Thiol Dependence of Nitric Oxide Synthase†

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ABSTRACT: Nitric oxide synthases (NOS) require NADPH and tetrahydrobiopterin (H_4biopterin) to convert L-arginine to L-citrulline. The additional requirement and effects of thiols during purification and activity assays of NOS are unclear; for example, glutathione (GSH) has been reported to stimulate or, in the presence of catalase, to inhibit enzyme activity. We therefore studied the effects of different thiols, thiol reagents, antioxidants, and H_4biopterin-regenerating systems on purified porcine cerebellum NOS. GSH in the presence of catalase did not inhibit NOS. In contrast, GSH and, to a lesser degree, several other thiols consistently stimulated total L-arginine turnover up to 4-fold. In the presence of GSH, V_{max} of NOS was increased, the usually observed loss of activity during the 15 min assay was less dramatic, and the apparent $S_{0.5}$ value for H_4biopterin decreased. Stabilization of NOS activity by GSH was augmented by protein disulfide isomerase (PDI), indicating that, at least in part, GSH acted by reductive protection of NOS protein thiols. Consistent with this, four different protein thiol reagents abolished NOS activity. In other experiments, specific allosteric binding was excluded as a potential mechanism of GSH regulation of NOS. In addition, GSH may affect NOS kinetics by recycling or preventing the autoxidation of H_4-biopterin. In support of this, the non-thiol reductant ascorbate and dihydropteridine reductase mimicked the effects of GSH on NOS kinetics, but not on NOS stability. Thus, NOS activity depends on both H_4biopterin and the reduced state of essential protein thiols.

Mammalian nitric oxide (NO)[1] formation from L-arginine (Iyengar et al., 1987; Palmer et al., 1988; Schmidt et al., 1988) is catalyzed by NO synthases (NOS; L-arginine,NADPH: oxygen oxidoreductase (nitric oxide-forming); EC 1.14.13.39), an enzyme family with at least three isozymes and corresponding genes (NOS I−III; also termed ncNOS, iNOS, and ecNOS; Nathan & Xie, 1994). The NADPH-dependent electron flow within NOS involves its flavin and heme moieties and is regulated by calmodulin binding (Abu-Soud & Stuehr, 1993). Physiologically, NO synthesis is regulated at the level of gene expression and, in concert with calmodulin, by the intracellular free calcium concentration (Schmidt et al., 1992a; Marletta, 1993; Schmidt et al., 1993; Nathan & Xie, 1994). Additional mechanisms, which may modulate NO synthesis to fine tune between its biological roles in signal transduction and cytotoxicity (Schmidt & Walter, 1994), include the following: autoinhibition (Rogers & Ignarro, 1992), endogenous inhibitory N^{ω}-methylated L-arginines (Vallance et al., 1992), protein phosphorylation (Nakane et al., 1991; Dawson et al., 1993), subcellular localization (Michel et al., 1993), and subunit dimerization (Schmidt et al., 1991). In addition, stimulation of NOS activity by H_4biopterin (Kwon et al., 1989; Tayeh & Marletta, 1989) depends on the amount of enzyme-bound H_4biopterin (Mayer et al., 1991; Schmidt et al., 1992b). However, it is still unclear whether H_4biopterin is directly involved in NOS catalysis, i.e., redox cycles (Stuehr et al., 1990), or is an allosteric regulator of the enzyme (Giovanelli et al., 1991).

Thiol dependence of NOS has remained controversial and is mechanistically unclear. In vivo, thiol depletion does affect NO formation (Murphy et al., 1991; Hecker et al., 1992; Ghigo et al., 1993); however, the specificity of this effect and relevance of non-thiol antioxidants (e.g., ascorbic acid and vitamin E; Buettner, 1993; Meister, 1994) is uncertain. In vitro, most published protocols for the purification of NOS use millimolar buffer concentrations of thiols such as β-mercaptoethanol (β-ME; Bredt & Snyder, 1990; Mayer et al., 1990; Schmidt et al., 1991) or DTT (Knowles et al., 1990; Hevel et al., 1991; Stuehr et al., 1991); however, no study has yet addressed whether the enzyme contains protein thiols that are both essential for activity and sensitive to reductants and thiol reagents. Furthermore, thiols are frequently included in NOS activity assays. In one report (Stuehr et al., 1990), enhancement of total L-arginine turnover by partially purified NOS-II by GSH was similar in magnitude to that by dihydropteridine reductase (DHPR), and both effects were not additive. It was, thus, concluded that GSH is required in the NOS-II activity assay solely to chemically recycle H_4biopterin. However, later a redox role for H_4biopterin in NOS catalysis and, thus, enzymic generation of q-H_2biopterin, H_2biopterin, or other oxidized species were disputed (Giovanelli et al., 1991); in the same study,

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[1] Abbreviations: AsO, arsenite anion; biopterin, 6-(L-*erythro*-1′,2′-dihydroxypropyl)pterin; BSA, bovine serum albumin; cGMP, cyclic guanosine 3′,5′-monophosphate; diamide, azodicarboxylic acid bis-[dimethylamide]; DHA, dehydroascorbate; DHFR, dihydrofolate reductase; DHPR, dihydropteridine reductase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GC-S, soluble guanylyl cyclase; GS-But, *S*-butyl-GSH; GS-Eth, *S*-ethyl-GSH; GS-Hex, *S*-hexyl-GSH; GS-Oct, *S*-octyl-GSH; HbO_2, oxyhemoglobin; H_2biopterin, (6*R*)-7,8-dihydrobiopterin; H_4biopterin, (6*R*)-5,6,7,8-tetrahydrobiopterin; $S_{0.5}$, concentration for half-maximal stimulation; β-ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide; NO, nitric oxide; NOS, NO synthase; [O], oxygen radical species; PDI, protein disulfide isomerase; q-H_2biopterin, (6*R*)-6,7-dihydrobiopterin; SOD, superoxide dismutase; TPOR, thiol: protein-disulfide oxidoreductase; Trolox-C, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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GSH was found to be inhibitory on NOS-I. We, therefore, addressed the question of thiol dependence of NOS using porcine cerebellum NOS-I in the absence or presence of reductive and oxidative protein thiol modification, low molecular weight thiols, other antioxidants, H_4biopterin, and pterin recycling systems. The data indicate that GSH stimulates L-arginine turnover by at least two mechanisms, protein thiol reduction and H_4biopterin regeneration.

