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IN VITRO PHOSPHORYLATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE: ANALYSIS OF ^{32}P-LABELED, INACTIVATED ENZYME[1]

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SUMMARY

Rat liver microsomal 3-hydroxy-3-methylglutaryl-CoA reductase was inactivated with Mg^{2+} and [γ-^{32}P]ATP, then solubilized and purified to homogeneity. The ^{32}P radioactivity was precipitated by antibody to homogeneous rat liver reductase and comigrated with nonprecipitated, homogeneous reductase on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Under nondenaturing conditions, ^{32}P radioactivity comigrated with reductase protein and activity on polyacrylamide gels. These results provide direct support for the concept that the enzyme is covalently phosphorylated during the *in vitro* incubation of microsomes with Mg^{2+} and ATP.

HMG-CoA[3] reductase [hydroxymethylglutaryl-CoA reductase (NADPH), EC 1.1.1.34], the key regulatory enzyme of hepatic cholesterogenesis (1) is interconvertible *in vitro* between forms of differing catalytic activity (2). Microsomal reductase is extensively inactivated *in vitro* in the presence of Mg^{2+}, ATP (2-5), and a protein present in microsomes and cytosol (2,3,5,6). Reductase activity is restored by treatment with a cytosolic activator protein (2,3,5,6), *E. coli* alkaline phosphatase (7), or potato acid phosphatase (8).

The phosphorylation of HMG-CoA reductase during incubation with Mg^{2+} and ATP is suggested by several lines of indirect evidence. Incorporation of ^{32}P from [γ-^{32}P]ATP into bulk microsomal protein accompanies a decrease in reductase activity (9,10). The release of ^{32}P from microsomes by partially purified phosphorylase phosphatase (9) or cytosol (10) is accompanied by restoration of reductase activity. Beg *et al.* (9) have shown the incorpora-

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[3]Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; TLC, thin-layer-chromatography.

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tion of ^{32}P into solubilized rat liver protein precipitated by antibody prepared against chicken liver reductase. However, their reported level of ^{32}P incorporation did not exclude the presence of contaminating phosphorylated proteins in the immunoprecipitate. Since direct evidence for phosphorylation of the pure reductase protein was lacking, it was this definitive question that we addressed. A preliminary indication of direct phosphorylation was reported by Nordstrom *et al.* (2).

To examine whether HMG-CoA reductase is covalently modified by phosphorylation, we inactivated microsomal reductase in the presence of sufficient Mg^{2+} and [γ-^{32}P]ATP to insure that, if labeling of reductase protein occurred, it could be detected, and then purified the enzyme to homogeneity. We determined: 1) whether the ^{32}P was associated with protein and reductase activity on polyacrylamide gels; 2) whether the ^{32}P was associated with homogeneous reductase monomer on SDS-polyacrylamide gels; and 3) whether the same was true after immunoprecipitation of homogeneous reductase by monospecific antibody.

MATERIALS AND METHODS

Chemicals - Chemicals from commercial sources included: [^{32}P]Orthophosphoric acid, carrier free (ICN Biochemicals); DL-[3-^{14}C]HMG-CoA and Formula 950A (New England Nuclear); D(-)3-phosphoglyceric acid (Na salt), glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), 3-phosphoglyceric acid kinase (EC 2.7.2.3) and sodium dodecyl sulfate (Sigma); cholestyramine resin (Questran) (Mead Johnson); Scintiverse (Fisher); Polygram cell 300 PEI cellulose (Brinkman). Other chemicals were from previously listed sources (2,11).

Reductase Activator - Reductase activator purified 10-fold from rat liver cytosol (2) was stored under liquid N_2 (stable for over 30 months).

