Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. (1988) *Science 239*, 487-491.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *In Molecular Cloning: A Laboratory Manual* (Nolan, C., Ed.) 2nd ed., pp 7.19-7.22, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Shaffer, A. L., Wojnar, W., & Nelson, W. (1990) *Anal. Biochem. 190*, 292-296.
Shimomaye, E., & Salvato, M. (1989) *Gene Anal. Tech. 6*, 25-28.
Tindall, K. R., & Kunkel, T. A. (1988) *Biochemistry 27*, 6008-6013.
Todd, J. A., Bell, J. I., & McDevitt, H. O. (1987) *Nature (London) 329*, 599-604.
Tse, W. T., & Forget, B. G. (1990) *Gene 88*, 293-296.
Veres, G., Gibbs, R. A., Scherer, S. E., & Caskey, C. T. (1987) *Science 237*, 415-417.
Wang, A. M., Doyle, M. V., & Mark, D. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9717-9721.

Articles

Biological Thiols Elicit Prolactin Proteolysis by Glandular Kallikrein and Permit Regulation by Biochemical Pathways Linked to Redox Control†

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ABSTRACT: Rat glandular kallikrein (GK), a trypsin-like serine protease, cleaves rat prolactin (PRL) in vitro to novel forms detectable in vivo and likely to be of physiological significance. PRL proteolysis by GK is thiol-dependent, with thiols acting upon PRL to refold the molecule into novel conformations that are GK substrates. This study compared several natural and synthetic thiols for their ability to elicit PRL proteolysis by GK. Rat PRL was incubated with rat GK in the presence of various thiols and 0.5% Triton X-100, which enhances thiol-elicited proteolysis. Cleavage was analyzed by gel electrophoresis under reducing and nonreducing conditions. In the presence of Triton X-100, all low molecular weight thiols elicited PRL cleavage by GK. The order of potency was dithiothreitol > mercaptoethanol > lipoic acid > cysteamine = glutathione (GSH) = coenzyme A > cysteine. In the absence of Triton, however, dithiothreitol, coenzyme A, and mercaptoethanol were most effective in eliciting GK proteolysis. Triton X-100 enhanced PRL cleavage by 4-19-fold, depending upon the thiol used. Folding isomers of processed PRL observed following cleavage included disulfide-liked homodimers, oxidized monomers, reduced monomers and mixed disulfides; the folding isomers generated varied depending upon the thiol used. GSH potency in eliciting PRL proteolysis increased 10-fold in the presence of biochemical pathways shuttling reducing equivalents to GSH disulfide (GSSG). PRL cleavage by GK could be controlled by substrates, enzymes, and cofactors making up the reducing shuttle when GSSG was used. Thioredoxin (a protein disulfide oxidoreductase) potently elicited PRL proteolysis by GK in the presence of a reducing shuttle and Triton X-100. Thioredoxin was about 400 times more potent than GSSG under such conditions. The results document that biological thiols can elicit PRL proteolysis by GK and permit control of the reaction by biochemical pathways linked to redox control.

Glandular kallikrein (GK;[1] EC 3.4.21.35) is the prototypical member of a distinct family of serine proteases that appears to function in the specific biosynthetic processing of bioactive peptides. Other members of this family include the γ-subunit of nerve growth factor and epidermal growth factor binding proteins (MacDonald et al., 1988). GK is a major estrogen-induced enzyme in the rat anterior pituitary (Powers & Nasjletti, 1984; Clements et al., 1986; Powers, 1986; Chao et al., 1987; Hatala & Powers, 1987). Anterior pituitary GK has been hypothesized to process prolactin (PRL) to novel forms with unique hormonal roles. This hypothesis has evolved from evidence documenting unique tissue-specific regulation paralleling PRL (Powers & Hatala, 1986; Powers, 1987; Hatala & Powers, 1988), a subcellular localization ideally suited for a role in precursor processing (Golgi apparatus and secretory granules) (Hatala & Powers, 1989), and a cellular localization in PRL-producing cells but not other anterior pituitary cell types (Vio et al., 1990; Kizuki et al., 1990; Kitagawa et al., 1990). However, pituitary GK predominantly exists as a latent zymogen that can be activated with trypsin (Powers, 1986) and does not undergo activation during estrogen or dopamine regulation (Powers, 1986; Powers & Hatala, 1986; Kitagawa et al., 1990), during secretion (Chao & Chao, 1988), or within specific organelles (Hatala & Powers, 1989). Such latency has obscured the role of GK in the pituitary.

In an effort to resolve the role of anterior pituitary GK, we recently examined the ability of active GK (purified from rat urine) to cleave PRL in vitro. GK did not cleave standard rat PRL, but proteolysis was elicited with the thiols dithiothreitol and mercaptoethanol, and the thiol effects were enhanced by nonionic detergents such as Triton X-100 (Powers & Hatala,

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[1] Abbreviations: GK, glandular kallikrein; GSH, glutathione; GSSG, glutathione disulfide; PRL, prolactin.

0006-2960/91/0430-7666$02.50/0

1990). Under such conditions, GK cleaved PRL from a 25K form to a 22K form in reduced gels. The cleavages occurred at three sites clustered in a highly conserved domain near the C-terminus (Arg_{174}–Arg_{175}, Lys_{185}–Phe_{186}, Arg_{188}–Cys_{189}) (Powers & Hatala, 1990), and each site possessed features typical of cleavage sites in other GK substrates. Thiols did not affect GK cleavage of synthetic substrates but markedly altered PRL conformation: this effect was enhanced by Triton X-100 (Powers & Hatala, 1990). Thus, thiols seemed to reduce or refold the disulfides bracketing the processing domain to yield PRL folding isomers that were GK substrates.

