Kono, T., Suzuki, K., Dansey, L. E., Robinson, F. W., & Blevins, T. L. (1981) *J. Biol. Chem. 256*, 6400–6407.
Kono, T., Robinson, F. W., Blevins, T. L., & Ezaki, O. (1982) *J. Biol. Chem. 257*, 10942–10947.
Resh, M. D. (1982a) *J. Biol. Chem. 257*, 6978–6986.
Resh, M. D. (1982b) *J. Biol. Chem. 257*, 11946–11952.
Resh, M. D. (1982c) Ph.D. Thesis, Harvard University, Cambridge, MA.
Resh, M. D., Nemenoff, R. A., & Guidotti, G. (1980) *J. Biol. Chem. 255*, 10938–10945.

3-Hydroxy-3-methylglutaryl-CoA Reductase: Solubilization in the Presence of Proteolytic Inhibitors, Partial Purification, and Reversible Phosphorylation–Dephosphorylation†

Peter J. Kennelly, Karl G. Brandt, and Victor W. Rodwell*

ABSTRACT: A growing body of evidence indicates that 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34, reductase) is degraded by proteolytic enzymes during solubilization by traditional freeze–thaw techniques. We have solubilized reductase in an active, stable form with nonionic detergents [Lubrol WX or poly(oxyethylene) ether type W-1]. Solubilization proceeded in high (>70%) yield in the presence of the proteolytic inhibitors leupeptin, phenylmethanesulfonyl fluoride, and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,-*N*′,*N*′-tetraacetic acid and was independent of prior freeze–thawing of the microsomes. We have purified detergent-solubilized reductase 40-fold in high yield by means of sucrose density gradient centrifugation and dye–ligand chromatography. Detergent-solubilized reductase is heat labile, unlike reductase solubilized by the freeze–thaw method. Detergent-solubilized reductase can be inactivated up to 90% by use of reductase kinase. This inactivation requires both adenosine 5′-triphosphate and adenosine 5′-diphosphate, as has been previously observed for both microsomal and freeze–thaw solubilized reductase. Inactivation is reversed by subsequent treatment with a phosphoprotein phosphatase.

3-Hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34, reductase[1]), the rate-limiting enzyme in mammalian polyisoprenoid biosynthesis (Rodwell et al., 1976), traditionally has been solubilized by procedures that subject rat liver microsomes to freeze–thawing (Brown et al., 1973; Heller & Gould, 1973). Reductase solubilized in this manner has been purified to homogeneity, and its properties have been studied in several laboratories [for a review, see Kleinsek et al. (1981)]. Ness et al. (1981) observed, however, that inclusion of the sulfhydryl protease inhibitor leupeptin or careful removal of lysosomes from the microsomal fraction prior to freeze–thawing blocked subsequent freeze–thaw solubilization. Moreover, addition of lysosomes to lysosome-depleted microsomes restored the susceptibility of reductase to solubilization by freeze–thawing. They therefore concluded that freeze–thaw solubilization relies upon cleavage of reductase by lysosomal proteases. This observation has been confirmed by Chin et al. (1982a). Using immunodetection techniques, they observed that the subunit relative molecular mass of reductase from UT-1 Chinese hamster ovary cells extracted in the presence of leupeptin was larger (62 000) than that observed when leupeptin was omitted (50 000–55 000). They later reported that inclusion of EGTA, in addition to leupeptin, yielded a reductase subunit M_r of 90 000, a relative molecular mass that matched that from immunoprecipitates of in vitro translation products of total UT-1 cellular polyadenylated RNA (Chin et al., 1982b). We have developed a method for solubilizing reductase in high yield in the presence of inhibitors of proteolysis without prior freeze–thawing of microsomes, have purified the reductase thus solubilized 40-fold, and have studied its properties.

Materials and Methods

Materials. Purchased materials included the following: Lubrol WX, poly(oxyethylene) ether type W-1, leupeptin, PMSF, EGTA, octyl glucoside, Amberlite XAD-2, and Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO); Quick Seal centrifuge tubes (Beckman Instruments, Palo Alto, CA); phenoxyacetylcellulose (Regis Chemical Co., Morton Grove, IL); Matrex Gel Green A and ultrafiltration membranes (Amicon Corp., Danvers, MA); Biobeads SM-2 (Bio-Rad Laboratories, Richmond, CA); fluorescamine (Roche Diagnostics, Nutley, NJ); deoxycholate (Difco Laboratories, Detroit, MI). [3-^{14}C]HMG-CoA was prepared as previously described at a specific activity of 1–2 cpm/pmol (Williamson & Rodwell, 1981). Other materials were from previously listed sources (Harwood & Rodwell, 1982; Nordstrom et al., 1977).