[γ-^{32}P]*ATP* - [γ-^{32}P]ATP was prepared by a modification of the method of Glynn and Chapel (12). The exchange mixture contained, in 1.0 ml: 0.3 µmol Na_2HPO_4, 2.0 µmol 3-phosphoglycerate (pH 7.0), 6.0 µmol ATP (pH 7.0), 10 µg phosphoglycerate kinase, 600 µg glyceraldehyde 3-phosphate dehydrogenase, 0.1 µmol NAD, 10 µmol glutathione, 6.0 µmol $MgCl_2$, 50 µmol Tris·HCl (pH 8.0) and 10 mCi carrier-free H_3PO_4. The mixture, pH 7-8, was incubated at room temperature for 2-4 h, heated at 100° for 5 min, and then used for inactivation of reductase. ^{32}P in the ATP exchange reaction and in fractions from PEI-cellulose TLC of ATP was measured in 4 ml of Scintiverse. As judged by PEI-TLC in 1.0 M LiCl (13), the yield of [γ-^{32}P]ATP was about 8 mCi.

Buffered Solutions - Buffer A contained 25 mM K_xPO_4, pH 7.0, 1.0 mM dithiothreitol, 1.0 mM EDTA, and 10% (w/v) sucrose. Other buffers contained combinations of: 50 mM K_xPO_4, pH 7.5 (P); 40 mM Tris·HCl, pH 7.5 (T); 1.0 mM EDTA (E); 5.0 mM dithiothreitol (D); 50 mM NaF (F); 100 or 300 mM sucrose (S_{100}, S_{300}); 50, 1,000 or 1,250 mM KCl (K_{50}, $K_{1,000}$, $K_{1,250}$); 30% or 50% (w/v) glycerol (G_{30}, G_{50}).

Animals - Female, Wistar strain rats, housed in a windowless room darkened from 0300-1500 h, were fed water and 3% (w/w) cholestyramine in rodent chow (Purina) *ad libitum* for at least four days prior to use.

Protein - Protein was determined either by the method of Bradford (14) or by that of Sedmak and Grossberg (15), using bovine serum albumin as standard.

Assay of HMG-CoA Reductase Activity - Assays were conducted essentially as described by Shapiro *et al.* (4) in PED-K_{70}, using a final assay volume of 75 µl. Reductase activity is expressed as nmol or pmol mevalonate formed per min at 37°.

Apparent Reductase Activity (R_a) - R_a measures reductase activity prior to activation. Reductase, 5-10 µl, was mixed with 5 µl 1.0 M NaF, 10 µl bovine serum albumin (20 µg/µl) and PED-K_{70} to a volume of 50 µl and incubated at 37° for 30 min. Reductase activity was then assayed immediately.

Total Reductase Activity (R_t) - R_t measures reductase activity after full activation. Reductase, 5-10 µl, was mixed with 10 µl 80 mM $MgCl_2$ (to precipitate traces of F^-), 10 µl reductase activator (14 µg protein/µl) and PED-K_{70} to a volume of 50 µl and incubated at 37° for 30 min. Reductase activity was then assayed immediately.

Fraction of Reductase Present in Active Form (R_a/R_t) - The ratio of apparent to total activity (R_a/R_t) measures the fraction of reductase present in an active form. R_a/R_t is zero for completely inactive, and 1.0 for fully active reductase.

Preparation of Microsomes - Fifteen rats were killed at 0900 h by cervical dislocation. Unless otherwise noted, subsequent operations were at 0-4°. Livers (140 g) were excised into and rinsed in S_{300}, weighed, homogenized in 2.0 ml $S_{300}D$ per g liver (60 s, Waring blender), centrifuged (12,000 x g; 15 min), and the precipitate was discarded. The supernatant liquid was diluted to 390 ml with $S_{300}D$ and centrifuged (178,000 x g; 90 min). The supernatant liquid was discarded. The microsomal pellet was suspended in 116 ml TED-$S_{100}K_{50}F$. After removal of a portion for analysis, this was used immediately to prepare Inactivated Microsomes. All subsequent buffers contained 50 mM NaF to retard activation of reductase.

