HMG-CoA reductase or increase in phosphorylation of reductase was observed with purified reductase kinase and cAMP.

In order to demonstrate specific phosphorylation of reductase kinase, purified reductase kinase was incubated with reductase kinase kinase and [γ-^{32}P]ATP plus Mg^{2+} for 1 hr at 37°C. Analysis of the incubation mixture by polyacrylamide gel electrophoresis revealed that all protein-bound ^{32}P radioactivity (>95%) was present in the position corresponding to the protein band of purified reductase kinase (Fig. 5). Dephosphorylation of ^{32}P-labeled reductase kinase with phosphoprotein phosphatase was associated with significant loss (70%) of radioactivity in the protein band. Incubation of reductase kinase without reductase kinase kinase resulted in no ^{32}P incorporation.

To demonstrate that reductase kinase activity was modulated by covalent modification *in vivo*, rats (250 g) were injected intraperitonially with carrier-free $^{32}PO_4$ (1.0 mCi; 1 Ci = 3.7 × 10^{10} becquerels). Rats were sacrificed 30 min after injection and the reductase kinase was solubilized from liver microsomes

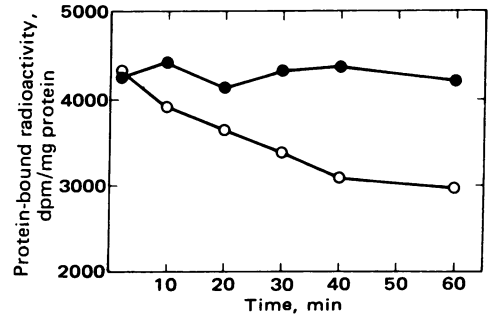

FIG. 6. Dephosphorylation of reductase kinase labeled *in vivo* with ^{32}P. Solubilized reductase kinase was isolated from hepatic microsomes of rats injected with 1 mCi of carrier-free ^{32}P. Solubilized reductase kinase (680 μg; 4500 dpm/mg of protein) was incubated with phosphoprotein phosphatase (800 μg) with (●) or without (○) NaF at 37°C. At the end of the 60-min incubation, 25 mM NaF was added and reductase kinase activity was determined. Reductase kinase inhibited the enzymic activity of HMG-CoA reductase by 55% at 0 min; however, after the 60-min incubation with phosphoprotein phosphatase the inhibition by reductase kinase decreased to 4% (±5%) of control.

(≈4500 dpm/mg of protein). Solubilized reductase kinase inactivated microsomal HMG-CoA reductase (55% of control) and phosphorylated histone 2A (68% > control). Incubation of solubilized reductase kinase with phosphoprotein phosphatase was associated with a time-dependent release of protein-bound ^{32}P radioactivity that was blocked by NaF (Fig. 6) and a decrease in the capacity of solubilized reductase kinase to phosphorylate histone 2A (17% > control) or inactivate HMG-CoA reductase (4% of control).

DISCUSSION

It is well recognized that the hepatic regulation of HMG-CoA reductase and cholesterogenesis is under complex control (8–10). Our laboratory has recently demonstrated the existence of HMG-CoA reductase in interconvertible active and inactive forms (1). The interconversion of the active and inactive forms of HMG-CoA reductase is achieved by a phosphorylation–dephosphorylation reaction sequence, which provides a mechanism for the rapid short-term regulation of the pathway for cholesterol biosynthesis. We have also recently shown that the enzymic activity of solubilized and purified HMG-CoA reductase from avian liver is modulated noncovalently by various effectors including acetyl CoA, acetoacetyl CoA, and 3-hydroxy-3-methylglutaric acid (11). The present studies provide evidence that reductase kinase, the enzyme that catalyzes the phosphorylation and inactivation of HMG-CoA reductase, also exists in active and inactive forms. Reductase kinase purified from hepatic microsomes catalyzed the phosphorylation and inactivation of HMG-CoA reductase.

The enzymic activity of reductase kinase was significantly decreased after incubation with phosphoprotein phosphatase. Activity was restored by a cAMP-insensitive protein kinase, reductase kinase kinase, present in microsomes and cytosol. In the presence of [γ-^{32}P]ATP plus Mg^{2+}, parallel increase in protein-bound ^{32}P radioactivity and in reductase kinase enzymic activity were observed. Incubation of *in vitro* or *in vivo* ^{32}P-labeled reductase kinase with phosphoprotein phosphatase resulted in a time-dependent release of protein-bound ^{32}P radioactivity and decrease in reductase kinase enzymic activity. Incubation of purified reductase kinase with [γ-^{32}P]ATP plus Mg^{2+} and reductase kinase kinase followed by polyacrylamide gel electrophoresis confirmed that virtually all radioactivity and enzymic activity were associated with the single electrophoretic band of purified reductase kinase.