Western blot analysis of rat pituitaries revealed 22K PRL variants that were estrogen-dependent (like GK) and appeared to correspond to products of PRL cleavage by GK (Powers & Hatala, 1990). Indeed, others had previously reported novel 22K PRL variants in rat pituitary extracts (Oetting & Walker, 1985; Sinha & Jacobsen, 1988; Shah & Hymer, 1989). Moreover, 22K PRL variants can be secreted during cell culture (Oetting & Walker, 1985) and have been detected in human plasma (Lui et al., 1990), which is consistent with a hormonal role for these products. In addition, increased levels of the 22K variant have been reported following cysteamine treatment during pituitary cell culture (Oetting et al., 1989). We found that cysteamine also increased the 22K PRL variant after in vivo dosing, and the increases were estrogen-dependent and specific for the 22K form (Powers & Hatala, 1990). Cysteamine is a biological thiol known to alter PRL conformation in vivo (Sagar et al., 1985; Scammell et al., 1985); thus, its ability to increase 22K PRL variants strongly suggests that thiol-dependent PRL cleavage by GK is of physiological relevance and participates in the production of 22K PRL variants in vivo. Conversely, the above evidence also implies that novel mechanisms exist for reversibly activating GK within lactotrophs, thus enabling PRL processing.

Many elements of the hypothesis that GK may function as a PRL-processing protease are unresolved or require further testing. For example, the mechanism through which zymogen GK may be reversibly activated is unknown, as is the identity of biological molecules that may mimic Triton X-100. In addition, the thiol dependence of PRL proteolysis by GK is unusual and presents a novel mechanism for protein processing. Our initial in vitro studies of PRL cleavage by GK used the synthetic thiols dithiothreitol and mercaptoethanol and did not examine the ability of biological thiols to support the reaction. Therefore, the present study compared several natural thiols for their ability to elicit PRL proteolysis by GK in vitro. In addition, the thiol-dependent chemistry raised the possibility of reaction control by biochemical pathways that generate reducing equivalents for thiol reduction. Thus, regulation of the in vitro reaction by biochemical pathways linked to redox control was explored.

Experimental Procedures

Biological Materials. Rat PRL (preparation B-6 for biological studies) was obtained from the NIDDK National Pituitary Program (Baltimore, MD). Rat GK was purified from rat urine as previously described (Powers & Hatala, 1990). Thioredoxin reductase from *Escherichia coli* was obtained from IMCO (Stockholm, Sweden), and thioredoxin from *E. coli* was obtained from Schweizerhall (Piscataway, NY). Glucokinase (from *Bacillus stearothermophilus*), glutathione reductase and glucose-6-phosphate dehydrogenase (from Baker's yeast), NADPH (tetrasodium salt), and other biological reagents were from Sigma Chemical Co. (St. Louis, MO). The following thiol reagents were also purchased from Sigma: DL-1,4-dithiothreitol, 2-mercaptoethanol, cysteamine (2-mercaptoethylamine), lipoic acid (DL-6,8-thioctic acid, reduced form), coenzyme A (yeast, sodium salt), glutathione (GSH, free acid), and glutathione disulfide (GSSG, free acid).

PRL incubations with GK. PRL (2 mg/mL; 91 μM) was incubated with GK (7.5–25 μg/mL; 0.17–0.69 μM) at 37 °C in 0.1 M Tris-HCl/1 mM EDTA (pH 8) containing the indicated concentrations of thiols either with or without 0.5% Triton X-100 (as noted). The total reaction volume was 25 μL, and the incubation time was 15–24 h, as noted. At the end of the incubation the reaction was stopped by adding 100 μL of the electrophoresis sample buffer of Laemmli (1970) containing 150 mM dithiothreitol and heating at 100 °C for 5 min. For some experiments the reaction volume was divided into two 12.5-μL aliquots: one was heated in 50 μL of electrophoresis sample buffer containing dithiothreitol, and the other received 2.5 μL of 250 mM iodoacetamide (to alkylate thiols and trap disulfides). The reaction volume was incubated 10 min at room temperature and was then heated in 50 μL of electrophoresis sample buffer without dithiothreitol.

Some PRL incubations with GK were conducted in the presence of biochemical pathways capable of shuttling reducing equivalents to oxidized thiol reagents. The reducing shuttles employed are described in Figure 3. All components of the shuttles were tested for protease contamination prior to use. The only reagent exhibiting protease activity was GSSG; this was inhibited with 200 μM (*p*-amidinophenyl)methanesulfonyl fluoride (Laura et al., 1980).

It should be noted that the PRL and GK concentrations used are likely to be physiologically relevant. The female rat anterior pituitary (10-mg wet weight, 10-μL volume) contains at least 50 ng of GK and 70 μg of PRL (Hatala & Powers, 1988). Also, 50% of anterior pituitary cells (5-μL volume) are lactotrophs (Luque et al., 1986), and 8 and 4% of the lactotroph cell volume constitutes the Golgi apparatus (0.4 μL) and secretory granules (0.2 μL), respectively (Farquhar et al., 1978). Subcellular fractionation studies have shown that roughly 75 and 25% of GK, and 5 and 95% of PRL, are concentrated in the Golgi apparatus and secretory granules, respectively (Hatala & Powers, 1989). On the basis of a molecular weight of 22K for PRL, concentrations of 357 μM and 15.1 mM are predicted in the Golgi apparatus and secretory granules, respectively; the 91 μM PRL concentration used in our study is less than those predicted. On the basis of a molecular weight of 36K for GK, concentrations of 2.6 and 1.7 μM are predicted in the Golgi apparatus and secretory granules, respectively; the highest GK concentration used in this study is only 0.69 μM. However, it should be borne in mind that active GK was used in the present study, although anterior pituitary GK predominantly exists as a latent zymogen. For the purposes of this study it is assumed that latent pituitary GK can be activated within lactotrophs to a form that is enzymatically indistinguishable from active rat urinary GK.