Enzymes. Low molecular weight phosphoprotein phosphatase was purified through the ammonium sulfate fractionation as described by Nordstrom et al. (1977). Reductase kinase was purified through the Blue Sepharose step (0.44 mg of protein/mL, 0.2 picounit of microsomal reductase inactivated min^{-1} mg^{-1}) as described by Harwood (1982). Freeze–thaw solubilized reductase was purified through the heat fraction as described by Rogers et al. (1980).

† From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907. *Received March 4, 1983.* Supported by Grant HL 19223 and by a National Science Foundation predoctoral fellowship (8166394) granted to P.J.K. Journal Paper No. 9367 from the Purdue University Agricultural Experiment Station.

[1] Abbreviations: reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; Lubrol, Lubrol WX; PEW-1, poly(oxyethylene) ether type W-1; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HMG, 3-hydroxy-3-methylglutaric acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; NADP$^+$, nicotinamide adenine dinucleotide phosphate; CoASH, coenzyme A sulfhydrylated; M_r, relative molecular mass.

0006-2960/83/0422-2784$01.50/0

Assays. Protein was assayed either by the fluorescamine method of Böhlen et al. (1973) or, where indicated, by optical density at 280 nm. Reductase activity was measured as described by Shapiro et al. (1974). One unit of reductase activity equals 1 mol of mevalonate formed min^{-1}. Reductase kinase activity was assayed as described by Harwood & Rodwell (1982).

Buffered Solutions. Buffer A contained 50 mM $K_xH_xPO_4$, 5.0 mM DTT, 70 mM KCl, 1.0 mM EDTA, 1.0 mM EGTA, and 1.0 mM PMSF. Buffer B consisted of 50 μM leupeptin in buffer A (pH 7.5). Buffer C consisted of 0.2% (v/v) PEW-1 in buffer A (pH 6.6). Buffer D contained 70 mM Tris (pH 7.7), 5.0 mM DTT, 1.0 mM EDTA, 250 mM sucrose, 200 mM KCl, and 50 mM NaF. Buffer E contained 50 mM $K_xH_xPO_4$ (pH 7.5), 5.0 mM DTT, 1.0 mM EDTA, 250 mM sucrose, and 250 mM KCl. Buffer F contained 25 mM Tris (pH 7.7), 2.5 mM DTT, 0.5 mM EDTA, 125 mM sucrose, and 60 mM $MgCl_2$. Buffer G consisted of 1% (v/v) glycerol, 1.0 mM sodium pyrophosphate, and 1.0 mM PMSF in buffer C. Lubrol or PEW-1 was added as 5% (v/v) stock solutions prepared by melting the detergents at 100 °C.

Preparation of Microsomes. Female Wistar-strain rats (150–200 g) were housed in a room darkened from 0300 to 1500 h for at least 3 weeks. For at least 4 days prior to sacrifice they were fed 3% (w/w) cholestyramine in powdered lab chow. Rats were killed at the diurnal peak (0900 h), and their livers were excised into chilled 300 mM sucrose (20 mL/liver). All subsequent operations were at 4 °C. Livers were washed twice with 300 mM sucrose (20 mL/liver), weighed, and homogenized in 5.0 mM DTT in 300 mM sucrose[2] (2 mL/g of liver tissue, Waring Blendor, 60 s). Alternatively, livers were passed through a Harvard tissue press into 5.0 mM DTT in 300 mM sucrose[2] (2 mL/g of liver tissue) and then homogenized by 10 strokes of a Dounce homogenizer. Liver homogenates were centrifuged (12000*g*, 15 min) and the pellet was discarded. The supernatant liquid was decanted and centrifuged (141000*g*, 60 min). The high-speed supernatant liquid was discarded. The pellet was resuspended in buffer B (20 mL/liver, glass–Teflon homogenizer) to give Unwashed Microsomes. Unwashed Microsomes were centrifuged (141000*g*, 60 min), the supernatant liquid was discarded, and the pellet (Washed Microsomes) was either used fresh or stored in liquid N_2.

