Regulation of vertebrate liver HMG-CoA reductase via reversible modulation of its catalytic activity

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Abstract We have investigated the comparative biochemistry of in vitro regulation of HMG-CoA reductase (EC 1.1.1.34) in microsomal preparations from the livers of nine vertebrates. In all instances, reductase activity was rapidly and profoundly decreased by addition of MgATP. Reductase activities were restored to near or above initial levels after removal of MgATP and incubation with a crude, low molecular weight phosphatase preparation from rat liver cytosol. Restoration of reductase activity was inhibited both by NaF and by pyrophosphate, known inhibitors of phosphoprotein phosphatase activity. Liver cytosol of species other than the rat exhibits reductase phosphatase activity. The converter enzymes that catalyze modulation of HMG-CoA reductase activity (reductase kinase and reductase phosphatase) thus appear to be ubiquitous in vertebrate liver. Interconversion in vitro of active and inactive forms of reductase probably is general for vertebrate liver also. The majority of the reductase present in vertebrate liver may be present in a catalytically inactive or latent form in vivo. Under the experimental conditions used, the fraction present in the active form is, for a given species, quite constant. Species to species, from 20–45% of the reductase appears to be present in the active form.—**Hunter, C. F., and V. W. Rodwell.** Regulation of vertebrate liver HMG-CoA reductase via reversible modulation of its catalytic activity. *J. Lipid Res.* 1980. **21:** 399–405.

Supplementary key words phosphoproteins · protein phosphatase · protein kinase · cholesterogenesis · reductase kinase · reductase kinase kinase · reductase phosphatase

The reaction catalyzed by HMG-CoA reductase is rate-limiting for biosynthesis of isopentenyl pyrophosphate, and hence for diverse polyisoprenoids, including cholesterol (1). In primates, rodents, and probably all vertebrates, carbon flow from acetyl-CoA to isopentenyl pyrophosphate is stringently regulated via control of the rate of synthesis of reductase protein (2).

HMG-CoA reductase is also regulated in vitro by reversible inactivation–reactivation of its catalytic activity (**Fig. 1**). In vitro inactivation of both microsomal (3–19) and solubilized (6, 7, 17) reductase by MgATP, catalyzed by reductase kinase (8, 9, 13), is accompanied by incorporation of ^{32}P from [γ-^{32}P]ATP into protein precipitated by antibody to reductase protein (11, 19) and into homogeneous reductase monomer (19). Reactivation of MgATP-inactivated reductase is catalyzed by a variety of broad specificity phosphoprotein phosphatases from liver and other sources (6, 8–12, 15, 17–19). Like reductase, reductase kinase is also an interconvertible enzyme. Its activity is stimulated by MgATP and is decreased by addition of phosphatase preparations (9, 13, 18). Activation of reductase kinase (9, 13, 18) by phosphorylation by the γ-phosphate of ATP (13) is catalyzed by a third converter protein, reductase kinase kinase (9, 13, 18).

To shed light on the possible physiological significance of modulation of reductase activity by phosphorylation–dephosphorylation, we asked whether this was general for vertebrate reductases.

MATERIALS AND METHODS

Chemicals

Chemicals from commercial sources included 3-hydroxy-3-methyl[3-^{14}C]glutaric acid, 54.9 Ci/mol, and DL-[5-^{3}H]mevalonic acid, DBED salt, 6.7 Ci/mmol (New England Nuclear); 3-hydroxy-3-methylglutaric acid and *N,N'*-dicyclohexylcarbodiimide (Schwarz/Mann); coenzyme A, trilithium salt (Boehringer); ATP and bovine serum albumin (Sigma); dithiothreitol (Calbiochem); NaF and EDTA (J. T. Baker).