MATERIALS AND METHODS

Materials. FAD, FMN, NAD^+, NADH, $NADP^+$, GSH, GSSG, and Cu-containing superoxide dismutase (SOD) were obtained from Boehringer-Mannheim (Mannheim, Germany); L-[2,3,4,5-^{3}H]arginine hydrochloride (specific activity 2.85 TBq/mmol) from Amersham (Braunschweig, Germany); Dowex AG 50 WX-8 (100−200 mesh, Na^+ form) and Polyprep chromatography columns from Bio-Rad Laboratories (München, Germany); (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4biopterin) from Dr. Schircks Laboratories (Jona, Switzerland); 2′,5′-ADP-Sepharose 4B and Sephadex G-25 from Pharmacia (Freiburg, Germany); *Spirulina* species thioredoxin, *N*-ethylmaleimide (NEM), azodicarboxylic acid bis[dimethylamide] (diamide), sodium arsenite (AsO), phosphodiesterase 3′,5′-cyclic nucleotide activator (calmodulin), 3-[(cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), *S*-alkyl derivatives of GSH (GS-Eth, *S*-ethyl-GSH; GS-But, *S*-butyl-GSH; GS-Hex, *S*-hexyl-GSH; GS-Oct, *S*-octyl-GSH), sheep liver dihydropteridine reductase (DHPR), bovine liver dihydrofolate reductase (DHFR), *Escherichia coli* Mn-containing SOD, bovine hemoglobin, glutathione agaroses, and N^{ω}-nitro-L-arginine (NO_2Arg) from Sigma Chemicals (Deisenhofen, Germany); NADPH from Pharma Waldhof (Düsseldorf, Germany), Boehringer-Mannheim (Mannheim, Germany), and Sigma Chemicals (Deisenhofen, Germany); 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one (ebselen) from Biomol (Hamburg, Germany); sodium ascorbate, dehydroascorbate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox-C), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) from Aldrich-Chemie (Steinheim, Germany); protein disulfide isomerase (PDI; EC 5.3.4.1) from Takara/ITC Biomedicals (Heidelberg, Germany); *E. coli* thioredoxin was a kind gift of Dr. Follmann (University of Kassel, Germany). All other chemicals, reagents, and solvents were of analytical grade and provided either by Merck AG (Darmstadt, Germany) or by Sigma Chemicals (Deisenhofen, Germany). Water was deionized to 18 MΩ cm (Milli-Q; Millipore, Eschborn, Germany).

Thiol compounds and other reductants including H_4-biopterin were dissolved in sonicated, degassed and Ar-gassed water and adjusted to pH 7.0. Ultrasonic treatment (5−10 s) was necessary to dissolve *S*-alkyl derivatives of GSH. All thiol stock solutions were used immediately. Hemoglobin was reduced with sodium dithionite; excess reducing agent was removed by gel filtration on prepacked Sephadex G-25 columns (Pharmacia, Freiburg, Germany), and oxyhemoglobin (HbO_2) was eluted with phosphate buffer (67 mM Na_2HPO_4/KH_2PO_4, pH 7.4). The identity and quantity of HbO_2 were determined spectrophotometrically ($\epsilon_{415\ nm}$ = 131 mM^{-1} cm^{-1}). Thioredoxin From *Spirulina* species or *E. coli* was reduced by preincubation for 90 min in 2 mM DTT (Trumper et al., 1994) and diluted 1:10 immediately before use in the NOS assay.

Preparation of NOS-I. The procedure to prepare thiol-free NOS-I was adapted from two previously described methods for rat (Schmidt et al., 1991) and porcine cerebellum (Mayer et al., 1990). Porcine cerebella (1 kg wet weight) were obtained from a local slaughter house, stored on ice for up to 1 h, washed in 0.9% NaCl, and homogenized in 3 L of buffer 1 (50 mM triethanolamine hydrochloride and 0.5 mM EDTA, pH 7.5) using a Waring blender (10 s; Dynamics Corp., New Hartford, CT) and an Ultra-Turrax (3 min; Janke & Kunkel, Staufen, Germany). The homogenate was centrifuged for 45 min at 4000*g*. Solid ammonium sulfate (176 g/L; 30% saturation) was added in small aliquots over 30 min to the stirred supernatant fraction. The suspension was centrifuged (30 min; 27 500*g*), and the pellet was resuspended for 30 min (10 s; Ultra-Turrax) in 1.2 L of buffer 1 containing 211 g of ammonium sulfate (30% saturation). This suspension was centrifuged (15 min; 27 500*g*), and the resulting pellet was resuspended for 30 min in 1.2 L of buffer 2 (20 mM triethanolamine hydrochloride and 0.5 mM EDTA, pH 7.5). This suspension was centrifuged (60 min; 27 500*g*), and the resulting clear supernatant fraction was first filtered through glass wool and then applied to a glass-fritted chromatography column filled with 5 mL of 2′,5′-ADP-Sepharose preequilibrated with buffer 2. The flow through was recirculated, and the column was then washed with 100 mL of buffer 2, 40 mL of buffer 2 containing 0.6 M NaCl, and 40 mL of buffer 2 containing 10 mM NADH. NOS-I was eluted with 26 mL of buffer 2 containing 10 mM NADPH and 0.1% CHAPSO. The eluate was immediately concentrated (Centriprep 30, Amicon, Witten, Germany) to about 0.5 mL. NADPH was removed by repeatedly diluting the concentrate with buffer 2 and reconcentrating. The yield of this purification method was about 1 mg of NOS-I with ≥95% purity and specific activity of 112 nmol mg^{-1} min^{-1}. Purified NOS-I was stored frozen at −70 °C in 50 μL aliquots containing 5 μL of glycerol. The data presented in this study were obtained with three different preparations of NOS-I isolated by this procedure. For control experiments, NOS-I was also purified according to the same protocol except that all buffers contained 7 mM GSH. The yield and purity of NOS-I by this protocol were similar, and the specific activity was 125 nmol mg^{-1} min^{-1}.

NOS Activity Assay. Catalytic activity of NOS-I was assayed by the calcium/calmodulin-dependent conversion of L-arginine to L-citrulline (Bredt & Snyder, 1990). Purified NOS-I (0.25 μg, 15.6 nmol of NOS-I monomer L^{-1}) was incubated for 15 min (if not otherwise indicated) at 37 °C in a total volume of 0.1 mL of buffer 3 containing 0.5 μM calmodulin, 226 μM $CaCl_2$, 477 μM $MgCl_2$, 0.5 mM EDTA, 5 μM FAD, 5 μM FMN, 5 μM H_4biopterin, 20 μM L-arginine (including 5.55 kBq of L-[2,3,4,5-^{3}H]arginine), and 0.1 mM NADPH. The reaction was stopped with 0.9 mL of buffer 4 (20 mM sodium acetate, 2 mM EDTA, pH 5.5). Citrulline was extracted by chromatography on a 0.8 mL cation exchange column (Dowex AG 50 WX-8 resin, Na^+ form) preequilibrated with buffer 4. The combined column flow through (1 mL) and water eluate (2 mL) were measured for ^{3}H radioactivity by liquid scintillation counting (Tri-Carb 2500 TR, Packard, Frankfurt am Main, Germany); results for samples containing DTNB were quench-corrected.