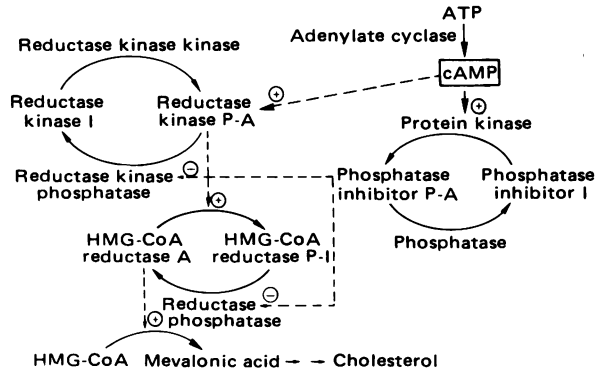

FIG. 7. Schematic representation of the modulation of enzymic activity of hepatic reductase kinase and HMG-CoA reductase. The active, inactive, and phosphorylated forms of the enzymes are designated A, I, and P, respectively. The enzymically active form of HMG-CoA reductase is dephosphorylated, whereas the active form of reductase kinase and phosphatase inhibitor protein is phosphorylated. cAMP would be expected to modulate the bicyclic cascade system by increasing the activity of the cAMP-dependent reductase kinase and to inhibit reductase kinase and HMG-CoA reductase phosphatases by increasing the activity of the phosphatase inhibitor protein.

Based on the combined results presented in this report we conclude that the enzymic activity of reductase kinase, like that of HMG-CoA reductase, is modulated by a phosphorylation–dephosphorylation reaction sequence.

The enzymic activity of solubilized and partially purified reductase kinase activity was increased by cAMP. The catalytic activity of purified reductase kinase utilized in these studies, however, was not modulated by cAMP. These results have been interpreted to indicate that cAMP-dependent and -independent reductase kinases are present in the microsomes.

Our current concept of the regulation of the enzymic activity of HMG-CoA reductase is depicted in Fig. 7. Regulation is by a bicyclic cascade with the activities of HMG-CoA reductase and reductase kinase regulated by phosphorylation–dephosphorylation. HMG-CoA reductase is enzymically active when dephosphorylated, whereas reductase kinase is enzymically active when phosphorylated. This bicyclic cascade system should be sensitive to endocrine signals (e.g., glucagon, insulin, etc.) and other metabolic effectors. Several effectors may regulate cholesterogenesis by altering the concentration of the enzymically active forms of the two enzymes. The *in vitro* inhibitory effect of cAMP on HMG-CoA reductase activity (12) may result from an increase in the activity of the cAMP-sensitive reductase kinase. The mechanism by which insulin increases and glucagon decreases the activity of HMG-CoA reductase *in vivo* (13) remains to be established. Based on our results, hormonally induced changes in cellular cAMP would be expected to alter the activity of reductase kinase and thereby regulate the enzymic activity of HMG-CoA reductase. In addition, changes in cellular concentration of cAMP may alter the activity of the phosphoprotein phosphatase inhibitor protein (14, 15), thereby influencing the dephosphorylation of both reductase kinase and HMG-CoA reductase. Phosphorylase phosphatase inhibitor protein which is capable of being phosphorylated (active form) by a cAMP-dependent protein kinase has been shown to be present in dog liver (14) and rabbit skeletal muscle (15). It is also clear from various studies that the administration of insulin is associated with dephosphorylation of many key hepatic interconvertible enzymes (16–19). Khandelwal *et al.* (19) have demonstrated that the hepatic activities of phosphorylase a and b, phosphorylase kinase, and glycogen synthase (I form) were significantly decreased in diabetic rats. The enzyme levels were restored to control values by insulin therapy. Insulin treatment was also associated with a concomitant increase in phosphoprotein phosphatase activity and a decrease in the heat-stable phosphatase inhibitor activity. Conversely, glucagon would be expected to restore the activity of inhibitor protein through a cAMP-dependent protein kinase (phosphorylation).

In the present studies, phosphorylase phosphatase enzyme has been utilized for both HMG-CoA reductase and reductase kinase dephosphorylation reactions. Therefore, it is likely that the regulation of phosphatase activity, as described above, is involved in the modulation of both HMG-CoA reductase and reductase kinase activities. Additional studies will be required to delineate the multifaceted control of the enzymic pathway for cholesterol biosynthesis.

We wish to express our appreciation to Dr. Kuo-Ping Huang, Section of Developmental Enzymology, National Institutes of Health, for many helpful discussions related to this research.

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