R,S-[3-^{14}C]HMG-CoA was prepared essentially as described by Goldfarb and Pitot (20). Low specific activity HMG-CoA prepared as described by Nordstrom, Rodwell, and Mitschelen (6) was diluted to a specific activity of 2 cpm/pmol for use. For preparation of high specific activity material (6.9 cpm/pmol), the order of addition of reactants in the preparation of HMG-CoA was reversed (CoA was added to a solution of HMG-anhydride). Both preparations were

Abbreviation: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

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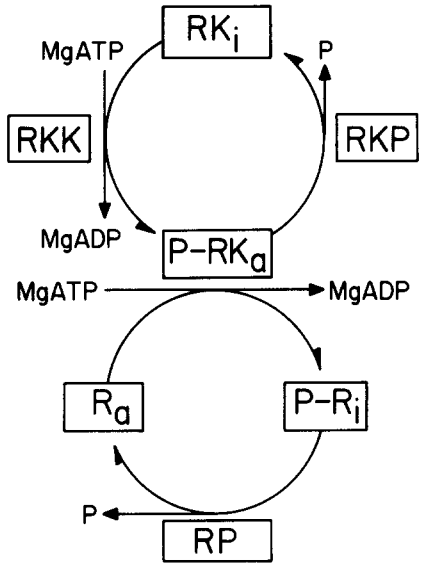

Fig. 1. Known cofactors and converter proteins that participate in reversible phosphorylation–dephosphorylation of HMG-CoA reductase. Enzymes, enclosed in squares, are: R = HMG-CoA reductase, RK = reductase kinase, RKK = reductase kinase kinase, RP = reductase phosphatase, and RKP = reductase kinase phosphatase. The identity/nonidentity of RP and RKP has not been established. The interconvertible proteins R and RK exist in phosphorylated (P-R, P-RK) and dephospho forms (R, RK). The subscripts indicate their catalytically active (a) and inactive (i) forms. The diagram incorporates data from many laboratories (3–20).

purified by descending paper chromatography prior to use (6).

Buffered solutions

PEDK contained 50 mM K_xPO_4 (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol (added just prior to use) and 70 mM KCl. TEDK contained 40 mM Tris–HCl (pH 7.5), 1.0 mM EDTA, 10 mM dithiothreitol (added just prior to use), and 70 mM KCl. TEDKF contained, in addition, 50 mM NaF. ATP solutions were adjusted to pH 7.5 with KOH.

Reductase phosphatase

An approximately ten-fold purified preparation of low molecular weight rat liver cytosol reductase phosphatase (6), 12–15 mg per ml in PEDK, was stored in liquid N_2. Under these conditions, phosphatase activity is retained for at least 30 months.

Animals

Female Wistar strain rats (150–200 g) from our department colony were used. They were fed commercial lab chow and water ad libitum and housed in a windowless room, dark from 1700–0300 hr and illuminated from 0300–1700 hr. The animals were killed at the time of maximal reductase activity (0900 hr). All other animals were killed between 0900–1100 hr. Male and female DBA/2 strain mice (a gift of Dr. Edward Golub), Mongolian gerbils (a gift of Dr. Fred Regnier), an albino hamster (Plantenga Pet Palace, Lafayette, IN), male New Zealand white rabbits (Buckles Feed Depot, Lafayette, IN) and a male Berkshire pig (Purdue Univ. Animal Sciences Center) were housed in a room illuminated from 0800 to 2000 hr and fed pelleted lab chow and water ad libitum. Animals killed immediately after delivery included mature white hens (Purdue Univ. Poultry Farm), female bullfrogs (Mogul-Ed, Oshkosh, WI), and a black bullhead catfish in water at 18°C (a gift of Dr. Ann Spacie).

Liver microsomal preparations

Fresh liver tissue, 2–10 g, was excised into chilled buffer (TEDK or TEDKF), weighed, passed through a chilled Harvard tissue press, mixed with 1.5 ml of TEDK or TEDKF per g of wet tissue, and homogenized with 15 strokes of a Dounce homogenizer pestle. Subsequent operations were at 4°C. The homogenate was centrifuged for 5 min at 1,000 *g*, then, without stopping the centrifuge, for 15 min at 12,000 *g*. The pellet was discarded. The decanted supernatant liquid was centrifuged for 90 min at 105,000 *g*. The supernatant liquid was discarded. The pellet, suspended in 1.0–2.5 ml of TEDK or TEDKF by gentle homogenization with a motor-driven Potter-Elvehjem pestle, was used directly as a source of HMG-CoA reductase.

