**Redox Regulation of Methionine Aminopeptidase 2 Activity***

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**Background:** The N-terminal methionine in new eukaryotic proteins is removed by methionine aminopeptidases, but how these enzymes are regulated is not known.

**Results:** Methionine aminopeptidase 2 contains a single disulfide bond that exists in oxidized and reduced states and influences enzyme function.

**Conclusion:** MetAP2 is regulated by an allosteric disulfide bond.

**Significance:** This has implications for MetAP2 substrate proteins and other similar enzymes.

Protein translation is initiated with methionine in eukaryotes, and the majority of proteins have their N-terminal methionine removed by methionine aminopeptidases (MetAP1 and MetAP2) prior to action. Methionine removal can be important for protein function, localization, or stability. No mechanism of regulation of MetAP activity has been identified. MetAP2, but not MetAP1, contains a single Cys228-Cys448 disulfide bond that has an −RHStaple configuration and links two β-loop structures, which are hallmarks of allosteric disulfide bonds. From analysis of crystal structures and using mass spectrometry and activity assays, we found that the disulfide bond exists in oxidized and reduced states in the recombinant enzyme. The disulfide has a standard redox potential of −261 mV and is efficiently reduced by the protein reductant, thioredoxin, with a rate constant of 16,180 m−1 s−1. The MetAP2 disulfide bond also exists in oxidized and reduced states in glioblastoma tumor cells, and stressing the cells by oxygen or glucose deprivation results in more oxidized enzyme. The Cys228-Cys448 disulfide is at the rim of the active site and is only three residues distant from the catalytic His231, which suggested that cleavage of the bond would influence substrate hydrolysis. Indeed, oxidized and reduced isoforms have different catalytic efficiencies for hydrolysis of MetAP2 peptide substrates. These findings indicate that MetAP2 is post-translationally regulated by an allosteric disulfide bond, which controls substrate specificity and catalytic efficiency.

In eukaryotes, protein translation is initiated with methionine, and about 80% of proteins have their N-terminal methionine removed soon after maturation (1). Removal of the initiator methionine is important for the activity, stability, or compartmentalization of many proteins within the cell (2, 3).

The methionine cleavage is catalyzed by two cobalt-containing metalloproteases, methionine aminopeptidase 1 (MetAP1) and 2 (MetAP2) (4). Bacteria and archaea contain only one MetAP. Interestingly, mammalian MetAP1 is homologous to the bacterial enzyme, and mammalian MetAP2 is homologous to the archaeal enzyme (5). MetAP1 and MetAP2 share a similar tertiary structure despite only an 18% amino acid positional identity. The shed methionine is reused in new protein synthesis. Methionine is an essential nutrient (*i.e.* it cannot be synthesized) and is highly regulated in mammalian cells, with excess methionine leading to growth inhibition (6).

MetAP function is essential for cell proliferation and viability. The MetAPs are encoded by the *map* genes. In yeast, the deletion of either *map* gene results in reduced growth rate, but the loss of both genes is lethal (7). In mammalian cells, inhibition of MetAP1 activity results in cell cycle arrest at G2/M, whereas loss of MetAP2 leads to proliferation arrest at G1 (8, 9). Inhibition of either enzyme can also result in apoptotic cell death (10).

Eukaryote proteases are typically regulated either by protease inhibitors or by compartmentalization, and neither mechanism applies to the MetAPs. Secreted enzymes, such as the serine proteases and metalloproteases, are controlled by protease inhibitors in the extracellular milieu, whereas cellular protease activity is often controlled by sequestering of the enzymes in discrete compartments, such as organelles or membranes. The mammalian acid hydrolases, for instance, are found in lysosomes that constrain their distribution and provide the acidic environment required for their efficient activity, whereas the intramembrane proteases only cleave other integral membrane proteins. No endogenous inhibitors of the MetAPs have been identified, and the enzymes are not compartmentalized in the cytosol. Whether and how these enzymes are regulated are open questions. The MetAPs have different substrates. The metalloproteases generally prefer amino acids with small and uncharged side chains at the second residue (P1') of the sub-