Gel Electrophoresis. Samples were analyzed by use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis with the buffer system of Laemmli (1970). The 0.75-mm gels contained polyacrylamide concentrations of 13% or 15% in the separating gel (8 cm) and 6% in the stacking gel (1 cm); 2–3 μg of PRL was loaded per lane. After electrophoresis, gels were stained with Coomassie blue R-250, destained, dried, and analyzed with a Hoefer GS 300 scanning densitometer (San Francisco, CA).

Results

Potencies of Different Thiols in Eliciting PRL Proteolysis. Figure 1 shows the ability of various thiols to elicit PRL

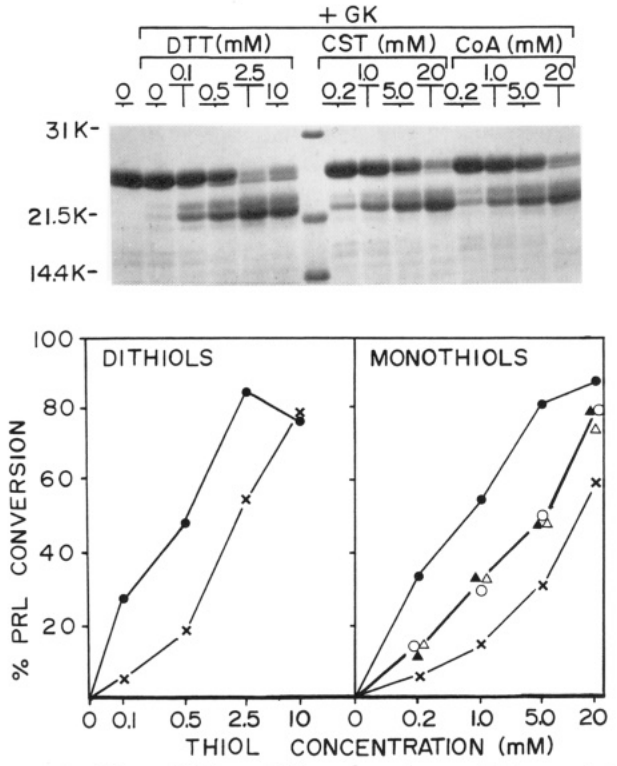

FIGURE 1: Effect of different thiol concentrations on PRL proteolysis by GK. PRL (2 mg/mL) was incubated for 15 h at pH 8, 37 °C, with 7.5 μg/mL GK, 0.5% Triton X-100, and the indicated concentrations of thiols. The upper panel presents a gel showing the products of PRL proteolysis by GK elicited with various concentrations of dithiothreitol (DTT), cysteamine (CST), and coenzyme A (CoA). The lower panel shows the results of scanning densitometry of such gels evaluating the seven different thiols. The dithiols were dithiothreitol (●) and lipoic acid (×). The monothiols were mercaptoethanol (●), cysteamine (○), coenzyme A (▲), GSH (△), and cysteine (×).

Table I: Thiol Potencies in Eliciting PRL Proteolysis by GK[a]

thiol	concn giving 50% PRL cleavage (mM)	thiol	concn giving 50% PRL cleavage (mM)
dithiothreitol	0.5	glutathione	5.3
mercaptoethanol	0.7	coenzyme A	5.5
lipoic acid	2.1	cysteine	12.8
cysteamine	4.7		

[a] Values were determined by interpolation from the concentration dependence curves shown in the lower panel of Figure 1.

proteolysis by GK. Negligible PRL was processed by GK in the presence of Triton X-100 alone, but different thiols produced concentration-dependent increases in PRL processing from a 25K form to a 22K form. (Note that intact PRL migrates as a tight doublet of isomers that persist in processed PRL.) The concentrations required for 50% processing (EC_{50}) are given in Table I. It should be noted that the EC_{50} values are not absolute and vary with GK concentration, length of incubation, Triton X-100 concentration, and other variables. The values shown were derived from a single experiment in which the only variables were thiol type and concentration. All low molecular weight thiols tested were effective in eliciting PRL proteolysis. However, potency markedly varied: for example, dithiothreitol was 23 times more potent than cysteine. Among natural thiols (coenzyme A, cysteamine, cysteine, GSH, and lipoic acid), lipoic acid was about 2.5 times more potent than cysteamine, GSH, and coenzyme A, all of which had similar potencies; cysteine was the least potent thiol tested.

The above experiments examined thiol effects in the presence of 0.5% Triton X-100. Table II presents results from

Table II: Effect of Triton X-100 on PRL Proteolysis Elicited by Different Thiols[a]

thiol	concn (mM)	% proteolysis − Triton X-100	% proteolysis + Triton X-100
dithiothreitol	5	27	>95
coenzyme A	20	24	92
mercaptoethanol	10	20	>95
glutathione	20	13	91
lipoic acid	10	8	82
cysteamine	20	5	>95
cysteine	20	<5	60

[a] PRL (2 mg/mL) was incubated for 18 h with 15 μg/mL GK at pH 8, 37 °C, with the indicated concentrations of thiols in either the presence or absence of 0.5% Triton X-100. Reactions were then stopped and samples were subjected to gel electrophoresis. Values for percent proteolysis were determined by scanning densitometry of stained gels.