Noncatalytic Inactivation. For some experimets, NOS-I was pretreated in the absence or presence of GSH, DHPR, or PDI by preincubating at 37 °C in 30 μL of buffer 3 (50

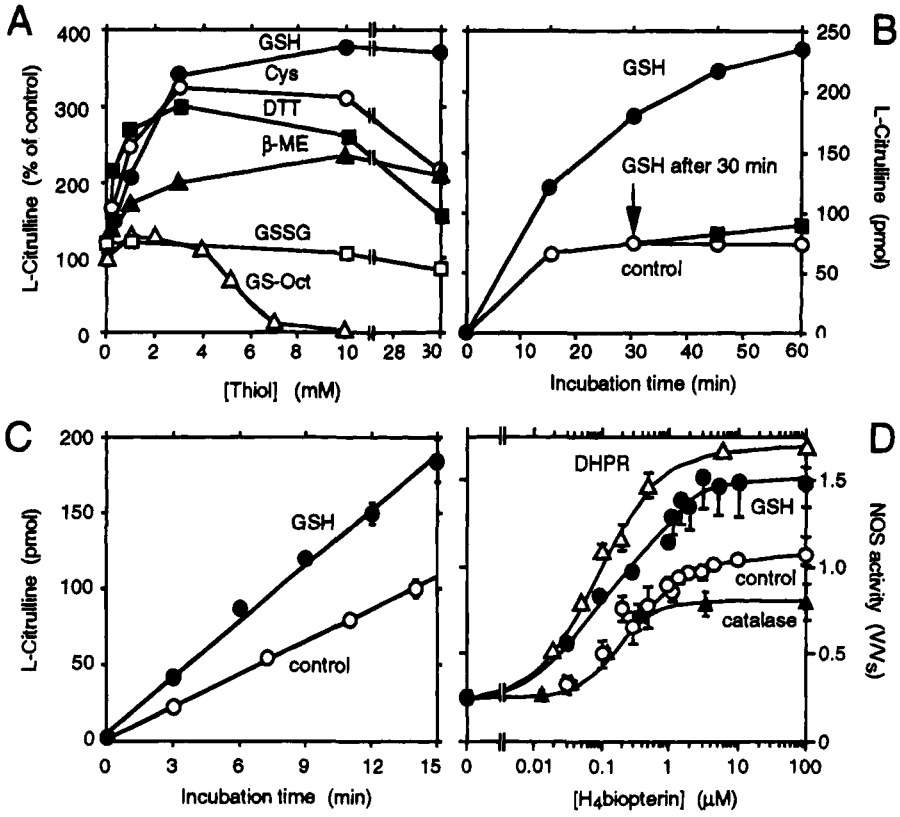


FIGURE 1: Effects of different thiol compounds on NOS activity and kinetics. NOS-I was incubated as described under Materials and Methods: in A, for 25 min in the absence or presence of different concentrations (mM) of GSH (●), Cys (○), DTT (■), β-mercaptoethanol (▲), GSSG (□) and GS-Oct (△); in B, for different times between 0 and 60 min in either the absence (○) or presence of 7 mM GSH (●, added at $t = 0$ min; ■, added at $t = 30$ min, see arrow); in C, for different times between 0 and 15 min in the absence (control, ○) or presence of 7 mM GSH (GSH, ●); in D, for 15 min with different concentrations of H_4biopterin in the absence (control, ○) and presence of 7 mM GSH (GSH, ●), 4 units mL^{-1} of DHPR and 1 mM NADH (△), or 5000 units of catalase mL^{-1}; (▲) L-Citrulline formation (in picomoles or percent of control) or specific activity (initial rate ratio V/V_S) is reported. V_S values were determined under standard assay conditions, i.e, without preincubation and without addition of GSH. The results shown represent one experiment (A–C, four separate experiments) or the average of four experiments (D) and the mean of triplicates of each data point.

mM triethanolamine hydrochloride, pH 7.0) in the absence of arginine and cofactors, i.e., catalytically inactive. Aliquots were withdrawn at different time intervals and assayed for NOS activity.

Kinetic Analysis. NOS activity is reported either as total product accumulation (in picomoles of citrulline or percent of control) or as the ratio of initial velocities under experimental and standard assay conditions (V/V_S). To determine K_m, $S_{0.5}$, and V_{max} values, the standard assay time was 15 min. Calculations were done either by double-reciprocal Lineweaver–Burk plots (for L-arginine and NADPH kinetics; Lineweaver & Burk, 1934) or by nonlinear regression analysis (for H_4biopterin kinetics, Gauss–Newton method) according to the equation $V = V_S + (V_{max} - V_S)/(1 + K_m/S)^{n_H}$).

Catalytic Inactivation. To study catalytic inactivation of NOS-I, assay times were increased to >15 min and the effect of different additives on total L-citrulline accumulation measured.

Rechromatography of NOS-I on GSH Matrices. Poly-prep columns (Bio-Rad) were filled with 0.5 mL of preswollen agarose cross-linked (*S*, thiol-linked; *N*, α-amino-linked) via 12 carbon spacers to GS-Oct (*N*) or GSH (*S*), and equilibrated with 10 mL of buffer (20 mM Tris-HCl, pH 7.8). Purified NOS-I (28.4 μg) was diluted in 0.5 mL of buffer 5 and loaded onto each column; each flow through was reapplied twice. Each column was washed with buffer 5 and buffer 5 containing 0.2 M NaCl before NOS-I was eluted with buffer 5 containing 0.2 M NaCl and either 14 mM GSH or 5 mM GS-Oct.

Table 1: Effect of the GSH–GSSG Redox Potential on Citrulline Formation

thiol concn (mM)			ratio	redox potential[a]	citrulline formation[b]
GSSG	GSH	total	GSSH:GSH	(mV)	(% of control)
0	0	0			100 ± 4.1
10	0	10			105 ± 7.3
5	5	10	1:1	−169	190 ± 17.5
3.33	6.67	10	1:2	−182	197 ± 4.1
2.5	7.5	10	1:4	−189	188 ± 11.3
1	9	10	1:10	−206	203 ± 0
0.5	9.5	10	1:20	−217	187 ± 19.6
0.25	9.75	10	1:40	−227	196 ± 4.1
0	10	10			192 ± 6.7

[a] Redox potentials were calculated by the equation of Nernst: $E_h = E_0 + 2.303RT/nF \log([GSSG]/[GSH]^2)$; $E_{0,GSH} = -0.240$ V (Hwang et al., 1992). [b] L-Citrulline formation was measured for 25 min in the absence or presence of different concentrations and ratios of added GSH and GSSG and is reported as percent of control, i.e., standard assay conditions without addition of thiol. The results shown represent one experiment and the mean of triplicates of each value. Similar results were obtained in three separate experiments.

Protein Determination. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

Table 2: Effects of Different Additives during Purification and Activity Assay on Citrulline Formation by NOS-I

purification[a]	activity assay[b]		citrulline formation			
			per 15 min		per 60 min	
GSH (mM)	H_4biopterin (μM)	GSH (mM)	nmol mg^{-1}	% of control	nmol mg^{-1}	% of control
0	5	0	407		444	
0	5	7	572	141	1085	244
0	55	0	476		633	
0	55	7	633	133	1179	186
7	5	0	1171		1282	
7	5	7	1380	118	2853	222

[a] NOS-I was purified in either the absence or presence of 7 mM GSH. [b] NOS activity was assayed for 15 or 60 min in either the absence or presence of 7 mM GSH and 5 or 55 μM H_4biopterin. The results shown represent the mean of triplicates of one experiment. Similar results were obtained in two separate experiments.