Sucrose Density Gradients. Sucrose density gradients were prepared by placing 90 mL of buffer C that contained 300 mM KCl and 30% (w/v) sucrose in a 1.5 × 4 in. polyallomer Quick Seal tube and freeze–thawing once (Baxter-Gabbard, 1972).

Results

Leupeptin Blocks Solubilization of Reductase by Freeze–Thawing. We confirm the observation of Ness et al. (1981) that inclusion of leupeptin during the isolation and freeze–thawing of microsomes blocks solubilization of reductase by freeze–thawing. The effect was dependent upon the concentration of leupeptin. Complete (99%) blockage was achieved with 50 μM leupeptin. Leupeptin, 50 μM, was therefore included throughout all subsequent microsome preparations and detergent solubilizations.

Table 1: Efficiency of Detergent Solubilization of Reductase in the Presence of Inhibitors of Proteolysis[a]

detergent	reductase activity (nanounits/liver) Washed Microsomes	Soluble Extract	fraction solubilized (%)
PEW-1[b]	223	208	93
PEW-1[c]	264, 485	210, 331	80, 68
PEW-1[d]	357, 356	252, 279	71, 78
Lubrol[e]	353	267	76
Lubrol[d]	393, 105	258, 64	73, 60
Lubrol[f]	146	83	57
Lubrol[g]	97	80	83
Lubrol[h]	656, 220	470, 132	72, 60

[a] Microsomes were isolated and solubilizations were conducted in the presence of 50 μM leupeptin, 1.0 mM EGTA, and 1.0 mM PMSF as described in the text. KCl concentration, buffer identity, pH, method of tissue homogenization (Waring Blendor or Dounce homogenizer), and use of fresh or freeze–thawed microsomes were varied as indicated in the accompanying footnotes. The mean fraction of reductase solubilized by all variants of this procedure was 73%. [b] 70 mM KCl in 50 mM $K_xH_xPO_4$ (pH 6.6), Waring Blendor, fresh microsomes. [c] 70 mM KCl in 50 mM $K_xH_xPO_4$ (pH 7.5), Waring Blendor, fresh microsomes. [d] 70 mM KCl in 50 mM $K_xH_xPO_4$ (pH 7.5), Dounce homogenizer, fresh microsomes. [e] 15 mM $K_xH_xPO_4$ (pH 7.5), Dounce homogenizer, fresh microsomes. [f] 15 mM $K_xH_xPO_4$ (pH 7.5), Waring Blendor, freeze–thawed microsomes. [g] 70 mM KCl in 40 mM Tris (pH 7.5), Waring Blendor, freeze–thawed microsomes. [h] 25 mM KCl in 15 mM $K_xH_xPO_4$ (pH 7.5), Waring Blendor, freeze–thawed microsomes.

Reductase Can Be Solubilized with Nonionic Detergents without Freeze–Thawing. Reductase can be solubilized in high (>70%) yield in the presence of the proteolytic inhibitors leupeptin, EGTA, and PMSF when PEW-1 or Lubrol is used. All manipulations were at room temperature due to the low solubility of these detergents in the cold. Washed Microsomes were suspended in buffer B that contained 0.5% (v/v) Lubrol or PEW-1 (20 mL/liver, 10 strokes of a Dounce homogenizer). After 1 h, the homogenate was centrifuged (141000*g*, 60 min). The pellet was discarded. The supernatant liquid (Soluble Extract) contained at least 70% of the reductase activity and 30–50% of the protein of Washed Microsomes and represented 1.5–2.5-fold purification. Solubilization efficiency was not improved when the detergent concentration was increased from 0.5 to 2.0% (v/v). Solubilization was independent of whether the detergent used was Lubrol or PEW-1 (both consist of C16–C18 aliphatic alcohols attached to polyethoxy head groups), whether Tris or phosphate buffer was used, whether liver tissue was homogenized with a Waring Blendor or a Dounce homogenizer, and whether or not the Washed Microsomes were fresh or freeze–thawed (Table I). Raising the leupeptin concentration to 400 μM did not affect solubilization efficiency.

Partial Purification of Detergent-Solubilized Reductase. We used two schemes to partially purify reductase solubilized from microsomes with nonionic detergents. For method 1, Soluble Extract was chromatographed directly on a dye–ligand column of Matrex Gel Green A as detailed below. For method 2, Soluble Extract was fractionated by sucrose density gradient ultracentrifugation and then chromatographed on Matrex Gel Green A. Identical conditions were used in both methods for the Matrex Gel Green A column.