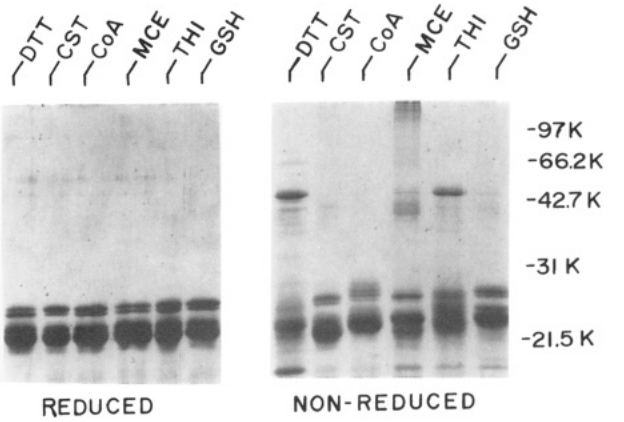

FIGURE 2: Variations in the folding state of processed PRL elicited with different thiols. PRL (2 mg/mL) was incubated for 15 h at pH 8, 37 °C, with 7.5 μg/mL GK, 0.5% Triton X-100, and one of the following thiols: dithiothreitol (DTT, 2 mM), glutathione (GSH, 20 mM), cysteamine (CST, 20 mM), lipoic acid (THI, 10 mM), or coenzyme A (CoA, 20 mM). At the end of the reaction, aliquots were either heated in electrophoresis sample buffer containing 150 mM DTT (reduced) or alkylated with 50 mM iodoacetamide and heated in electrophoresis sample buffer lacking DTT (nonreduced).

experiments comparing thiol effects in both the presence and absence of Triton X-100. PRL proteolysis elicited with all thiols was markedly enhanced by Triton X-100. However, the different thiols varied in their ability to elicit PRL proteolysis in the absence of Triton X-100. For example, in the absence of Triton X-100, dithiothreitol yielded over 5 times more cleavage than cysteamine. Among natural thiols, coenzyme A elicited the greatest cleavage in the absence of Triton X-100, followed by GSH, lipoic acid, cysteamine, and cysteine.

Folding Isomers of Processed PRL Elicited by Different Thiols. Samples were reduced with dithiothreitol prior to electrophoresis in the above experiments. Figure 2 compares the gel patterns of reduced samples with those of nonreduced samples. All thiols yielded equivalent PRL proteolysis in this experiment (see reduced samples), but the folding isomers of processed PRL in nonreduced samples markedly varied depending upon the thiol used. For example, prominent 50K bands were seen only with the dithiols dithiothreitol and lipoic acid; 18K bands were most prominent with dithiothreitol and were absent in reactions run with cysteamine. The 50K band probably represents a disulfide-linked homodimer and the 18K band an oxidized monomer of processed PRL, since both bands were transformed to 22K processed PRL in reduced samples. Each thiol produced a unique pattern of bands of processed PRL in the 22–24K region in nonreduced samples; the differences may reflect either distinct mixtures of mixed disulfides (e.g., GSH–PRL, coenzyme A–PRL, etc.) or monomers with

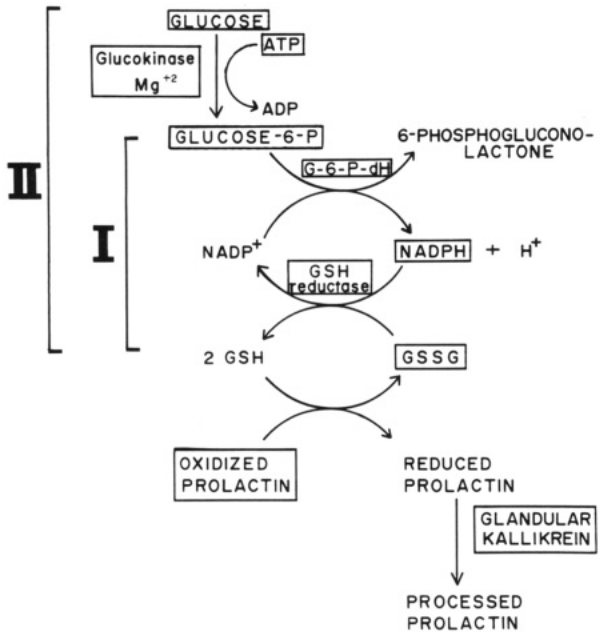

FIGURE 3: Diagram showing the biochemical pathway used to shuttle reducing equivalents to GSSG to regenerate reduced GSH. Reducing shuttle I included all boxed reagents except glucose, ATP, glucokinase, and Mg^{+2}. Reducing shuttle II had all boxed reagents except glucose 6-phosphate (glucose-6-P). Concentrations of the various components were as follows: 6 mM glucose, 6 mM ATP, 6 mM $MgCl_2$, 1.0 μg/mL glucokinase, 5 mM glucose-6-P, 1.0 μg/mL glucose-6-phosphate dehydrogenase (G-6-P dH), 240 μM NADPH, and 1.0 μg/mL GSH reductase. GSH and GSSG concentrations were as noted. Reducing shuttles were prepared in 0.1 M Tris-HCl, pH 8, containing 0.5% Triton X-100.

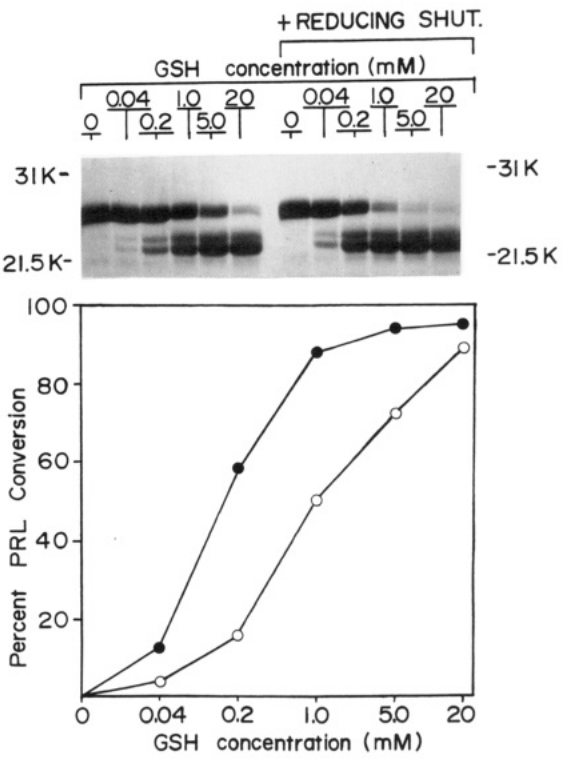

FIGURE 4: Effect of a reducing shuttle on GSH potency in eliciting PRL proteolysis by GK. PRL (2 mg/mL) was incubated for 20 h at pH 8, 37 °C, with 20 μg/mL, GK, 0.5% Triton X-100, and the indicated concentrations of GSH in either the presence or absence of reducing shuttle I (Figure 3). The upper panel presents a gel showing the PRL processing obtained under the various conditions. The lower panel shows the results of quantitative analysis of the gel using scanning densitometry: GSH alone (○), GSH plus reducing shuttle I (●).

varying disulfide-bonding patterns, since the differences were obliterated in samples that were reduced prior to electrophoresis.