Table 3: The $S_{0.5}$ Value of NOS-I for H_4biopterin in the Presence of Antioxidants and Pterin Recycling[a]

condition	$S_{0.5}$ (nM)	V_{max} (V/V_S)
control	300	1
GSH (7 mM)	151	1.34−1.85
ascorbate	68	1.58−1.79
DHPR	90	1.58
DHFR	no effect	no effect
catalase	200	0.74

[a] NOS activity was assayed for 15 min under different conditions and is reported as specific activity (initial rate ratio V/V_S); V_S was determined under standard assay conditions, i.e., without additives. Results represent the mean values of one to four experiments (each in triplicate).

RESULTS

Thiols Increase L-*Citrulline Formation.* Total L-arginine turnover by NOS-I, expressed as accumulated L-citrulline (picomoles or percent of control), was consistently increased by GSH. Several other thiols also increased L-citrulline formation; however, their effectiveness, apparent $S_{0.5}$ values, and concentration dependence varied from GSH (Figure 1A). GSH caused the greatest increase in L-citrulline formation both at physiological ($S_{0.5}$ = 1.1 mM) and at supramaximal (>7 mM) concentrations. Cys and DTT reached their maximal effect at slightly lower concentrations ($S_{0.5}$ = 0.46 and 0.34 mM, respectively) but with biphasic concentration−effect curves in both the absence and presence of SOD. Of all four tested thiol compounds, β-ME caused the smallest stimulation but, like GSH, with a monophasic concentration−effect curve ($S_{0.5}$ = 0.59 mM). At different redox potentials (between −0.169 and −0.227 V), GSSG had no effect on NOS-I, while the GSH effect was still observed and not modified by GSSG (Table 1).

NOS Kinetics. The effects of thiols on NOS-I kinetics were studied in more detail for GSH. In the absence of GSH, the rate of L-citrulline formation dropped dramatically after 15 min of incubation and virtually ceased after 30−45 min (Figure 1B). This drop in activity was unaffected by scavengers of NO or superoxide anions, HbO_2 (150 μM) and SOD (up to 1000 units mL^{-1}), respectively (not shown). In the presence of GSH, L-citrulline formation remained often linear for up to 20 min and the subsequent drop in activity was less pronounced. Moreover, GSH increased the initial velocity of NOS catalysis compared to standard assay conditions (V/V_S) by a factor of up to 1.85 (Figure 1C). Under assay conditions identical to those reported by Giovanelli et al. (1991; 250 mM HEPES, pH 6.4, 1 mg of BSA, and 8.67 kilounits of catalase per mL), inhibition of NOS-I by GSH was never observed; instead, GSH also stimulated initial velocity here 1.15-fold. The combined increase in initial velocity and the attenuated drop in catalytic rate by GSH resulted in a 4-fold increase in L-citrulline formation for 60 min incubations. When GSH was added 30 min after the incubation had commenced, no effect on subsequent L-citrulline formation was observed (Figure 1B). All effects of GSH on NOS-I were observed with several

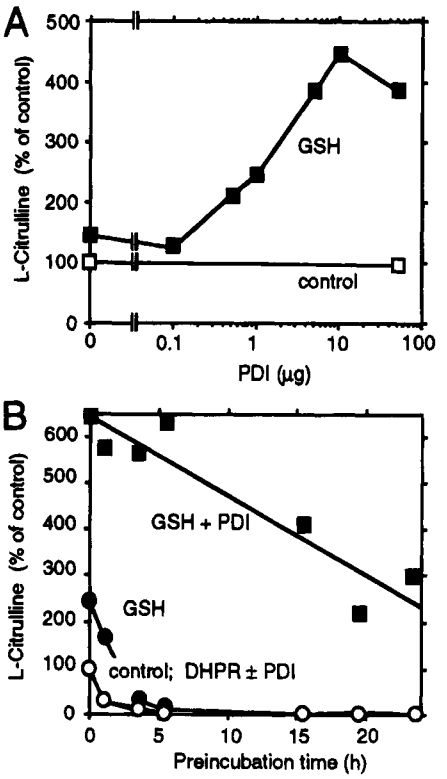


FIGURE 2: Effects of protein disulfide isomerase (PDI) and GSH. NOS-I was assayed for 25 min: in A, after a 4 h preincubation at 37 °C in the absence (□) or presence (■) of 7 mM GSH and with different amounts of PDI; in B, after a preincubation for different time points (at room temperature) without additions (○, control) or in the presence of 7 mM GSH (●, GSH), GSH and 5 μg of PDI (■, GSH+PDI), 4 units mL^{-1} of DHPR (△), or DHPR and 5 μg of PDI (▲, PDI). L-Citrulline formation (in percent of control) is reported; control values were determined under standard assay conditions; i.e., without preincubation and addition of GSH or PDI, as described under Materials and Methods. The results shown represent one experiment and the mean of triplicates of each data point. Similar results were obtained in three separate experiments. Values for control, DHPR, and DHPR and PDI in panel B were superimposable (only the control symbols are shown).