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2 The abbreviations used are: MetAP, methionine aminopeptidase; eNOS, endothelial nitric oxide synthase; MPB, 3-(N-maleimido-propionyl)biocytin; RFU, relative fluorescence units; TCEP, tris(2-carboxyethyl)phosphine; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; AMC, 7-amino-4-methylcoumarin; PDB, Protein Data Bank.
Redox Regulation of Methionine Aminopeptidase 2 Activity

substrate for both MetAPs but is preferentially cleaved by 56 °C. The gel slices were washed with 25 mM ammonium bicarbonate, 50% (v/v) acetonitrile and dried before digestion with 12.5 µg/ml trypsin (Promega) in 25 mM ammonium bicarbonate buffer overnight at 30 °C. Trypsin digestion was stopped by adding formic acid to 5% (v/v). Peptides were eluted from gel slices with 5% (v/v) formic acid, 50% (v/v) acetonitrile for 30 min at 25 °C, and mass spectrometry analysis was performed as described previously (20). The data were searched using Mascot (version 2.4.01, Matrix Science) against the Human Uniprot database (2013_02) with the following parameters: 10 ppm for precursor tolerance; ±0.4 Da for product ion tolerances; digestion with trypsin with up to three missed cleavages; and oxidation (Met), carboxyamidomethyl (Cys), and methylisulfide (Cys) selected as variable modifications. To determine the extent of MetAP2 disulfide cleavage by thioredoxin, the relative ion abundance of peptide containing Cys-carboxyamidomethyl and Cys-methylisulfide was used. The relative ion abundance of peptides was calculated using the area of the peak detected from the extracted ion chromatograms generated by the XCalibur Qual Browser software (version 2.0.7, Thermo Scientific). The ratio of carboxyamidomethyl to methylisulfide labeling represents the fraction of the cysteine in the population that is in the reduced state.

Rate of Reduction of the MetAP2 Disulfide Bond—The activity of MetAP2 was measured using a coupled enzyme assay (21). MetAP2 (0.1 µM) was incubated with 0.5 mM Met-Gly-Pro-AMC (R&D Systems) and 0.0125 units/ml porcine dipeptidyl peptidase in 50 mM Hepes, pH 7.4, buffer containing 100 mM NaCl, 0.1 mM CoCl2, and 1 mg/ml PEG 6000 (Hepes buffer). The rate of increase in fluorescence (λex = 335 nm; λem = 460 nm) was measured at 25 °C using a Fluoroskan Ascent microplate fluorimeter (Thermo Scientific). MetAP2 was oxidized or reduced by incubating 0.2 µM enzyme with 200 µM diamide (Sigma) or 10 µM thioredoxin, respectively, in Hepes buffer for 1 h at 25 °C in the dark. The rate of reduction of the MetAP2 Cys228-Cys448 disulfide bond by thioredoxin was measured by incubating 0.1 µM MetAP2 with 1 µM thioredoxin in Hepes buffer. The reaction was quenched at discrete times by adding 10 mM iodoacetamide, and the MetAP2 activity was measured in the coupled enzyme assay.

Redox Potential of the MetAP2 Disulfide Bond—The standard redox potential of the Cys228-Cys448 disulfide bond was determined using either oxidized and reduced dithiothreitol or thioredoxin. For the experiments using dithiothreitol, unpaired cysteine thiolis in MetAP2 were blocked with 10 mM iodoacetamide and 10 mM N-ethylmaleimide for 1 h at 25 °C, and the alkylators were removed using a Zea spin desalting column equilibrated with deoxygenated sodium phosphate, pH 7.4, buffer containing 10 mM EDTA. The blocked MetAP2 (250 mM) was incubated with 1 mM oxidized dithiothreitol (DTTred trans-4,5-dihydroxy-1,2-dithiane; Sigma) and various concentrations of reduced dithiothreitol (DTTred) for 18 h at 30 °C in the phosphate buffer. The unpaired MetAP2 Cys228 and Cys448 thiols were labeled with 2 mM 3-(N-maleimido-propioloyl)biocytin (MPB; Invitrogen) for 30 min at 25 °C, and unreacted MPB was quenched with 5 mM glutathione for 10
min at 25 °C. Samples were resolved on NuPAGE Novex 4–12% BisTris gel with MOPS running buffer under non-reducing conditions, and the MetAP2 was transferred onto polyvinylidene fluoride membrane and blotted either with anti-MetAP2 monoclonal antibody (Abcam) and goat anti-rabbit peroxidase antibody (Dako) or with streptavidin-peroxidase to detect the MPB label. The blots were visualized using chemiluminescence (PerkinElmer Life Sciences), and band intensity was quantified using ImageQuant TL version 8.1 software (GE Healthcare). The fraction of reduced Cys224-Cys448 disulfide bond was measured from the intensity of MPB labeling with reference to labeling of the fully reduced enzyme. For the experiments using thioredoxin, 1/9262 M MetAP2 was incubated with a 50/9262 M concentration of different ratios of oxidized (Trxox) and reduced (Trxred) thioredoxin for 18 h at 30 °C in deoxygenated sodium phosphate, pH 7.4, buffer containing 10 mM EDTA. MetAP2 activity was measured from the initial rate of hydrolysis of Met-Gly-Pro-AMC, and the fraction of reduced enzyme was calculated from the activity of fully oxidized or reduced enzyme. Reduced thioredoxin was prepared by incubating with TCEP-agarose (Pierce) for 30 min at room temperature, whereas oxidized thioredoxin was prepared by exposure to ambient air for 3 days. The thioredoxin was desalted using a Zeba spin desalting column equilibrated with the phosphate buffer. Oxidation of thioredoxin was quantified from the loss of the two catalytic thiols using 1 mM thiol fluorescent probe IV (Calbiochem) and a Fluoroskan Ascent microplate fluorimeter (ex 400 nm; em 465 nm).