Sucrose Density Gradient Ultracentifugation. Soluble

[2] Proteolytic inhibitors were not included at this stage because existing evidence indicated that lysosomal rupture is a necessary prerequisite for the release of the proteases, which proteolytically solubilize and degrade reductase, and that such rupture must be induced by freeze–thawing or other stringent treatments (Ness et al., 1981). We have recently altered our procedure by including leupeptin (50 μM), EGTA (5.0 mM), and PMSF (1.0 mM) in the liver homogenization buffer. This has not affected the ability to solubilize reductase in high efficiency by the procedures listed below. Moreover, this modification does not alter the properties of the reductase thus solubilized from those reported herein (heat stability, sedimentation on sucrose density gradient ultracentrifugation, chromatography on Matrex Gel Green A, ability to be inactivated by reductase kinase and reactivated by low molecular weight phosphoprotein phosphatase).

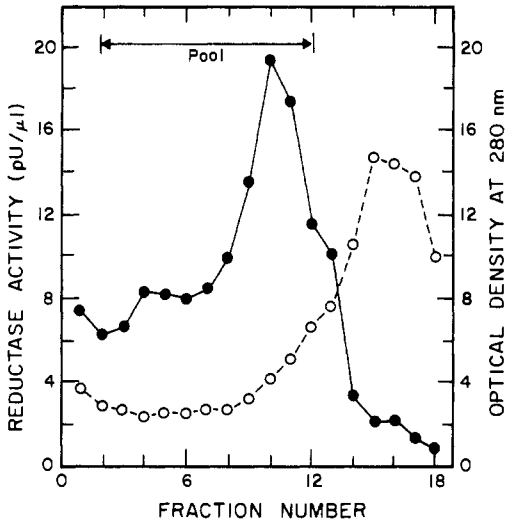

FIGURE 1: Sucrose density gradient centrifugation of detergent-solubilized reductase. Soluble Extract (2.2 nanounits/mg, 5.0 mg of protein/mL) was concentrated 8-fold by ultrafiltration. A portion, 9.0 mL, was loaded onto a sucrose density gradient, centrifuged (155000*g*, 12 h, 20 °C), and fractionated. Fractions, 5.7 mL, were then assayed for reductase activity (●) and for protein (○) and pooled as indicated. The Sucrose Pool contained 75% of the applied reductase activity purified 2.8-fold (6.3 nanounits/mg).

Extract was concentrated 5–10-fold (Amicon ultrafiltration cell, PM-10 membrane). Portions, 10 mL, were applied to sucrose density gradients containing 0.2% (v/v) PEW-1, centrifuged (20 °C, 155000*g*, 12–14 h), and then divided into 16–20 fractions. The Sucrose Pool is generally 2–4-fold purified over the Soluble Extract with 70–90% recovery of activity (Figure 1).

Matrex Gel Green A Chromatography. Soluble Extract (method 1) or Sucrose Pool (method 2) was applied to a column of Matrex Gel Green A (10 mL of resin/liver) equilibrated in buffer C. The column was washed with 3–5 column volumes of buffer C and then with 3–5 column volumes of 400 mM KCl in buffer C. Reductase was eluted with 5 column volumes of 1000 mM KCl in buffer C. Active fractions were pooled to give the Green A Fraction (Figure 2). This generally gave 3–10-fold purification with 70–100% recovery of activity. The overall purification by method 1 was 10–20-fold, and by method 2, 20–40-fold, over the Unwashed Microsomes. Typical overall recovery of activity from Unwashed Microsomes was 60–80% for method 1 and 50–65% for method 2.

Stability of Detergent-Solubilized Reductase. Reductase solubilized with either Lubrol or PEW-1 in the presence of proteolytic inhibitors was most stable at pH 6.6 (Figure 3) in 1 M KCl. All efforts to remove detergent from detergent-solubilized reductase (solvent extraction, acetone precipitation, exchange into nondetergent buffers via sucrose density gradient centrifugation or dye–ligand chromatography, adsorption of detergent onto Sephadex LH-20, Amberlite XAD-2, Biobeads SM-2, or phenoxyacetylcellulose) either inactivated detergent-solubilized reductase or dramatically reduced its stability. Addition of Triton X-100, octyl glucoside, or deoxycholate to detergent-solubilized reductase from which the Lubrol had been removed by sucrose density gradient centrifugation failed to restore stability. Addition of 30% glycerol, 1 mg/mL bovine serum albumin, or 0.1% (v/v) Lubrol enhanced stability; addition of 10 mM HMG, CoASH, or $NADP^+$ or of 15% (w/v) sucrose had no beneficial effect.