Protein determination

Protein was measured by the method of Bradford (21) using bovine serum albumin as standard.

HMG-CoA reductase activity

HMG-CoA reductase activity was assayed essentially as described by Shapiro et al. (4). Samples to be assayed, 50 μl, were mixed with 25 μl of cofactor-substrate solution that contained 2.25 μmol of glucose-6-phosphate, 0.23 μmol of $NADP^+$, 0.15 IU of glucose-6-phosphate dehydrogenase, 1.8 μmol of EDTA, 28 nmol (1.72×10^5 cpm) of *R,S*-[3-^{14}C]HMG-CoA, 1.3×10^4 dpm of *R,S*-[5-^{3}H]mevalonate as an internal standard, and incubated at 37°C. After 30 min, the reaction was stopped by adding 10 μl of 6N HCl. Samples were incubated at 37°C for a further 15 min to convert mevalonate to mevalonolactone. Samples were then chromatographed as described by Shapiro et al. (4). Radioactive mevalonolactone was removed and counted in 4.0 ml of scintillation fluid (21 g of 2-(4′-t-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole, 240 g of naphthalene, 1200 ml of ethylene glycol and 1800 ml of toluene). We express reductase activity in picounits (pU), the picomoles of mevalonate formed per minute. Specific activities are given as pU per mg protein.

Determination of the fraction of HMG-CoA reductase present in the active form

Inclusion of 50 mM NaF at all stages during isolation and assay of microsomal preparations severely inhibits conversion of reductase to the active form (6). To estimate the fraction of HMG-CoA reductase present in an active form, we therefore isolated and assayed microsomal preparations in TEDKF. To determine the total quantity of reductase present, a parallel preparation from the same liver but in TEDK was assayed for reductase activity both directly and after treatment with reductase phosphatase. From these data, we calculate the ratio R_A/R_T, a dimensionless variable that expresses the fraction of HMG-CoA reductase present in the active form.

RESULTS

Characterization of the reaction product as mevalonic acid

To establish that microsomal preparations from all nine vertebrate livers studied catalyzed the HMG-CoA reductase reaction, and to validate the conditions used to assay reductase activity, we isolated the material that migrated in the mevalonolactone region in the standard (benzene–acetone) solvent system and rechromatographed it in additional solvents. Inasmuch as incubations contained authentic [5-^{3}H]mevalonolactone, our criterion that the ^{14}C-labeled product counted in the standard assay was indeed mevalonolactone was coincidence in the ^{3}H/^{14}C ratio throughout the peak region of the chromatograms. **Fig. 2** depicts typical data for four chromatographic systems and four vertebrates. From data of this type, we established that, for all nine vertebrates, the product formed enzymically from HMG-CoA and counted in the standard assay was indeed >95% mevalonolactone.

Kinetics of inactivation, reactivation, and subsequent inactivation of microsomal HMG-CoA reductase in nine species of vertebrates

We asked whether the hepatic reductase of animals other than the rat could be inactivated by incubation with MgATP, and if so, whether activity could be restored by treatment with reductase phosphatase (6). **Fig. 3** shows kinetic data for three animals having high, intermediate, and low hepatic reductase activity. Comparable data were obtained for the remaining six species. **Fig. 4** depicts, in abbreviated form, the data for all nine vertebrates. In all instances, incubation of microsomal preparations at 37°C increased reductase activity. Following the addition of MgATP,