Preparation of Inactivated Microsomes - The microsomal resuspension was mixed with 0.48 mmol $MgCl_2$ and either 1.2 mmol (2.26 mCi) (Exp. 1), or 0.3 mmol (8.8 mCi) (Exp 2) [γ-^{32}P]ATP at a final volume of 120 ml, incubated at 25° for 30 min, and then diluted to 390 ml with TED-$S_{100}K_{50}F$ to yield Inactivated Microsomes. After removal of a portion for analysis, the Inactivated Microsomes were centrifuged (178,000 x g; 90 min). The supernatant liquid was discarded and the pellet was frozen overnight in liquid N_2.

Solubilization - Reductase was solubilized as described by Brown *et al.* (16). The pellets were thawed at 25°, suspended in 0.12 ml TED-$G_{50}F$ per g liver, incubated at 25° for 1 h, and diluted to 390 ml with TED-$K_{1,250}F$. The suspension was centrifuged (178,000 x g; 90 min), and the supernatant liquid was retained as the soluble extract.

Purification of Partially Inactivated HMG-CoA Reductase - HMG-CoA reductase was purified to homogeneity from the soluble extract by affinity chromatography as described elsewhere (11).

RESULTS AND DISCUSSION

Four experiments involving the inactivation of microsomal reductase from rat liver by Mg^{2+} and [γ-^{32}P]ATP were performed. Data from two are reported here.

Reductase in the microsomes was not fully active, as shown by the R_a/R_t (0.53 and 0.69, Table I). After incubation with Mg^{2+} and [γ-^{32}P]ATP, R_a/R_t decreased markedly in the two microsomal preparations, to 0.08 and 0.20 respectively (Table I). The R_a/R_t increased somewhat during solubilization and purification through heat treatment, but remained near that level (0.35 and

TABLE I

SUMMARY OF PURIFICATION OF [γ-^{32}P]ATP-INACTIVATED HMG-CoA REDUCTASE[a]

Fraction	Exp.[b]	R_a	R_t	R_a/R_t	Protein	Enrichment	Recovery	Specific[c] Activity
		nmol/min			mg	-fold	%	nmol/min/mg
Microsomes	1	3,031	5,719	0.53	2,772	(1.0)	(100)	2.1
	2	6,287	9,112	0.69	4,462	(1.0)	(100)	2.0
Inactivated Microsomes	1	387	4,836	0.08	2,925	0.83	85	1.7
	2	2,246	11,232	0.20	4,017	1.4	123	2.8
65^0 Fraction	1	1,081	3,089	0.35	12	39	46	139
	2	2,145	4,564	0.47	33.6	68	50	137
CoA Fraction	1	557	1,466	0.38	1.3	326	47	1,175
	2	1,080	1,895	0.57	2.0	468	21	935
Blue Dextran Fraction	1	331	870	0.38	0.16	1,529	15	5,505
	2	937	1,912	0.49	0.12	8,027	21	16,054

[a]Details of the purification method are described in reference 11.

[b]Experiment 1 - 10.0 mM [γ-^{32}P]ATP (1.9 Ci/mol); Experiment 2 - 2.5 mM [γ-^{32}P]ATP (28.8 Ci/mol).

[c]Specific activities are based on R_t.

0.47, respectively) throughout purification to the final (Blue Dextran) fraction (Table I). This apparent partial loss of inactivation may result from the action of a fluoride-insensitive phosphatase which is removed by the heat treatment step. Table II lists the inactivation conditions and the quantities of reductase and of ^{32}P present in the Blue Dextran fractions.

SDS-polyacrylamide gels of the Blue Dextran fraction stained for protein exhibited a single band which migrated with the R_f of reductase monomer (M_r 52,000) (11, 17, 18 and Fig. 1A).