Heating detergent-solubilized reductase results in rapid loss of activity (Figure 4). By contrast, freeze–thaw-solubilized

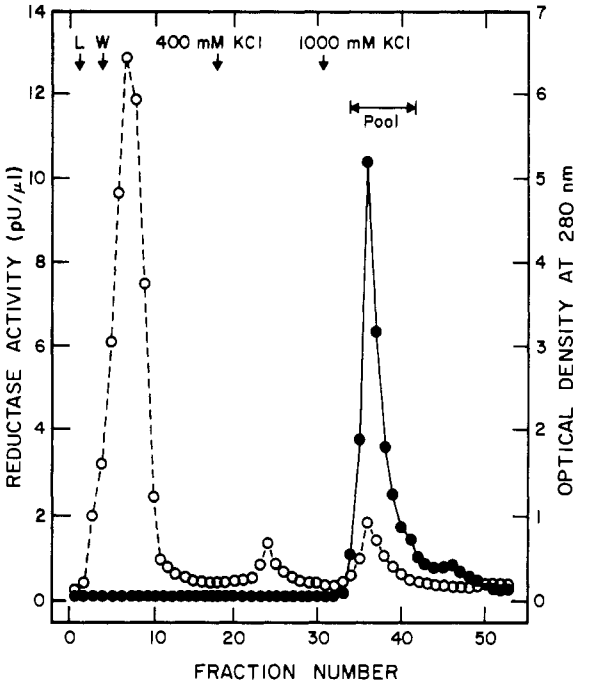

FIGURE 2: Chromatography of detergent-solubilized reductase on Matrex Gel Green A. Soluble Extract (21 mL, 285 nanounits, OD_{280} 12.0) was loaded (arrow L) onto a 2.5 × 8.0 cm column of Matrex Gel Green A equilibrated in buffer C. The column was then washed (arrow W) with 100 mL of buffer C and then with 90 mL of 400 mM KCl in buffer C. Reductase was then eluted with 150 mL of 1000 mM KCl in buffer C. Fractions, 7.1 mL, were assayed for reductase activity (●) and for protein (○) and pooled as indicated. The Green A Pool contained 95% of the applied reductase activity purified 10-fold.

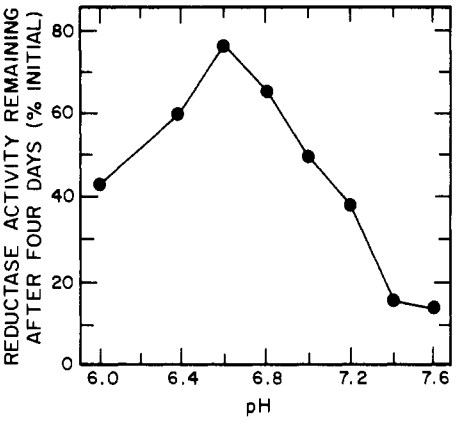

FIGURE 3: pH stability of detergent-solubilized reductase. Green A Fraction prepared by method 1 (4.0 nanounits/mg, 0.60 mg of protein/mL) titrated to the indicated pH values was incubated at 37 °C to accelerate denaturation. Portions were removed daily and assayed for reductase activity. Shown is the fraction of initial reductase activity remaining after 4 days.

reductase was stable at 65 °C, even in the presence of 0.25% (v/v) Lubrol.

Detergent-Solubilized Reductase Can Be Reversibly Inactivated with Reductase Kinase. When partially purified reductase kinase from rat liver cytosol is used, detergent-solubilized reductase can be inactivated up to 90% (Table II). Inactivation required both ATP and ADP (Table III) and was reversed by treatment with low molecular weight phosphoprotein phosphatase (Table II).