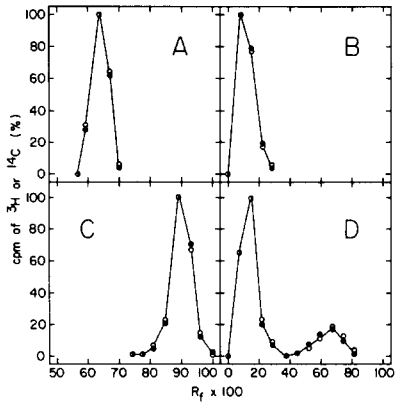

Fig. 2. Characterization of the reaction product. For each of the nine vertebrates studied, the product formed when microsomes were incubated with [3-^{14}C]HMG-CoA was characterized by thin-layer chromatography. Eight deproteinized reaction mixtures were applied to eight channels (each 2.5 × 17.5 cm) of an activated Silica gel G sheet and chromatographed in benzene acetone 1:1 (v/v) (4). The top half of one channel was sectioned at 0.5 cm intervals and the ^{14}C and ^{3}H in each channel was determined (A). The region $R_f \times 100 = 50–75$ was scraped from the remaining seven channels, pooled, and extracted with acetone or methanol. The extract was then chromatographed under the following conditions: after treatment with 0.1 M NaOH (15 min; 37°C) to convert mevalonolactone to mevalonic acid, on cellulose in 1-butanol–NH_4OH–water 20:1:1 (v/v), (B); on cellulose as mevalonolactone in isobutyric acid–NH_4OH–water 66:1:33 (v/v), (C); or after treatment with 15 N NH_4OH (15 min; 37°C) to convert mevalonolactone to a mixture of mevalonic acid ($R_f \times 100 = 15$) and mevalonic acid amide ($R_f \times 100 = 65$), on cellulose in the same solvent as in B, above, (D). Each chromatogram was sectioned at 1.0 cm intervals and ^{14}C and ^{3}H were measured. Symbols are: ^{3}H from the [5-^{3}H]mevalonolactone internal standard (●) and ^{14}C from the reaction product (○). Representative data for the pig (A), catfish (B), rat (C), and rabbit (D) are expressed as the fraction of the peak ^{3}H or ^{14}C present at a particular position on the chromatogram.

reductase activity decreased precipitously, but was restored close to or above initial levels after removal of MgATP by high speed centrifugation and treatment with reductase phosphatase. Subsequent addition of MgATP in all cases resulted in a second precipitous decrease in reductase activity. While quantitative differences are apparent, the reductases of all species respond in a manner similar to that of rat liver. This is further shown in **Table 1,** where treatment with reductase phosphatase increased the activity of MgATP-inactivated reductases from 3.8- to 12.5-fold.

Requirements for inactivation and reactivation of reductase

Inactivation of hepatic reductase from all nine vertebrate livers examined requires both Mg^{2+} and ATP (**Fig. 5**). The activity of MgATP-inactivated hepatic reductase from all nine vertebrates is unaffected by addition of serum albumin, but is restored close to or above initial levels by incubation with reductase phosphatase (**Fig. 6**).

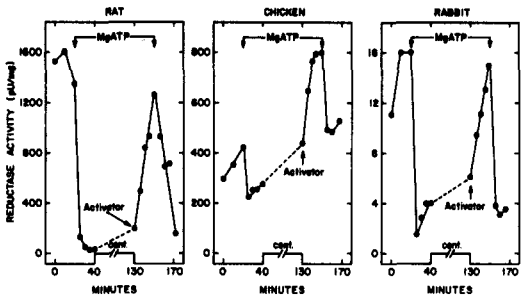

Fig. 3. Kinetics of inactivation, reactivation, and subsequent inactivation of microsomal HMG-CoA reductase. Microsomes suspended in TEDKF were incubated at 37°C. Portions were removed for assay of reductase activity at 0, 10, and 19 min. At 20 min, solutions were made 4.0 mM in $MgCl_2$ and 2.0 mM in ATP. Portions were removed for assay at 25, 30, and 39 min. At 40 min, the suspensions were transferred to centrifuge tubes, centrifuged (4°C; 105,000 *g*; 60 min) to separate microsomes from MgATP, and suspended in TEDK. A portion was removed for assay at 129 min. At 130 min, samples were mixed with 0.1 volume of reductase phosphatase, 10 mg per ml, and incubated at 37°C. Samples were removed for assay at 135, 140, 145 and 149 min. At 150 min, solutions were again made 4.0 mM in $MgCl_2$ and 2.0 mM in ATP and incubation at 37°C was continued. Samples were removed for assay at 155, 160, 166 and (for the rat data) 171 min. Data shown are for animals exhibiting high, intermediate, and low reductase activity. Identical experiments were conducted for all nine species of animal. These data are summarized in Fig. 4.