TABLE II

INCORPORATION OF ^{32}P RADIOACTIVITY INTO PURIFIED HMG-CoA REDUCTASE

Exp.	[γ-^{32}P]ATP		Blue Dextran fraction	
	Concentration during Inactivation	Specific Activity	Quantity of Tetramer	Total ^{32}P Present
	mM	Ci/mol	pmol	cpm
1	10.0	1.9	769	3,892
2	2.5	28.8	577	14,934

Data are for experiments 1 and 2 (Table I). The quantity of tetramer present was calculated from the amount of purified reductase protein and an assumed molecular weight of 208,000. ^{32}P cpm were calculated by counting 100-250 µl of Blue Dextran fraction in 10 ml Formula 950A fluor.

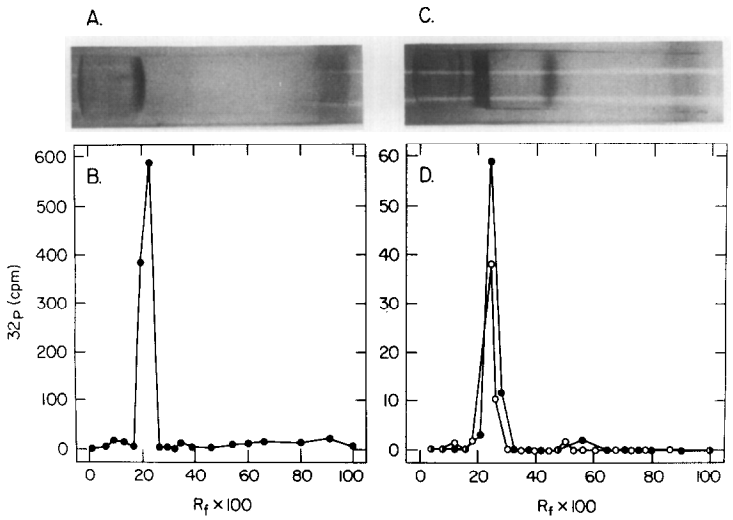

FIG. 1. Analysis of the Blue Dextran fraction by SDS-polyacrylamide gel electrophoresis. A and C - stained protein. A-Blue Dextran fraction, Exp. 2. C - Immunoprecipitate of Blue Dextran fraction, Exp. 1. B and D - ^{32}P in gel slices. LEFT: Exp. 2, (●) - ^{32}P profile in 26 µg of Blue Dextran fraction applied directly to gel. RIGHT: Exp. 1, (●) - ^{32}P profile for 10 µg of Blue Dextran fraction applied directly to gel, (○) - ^{32}P profile of 2/3 of a gel containing material precipitated from 10 µg of Blue Dextran fraction by antibody to homogeneous rat liver reductase (the remainder of the gel was stained for protein). Antiserum was obtained from a rabbit immunized with purified rat liver HMG-CoA reductase (11). The IgG fraction, partially purified by the method of Goding (20), was used in an immunoprecipitation experiment. Ten µg of pure, partially inactivated reductase (Blue Dextran fraction) was incubated for 30 min at 37°, then for 36 h at 4°, in the presence of 280 µg of IgG in phosphate-buffered saline (20 mM Na_xPO_4, pH 8.0; 130 mM NaCl). The immunoprecipitate was collected by centrifugation (Beckman Microfuge) and washed four times with 0.5 ml phosphate-buffered saline with thorough mixing and centrifugation between each wash. The immunoprecipitate was then processed for SDS-polyacrylamide gel electrophoresis by the method of Weber and Osborn (21), using 10% gels. Gels were sliced prior to counting in Formula 950A fluor.

^{32}P Radioactivity comigrated exclusively with the protein band on SDS-gel electrophoresis (Fig. 1, B and D). The majority of the ^{32}P was in this peak in both experiments (97% Exp. 1; 91% Exp. 2).

^{32}P Immunoprecipitated by antibody to homogeneous rat liver reductase comigrated with non-immunoprecipitated ^{32}P (Fig. 1, C and D), demonstrating that the ^{32}P-labeled protein in the Blue Dextran fraction was identical with that from immunoprecipitation. The recovery was nearly 100%. The gel in Fig. 1C shows the subunit band pattern of the immunoprecipitate. The major high molecular weight subunit of the IgG in the immune complex migrated with the same R_f as the purified enzyme, and thus the reductase monomer band appears broader and more heavily stained.