The results were expressed as the ratio of reduced to oxidized protein and fitted to Equation 1,

\[
R = \frac{[\text{Reductant}_{\text{red}}]}{[\text{Reductant}_{\text{ox}}]} \frac{[\text{Reductant}_{\text{red}}]}{[\text{Reductant}_{\text{ox}}]} K_{eq}
\]

(Eq. 1)

where \( R \) is the fraction of reduced protein at equilibrium, and \( K_{eq} \) is the equilibrium constant. The standard redox potential (\( E^0_{\text{DTT}} \)) of the Cys224-Cys448 bond was calculated using the Nernst equation (Equation 2),

\[
E^0_{\text{DTT}} = \frac{RT}{2F} \ln K_{eq}
\]

(Eq. 2)

**FIGURE 1. MetAP2 contains a single RH Staple disulfide bond.** A, ribbon structure of human MetAP2 (PDB identifier 1B59) (26). The single Cys228-Cys448 disulfide bond (yellow) is at the rim of the active site, and the Cys228 residue of the bond is only three residues distant from the catalytic His231 (pink). B, close up of the Cys228-Cys448 disulfide bond and catalytic His231. The disulfide bond has a RH Staple configuration and links two β-loop structures. C, evolutionary acquisition of the disulfide bond in MetAP2. The MetAP2 disulfide bond was acquired early in the evolution of the protein. Loss of a cysteine residue in plants was a result of speciation from the metazoans. Among the fungal species, S. cerevisiae lost one cysteine residue in the process of speciation from S. pombe, and the second cysteine was lost during speciation from S. cerevisiae to C. albicans. MetAP1 never acquired both of the corresponding cysteines.
using a value of $-307\ \text{mV}$ for the redox potential of the DTT disulfide bond (22) and $-270\ \text{mV}$ for the redox potential of the thioredoxin disulfide bond (23).

ELISA for the Quantification of Oxidized MetAP2—Alkylated MetAP2 or bovine serum albumin (0.1 mM) was incubated without or with 1 mM TCEP for 1 h at 25 °C, the reduced Cys$^{228}$ and Cys$^{448}$ cysteines were labeled with 1 mM MPB for 30 min at 25 °C in the dark, and the unreacted MPB was quenched with 3 mM reduced glutathione for 10 min at 25 °C. The human glioblastoma U251 cell line was cultured in DMEM containing 10% fetal calf serum (Invitrogen) with or without 5 mM glucose for 24 h or minimum essential medium containing 10% fetal calf serum in normoxic (21% O$_2$, 5% CO$_2$) or hypoxic (1% O$_2$, 5% CO$_2$) conditions for 3 days. Lysate from 4 × 10$^6$ cells was prepared using 50 mM sodium phosphate, pH 7.2, buffer containing 150 mM NaCl, 1% (v/v) Triton X-100, 5 mM PMSF, 0.8 mM aprotinin, 50 mM bestatin, 20 mM leupeptin, and 100 mM iodoacetamide on ice for 30 min. Cell debris was removed by centrifugation, supernatant was incubated with 100 mM N-ethylmaleimide for 30 min at 25 °C in the dark, and the thiol alkylators were removed using a Zeba spin desalting column equilibrated with phosphate-buffered saline. The lysates (300 μg in 50 μl) were incubated without or with 1 mM TCEP for 1 h at 25 °C, the reduced cysteines were labeled with 2 mM MPB for 30 min at 25 °C in the dark, and the unreacted MPB was quenched with 5 mM reduced glutathione for 10 min at 25 °C. The lysates were diluted in phosphate-buffered saline to 1 mg/ml, and 100 μg were used in ELISA.