Inhibition of reactivation of reductase by pyrophosphate

Reactivation of MgATP-inactivated microsomal reductase of all nine vertebrates is blocked by NaF (**Table 2**) or by pyrophosphate (Fig. 6), an observation consistent with reactivation by a phosphoprotein phosphatase (22).

Activation state of reductase

As may be inferred from the rise in reductase activity that accompanies incubation of microsomal suspensions at 37°C (Figs. 3 and 4), HMG-CoA reductase as initially isolated appears to be present in a partially inactive state. To determine the fraction of reductase present in a latent form, livers from each animal were divided in two equal portions at the time the animal was killed, and homogenized in the presence or absence of NaF. The microsomes were then assayed for reductase activity. In addition, the microsomes in fluoride-free buffer were also incubated with reductase phosphatase to convert reductase to the fully active form prior to assay. The reductase specific activity of microsomes isolated and assayed in the presence of fluoride was in all instances far lower than the activity of suspensions isolated in the absence of fluoride, whether or not these had been treated with reductase phosphatase (Table 2). From these data, we calculated the ratio R_A/R_T, where R_A is the specific activity of reductase present in the active form (assayed in NaF) and R_T is the reductase specific activity measured in the absence of NaF with or without prior treatment with reductase phosphatase (Table 2).

By either method for calculation of R_A/R_T, the reductase of all nine species appears to be present in vivo predominantly in an inactive form. Although both methods of calculation yield similar conclusions, we favor calculating R_A/R_T from data obtained using reductase phosphatase, as this presumably insures complete conversion to the active form. Despite variations between preparations, the ratio R_A/R_T is essentially constant for a given species. R_A/R_T ratios vary from approximately 0.15 to 0.55 for the animals tested, suggesting that while all reductases may be present in a partially inactive state in vivo, there may be quantitative differences between species.

DISCUSSION

We have detected HMG-CoA reductase activity in hepatic microsomes from nine vertebrates that range in evolutionary complexity from fish to mammals and

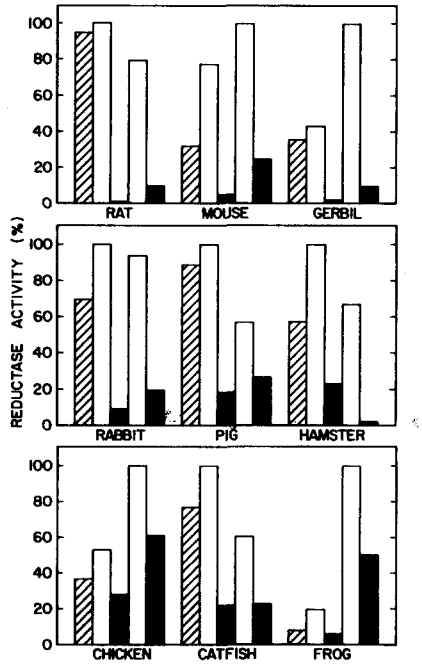

Fig. 4. Inactivation–reactivation–inactivation of HMG-CoA reductase from livers of nine vertebrates. The data were obtained from kinetic experiments of the type shown in Fig. 3. For each animal, the bar heights represent, from left to right, the HMG-CoA reductase activity at 0 min, 19 min (before MgATP), 39 min (after MgATP), 149 min (after reductase phosphatase), and 166 min (after a second addition of MgATP). To permit representation of divergent activities, data are normalized to maximal reductase activity for each animal. Absolute reductase activities corresponding to 100% were: 1,900 (rat), 171 (mouse), 1,270 (gerbil), 16 (rabbit), 230 (pig), 30 (hamster), 808 (chicken), 147 (catfish), and 22 (frog) pU per mg protein.

