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Effects of Gigapascal Level Pressure on Protein Structure and Function

Wei-Qiang Chen,[†] Gunter Heymann,[‡] Petri Kursula,[§,∥] Margit Rosner,[⊥] Markus Hengstschläger,[⊥] Hubert Huppertz,[‡] and Gert Lubec*[,†]

[†]Department of Pediatrics, Medical University of Vienna, Währinger Gürtel 18, 1090 Vienna, Austria

[‡]Institute of General, Inorganic and Theoretical Chemistry, University of Innsbruck, Innrain 52; 6020 Innsbruck, Austria

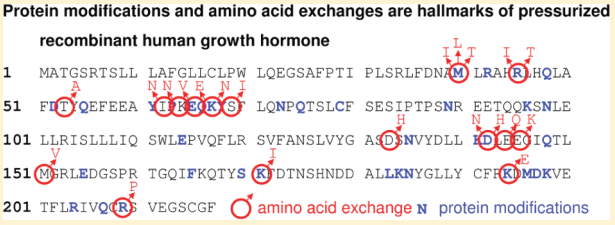
[§]Department of Biochemistry, University of Oulu, PL3000; 90014 Oulu, Finland

[∥]Centre for Structural Systems Biology (CSSB-HZI), German Electron Synchrotron (DESY), Notkestraße 85, 22607 Hamburg, Germany

[⊥]Institute of Medical Genetics, Medical University of Vienna, Währinger Straße 10, 1090 Vienna, Austria

Supporting Information

ABSTRACT: Information on very high pressure (VHP) effects on proteins is limited and therefore effects of VHP on chemistry, structure and function of two model proteins in medical use were studied. VHP (8 GPa) application to L-asparaginase (L-ASNase) resulted in faster mobility on clear native gels. VHP induced generation of lower-MW forms of L-ASNase but VHP treatment did not deteriorate asparaginase activity. Electrophoretic patterns in native and denaturing gels were comparable for untreated and pressurized recombinant human growth hormone (rhGH). rhGH function, however, was deteriorated as shown by a bioassay. In L-ASNase and rhGH a series of protein modifications and amino acid exchanges (indicating cleavage of covalent bonds) were revealed that may probably lead to functional and conformational changes. The findings have implications in protein chemistry, structure, and function and are useful for designing biotechnological applications of protein products.



INTRODUCTION

Application of high pressure on the order of hundreds of MPa (HP) on food,[1−8] complex mixtures,[9] crystals,[10,11] as well as viruses and bacteria[12−15] have been described. Pressurization is of relevance for industry, stabilizing food, dissociating aggregates[16] from complex mixtures such as micelles[17−19] and fibrils,[20] melting crystals, and killing microorganisms but also for developing new ways for synthetic discoveries.[21] While application of HP on organisms and complex mixtures is mainly relevant for applied science, pressurizing individual proteins may focus on direct HP effects, ruling out interactions with other molecules.

Kalbitzer and co-workers showed that 200 MPa pressurization of the histidine containing protein from *Staphylococcus carnosus* led to conformational changes and intramolecular effects, as the four stranded beta-pleated sheet of this protein is the least compressible part of the structure.[22] Infectious prion proteins show pressure-sensitive and pressure-insensitive epitopes as detected by resistance against proteinase K treatment.[23] Floriano et al. revealed that HP-induced metmyoglobin unfolding of the alpha-helices follows the sequence of migrating hydrogen bonds.[24] Iwasaki and Yamamoto[25] observed HP-induced global structural changes in the S1 fragment of myoglobin at about 150 MPa and local structural changes of binding sites for ATPase and actin at higher pressures up to 300 MPa. Using fluorescence line-narrowing spectroscopy, vibrational frequencies shifted with pressure increase and indicated that horseradish peroxidase is less compressible at higher pressures.[26] Comparing the intrinsic compressibility of five proteins by molecular dynamics simulations indicated differences between volume fluctuations and internal cavity formation.[27] Arnold and co-workers[28] showed that ^{1}H-NMR detected HP-induced chemical shifts in a tetrapeptide as observed for the backbone amide protons. 150 MPa was sufficient to irreversibly change the quaternary structure of tetrameric urate oxidase; at this pressure, the tetrameric nature was still preserved in the soluble fraction, another fraction aggregated, but activity was decreasing until it was completely lost at 200 MPa. The pressurized protein showed expansion of its substrate binding pocket at the expense of a large neighboring hydrophobic cavity. X-ray crystallography, small-angle X-ray scattering (SAXS) and fluorescence spectroscopy were used for these experiments.[29] Studies on HP effects on pectin methylesterase using Fourier transform infrared (FTIR) spectroscopy showed that 1 GPa was necessary to induce changes in the enzyme secondary structure and revealed unusual pressure stability of beta-helices.[20] Pressure-induced denaturation and conformational loss depend on the oligomeric state of beta-lactalbumin and is partially reversible up to 300 MPa.[30] FTIR spectroscopy was also used to study

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lipoxygenase with HP, and changes in the amide I band suggested structural changes in the protein.[31]

There is limited information on effects of very high pressure (VHP) on proteins: Using HP infrared spectroscopy on human serum albumin, Smeller and co-workers observed that it was completely unfolded at 1 GPa.[32] The strongly hydrophobic integral membrane proteins from photosynthetic bacteria were losing secondary and tertiary structure at 2.5 GPa.[33] Studies on the active site of deoxyhemoglobin and myoglobin revealed that pocket collapse was observed at 3.4 GPa.[34] Reversibility of VHP effects on pancreatic trypsin inhibitor was studied using FTIR spectroscopy: 1.5 GPa induced changes in the secondary structure of this enzyme, but this was proposed to be reversible.[35]

Hemley reported the effects of HP on molecules in an excellent review article.[36] The free-energy charge (pressure−volume work) associated with the high degree of compression (compression on the energetics of electrons) can be in excess of 10 eV, which exceeds the strength of the strongest molecular bond. Therefore, one may realistically expect that protein conformation and function can be significantly affected by very high pressure.