Discussion

We have solubilized reductase under conditions that avoid freeze–thawing of microsomal membranes in the presence of

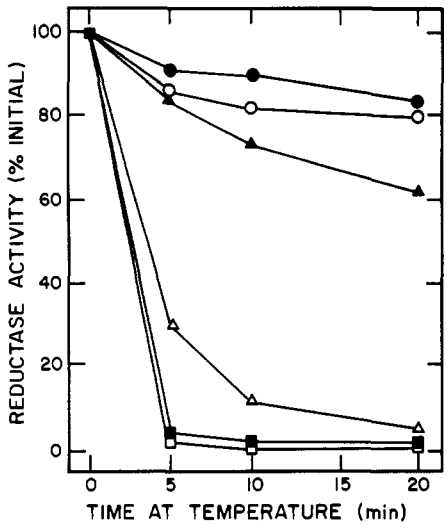

FIGURE 4: Heat stability of detergent-solubilized reductase. Green A Fraction prepared by method 1 (4.0 nanounits/mg, 0.60 mg of protein/mL) in polyethylene tubes was heated to 45 (●), 50 (○), 55 (▲), 60 (△), 65 (■), or 70 °C (□). At the indicated times, portions were removed, cooled to room temperature, centrifuged to remove denatured protein, and assayed for reductase activity.

Table II: Detergent-Solubilized Reductase Is Reversibly Inactivated by Reductase Kinase and Phosphoprotein Phosphatase[a]

additions to incubation 1		reductase activity (picounits)	
reductase kinase	ATP + ADP	without phosphoprotein phosphatase	with phosphoprotein phosphatase
−	−	71 ± 7	83 ± 3
−	+	70 ± 5	83 ± 2
+	−	71 ± 1	81 ± 2
+	+	7 ± 1	77 ± 2

[a] Detergent-solubilized reductase prepared by method 2 (10 nanounits/mg, 0.80 mg of protein/mL) was dialyzed (3 h, 25 °C) against 100 volumes of 2.0 mM DTT, 1.0 mM EDTA, 0.2% (v/v) PEW-1, and 250 mM KCl in 40 mM Tris (pH 7.5). Reductase kinase was dialyzed (3 h, 4 °C) against 50 volumes of buffer D to remove pyrophosphate and then concentrated 4-fold by ultrafiltration (Amicon UM-10 membrane, 1.55 mg of protein/mL). The experiment involved two successive incubations. Detergent-solubilized reductase, 10 μL, was mixed with (a) 22.5 μL of buffer D plus 100 mM NaF in 5 μL of buffer F, (b) 22.5 μL of buffer D plus 15 mM ATP, 15 mM ADP, and 100 mM NaF in 5 μL of buffer F, (c) 22.5 μL of reductase kinase plus 100 mM NaF in 5 μL of buffer F, and (d) 22.5 μL of reductase kinase plus 15 mM ATP, 15 mM ADP, and 100 mM NaF in 5 μL of buffer F. Incubation was for 30 min at 30 °C (incubation 1). Samples were next mixed with either 32.5 μL of buffer E or 32.5 μL of low molecular weight phosphoprotein phosphatase in buffer E and incubated for 30 min at 37 °C (incubation 2). Reductase activity was then determined (Harwood & Rodwell, 1982). Data are mean values for three determinations ± the standard error.

Table III: Inactivation of Detergent-Solubilized Reductase by Cytosolic Rat Liver Reductase Kinase Requires both ATP and ADP[a]

nucleotides present during incubation 1	reductase activity (picounits) with reductase kinase	reductase activity (picounits) without reductase kinase	inactivation (%)
none	90 ± 3	87 ± 4	−3
2 mM ADP	98 ± 4	99 ± 6	1
2 mM ATP	91 ± 4	92 ± 6	2
2 mM ATP + 2 mM ADP	75 ± 4	99 ± 2	24

[a] Dialyzed, detergent-solubilized reductase (see Table II), 12.5 μL, was mixed with either (a) 20 μL of buffer G plus 408 mM NaF in 5 μL of buffer F, (b) 20 μL of reductase kinase plus 408 mM NaF in 5 μL of buffer F, (c) 20 μL of buffer G plus 15 mM ADP and 408 mM NaF in 5 μL of buffer F, (d) 20 μL of reductase kinase plus 15 mM ADP and 408 mM NaF in 5 μL of buffer F, (e) 20 μL of buffer G plus 15 mM ATP and 408 mM NaF in 5 μL of buffer F, (f) 20 μL of reductase kinase plus 15 mM ATP and 408 mM NaF in 5 μL of buffer F, (g) 20 μL of buffer G plus 15 mM ATP, 15 mM ADP, and 408 mM NaF in 5 μL of buffer F, or (h) 20 μL of reductase kinase plus 15 mM ATP, 15 mM ADP, and 408 mM NaF in 5 μL of buffer F. Samples were then incubated for 30 min at 30 °C (incubation 1). Reductase activity was then determined (Harwood & Rodwell, 1982). Data are mean values of three determinations ± the standard error. Percent inactivation refers to activity determined following incubation with reductase kinase relative to that measured following incubation with buffer.

inhibitors of proteolysis (leupeptin, EGTA, PMSF), one of which (leupeptin) blocks solubilization by conventional freeze–thaw techniques. The procedure is efficient, adaptable to scaleup, and yields stable, active reductase. We have achieved partial purification of reductase solubilized in this manner by preparative sucrose density gradient ultracentrifugation and dye–ligand chromatography.