TABLE 1. Reactivation by reductase phosphatase of MgATP-inactivated reductase from nine vertebrate livers

Animal	Specific Activity of HMG-CoA Reductase		Activation (Col. 2/Col. 1)
	After MgATP	After Reductase Phosphatase	
	pU/mg		*-fold*
Rat	86	504	5.9
Mouse	100	792	7.9
Gerbil	53	313	5.9
Rabbit	3.2	16	5.0
Pig	17	64	3.8
Hamster	13	133	10.2
Chicken	39	210	5.4
Catfish	46	393	8.5
Frog	2.0	25	12.5

Hepatic microsomal pellets from the indicated animals were suspended in TEDKF, made 4.0 mM in $MgCl_2$ and 2.0 mM in ATP, incubated at 37°C for 20 min, and then transferred to centrifuge tubes. The microsomal pellets were collected by centrifugation (4°C; 105,000 *g*; 60 min), suspended in TEDK, mixed with 0.1 volumes of reductase phosphatase, 10 mg per ml, and incubated at 37°C for 20 min. HMG-CoA reductase activity was then determined in the usual manner. Shown are the activities just prior to removal of ATP by centrifugation (after MgATP), and after incubation with reductase phosphatase at 37°C for 20 min.

have provided preliminary evidence that the assay conditions employed provide a valid estimate of reductase activity. Except for the rat, we did not, however, attempt to study reductase activity at the optimal time of day. Under these conditions, reductase activities ranged from 16 (rabbit) to 1,900 pU per mg of protein (rat).

Microsomal reductase activity of all species examined was inactivated on addition of MgATP and was restored to or above initial levels following removal of ATP and addition of reductase phosphatase. A second addition of MgATP again decreased reductase activity in all instances. Both Mg^{2+} and ATP are required for inactivation, and the reactivation process is blocked by either NaF or sodium pyrophosphate, both known inhibitors of phosphoprotein phosphatase activity (22). Since we detected no requirement for extramicrosomal proteins during inactivation of any microsomal reductase, we infer that reductase kinase and reductase kinase kinase are present and associated with the hepatic microsomes of all vertebrates.

In the absence of added inhibitors of phosphoprotein phosphatase activity, the activity of MgATP-inactivated reductases tended to rise, suggesting the presence in, or associated with, microsomes of an enzyme, presumably reductase phosphatase, that catalyzes dephosphorylation of HMG-CoA reductase. This latter inference was confirmed by detection of reductase phosphatase activity in the hepatic cytosol of several species when tested against MgATP-inactivated rat liver microsomal reductase.[2] Taken together, these observations suggest that reversible interconversion of HMG-CoA reductase between forms of widely differing activity is ubiquitous in vertebrate livers. These interconversions presumably involve cyclical phosphorylation–dephosphorylation of reductase protein (Fig. 1).

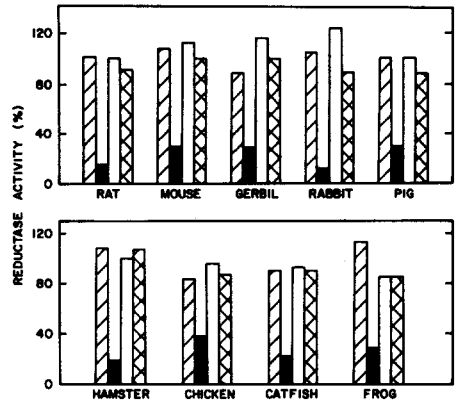

Fig. 5. Dependence of inactivation on both Mg^{2+} and ATP. Bar heights represent HMG-CoA reductase activity of TEDKF suspensions of hepatic microsomes. Bars represent, from left to right, addition of: TEDKF, 4.0 mM $MgCl_2$ + 2.0 mM ATP in TEDKF, 4.0 mM $MgCl_2$ in TEDKF, and 2.0 mM ATP + 2.0 mM EDTA in TEDKF. Data are normalized to permit representation of divergent specific activities.