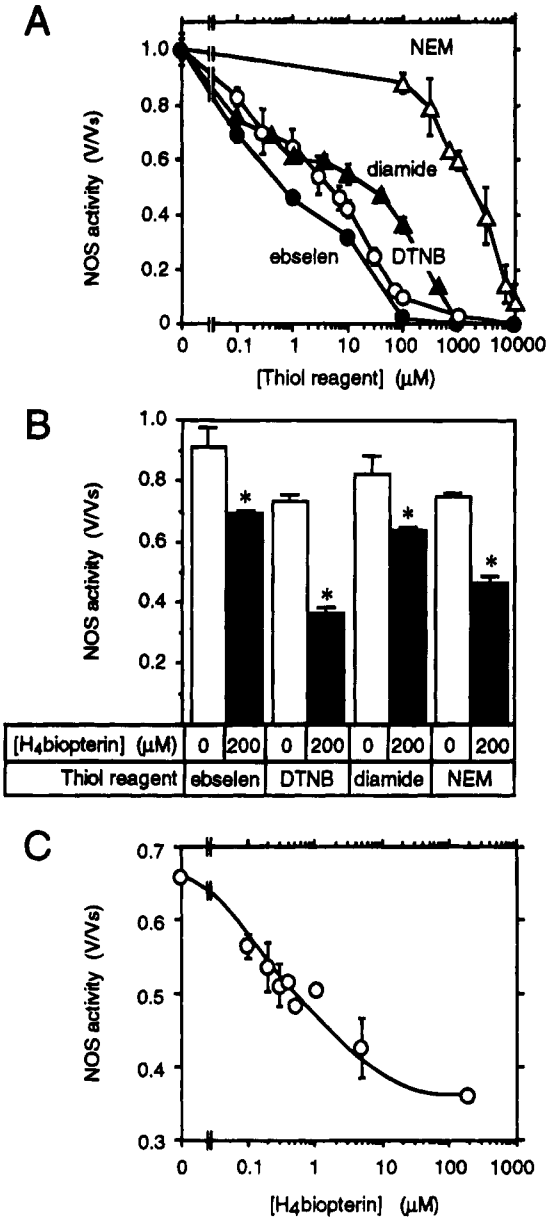


FIGURE 3: Inhibition of NOS-I by different thiol reagents. NOS-I was assayed for 15 min: in A, with different concentrations of *N*-ethylmaleimide (NEM, △), diamide (▲), DTNB (○), or ebselen (●); in B, with the thiol reagents NEM (500 μM), diamide (0.5 μM), DTNB (0.5 μM), or ebselen (0.1 μM) in the absence (open bars) or presence of H_4biopterin (200 μM, closed bars); in C, with DTNB (○, 0.5 μM) in the presence of different concentrations of H_4biopterin. Specific NOS activity (initial rate ratio V/V_S) is reported; V_S values were determined under standard assay conditions, i.e., without addition of thiol reagents as described in Materials and Methods. The results shown represent one experiment and the mean of triplicates of each data point. Similar results were obtained in two separate experiments.

NOS-I preparations and irrespective of whether GSH was present during the purification or not (Table 2). Moreover, GSH decreased the $S_{0.5}$ value for H_4biopterin (Figure 1D; Table 3) while the apparent K_m value for L-arginine was unchanged (7.4 and 7.1 μM, in the absence or presence of 7 mM GSH, respectively).

Protein Thiol Reduction and Enzyme Stabilization. First, modulation of essential protein thiols of NOS-I was considered as a mechanism of action of GSH. In a concentration-dependent manner ($S_{0.5}$ = 10 μg mL^{-1}; i.e., 14-fold molar excess), PDI increased total L-citrulline formation and

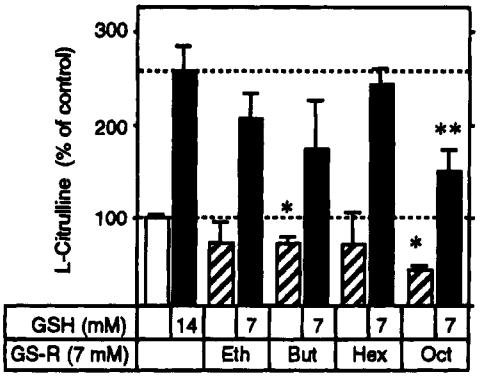


FIGURE 4: Effects of *S*-alkyl derivatives of GSH. NOS-I was incubated for 40 min without additives (open bar), in the presence (7 mM) of different GS-R (hatched bars), GSH (closed bar), or combinations of GS-R and GSH (closed bars). L-Citrulline formation (in percent of control) is reported; control values were determined under standard assay conditions, i.e., without addition of thiol, as described in Materials and Methods. The results shown represent one experiment and the mean of triplicates of each data point. Similar results were obtained in three separate experiments.

attenuated noncatalytic inactivation (Figure 2A) but only in the presence of GSH. PDI alone was ineffective; denatured PDI did not augment the GSH effect; BSA at the same concentration (micrograms of protein per milliliter) did not replace PDI. Augmentation of the GSH effect by PDI was independent of the GSH:GSSG ratio and redox potential. During noncatalytic preincubation, GSH and PDI stabilized NOS-I, resulting in a 40-fold increase of the apparent half-life of NOS-I at room temperature from 0.5 to 18 h (Figure 2B). Furthermore, four different thiol reagents abolished NOS activity in a concentration- and time-dependent manner (Figure 3A): NEM, an alkylans which at higher concentrations may also react with amino and imidazole functions; diamide, a thiol oxidant; DTNB (Ellman's reagent), which forms thionitrobenzoate−protein mixed disulfides; and ebselen, a seleno organic compound which exhibits both GSH peroxidase and antioxidant activity. The rank order of potency (IC_{50}) was ebselen (0.7 μM) > DTNB (5 μM) > diamide (25 μM) > NEM (1.1 mM). The inhibition of NOS-I by ebselen (0.32 ± 0.02 and 0.02 ± 0.02 V/V_S, at 10 and 100 μM ebselen, respectively) was almost fully overcome by 7 mM GSH (control, 1.72 ± 0.09 V/V_S; 10 μM ebselen, 1.69 ± 0.03 V/V_S; 100 μM ebselen, 1.56 ± 0.10 V/V_S). Conversely, AsO, a complexing reagent for vicinal dithiols but not for monothiols (Zahler & Cleland, 1968; Petronilli et al., 1994) was only slightly inhibitory (85.9% ± 3.2% of control at 1 mM). When a submaximal concentration of ebselen, DTNB, diamide, or NEM was used, inhibition of NOS was increased in a concentration-dependent manner by H_4biopterin (Figure 3C). The corresponding IC_{50} value for H_4biopterin-induced inhibition under these conditions (0.35 μM) was in very good agreement to its $S_{0.5}$ value (0.30 μM) for stimulation of NOS-I in the absence of these protein thiol reagents.

GSH Antagonists and Chromatography. GS-Oct and other S-alkylated GSH derivatives, which competitively displace GSH from GSH protein-binding sites, inhibited NOS-I activity in a concentration-dependent manner (IC_{50} = 50 mM; see Figures 1A and 4). Moreover, this inhibition was partially overcome by an equimolar concentration of GSH but was not competitive ($K_{a,GSH}$, 2.0 and 1.5 mM in the absence and presence of 7 mM GS-Oct, respectively). In

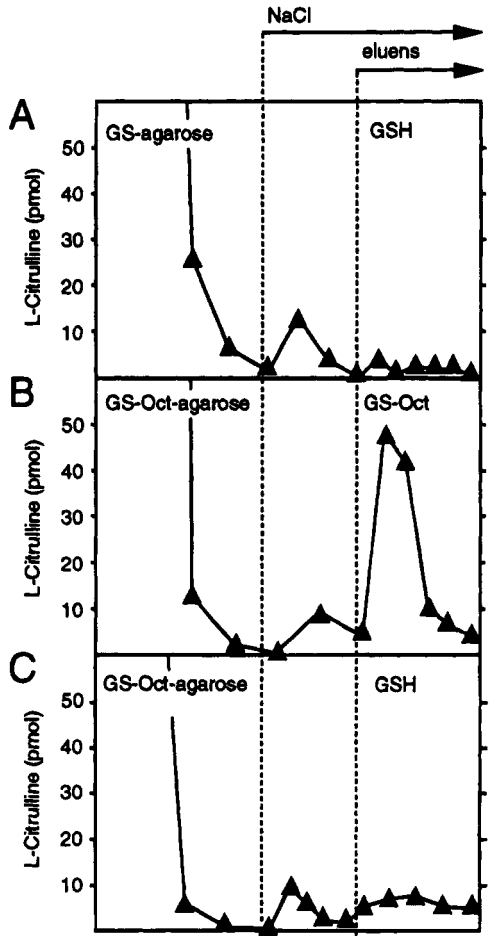


FIGURE 5: Affinity chromatography of NOS-I on GSH and GS-Oct matrices. Purified NOS-I (28.4 μg) was loaded in 0.5 mL of buffer 5 (see Materials and Methods) on 0.5 mL of epoxy-activated, cross-linked 4% beaded agarose matrices which were linked via the thiol function through a 12-carbon spacer to GSH (A) or GS-Oct (B, C). The columns were washed with 5 column volumes of buffer 5, before nonspecifically bound protein was removed by washing with buffer 5 containing 0.2 M sodium chloride (NaCl). NOS-I was eluted with 20 mM GSH (A, C) or 5 mM GS-Oct (B), collecting 0.5 mL fractions (▲). Each fraction was assayed for 15 min in the presence of 7 mM GSH and $^1/_{10}$ of the eluent concentration. L-Citrulline formation (pmol) is reported and was determined as described in Materials and Methods. The results shown represent one experiment and the mean of triplicates of each data point.