Effect of Coupling GSH to a Reducing Shuttle. GSH is well-known to undergo rapid, spontaneous oxidation to GSSG in solutions at neutral or alkaline pH, whereas synthetic thiols, such as dithiothreitol, are resistant to spontaneous oxidation. Thus, it was of interest to compare GSH potency in eliciting PRL cleavage in the presence and absence of a biochemical pathway capable of shuttling reducing equivalents to GSSG. Initial experiments employed reducing shuttle I (diagrammed in Figure 3), which employs the first step of the pentose phosphate pathway. The presence of a reducing shuttle markedly increased the potency of GSH in eliciting PRL proteolysis (Figure 4). The GSH concentration required for 50% processing decreased from 1 to 0.15 mM in the presence of the reducing shuttle, and concentrations needed for near complete cleavage decreased from 20 to 1 mM.

The striking effects of the reducing shuttle on GSH potency indicated that the reaction might be regulated by any one of the substrates, enzymes, and cofactors making up the reducing shuttle. GSSG was employed in experiments to document this point. Negligible PRL proteolysis by GK was elicited with 2 mM GSSG in the absence of a reducing shuttle. In the presence of a reducing shuttle, however, 0.3 mM GSSG yielded 50% PRL cleavage and nearly full processing was obtained with 1 mM GSSG (Figure 5). Furthermore, proteolysis elicited with GSSG was dependent upon each component of the reducing shuttle (Figure 6). Thus, a wide array of biological molecules could control the reaction when run with GSSG. The versatile nature of such control was further explored by replacing glucose 6-phosphate of reducing shuttle I with glucose, glucokinase, ATP, and magnesium, to give reducing shuttle II (Figure 3). Cleavage could be regulated

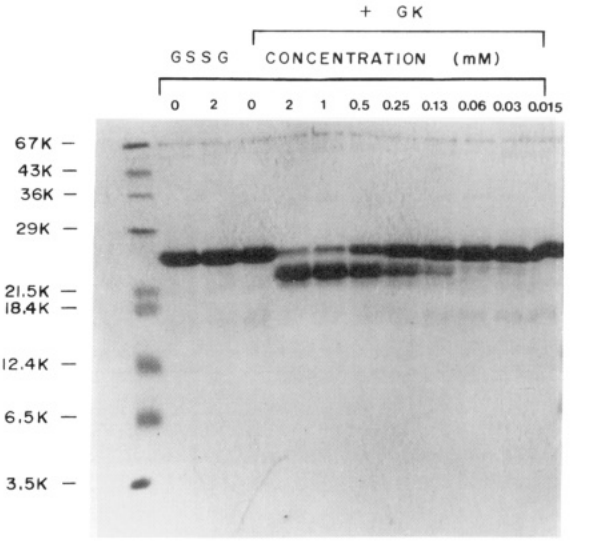

FIGURE 5: Potency of GSSG in eliciting PRL proteolysis by GK in the presence of reducing shuttle I. PRL (2 mg/mL) was incubated for 24 h at 37 °C, pH 8, with 25 μg/mL, GK, 0.5% Triton X-100, reducing shuttle I (Figure 3), and various concentrations of GSSG.

by each one of the new reagents (data not shown).

Effect of Thioredoxin Coupled to a Reducing Shuttle. The ability of thioredoxin (a protein disulfide oxidoreductase) to elicit PRL proteolysis by GK was also examined. In these experiments, reactions were driven with reducing shuttle I in which thioredoxin reductase replaced GSH reductase and thioredoxin (oxidized) replaced GSH. Thioredoxin potently elicited PRL proteolysis by GK in the presence of Triton X-100

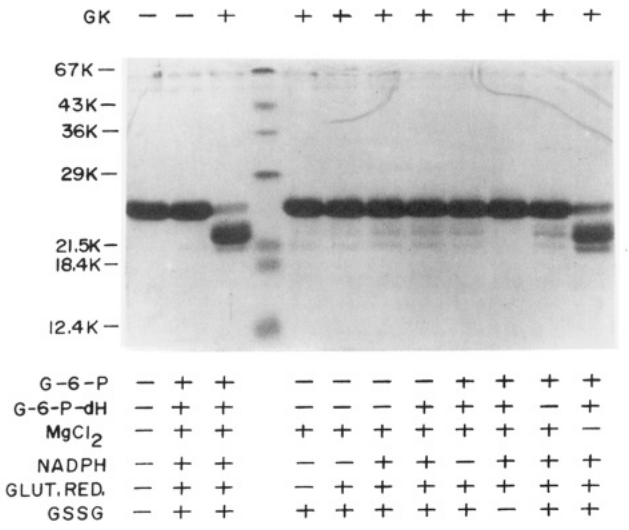

FIGURE 6: PRL proteolysis elicited with GSSG dependent upon each component of reducing shuttle I. PRL (2 mg/mL) was incubated for 24 h at 37 °C, pH 8, with 25 μg/mL GK, 0.5% Triton X-100, 2 mM GSSG, and the indicated components of reducing shuttle I (Figure 3).