The properties of detergent-solubilized reductase differ significantly from those of the proteolytically degraded form obtained by freeze–thaw techniques. Unlike freeze–thaw-solubilized reductase, detergent-solubilized reductase is unstable in the absence of detergent. The two forms of reductase differ dramatically in their temperature stability properties. Freeze–thaw-solubilized reductase is stable to heating; detergent-solubilized reductase rapidly loses activity when heated. This probably reflects a difference in the proteins since addition of detergent to freeze–thaw-solubilized reductase did not adversely affect its stability at elevated temperatures.

Detergent-solubilized reductase retains the ability to be regulated by reversible phosphorylation–dephosphorylation. Partially purified reductase kinase inactivated partially purified detergent-solubilized reductase up to 90%. The ease with which this high degree of inactivation was achieved is in marked contrast to the difficulty with which freeze–thaw-solubilized reductase is inactivated (Brown et al., 1975; Harwood, 1982) and approximates the facility with which microsomal reductase may be inactivated. As was previously observed with both microsomal and freeze–thaw-solubilized reductase (Brown et al., 1975; Harwood, 1982; Nordstrom et al., 1977), cytosolic reductase kinase requires both ATP and ADP for inactivation of detergent-solubilized reductase. Subsequent phosphoprotein phosphatase treatment completely reversed this inactivation. We therefore conclude that detergent-solubilized reductase represents a distinct form of reductase, which more closely approaches the native form of the enzyme than that solubilized by freeze–thaw techniques.

Acknowledgments

We are grateful to Drs. Michael L. Keith and David J. Shapiro of the Department of Biochemistry, University of Illinois, Urbana, IL, for many helpful discussions.

Registry No. Reductase, 37250-24-1; Lubrol WX, 11138-41-3; PEW-1, 71611-21-7; PMSF, 329-98-6; EGTA, 67-42-5; reductase kinase, 72060-32-3.

References

Baxter-Gabbard, K. L. (1972) *FEBS Lett. 20*, 117–119.

Böhlen, P., Stein, S., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys. 155*, 213–220.

Brown, M. S., Dana, S. E., Dietschy, J. M., & Siperstein, M. D. (1973) *J. Biol. Chem. 248*, 4731–4738.

Brown, M. S., Brunschede, G. Y., & Goldstein, J. L. (1975) *J. Biol. Chem. 250*, 2502-2509.
Chin, D. J., Luskey, K. L., Anderson, R. G. W., Faust, J. R., Goldstein, J. L., & Brown, M. S. (1982a) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1185-1189.
Chin, D. J., Luskey, K. L., Faust, J. R., MacDonald, R. J., Brown, M. S., & Goldstein, J. L. (1982b) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7704-7708.
Harwood, H. J. (1982) Ph.D. Thesis, Purdue University, West Lafayette, IN.
Harwood, H. J., & Rodwell, V. W. (1982) *J. Lipid Res. 23*, 754-761.
Heller, R. A., & Gould, R. G. (1973) *Biochem. Biophys. Res. Commun. 50*, 859-865.
Kleinsek, D. A., Dugan, R. E., Baker, T. A., & Porter, J. W. (1981) *Methods Enzymol. 71*, 462-479.
Ness, G. C., Way, S. C., & Wickham, P. S. (1981) *Biochem. Biophys. Res. Commun. 102*, 81-85.
Nordstrom, J. L., Rodwell, V. W., & Mitschelen, J. J. (1977) *J. Biol. Chem. 252*, 8924-8934.
Rodwell, V. W., Nordstrom, J. L., & Mitschelen, J. J. (1976) *Adv. Lipid Res. 14*, 1-74.
Rogers, D. H., Panini, S. R., & Rudney, H. (1980) *Anal. Biochem. 101*, 107-111.
Shapiro, D. J., Nordstrom, J. L., Mitschelen, J. J., Rodwell, V. W., & Schimke, R. T. (1974) *Biochim. Biophys. Acta 370*, 369-377.
Williamson, I. P., & Rodwell, V. W. (1981) *J. Lipid Res. 22*, 184-187.