Maxi-sorp 96-well plates (Nunc) were coated with 100 μl of 2 μg/ml anti-MetAP2 polyclonal antibody D-20 (Santa Cruz) in 100 mM Na$_2$CO$_3$/NaHCO$_3$, pH 9.6, buffer overnight at 4 °C with shaking and blocked with 200 μl of Superblock blocking buffer (Pierce) for 1.5 h at 25 °C with shaking. The samples described above (100 μl) were incubated in wells for 1–2 h at 25 °C with shaking. Wells were washed seven times with Tris-buffered saline containing 0.05% Tween 20 and incubated with 100 μl of a 1:10,000 dilution of streptavidin-peroxidase for 1 h at 25 °C with shaking. Wells were washed seven times, and the
bound peroxidase activity was measured by the addition of 100 µl of 1.25 mm 3,3',5,5'-tetramethylbenzidine (Sigma) and absorbance at 650 nm.

Hydrolysis of MetAP2 Peptide Substrates—Octapeptides derived from the N-terminal sequences of thioredoxin-1, GAPDH, cyclophilin A, and eNOS were synthesized by Mimotopes, and stock concentrations were prepared in DMSO. MetAP2 was reduced by incubating 0.2 µM enzyme with 10 µM thioredoxin in Hepes buffer for 1 h at 25 °C. Untreated or reduced MetAP2 (0.1 µM) was incubated with 1 mm peptides in Hepes buffer, aliquots were sampled at discrete times, and the reaction was quenched with 5% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation for 10 min at 10,000 
 g and 4 °C, the supernatant was adjusted to pH 7–8 by the addition of saturated sodium carbonate, and the samples were diluted with an equal volume of 100 mM sodium tetraborate buffer, pH 9.6. Free methionine was quantified by reverse-phase HPLC following derivatization with o-phthaldialdehyde as described (24). Data analysis was performed using XCalibur Qual Browser software.

RESULTS

MetAP2 Contains a Single —RHStaple Disulfide Bond—MetAP2 contains a single disulfide bond in crystal structures of the enzyme (Fig. 1A), whereas MetAP1 is devoid of disulfide bonds (8). The MetAP2 Cys228-Cys448 disulfide bond is at the rim of the active site, and the Cys228 residue of the bond is only three residues distant from the catalytic His231 (Fig. 1B). The disulfide bond has an —RHStaple configuration in 14 crystal structures (PDB identifiers: 1B59, 1KQ9, 1QZY, 1R58, 1R5G, 1R5H, 1YW7, 1YW8, 1YW9, 2ADU, 2EA2, 2EA4, 2GA2, and 2OAZ) and links two β-loop structures (25), which are common features of allosteric disulfide bonds (14–16). The disulfide bond is conserved in lower eukaryotes, such as S. pombe and multicellular fungal species, and in the metazoans, including all mammals (Fig. 1C).

The MetAP2 Disulfide Bond Is Reduced in a Fraction of the Recombinant Enzyme—The redox state of the Cys228-Cys448 disulfide bond in the recombinant enzyme and its susceptibility to reduction by the strong protein reductant, thioredoxin, was determined using mass spectrometry. Differential cysteine labeling allows for an estimation of the fraction of a disulfide bond that is reduced in the protein preparation. The unpaired Cys228 and Cys448 cysteines were alkylated with iodoacetamide, and the Cys228, Cys448 disulfide bond cysteines alkylated with methyl methanethiol sulfonate following reduction with dithiothreitol. The ratio of iodoacetamide to methyl methanethiol sulfonate labeling represents the fraction of the disulfide in the protein preparation. The unpaired Cys228 cysteine was alkylated with iodoacetamide, and the Cys228-Cys448 disulfide bond was reduced in 20% of MetAP2 molecules in the preparation, which increased to ~70% following incubation of 1 µM enzyme with 10 µM thioredoxin (Fig. 2).