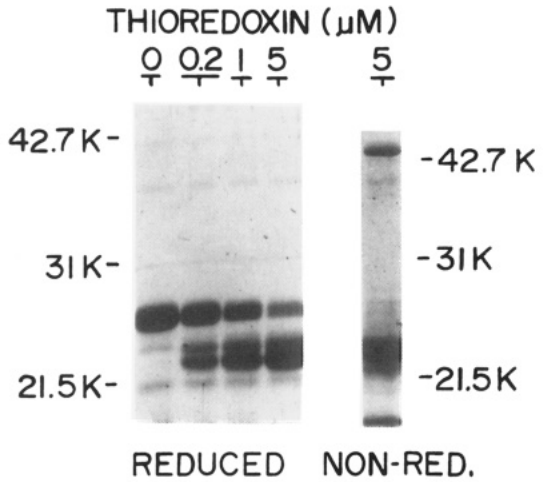

FIGURE 7: Thioredoxin potently elicits PRL proteolysis by GK. PRL (2 mg/mL) was incubated for 20 h at 37 °C, pH 8, with 20 μg/mL GK, 0.5% Triton X-100, and reducing shuttle I (Figure 4) in which GSH reductase was replaced with 180 ng/mL thioredoxin reductase and GSSG was replaced with the indicated concentrations of oxidized thioredoxin. Samples were subjected to electrophoresis after heating in electrophoresis sample buffer with 150 mM dithiothreitol (reduced), or after 10 min of incubation with 50 mM iodoacetamide followed by heating in electrophoresis sample buffer without dithiothreitol (nonreduced).

and a reducing shuttle (Figure 7). The thioredoxin concentration required for 50% PRL processing was 0.6 μM, and nearly complete processing was obtained with 5.0 μM. In the presence of a reducing shuttle, oxidized thioredoxin was about 400 times more potent than GSSG in eliciting PRL proteolysis (data not shown). The PRL proteolysis elicited by thioredoxin was completely dependent upon an intact reducing shuttle, and virtually no cleavage occurred in the absence of Triton X-100 (data not shown). Nonreduced samples revealed that thioredoxin produced folding isomers of processed PRL similar to those elicited with dithiothreitol (50K homodimers, 18K oxidized monomers, etc.) (Figure 7).

Discussion

Considerable evidence indicates that the disulfide bonds of PRL are labile and can undergo marked alterations in their redox state and bonding pattern within pituitary secretory granules in vivo. Thus, PRL has been reported to undergo thiol-dependent "depletion–transformation" events in which pituitary PRL immunoreactivity is transiently decreased during secretory events due to conformational changes in PRL (Mena et al., 1986). In bovine secretory granules, PRL predominantly exists as disulfide-linked oligomers that appear to be transformed to monomeric hormone prior to or during secretion (Lorenson, 1985). Further, cysteamine administration to rats causes striking decreases in PRL immunoreactivity by inducing conformational changes in PRL within secretory granules (Sagar et al., 1985; Scammell et al., 1985). The significance of these alterations in the redox state of PRL disulfides has been obscure. However, the recent discovery that thiols elicit PRL cleavage by GK suggests that the redox changes may produce conformational alterations in PRL required for processing to novel products that may have unique hormonal roles (Powers & Hatala, 1990). This discovery has also raised the question of the identity of biological thiols that may support this reaction in vivo. In the present study, all biological thiols tested could support PRL proteolysis by GK in vitro. However, the thiols varied in their intrinsic potencies, in the folding isomers of processed PRL that they generated, and in their ability to support PRL proteolysis in the absence of Triton X-100.

Among the low molecular weight thiols tested, cysteine had the least potency in supporting GK processing in the absence of a reducing shuttle. However, none of the low molecular weight biological thiols exhibited a remarkably high potency in eliciting PRL proteolysis under such conditions; their potency was less than 25% that of the synthetic thiol dithiothreitol. Although lipoic acid exhibited 2.5-fold greater potency than most other biological thiols, it should be borne in mind that lipoic acid is a dithiol—in contrast with the other low molecular weight biological thiols tested, which are monothiols. Thus, the potencies of lipoic acid and other biological thiols are almost identical when expressed in terms of free sulfhydryl content.

In the absence of Triton X-100, considerable differences in biological thiol potency in eliciting PRL processing were noted. Although all thiols required Triton X-100 for full efficacy, only coenzyme A approached dithiothreitol in its ability to elicit PRL proteolysis in the absence of Triton X-100. Interestingly, cysteamine exhibited little ability to elicit PRL proteolysis in the absence of Triton X-100 and was markedly less potent than mercaptoethanol in the presence of Triton X-100. Cysteamine corresponds to the structural domain of coenzyme A containing the active sulfhydryl and is identical with mercaptoethanol except for the substitution of an amine for an hydroxyl group. This clearly indicates that structural features of the thiol can dramatically affect its ability to react with PRL disulfides determining the conformation of the cleavage domain (PRL_{173}–PRL_{189}). It is also of note that thioredoxin, the most potent thiol reagent for eliciting PRL proteolysis, was entirely dependent upon Triton X-100 for its effects.

The ability of Triton X-100 (a nonionic detergent) to enhance thiol-elicited proteolysis would suggest that hydrophobic domains of PRL may mask critical disulfides. However, lipoic acid, a hydrophobic molecule soluble in organic solvents, exhibited low efficacy in eliciting PRL proteolysis in the absence of Triton X-100. Such results, together with the low efficacy of other nonionic detergents in enhancing PRL proteolysis (Powers & Hatala, 1990), suggest that Triton X-100 does not simply disrupt hydrophobic domains in PRL to facilitate thiol access but may produce more specific alterations in PRL

conformation that either enhance thiol access to key disulfides or yield PRL conformations that are particularly favored as GK substrates.