Distinct Heme–Substrate Interactions of Lactoperoxidase Probed by Resonance Raman Spectroscopy: Difference between Animal and Plant Peroxidases†

Teizo Kitagawa,*,‡ Shinji Hashimoto,‡ Junji Teraoka,‡ Shingo Nakamura, Hisa Yajima, and Toichiro Hosoya

ABSTRACT: Resonance Raman scattering from cow milk lactoperoxidase (LPO) and its complexes with various electron donors and inhibitors was investigated. The Raman spectrum of LPO is strikingly close to that of hog intestinal peroxidase but distinctly dissimilar to that of horseradish peroxidase (HRP). The ν_{10} frequency suggested the six-coordinate high-spin structure of heme for native LPO in contrast with the five-coordinate high-spin structure for HRP. For the ν_{10} band, benzohydroxamic acid caused a frequency shift with HRP but not with LPO. Guaiacol, *o*-toluidine, and histidine brought about a frequency shift of the ν_4 mode for LPO but not for HRP. The frequency shift was restored upon removal of the substrate or inhibitor by dialysis. The down shift of the ν_4 frequency is considered to represent an appreciable donation of electrons from the substrate or inhibitor to the porphyrin LUMO and thus their direct interaction with the heme group. From the relative intensity of the shifted and unshifted ν_4 lines, the dissociation constant was determined to be K_d = 52 mM for guaiacol and K_d = 87 mM for histidine at pH 7.4. The binding of histidine was relatively retarded in the presence of sulfate anion (K_d = 150 mM for 0.53 M sulfate present), and imidazole alone yielded no frequency shift, indicating the binding of the carboxyl group of histidine to the protein cationic site on one hand and a weak charge-transfer interaction between the imidazole group and the heme group on the other.

Lactoperoxidase (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7) is a heme-containing enzyme with a single peptide of M_r 77 000, catalyzing the oxidation of various aromatic molecules by H_2O_2 in a manner similar to plant peroxidases. The catalysis by bovine lactoperoxidase (LPO)[1] was demonstrated to proceed via the same sequential intermediates as those of horseradish peroxidase (HRP) (Kimura & Yamazaki, 1979), for which details of the catalytic mechanism have been discussed (Yamazaki et al., 1981). In contrast with a great deal of accumulated data for elucidating the interactions of the heme group with an electron acceptor (H_2O_2) and its temporal evolution during the catalytic reaction, little is known about an interaction of the peroxidase with an electron donor. The visible spectrum of the pyridine hemochrome of LPO is not of typical protoheme type (Morrison et al., 1957), but the presence of iron protoporphyrin IX was revealed through digestion of the protein by Pronase (Sievers, 1979). However, difference in the heme environments between the animal and plant peroxidases has not been fully investigated yet.

Resonance Raman (RR) spectra of peroxidase bring detailed structural information of the heme vicinity (Kitagawa & Teraoka, 1982). Combined analysis of the NMR (LaMar & deRopp, 1982) and RR data (Teraoka & Kitagawa, 1981) of HRP established the presence of a strong hydrogen bond between the proximal histidine and a surrounding amino acid residue. The strong hydrogen bond was noted to serve as a common characteristic of all plant peroxidases (J. Teraoka, D. Job, Y. Morita, and T. Kitagawa, unpublished results). We are curious to know whether such a characteristic is retained by an animal peroxidase and also to find how differently the

† From the Department of Molecular Physiological Chemistry, Osaka University Medical School, Nakanoshima, Kitaku, Osaka, 530 Japan (T.K., S.H., and J.T.), the Department of Agriculture, Hirosaki University, Hirosaki, Aomori, 036 Japan (S.N.), and the Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Chiba, 260 Japan (H.Y. and T.H.). *Received March 8, 1983.* This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture and Science of Japan (No. 56480370 to T.K.).

‡ Present address: Department of Molecular Dynamics, Institute for Molecular Science, Okazaki National Research Institutes, Myodaiji, Okazaki, Aichi, 444 Japan.

[1] Abbreviations: LPO, lactoperoxidase; HRP, horseradish peroxidase; IPO, intestinal peroxidase; BHA, benzohydroxamic acid; RR, resonance Raman; LUMO, lowest unoccupied π molecular orbital.

0006-2960/83/0422-2788$01.50/0