Crystal Structure of Oxidized and Reduced MetAP2—The structure of the oxidized (intact Cys228-Cys448 disulfide bond) and reduced (unpaired Cys228 and Cys448) enzyme were resolved in a study of the enzyme bound to the inhibitors, fumagillin, TNP-470, and ovalicin (26). Crystals of the unliganded enzyme (PDB identifier 1BN5) and the enzyme in complex with fumagillin (PDB identifier 1BOA) contained a mix of oxidized and reduced MetAP2. The oxidized enzyme in complex with ovalicin (PDB identifier 1B59) was solved, whereas the structure of the reduced enzyme in complex with TNP-470 was determined (PDB identifier 1B6A).

The active site residues that accommodate the methionine side chain have been defined in the crystal structure of MetAP2 with L-Met (27). L-Met sits in a hydrophobic tunnel lined by Phe219, Gly222, Ile338, Met384, and Ala414. The oxidized (PDB identifier 1B59) and reduced enzyme (PDB identifier 1BN5) and the enzyme in complex with thioredoxin are shown in Fig. 3 (28). The oxidized enzyme has a single disulfide bond in complex with the inhibitor, fumagillin (PDB identifier 1BOA) contained a mix of oxidized and reduced enzyme in complex with TNP-470 and ovalicin. The structure of oxidized MetAP2 (Fig. 3, A and B) was resolved in complex with ovalicin (PDB identifier 1B59) and reduced enzyme (Fig. 3, C and D) determined (PDB identifier 1BN5).

The redox state of the Cys228-Cys448 disulfide bond was determined by measuring the rate of reduction of the disulfide. MetAP2 (0.1 µM) was incubated with 10 µM thioredoxin or 200 µM diamide for 1 h at 25°C. Reduction of the single disulfide bond by thioredoxin increased the rate of hydrolysis of the tripeptide 2.4-fold (from 23 ± 0.1 to 56 ± 0.3 RFU/min; p < 0.0001 using Student’s paired t test). Oxidation of the disulfide by diamide decreased the rate of hydrolysis of the tripeptide 2.3-fold (from 23 ± 0.1 to 10 ± 0.1 RFU/min; p < 0.05 using Student’s paired t test). Data points and error bars, mean ± S.E. of three independent experiments.

The solid lines are the best least squares fit of the linear phase of the coupled enzyme assay (from 40 min). B, the change in catalytic efficiency of hydrolysis of Met-Gly-Pro-AMC with thioredoxin reduction of the Cys228-Cys448 disulfide bond was used to measure the rate of reduction of the disulfide. MetAP2 (0.1 µM) was incubated with thioredoxin (1 µM) for discrete times, the reaction was quenched with iodoacetamide (10 mM), and the initial rate of hydrolysis of Met-Gly-Pro-AMC was measured as described in A. The solid line represents the best non-linear least square fit of the data to a single exponential. The calculated kobs for the pseudo-first order reaction is 0.97 ± 0.35 min⁻¹. Data points and error bars, mean ± S.E. of three independent experiments.
identifier 1B59) and reduced (PDB identifier 1B6A) enzyme have been aligned with respect to the catalytic His231 (Fig. 3). The Phe219, Gly222, Ile338, Met384, and Ala414 residues that define the L-Met tunnel are displaced slightly in the two structures.

The MetAP2 Disulfide Bond Is Efficiently Reduced by Thioredoxin—Reduction of the MetAP2 Cys228-Cys448 disulfide bond by thioredoxin or oxidation of the dithiol using diamide changes the catalytic efficiency of hydrolysis of a tripeptide substrate (Fig. 4A). Reduction of the single disulfide bond increased the initial rate of hydrolysis of the tripeptide from 23 ± 0.1 to 56 ± 0.3 relative fluorescence units (RFU)/min, whereas oxidation of the dithiol decreased the rate of hydrolysis to 10 ± 0.1 RFU/min. Assuming that the enzyme is fully reduced or oxidized by these treatments, the fraction of reduced enzyme in the untreated MetAP2 is estimated to be 28%. This is in the range of the reduced fraction calculated by mass spectrometry, which is a validation of both measures.

The change in catalytic efficiency of hydrolysis of the tripeptide following thioredoxin reduction of the Cys228-Cys448 disulfide bond was employed to measure the second order rate constant for reduction of the disulfide (Fig. 4B). The k_{obs} for the pseudo-first order reduction of MetAP2 by thioredoxin was 0.97 ± 0.35 min⁻¹, which equates to a rate constant of 16,180 M⁻¹ s⁻¹.