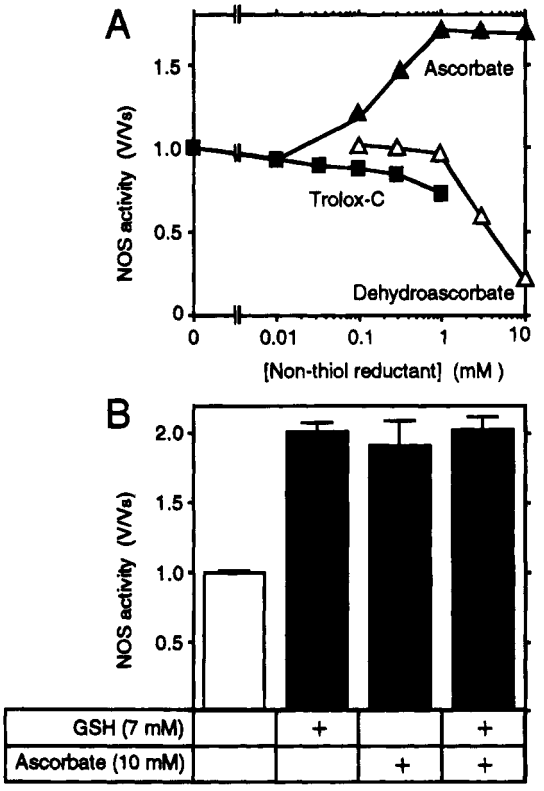


FIGURE 6: Effects of non-thiol antioxidants on NOS kinetics. NOS-I was incubated: in A, for 25 min in the presence of different concentrations of ascorbate (▲), dehydroascorbate (△) or Trolox-C (■); in B, for 15 min in the absence (open bar, control) or presence (closed bars) of 7 mM GSH or 10 mM ascorbate or both. Specific NOS activity (initial rate ratio V/V_S) is reported; V_S values were determined under standard assay conditions, i.e., without addition of ascorbate, Trolox-C or GSH, as described under Materials and Methods. The results shown represent one experiment and the mean of triplicates of each data point. Similar results were obtained in three separate experiments.

another set of experiments, NOS-I was found to bind to GS-Oct agarose (41% of total activity) and could be specifically eluted with 5 mM GS-Oct (14% recovery; Figure 5); however, NOS-I did not elute with up to 20 mM GSH. Moreover, NOS-I did not bind to S- or N-linked GSH-agaroses.

Partial Substitution of GSH by Ascorbate and DHPR. Several other reductants and pterine regenerating systems were examined. Ascorbate (EC_{50} = 0.2 mM; Figure 6A) partially substituted for GSH, i.e., it also increased V_{max}, attenuated catalytic inactivation, and decreased the $S_{0.5}$ value for H_4biopterin (see Table 3). Similar effects were observed with repurified[2] DHPR (Figure 7A–C), which enzymatically recycles q-H_2biopterin to H_4biopterin. Catalase and DHFR, which recycles H_2biopterin to H_4biopterin, were ineffective (in Figure 1D, shown for catalase). About one-third of the DHPR effect on L-citrulline formation was due to its cofactor NADH itself rather than to the enzyme (see Figure 7A). GSH, ascorbate, and DHPR effects were not additive (Figure 6B). The redox partner of ascorbate, DHA, was weakly inhibitory at concentrations 10-fold higher than for ascorbate-induced stimulation. Other reductants were either without effect (e.g., Trolox-C, a water-soluble analog of vitamin E, and thioredoxin, a 12 kDa thiol reductant) or less effective (e.g., NADH) than ascorbate and GSH. These data are summarized in Table 4. When ascorbate or DHPR was added 30 min after the incubation had commenced and the catalytic rate of NOS-I dropped, no stimulation of the subsequent L-citrulline was observed (in Figure 7B, shown for DHPR). When, instead of recycling H_4biopterin, the initial H_4biopterin concentration was increased 145-fold above its $S_{0.5}$ value to 55 μM, the effects of GSH or DHPR on NOS-I activity were still observed and were of similar magnitude (Table 2). However, DHPR did not substitute for the GSH-induced stabilization of NOS-I during noncata-

[2] When the commercially available preparation of DHPR (Sigma Chemicals, Deisenhofen, Germany) was used without further purification, it also stimulated NOS-I; however, when combined with 7 mM GSH, which by itself also stimulated NOS-I, a nearly complete inhibition of L-citrulline formation was observed. When DHPR was purified from low molecular weight contaminants (<10 kDa), its stimulation of NOS-I was preserved but the inhibition of NOS-I in combination with GSH was no longer observed. The low molecular weight fraction by itself inhibited NOS neither alone nor in combination with GSH. The difference between the commercial and repurified DHPR preparations remains thus unclear.