Polyacrylamide gel electrophoresis of the Blue Dextran fraction from Exp. 2 demonstrated that, under conditions that maintained reductase activity

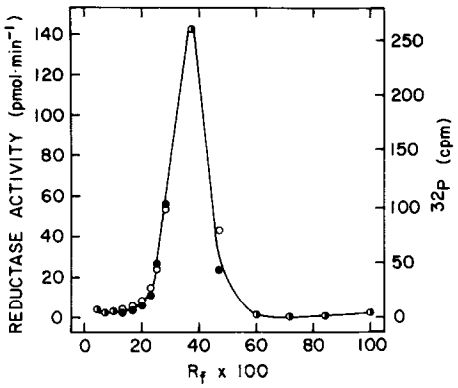

FIG. 2. Analysis of glycerol polyacrylamide gel electrophoresis of the Blue Dextran fraction from Experiment 2. (○) - reductase activity in gel slices, (●) - ^{32}P in gel slices. Polyacrylamide gel electrophoresis in 5% gels and the assay of HMG-CoA reductase activity in gel slices were performed as described (11). Two gels were run in parallel and one was stained for protein (not shown). The other was sliced approximately in half, longitudinally, prior to being sliced transversely at the indicated intervals. This provided pairs of half slices which were separated, one for enzyme assay and the other for ^{32}P determination as in Fig. 1.

(20% glycerol, 10 mM dithiothreitol), reductase activity was coincident with ^{32}P radioactivity and protein. Of the radioactivity in the gel, 91% comigrated with reductase activity and with protein (Fig. 2).

When microsomal reductase was incubated with Mg^{2+} and [γ-^{32}P]ATP, ^{32}P remained associated with reductase through solubilization and purification to homogeneity. Addition of reductase activator to homogeneous preparations restored reductase activity. ^{32}P was precipitated by antibody to homogeneous rat liver reductase, comigrated with reductase activity and protein during polyacrylamide gel electrophoresis, and comigrated with reductase monomer on SDS-polyacrylamide gel electrophoresis. Since ^{32}P remained associated with reductase through electrophoresis and SDS treatment, the bond between phosphate and reductase is probably covalent. Assuming that HMG-CoA reductase is a tetramer of approximately 200,000 molecular weight, preliminary calculations on the data from four experiments indicate that the apparent degree of phosphorylation of the enzyme was between 1 and 4 moles of phosphate per mole of tetramer.[4]

[4]If we assume that all phosphates incorporated into reductase have equivalent effects on activity, that the quantity of total phosphate (^{31}P plus ^{32}P) on reductase is inversely proportional to R_a/R_t, and that all phosphates on reductase are equally accessible to removal by reductase activator, we may calculate a preliminary stoichiometry from:

$$(F)\frac{\frac{(1/D)}{(A-B)}\quad\frac{(E)}{(1-C)}}{(1-B)} = \text{moles phosphate per mole of reductase}$$

We have shown unequivocally that HMG-CoA reductase is phosphorylated *in vitro* during inactivation by ATP. Reductase, thus, is one of several enzymes whose activity may be modulated by covalent modification. Phosphorylation/dephosphorylation of reductase represents a potential short-term mechanism for control of reductase activity whose physiological significance remains to be established. The relationship of stimulus and control mechanisms which may change phosphorylation state *in vivo* to reductase activity are under further investigation (19).

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where: A = R_a/R_t for unwashed microsomes (dimensionless); B = R_a/R_t for inactivated microsomes (dimensionless); C = R_a/R_t for the Blue Dextran fraction (dimensionless); D = specific activity of [γ-^{32}P]ATP (Ci x mol^{-1}); E = ^{32}P radioactivity in Blue Dextran fraction (Ci); and F = moles of reductase in Blue Dextran fraction.