Redox Potential of the MetAP2 Disulfide Bond—The standard redox potential of the Cys228-Cys448 disulfide bond was determined by employing either dithiothreitol or thioredoxin as the reducant. The labeling of fully reduced MetAP2 by the biotin-linked maleimide, MPB, was used to calculate the ratio of reduced to oxidized enzyme as a function of the ratio of reduced (DTT_red) to oxidized (DTT_ox) dithiothreitol. Biotin incorporation was measured by SDS-PAGE and blotting with streptavidin-peroxidase. The plot of the ratio of reduced to oxidized MetAP2 as a function of the ratio of reduced to oxidized DTT. The solid line represents the best non-linear least squares fit of the data to Equation 1. The calculated equilibrium constant is 0.028 (95% confidence interval: 0.012–0.044). From the Nernst equation, the standard redox potential of the Cys228-Cys448 disulfide is −261 mV. Data points and error bars, mean ± S.E. of three independent experiments. C, the initial rate of hydrolysis of Met-Gly-Pro-AMC by fully oxidized or reduced MetAP2 was used to calculate the ratio of reduced to oxidized enzyme as a function of the ratio of reduced (Trx_red) to oxidized (Trx_ox) thioredoxin. The solid line represents the best non-linear least squares fit of the data to Equation 1. The calculated equilibrium constant is 0.740 (95% confidence interval: 0.600–0.688), which equates to a standard redox potential of −266 mV for the Cys228-Cys448 disulfide bond. Data points and error bars, mean ± S.E. of three independent experiments.

FIGURE 5. Redox potential of the MetAP2 disulfide bond. A, the labeling of fully reduced MetAP2 by the biotin-linked maleimide, MPB, was used to calculate the ratio of reduced to oxidized enzyme as a function of the ratio of reduced (DTT_red) to oxidized (DTT_ox) dithiothreitol. B, plot of the ratio of reduced to oxidized MetAP2 as a function of the ratio of reduced to oxidized DTT. The solid line represents the best non-linear least squares fit of the data to Equation 1. The calculated equilibrium constant is 0.028 (95% confidence interval: 0.012–0.044). From the Nernst equation, the standard redox potential of the Cys228-Cys448 disulfide is −261 mV. Data points and error bars, mean ± S.E. of three independent experiments. C, the initial rate of hydrolysis of Met-Gly-Pro-AMC by fully oxidized or reduced MetAP2 was used to calculate the ratio of reduced to oxidized enzyme as a function of the ratio of reduced (Trx_red) to oxidized (Trx_ox) thioredoxin. The solid line represents the best non-linear least squares fit of the data to Equation 1. The calculated equilibrium constant is 0.740 (95% confidence interval: 0.600–0.688), which equates to a standard redox potential of −266 mV for the Cys228-Cys448 disulfide bond. Data points and error bars, mean ± S.E. of three independent experiments.

3 K. M. Cook and P. J. Hogg, unpublished observations.
FIGURE 6. MetAP2 exists in oxidized and reduced states in human glioblastoma cells. A, an ELISA was developed to measure the oxidized fraction of MetAP2. The unpaired cysteine thiol in recombinant MetAP2 were alkylated, the Cys<sup>228</sup>-Cys<sup>448</sup> disulfide bond was reduced with TCEP, and the reduced cysteines were labeled with MPB. The biotin labeling was quantified by capturing the labeled enzyme on antibody-coated wells and incubating with streptavidin-peroxidase. The MPB labeling of reduced MetAP2 was 4.8-fold higher than the labeling of non-reduced enzyme (**, p < 0.0001 using Student’s paired t test). There was no significant signal in the ELISA if control bovine serum albumin was used in place of MetAP2. B, human glioblastoma U251 cells were cultured in medium with or without glucose or under normoxic or hypoxic conditions for 3 days. Cell lysates (80 μg) were resolved on SDS-PAGE and immunoblotted for MetAP2 or GAPDH as a loading control. Molecular size markers in kDa are indicated at the left. C, U251 cells were cultured in medium with or without 5 mM glucose for 3 days. The unpaired cysteine thiols in MetAP2 in cell lysates were alkylated, the Cys<sup>228</sup>-Cys<sup>448</sup> disulfide bond was reduced with TCEP, the reduced cysteines were labeled with MPB, and the biotin incorporation was measured by ELISA as described in A. Background labeling in the absence of TCEP was subtracted from labeling following reduction of MetAP2, which also controlled for any small differences in total MetAP2 in the assay. Glucose deprivation triggered an increase in the oxidized fraction of cellular MetAP2 (**, p < 0.001 using Student’s paired t test). D, U251 cells were cultured under normoxic (21% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) conditions for 3 days. The unpaired cysteine thiols in MetAP2 in cell lysates were alkylated, the Cys<sup>228</sup>-Cys<sup>448</sup> disulfide bond was reduced with TCEP, the reduced cysteines were labeled with MPB, and the biotin incorporation was measured by ELISA as described in A. Hypoxic stress triggered an increase in the oxidized fraction of cellular MetAP2 (**, p < 0.01 using Student’s paired t test). Data points and error bars, mean ± S.E. of 3–4 independent experiments.