Vertebrate liver reductase may exist primarily in a catalytically latent form in vivo. As much as 80%, and no less than 55% of reductase appears to be present in an inactive form when microsomes are isolated and assayed under conditions that severely inhibit reductase phosphatase activity.

[2] Brown, W. Unpublished observations.

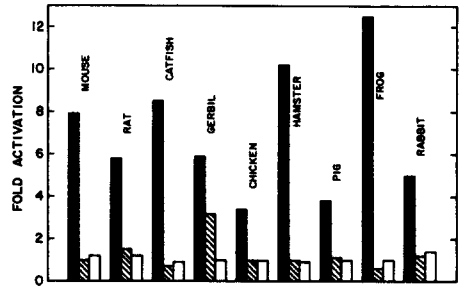

Fig. 6. Ability of reductase phosphatase, but not of serum albumin, to activate reductase from livers of all nine species of animal, and of pyrophosphate to block activation. Microsomal preparations previously inactivated by incubation with MgATP were mixed with reductase phosphatase, 1.0 mg per ml (solid bars), reductase phosphatase, 1.0 mg per ml, plus sodium pyrophosphate, pH 7.5, at a final concentration of 25 mM (hatched bars), or bovine serum albumin, 1.0 mg per ml (open bars). Data represent the fold stimulation of reductase activity observed following a 30 min incubation at 37°C.

TABLE 2. Activation state of hepatic HMG-CoA reductase

Animal	HMG-CoA Reductase Activity of Microsomes Suspended in TEDKF a	TEDK Less Phosphatase b	TEDK Plus Phosphatase c	R_A/R_T a/b	R_A/R_T a/c
	pmoles/min/mg				
Rat	115	368	500	0.31	0.23
	216	600	710	0.36	0.30
Gerbil	37	120	103	0.31	0.36
	202	575	513	0.35	0.39
Mouse	79	217	209	0.36	0.38
	45	120	133	0.38	0.34
Hamster	32	91	89	0.35	0.36
	44	138	150	0.32	0.29
Rabbit	14	24	33	0.58	0.42
	9	18	20	0.50	0.45
Pig	76	264	285	0.29	0.27
Chicken	270	697	813	0.39	0.33
Frog	0.54	1.5	4.0	0.36	0.14
Catfish	14	75	61	0.19	0.23

Liver tissue from the animals was divided in two approximately equal portions. Each portion was then homogenized immediately (Dounce pestle) in 10 mM dithiothreitol–300 mM sucrose in the presence or absence of 50 mM NaF. The microsomal fraction was then isolated. Microsomes isolated in the presence of NaF were suspended in TEDKF. Those isolated in the absence of NaF were suspended in TEDK. Portions of each suspension were then assayed for HMG-CoA reductase activity. The TEDK suspension was also assayed after 20 min activation with reductase phosphatase.

Modulation of reductase activity is, in quantitative terms, a more constant property of mammalian liver reductase than is catalytic activity per se. Species to species, reductase activity varied over 100-fold. Far less divergence was noted for phosphorylation–dephosphorylation of reductase activity. Species to species, the activation observed when MgATP-inactivated reductases were treated with reductase phosphatase varied about 3-fold, with most species exhibiting 4–8-fold activation. Similarly, the fraction of reductase apparently present in an inactive form in vivo was reproducible for a given animal, and varied less than 4-fold between species.

Retention of presumed phosphorylation sites on reductase and of kinases and phosphatases for these sites throughout the eons that have elapsed subsequent to the divergence of species might be fortuitous. The phosphorylation site might, for example, form a portion of the catalytic site. An alternative interpretation is that retention of these characteristics provides strong presumptive evidence for an important physiological role for phosphorylation–dephosphorylation of reductase that has resisted all attempted deletion or functional modification throughout vertebrate evolution.[3]

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