With use of reduced samples, it was apparent that GK always yielded the same pattern of PRL cleavage products regardless of the thiol used to elicit proteolysis (i.e., 25–22K conversion). However, when nonreduced samples were examined, it was apparent that the folding state of processed PRL markedly varied depending upon the thiol used. Indeed, each thiol produced a unique pattern of folding isomers of processed PRL. Of considerable interest was the generation of a 50K homodimer complex when dithiols (dithiothreitol, lipoic acid, or thioredoxin) were used. A number of growth factors circulate as disulfide-linked homodimers (PDGF-A, TGF-β, activin A)—and these factors also arise from trypsin-like processing by as yet unidentified processing proteases. Thus, homodimers of processed PRL may be of particular interest with respect to evaluation of biological activity. In this regard, it should be noted that efforts are underway to identify the predominant folding isomer of processed PRL present in nonreduced extracts from rat pituitaries. It is possible that novel biological activity may reside in a specific folding isomer of processed PRL whose formation is favored in vivo. Production of such forms in vitro for testing for novel biological activities may critically depend upon the thiol used to elicit proteolysis.

Within cells, thiols that function to modulate the thiol–disulfide redox state of cellular proteins are defined as thiol–disulfide redox buffers [see Gilbert (1990) for review]. The redox state of these buffers, in turn, is regulated by metabolic pathways that generate reducing equivalents for their maintenance; these reducing equivalents are typically shuttled to the thiol–disulfide redox buffer by cofactors such as NADPH. Thiols of major importance as thiol–disulfide redox buffers include GSH, the predominant low molecular weight cellular thiol, and thioredoxin, a 12K protein containing a vicinal cysteine pair with redox activity. We evaluated GSH as a thiol–disulfide redox buffer for modulating PRL cleavage since GSH levels of 2 mM have been reported in rat secretory granules (Greenan et al., 1990)—a site well suited for an interaction with PRL. In initial experiments, GSH concentrations required for maximal rates of PRL processing ($\geq$ 20 mM) were 10 times higher than the levels found in granules. However, GSH potency in vitro appears to be strongly affected by rapid, spontaneous oxidation in solution. Reducing shuttles capable of regenerating GSH from GSSG produced a 10-fold increase in potency, and maximal PRL processing could be sustained with 1 mM GSH. Thus, in the presence of a reducing shuttle, physiological GSH levels appear capable of functioning as a thiol–disulfide redox buffer to regulate PRL processing. Moreover, under such conditions, PRL cleavage could be controlled by each component of the reducing shuttle, emphasizing the regulatory versatility this system permits. The results with thioredoxin were also striking. Oxidized thioredoxin was about 400 times more potent than GSSG in the presence of a reducing shuttle and was the only thiol to effectively catalyze the reaction at concentrations below that of PRL. A thioredoxin:PRL molar ratio of 1:19 gave almost full processing, whereas a GSH:PRL ratio of at least 11:1 was required. The results with thioredoxin are of particular interest since thioredoxin exhibits high protein disulfide oxidoreductase activity and has been suggested to catalyze protein folding (Holmgren, 1985; Pigiet & Schuster, 1986). Further, mammalian thioredoxin is present in most subcellular fractions (Holmgren & Luthman, 1978) and has been immunocytochemically detected in secretory granules (Rozell et al., 1985), a site allowing interaction with PRL.

In conclusion, the hypothesis that anterior pituitary GK may function as a PRL processing enzyme was developed on the basis of compelling physiological and anatomical evidence linking these proteins. The discovery of thiol-dependent PRL proteolysis by GK has identified the products of GK cleavage of PRL and permitted the identification of estrogen- and cysteamine-induced 22K PRL variants likely to have arisen from this reaction in vivo. The results of the present study further support the hypothesis that GK may function in PRL processing by documenting that biological thiols and redox buffers at physiologically relevant levels can support PRL proteolysis by GK. Nonetheless, definitive proof of such control will require further analysis at the cellular and subcellular level, and other essential elements of the in vivo reaction remain obscure (e.g., the mechanism of activation of zymogen pituitary GK). Also, it should be stressed that thiols other than those tested may be active in catalyzing PRL cleavage by GK. Studies with protein disulfide isomerase and glutaredoxin seem especially warranted given their structural kinship to thioredoxin. Similarly, many biochemical pathways might contribute reducing equivalents to thiol–disulfide redox buffers modulating PRL cleavage, which may permit novel control of the reaction by extracellular signals that alter cellular metabolic activity and redox status.

References

Chao, J., & Chao, L. (1988) *Mol. Cell. Endocrinol. 55*, 21–31.

Chao, J., Chao, L., Swain, C. C., Tsai, J., & Margolius, H. S. (1987) *Endocrinology 120*, 475–482.

Clements, J. A., Fuller, P. J., McNally, M., Nikolaidis, I., & Funder, J. W. (1986) *Endocrinology 119*, 268–273.

Farquhar, M. G., Reid, J. J., & Daniell, L. W. (1978) *Endocrinology 102*, 296–311.

Gilbert, H. F. (1990) *Adv. Enzymol. Relat. Areas Mol. Biol. 63*, 69–172.

Greenan, J., Lorenson, M. Y., Conconi, M. V., & Walker, A. M. (1990) *Endocrinology 126*, 512–518.

Hatala, M. A., & Powers, C. A. (1987) *Biochim. Biophys. Acta 926*, 258–263.

Hatala, M. A., & Powers, C. A. (1988) *Biol. Reprod. 38*, 846–852.

Hatala, M. A., & Powers, C. A. (1989) *Neuroendocrinology 49*, 537–544.

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237–271.

Holmgren, A., & Luthman, M. (1978) *Biochemistry 17*, 4071–4077.

Kitagawa, A., Kizuki, K., Moriya, H., Kudo, M., & Noguchi, T. (1990) *J. Biochem. 108*, 971–975.

Kizuki, K., Kitagawa, A., Takahashi, M., Moriya, H., Kudo, M., & Noguchi, T. (1990) *J. Endocrinol. 127*, 317–323.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laura, R., Robison, D. J., & Bing, D. H. (1980) *Biochemistry 19*, 4859–4864.