MetAP2 Exists in Oxidized and Reduced States in Human Glioblastoma Cells—An ELISA was developed to measure the oxidized fraction of MetAP2 in recombinant enzyme and in tumor cell lysate. Briefly, unpaired cysteine thiol in MetAP2 are alkylated, the Cys<sup>228</sup>-Cys<sup>448</sup> disulfide bond is reduced with TCEP, and the reduced cysteines are labeled with MPB. MPB incorporation is measured by capturing the labeled enzyme on antibody-coated wells and employing streptavidin-peroxidase to detect the biotin. TCEP was used as the reducing agent in this assay because it is a non-thiol-based reductant and thus does not interfere with MPB labeling. The validity of the assay was determined using purified recombinant MetAP2. The MPB labeling of reduced MetAP2 was 4.8-fold higher than the labeling of non-reduced enzyme, and there was no significant signal when an irrelevant control protein was used (Fig. 6A).

We next determined whether the redox state of MetAP2 changes in human glioblastoma cells following two different stresses associated with tumor growth: low nutrient supply in the form of glucose and hypoxia. Glioblastoma is the most common and most malignant brain tumor (29). Human glioblastoma U251 cells were cultured in medium with or without 5 mM glucose or under normoxic or hypoxic conditions for 3 days. These different culture conditions did not change MetAP2 protein levels (Fig. 6B) but did influence the ratio of reduced to oxidized enzyme in the cells. Glucose (Fig. 6C) or oxygen (Fig. 6D) deprivation led to a significant increase in oxidized MetAP2 in the cells (p < 0.01 and p < 0.001, respectively). These results indicate that MetAP2 exists in oxidized and reduced states in tumor cells and that this equilibrium is influenced by relevant cellular stresses.

Oxidized and Reduced MetAP2 Have Different Catalytic Efficiencies for Hydrolysis of Native Peptide Substrates—The MetAP2 Cys<sup>228</sup>-Cys<sup>448</sup> disulfide bond is at the rim of the active site and is nearby the catalytic His<sup>231</sup>. It was anticipated, therefore, that cleavage of the disulfide bond would influence the catalytic activity of the enzyme. This was tested using octopeptides representing the N-terminal residues of the MetAP2 substrates: GAPDH, cyclophilin A, eNOS, and thioredoxin-1. MetAP2 was incubated with thioredoxin to reduce the Cys<sup>228</sup>-
Cys\textsuperscript{448} disulfide bond, and methionine release from the peptides as a function of time was measured by HPLC (Fig. 7). The reduced enzyme cleaved the thioredoxin-1 and eNOS peptides more efficiently than did oxidized enzyme (3.4- and 2.3-fold, respectively) but cleaved the GAPDH and cyclophilin A substrates with an efficiency comparable with that of oxidized enzyme (Table 1). The magnitude of the difference in catalytic efficiency of the oxidized and reduced enzyme preparations is likely to be an underestimate because the untreated enzyme contains \( \sim 20\% \) reduced protein (see Fig. 4A), and thioredoxin may not completely reduce the disulfide in the enzyme preparation (see Fig. 2).

### DISCUSSION

Proteases in nature are generally regulated by protease inhibitors or by restricting the enzymes to certain subcellular compartments. No endogenous inhibitors of the MetAPs have been identified, and they are not restricted to any particular compartment in the cytosol. The results shown herein imply that MetAP2 activity is regulated through chemical modification of its single disulfide bond. This cysteine residue has all of the features of an allosteric disulfide (14-16).