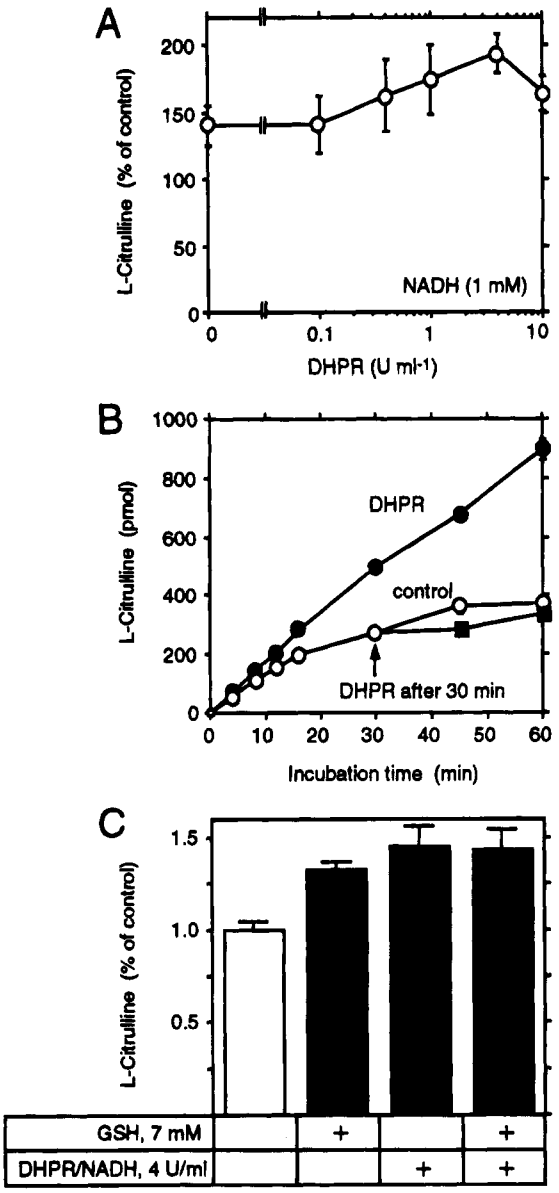


FIGURE 7: Effects of dihydropteridine reductase (DHPR). NOS-I was incubated as described under Materials and Methods: in A, for 30 min in the presence of different concentrations of DHPR and 1 mM NADH; in B, for different times between 0 and 60 min in either the absence (○) or presence of 4 units mL^{-1} of DHPR (●, added at $t = 0$ min; ■, added at $t = 30$ min, see arrow); in C, for 25 min in the absence (open bar) or presence (closed bars) of 7 mM GSH or 4 units mL^{-1} of DHPR + 1 mM NADH or both. L-Citrulline formation (in picomoles or percent of control), or specific activity (initial rate ratio V/V_S) is reported. V_S values were determined under standard assay conditions, i.e., without preincubation and without addition of GSH. The results shown represent one experiment and the mean of triplicates of each data point. Similar results were obtained in three separate experiments.

lytic preincubation, and none of its effects was potentiated by PDI (see Figure 2B).

DISCUSSION

We here report the thiol-dependence of NOS-I, i.e., both with respect to exogenous lower molecular weight thiols and endogenous NOS protein thiols. Two mechanisms by which GSH stabilizes NOS-I and enhances L-arginine turnover were identified. One involves reduction of essential NOS protein thiols and is specifically augmented by enzyme catalysis

Table 4: Effects of Reductants and Oxidants on Citrulline Formation[a]

compd		citrulline formation (% of control)		
redox activity	concn (mM)	15 min	30 min	60 min
reductant				
thiol				
GSH	7	161	158	325
cysteine	3		137	324
dithiothreitol	3		127	302
β-mercaptoethanol	10		108	235
cofactor				
NADH + H^+	1	169	131	
NADPH + H^+	1	165	137	
H_4biopterin	0.055	117	128	143
other				
Trolox-C	1	92	59	63
reduced thioredoxin (*E. coli*)	0.008		124[b]	
ascorbate	1	143	133	330
oxidant				
GSSG	10		105[b]	
cystine	1		72[b]	
dehydroascorbate	10		23	

[a] L-Citrulline formation was assayed for 15−60 min in the absence or presence of different reductants and is reported in percent of control, i.e., standard assay conditions without the addition of thiol and in the presence of 0.1 mM NADPH and 5 μM H_4biopterin. Each value represents the mean of triplicates. Similar results were obtained in three to six separate experiments. [b] 25 min incubation.

through PDI. By a second mechanism, GSH increased the apparent affinity of NOS for H_4biopterin and its V_{max}. These latter effects were mimicked by ascorbate or DHPR and, most likely, involve H_4biopterin recycling.

Previously, L-citrulline formation by partially purified NOS-II was reported to be enhanced by either GSH or DHPR in a nonadditive manner, suggesting H_4biopterin recycling as the sole mechanism of action for both GSH and DHPR (Stuehr et al., 1990). Conversely, GSH was reported to inhibit rat NOS-I (Giovanelli et al., 1991) when assayed at pH 6.4, limiting NADPH concentrations, in the presence of BSA, catalase, and both under V_{max} conditions (5 min incubation) or during prolonged incubations (20 min). However, we observed both under the aforementioned and our standard assay conditions (see Materials and Methods), that GSH consistently stimulates L-citrulline formation by porcine NOS-I. Thus, both NOS isoforms, types I and II, appear to have a similar requirement for thiols. Of several thiols tested, GSH was the most effective stimulator of NOS-I[3] and subsequently was studied in greater detail.

The GSH effects on NOS kinetics were complex. V_{max}, apparent affinity for H_4biopterin, both catalytic and thermal stability were increased; the latter and the V_{max} effect were potentiated by PDI. GSH could be replaced by ascorbate and several other reductants (Table 4), in agreement with a primarily reductive mechanism of action. The different sensitivity of NOS-I to these reductants did not correlate with their antioxidant pecking order, which may be explained by steric hindrance. The reverse effect, oxidative inhibition of NOS-I, was only observed with DHA and cystine but not with GSSG; again steric hindrance may explain this and also the fact that the GSH:GSSG redox potential was without effect. Several enzymes are regulated by GSH or ascorbate and served as working models [Table 5; confer Ziegler

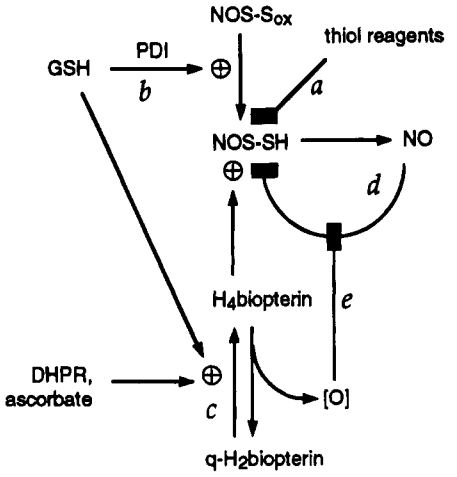
[3] Similar data for NOS-II are not available.

Table 5: Enzymes Modulated by GSH or Ascorbate

enzyme	modulator	mechanism	effect	refs
formaldehyde reductase	GSH	allosteric[a]	$V_{max}\uparrow$	Uotila & Mannervik, 1979
HMG-CoA reductase				
membranous	GSH	reductive, allosteric	$V_{max}\uparrow$, substrate ($K_m\uparrow$), NADPH (MM, $nH\downarrow$)	Roitelman & Shechter, 1984
solubilized	GSH	reductive, allosteric	$V_{max}\uparrow$, NADPH ($K_m\downarrow$)	Roitelman & Shechter, 1984
soluble guanylyl cyclase	GSH, ascorbate[b]	reductive	$V_{max}\uparrow$	Yoshikawa & Kuriyama, 1981; Niroomand et al., 1989
prolyl-4-hydroxylase[c]	ascorbate	reductive	stabilization	Myllylä et al., 1978
dopamine-β-monooxygenase	ascorbate	reductive	$V_{max}\uparrow$	Ahn & Klinman, 1987; Stewart & Klinman, 1991
indoleamine 2,3-dioxygenase	ascorbate	reductive	stabilization	Sono, 1989

[a] GSH also forms a hemimercaptal adduct with NAD^+ which functions as a substrate. [b] In some reports, ascorbate was described to act by an indirect mechanism (Goldberg et al., 1978) requiring H_2O_2 metabolism by catalase (Cherry & Wolin, 1989). [c] Related ascorbate-dependent enzymes, not discussed here, include prolyl-3-hydroxylase and lysyl hydroxylase. Abbreviations: MM, induction of Michaelis–Menten kinetics; n_H, Hill coefficient; $S_{0.5}$, concentration necessary to achieve half-maximal velocity.