Lorenson, M. Y. (1985) *Endocrinology 116*, 1399–1407.

Lui, J. H., Lee, D. W., & Markoff, E. (1990) *J. Clin. Endocrinol. Metab. 71*, 605–610.

Luque, E. H., Munoz de Toro, M., Smith, P. F., & Neill, J. D. (1986) *Endocrinology 118*, 2120–2144.

MacDonald, R. J., Margolius, H. S., & Erdos, E. G. (1988) *Biochem. J. 253*, 313–321.

Mena, F., Clapp, C., Aguayo, D., Lorenson, M. Y., & Martinez-Escalera, G. (1986) *Endocrinology 118*, 1795–1807.

Oetting, W. S., & Walker, A. M. (1985) *Endocrinology 117*, 1565–1570.

Oetting, W. S., Ho, T. W. C., Greenan, J. R., & Walker, A. M. (1989) *Mol. Cell. Endocrinol. 61*, 189-199.
Pigiet, V. P., & Schuster, B. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7643-7647.
Powers, C. A. (1986) *Mol. Cell. Endocrinol. 46*, 163-174.
Powers, C. A. (1987) *Endocrinology 120*, 429-431.
Powers, C. A., & Hatala, M. A. (1986) *Neuroendocrinology 44*, 462-469.
Powers, C. A., & Hatala, M. A. (1990) *Endocrinology 127*, 1916-1927.
Powers, C. A., & Nasjletti, A. (1984) *Endocrinology 114*, 1841-1844.
Rozell, B., Hansson, H.-A., Luthman, M., & Holmgren, A. (1985) *Eur. J. Cell Biol. 38*, 79-86.
Sagar, S. M., Millard, W. J., Martin, J. B., & Murchison, S. C. (1985) *Endocrinology 117*, 591-600.
Scammell, J. G., Burrage, T. G., Eisenfeld, A. J., & Dannies, P. S. (1985) *Endocrinology 116*, 2347-2354.
Shah, G. N., & Hymer, W. C. (1989) *Mol. Cell. Endocrinol. 61*, 97-107.
Sinha, Y. N., & Jacobsen, B. B. (1988) *Endocrinology 123*, 1364-1370.
Vio, C. P., Roa, J. P., Silva, R., & Powers, C. A. (1990) *Neuroendocrinology 51*, 10-14.

Secondary Structure Analysis of the Scrapie-Associated Protein PrP 27-30 in Water by Infrared Spectroscopy†

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ABSTRACT: A protease-resistant form of the protein PrP (PrP-res) accumulates in tissues of mammals infected with scrapie, Creutzfeldt-Jakob disease, and related transmissible neurodegenerative diseases. This abnormal form of PrP can aggregate into insoluble amyloid-like fibrils and plaques and has been identified as the major component of brain fractions enriched for scrapie infectivity. Using a recently developed technique in Fourier transform infrared spectroscopy which allows protein conformational analysis in aqueous media, we have studied the secondary structure of the proteinase K resistant core of PrP-res (PrP-res 27-30) as it exists in highly infectious fibril preparations. Second-derivative analysis of the infrared spectra has enabled us to quantitate the relative amounts of different secondary structures in the PrP-res aggregates. The analysis indicated that PrP-res 27-30 is predominantly composed of β-sheet (47%), which is consistent with its amyloid-like properties. In addition, significant amounts of turn (31%) and α-helix (17%) were identified, indicating that amyloid-like fibrils need not be exclusively β-sheet. The infrared-based secondary structure compositions were then used as constraints to improve the theoretical localization of the secondary structures within PrP-res 27-30.

Scrapie is a member of a group of neurodegenerative diseases of animals and humans called the transmissible spongiform encephalopathies. One hallmark of these diseases is the presence of abnormal fibrils in extracts of infected brain, as observed initially with scrapie-infected mice (Merz et al., 1981). Subsequent studies have shown that the major protein component of these amyloid-like fibrils is a disease-specific, protease-resistant form of PrP (PrP-res)[1] (Diringer et al., 1983; Prusiner et al., 1983; DeArmond et al., 1985; Merz et al., 1987). Unlike the normal proteinase K sensitive, detergent-soluble PrP (PrP-sen) of both uninfected and infected hosts, PrP-res was identified as the predominant protein of brain fractions enriched for scrapie infectivity (Bolton et al., 1982; Diringer et al., 1983; Gabizon et al., 1988). Because the infectious agents of scrapie and related neurodegenerative diseases are highly resistant to treatments harmful to nucleic acids, it has long been postulated that these agents are devoid of nucleic acid and composed primarily of protein (Alper et al., 1967; Griffith, 1967; Pattison & Jones, 1967; Prusiner, 1982). More recently, it was proposed that PrP-res, or the fibril it forms, is the transmissible agent (Bolton et al., 1982; Diringer et al., 1983; McKinley et al., 1983; Merz et al., 1983). The importance of PrP in these diseases has been underscored by molecular genetic data indicating that variations in the host PrP gene, which encodes both PrP-res and PrP-sen, appear to influence the incubation time (Carlson et al., 1986, 1988; Westaway et al., 1987; Race et al., 1990) and susceptibility to disease (Hsiao et al., 1989; Scott et al., 1989; Doh-ura et al., 1989; Goldgaber et al., 1989). However, it is not yet certain whether PrP-res is the transmissible agent itself, a component of the agent, or a byproduct of the disease which

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[1] Abbreviations: PrP-res, proteinase K resistant PrP; Prp-res 27-30, proteinase K digested PrP-res; PrP-sen, proteinase K sensitive PrP; Tris, tris(hydroxymethyl)aminomethane; PBS-S, phosphate-buffered saline with 0.5% sulfobetaine 3-14; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with 0.05% Tween 20.

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