The cysteine residue is characterized by five angles, and different combinations of these angles define 20 possible configurations (30, 31). Three of the 20 disulfide bond configurations are emerging as allosteric configurations: the \(-\text{RHStaple}, -\text{LHHook}, \text{and} -/+\text{RHHook} \) bonds (14). The most common secondary structures linked by allosteric disulfides are \( \beta \)-strands and/or \( \beta \)-loops (14). The MetAP2 Cys\textsuperscript{228}, Cys\textsuperscript{448} disulfide has a \(-\text{RHStaple} \) configuration in 14 crystal structures of the enzyme, the bond links two \( \beta \)-loops, and Cys\textsuperscript{228} of the disulfide is exposed to solvent.

The surface exposure of the MetAP2 disulfide is probably important for access to the bond by the cellular protein reductant, thioredoxin. The thioredoxin/thioredoxin reductase/NADPH redox system is well placed to regulate the redox state of the MetAP2 disulfide in the cytoplasm, and the thermodynamics of the reaction are favorable. The MetAP2 disulfide has a higher redox potential than the thioredoxin catalytic disulfide, \(-264 \text{ versus} -270 \text{ mV} \) (23), and the rate of the reaction is very efficient. For instance, thioredoxin reduces the MetAP2 disulfide 46 times faster than the \( \beta II \)-tryphtase allosteric disulfide bond \( (16,180 \text{ versus} 350 \text{ nmol L-Met/min/\mu g protein}) \) (28). It is interesting that newly synthesized thioredoxin itself is a substrate for MetAP2 (5).

Our results imply that thioredoxin-1 and eNOS are preferred substrates for reduced MetAP2, whereas GAPDH and cyclophilin A are cleaved by either isoform of the enzyme. Cleavage of the initiating methionine in thioredoxin-1, eNOS, GAPDH, and cyclophilin A leads to N-terminal modification of the proteins at the second residue. Thioredoxin-1 is \( N \)-acetylated at Val\textsuperscript{2}, eNOS is \( N \)-myristoylated at Gly\textsuperscript{2}, GAPDH is \( N \)-acetylated at Gly\textsuperscript{2}, and cyclophilin A is \( N \)-acetylated at Val\textsuperscript{2} (11, 32, 33). These modifications can affect localization, activity, and turnover of these proteins and will be regulated by MetAP2 activity and, we suggest, the redox state of the allosteric disulfide. The shift in substrate specificity of MetAP2 with cleavage of the Cys\textsuperscript{228}-Cys\textsuperscript{448} disulfide presumably results from a change in the orientation of the active site pocket. Crystal structures of MetAP2 with methionine (27) and small molecule inhibitors (26) in the active site pocket have been resolved but none so far with a polypeptide substrate. The residues that define the tunnel that accommodates L-Met in the MetAP2 active site are orientated slightly differently in the oxidized versus reduced enzyme. Structures of the enzyme isoforms in complex with, for example, the octapeptides of either eNOS or thioredoxin are required before the structural mechanism of the differential substrate specificity can be defined.

Solid tumor cells variably experience hypoxia, glucose deprivation, and growth factor withdrawal as they adapt to a limiting blood supply. Our results using glioblastoma cells imply that the ratio of oxidized to reduced MetAP2 is controlled in cancer cells by these stresses. Cellular stress leads to enhanced production of reactive oxygen species, some of which (hydrogen peroxide and hypochlorous acid) can oxidize dithiols to a disulfide bond (14). It is possible that the increase in ratio of oxidized to reduced MetAP2 in stressed tumor cells is a consequence of the increased production of reactive oxygen species.

In summary, our findings imply that MetAP2 activity is controlled by reduction of an allosteric disulfide bond rather than by an inhibitor or by compartmentalization. This scenario parallels with the regulation of the mast cell serine protease, \( \beta II \)-tryptase (28). No endogenous inhibitors of \( \beta II \)-tryphtase have been identified, and the secreted enzyme is not restricted to specific extracellular environments. As for MetAP2, \( \beta II \)-tryphtase activity is regulated by cleavage of a disulfide bond. The oxidized and reduced isoforms of \( \beta II \)-tryphtase have different specificity and catalytic efficiency for hydrolysis of substrates. It may be that cleavage of an allosteric disulfide bond is a mechanism of regulation of other enzymes that are not controlled by the conventional means.

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