Scheme 1



(1985)]. For example, human microsomal liver 3-hydroxy-3-methylglutaryl coenzyme A reductase (Roitelman & Shechter, 1984) shows Michaelis–Menten-type kinetics for NADPH only in the presence of GSH, which also lowers the Hill coefficient (n_H) for NADPH. Interestingly, GC-S, the target enzyme for NO-mediated signal transduction, is also regulated by GSH ($S_{0.5}$ = 0.5 mM; Böhme et al., 1974; Niroomand et al., 1989; Niroomand et al., 1991). Under reduced oxygen tension, the extent of GSH- and NO-induced activation of GC-S is additive and, thus is mediated by different mechanisms. The stimulation of GC-S by GSH is blocked by GS-Me and GSSG, which has been interpreted as a competitive mechanism on a putative GSH binding site (Niroomand et al., 1989). Thus on the basis of these established mechanisms of action of GSH (Table 5), NOS protein thiol reduction, GSH allosteric binding, and reductive interaction with a cosubstrate or cofactor (e.g., H_4biopterin) all appeared to be possible mechanisms of action for GSH on NOS-I.

First, we established that protein thiols are essential for NOS-I L-arginine turnover, which represents a new structural information on this complex multidomain enzyme. For example, four different monothiol reagents abolished NOS-I activity in a GSH-reversible manner (Scheme 1a). Moreover, the low effectiveness of the dithiol reagent AsO suggested that vicinal protein thiols are not essential for NOS-I activity (Zahler & Cleland, 1968; Petronilli et al., 1994). In agreement with this, stimulation of NOS-I by GSH was augmented by PDI (Scheme 1b), which catalyzes protein disulfide bond isomerization (PDI, EC 5.3.4.1) and thiol: protein-disulfide oxidoreductase (TPOR, EC 1.8.4.2). Both TPOR and PDI activities can be dissected in three chemical reactions: (i) protein disulfide formation, under oxidizing conditions, from reduced or partially reduced proteins, (ii) protein disulfide reduction, using low molecular weight thiol compounds, from any oxidized form including randomly joined disulfides (Lambert & Freedman, 1983), and (iii) protein disulfide isomerization, where both breakage and formation of disulfide groups occur toward the most stable folded conformation (Lambert & Freedman, 1983; Hawkins et al., 1991). In all three reactions, the chemical process catalyzed is thiol/disulfide interchange, i.e., attack of a thiolate anion ($R-S^-$) on a disulfide bond. Basically, any oxidation of a thiol group during catalysis or preincubation to the sulfenyl state ($R-S^+$) is a PDI substrate, provided it is sterically accessible (Axelsson & Mannervik, 1983). Enzymic augmentation of the GSH-dependent protein thiol reduction by PDI is specific; thioredoxin (reduced by DTT), which has also dithiol–disulfide oxidoreductase activity (Holmgren, 1979), was ineffective.

Second, several of our data suggested NOS-I may contain a GSH-binding site: neither GSSG nor GS-Oct substituted for GSH; GSH binding site antagonists such as GS-Oct inhibited NOS-I in a GSH-reversible manner; and NOS-I bound to a GS-Oct affinity column. However, the functional antagonism between GSH and GS-Oct was not competitive ($K_{a,GSH}$ unchanged) and NOS-I neither bound to GSH agaroses nor was eluted by GSH from GS-Oct agarose, making a specific GSH binding site on NOS-I highly unlikely.

Third, the complex interaction between H_4biopterin, GSH, and protein thiols suggested that protection of H_4biopterin from autoxidation by GSH and other reductants contributed also to the observed effects of GSH and would explain why several effects of DHPR and ascorbate on NOS kinetics were virtually identical to those of GSH (Scheme 1c). Artificially low steady state concentrations of H_4biopterin in the standard NOS assay will cause an apparently higher $S_{0.5}$ value of NOS for H_4biopterin and a drop in catalytic rate after 15 min in the absence of reducing agents. However, our kinetic control $S_{0.5}$ value for H_4biopterin is in excellent agreement with all published data. Moreover, stimulation of NOS-I by GSH

[4] The putative DHFR reductase 3-D homology module of NOS contains two conserved cysteines (Dr. J. Salerno, personal communication); moreover, in addition to the native proximal cysteine, the NOS heme can bind to a distal thiolate ligand (Dr. K. McMillan, personal communication).

was unchanged even at supramaximal H_4biopterin concentrations, suggesting that simple regeneration of H_4biopterin is not a sufficient explanation. Whether significant redox cycling (Giovanelli et al., 1991) or autoxidation (Mayer et al., 1995) of H_4biopterin occurs during NOS catalysis is still open. Potential products of these processes are H_2biopterin, q-H_2biopterin, and also H_3biopterin radical species (Pfleiderer, 1984). DHPR stimulated L-citrulline formation. q-H_2biopterin is the natural substrate of DHPR and may have been generated enzymatically during NOS catalysis or by H_4biopterin autoxidation; however, catalase which reduces peroxide-mediated autoxidation did not replace GSH. Moreover, q-H_2biopterin in instable and tautomerizes to H_2biopterin, a substrate for DHFR; however, DHPR, when given after 30 min, and DHFR were without effect on NOS kinetics. Thus different oxidation products, but not H_2biopterin, may accumulate in the absence of DHPR or GSH. Some of these may even inhibit the NOS pterin-binding site. Alternatively, the lower apparent $S_{0.5}$ value for H_4biopterin in the presence of GSH and ascorbate may also be the result of NOS thiol reduction in close vicinity to the NOS pterin-binding site. H_4biopterin increased the reactivity of NOS protein thiols toward all four monothiol reagents in a concentration-dependent fashion with an IC_{50} value similar to its $S_{0.5}$ value in the absence of these reagents (see Scheme 1c). Thus, a subset of the NOS essential protein thiols interacts with the pterin-binding site of NOS[4] and is apparently exposed to the protein surface upon H_4biopterin binding or *vice versa*.

Finally, NO may serve as a negative feedback regulator of NO biosynthesis (see Scheme 1d; Rogers & Ignarro, 1992; Rengasamy & Johns, 1993), and it was recently suggested that H_4biopterin autoxidation may interfere with this process by generating oxygen radicals which in turn scavenge NO and, thereby, prevent feedback inhibition of NOS (Scheme 1d,e; Mayer et al., 1995). Regenerating H_4biopterin may indirectly facilitate this process. However, under our conditions inactivation of catalytically active NOS was influenced neither by HbO_2 (NO scavenger) nor by SOD (superoxide scavenger), suggesting that feedback inhibition of NOS did not occur under our experimental conditions.

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