Although HP effects on proteins are well-documented, information on effects of VHP on proteins is limited. Moreover, no data on protein primary structure, i.e., protein sequence, modifications, and post-translational modification changes, have been reported so far. It was, therefore, the aim of the study to pressurize two native proteins, rhGH and L-ASNase, under VHP and to determine changes of primary structure, amino acid exchanges, changes of modifications, and post-translational modifications, degradation as well as functional properties. Chemical and functional changes were observed, indicating irreversible changes induced by very high pressure.

EXPERIMENTAL SECTION

Human growth hormone [rhGH (Nutropin, Genentech, Inc., South San Francisco, CA, USA)] is a recombinant protein expressed in *E. coli* and was purchased. rhGH was dialyzed into 10 mM phosphate buffer pH 7.0 overnight at 4 °C. The dialyzed rhGH was snap frozen in liquid nitrogen and lyophilized overnight. L-Asparaginase from *Erwinia chrysanthemi* (Erwinase; L-ASNase) was purchased in lyophilized powder form (EUSA Pharma, Oxford, U.K.), containing sodium chloride and glucose monohydrate.

Very High Pressure Treatment of Lyophilized Proteins Using the Multianvil Technique. Approximately 10 mg of the lyophilized proteins were pressurized under the following conditions. The boron nitride sample container is cylindrical, 5 mm high and 3 mm in diameter. Inside this BN crucible, protein samples were wrapped using a gold foil with the remaining space filled up with MgO. The synthesis pressure was 8 GPa (18/11 multianvil assembly[37,38]) and pressurization time was 250 min; subsequently, the pressure was decreased within 750 min to ambient pressure. Experiments were carried out in triplicate.

Native Polyacrylamide Gel Analysis (Native PAGE). Protein samples (rhGH 4.5 μg and L-ASNase 2 μg) were mixed with loading buffer (125 mM Tris−HCl, 20% glycerol, 0.2% (w/v) bromophenol blue, pH 6.8) and subsequently loaded onto wells of 10% (for rhGH) or 8% (for L-ASNase) native-PAGE. Electrophoresis was performed at a constant voltage of 50 V for 30 min and then 100 V for 2 h using a running buffer (25 mM Tris and 192 mM glycine, pH 8.3). Native protein markers (Invitrogen, Carlsbad, CA, U.S.A.) were loaded to one well. Immediately after electrophoresis, gels were fixed for 12 h in 50% methanol, containing 10% acetic acid, and the gels were stained with Colloidal Coomassie Blue (Invitrogen, Carlsbad, CA, U.S.A.) for 12 h on a rocking shaker. Excess of dye was washed out from the gels with distilled water and the gels were scanned with ImageScanner (Bioscience, Uppsala, Sweden).

SDS PAGE Analysis. Protein samples in the amount as given above were mixed with loading buffer (125 mM Tris−HCl, 20% glycerol, 2% SDS, 0.2% (w/v) bromophenol blue, pH 6.8) and subsequently loaded into wells of 12% SDS-PAGE. Electrophoresis was performed at a constant voltage of 50 V for 30 min and then 100 V for 2 h using a running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3). Standard protein markers (Bio-Rad Laboratories, Hercules, CA, U.S.A.) covering the range 10−250 kDa were loaded onto one well. The gels were stained and documented as described above.

Mass Spectrometrical Analysis by Nano-LC-ESI-CID/ETD-MS/MS. Nano-LC-ESI-CID/ETD-MS/MS analysis was performed essentially as reported.[39] Gel bands of interest were cut, put into a 1.5-mL tube, and washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate repeatedly. Addition of acetonitrile resulted in gel shrinking and the shrunk gel plugs were then SpeedVac-dried in a Speedvac Concentrator 5301 (Eppendorf, Germany). The dried gel pieces were reswollen and in-gel digested with 40 ng/mL trypsin (Promega, Madison, WI, U.S.A.) in digestion buffer (consisting of 5 mM octyl-b-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) overnight at 37 °C. Chymotrypsin digestion was performed by addition of 25 mM ammonium bicarbonate containing 25 ng/mL chymotrypsin (sequencing grade; Roche Diagnostic, Mannheim, Germany) for 30 min at 30 °C. AspN (Roche Diagnostics) digestion was done in 25 mM NH_4HCO_3 (pH 7.8) at 37 °C overnight. Peptide extraction was performed with 15 μL of 1% formic acid (FA) in 5 mM OGP for 30 min, 15 μL 0.1% FA for 30 min and 15 μL 0.1% TFA in 20% acetonitrile for 30 min. The extracted peptides were pooled for nano LC−ESI-CID/ETD-MS/MS analysis. The HPLC used was an Ultimate 3000 system (Dionex Corporation, Sunnyvale, CA, U.S.A.) equipped with a PepMap100 C-18 trap column (300 μm × 5 mm) and PepMap100 C-18 analytic column (75 μm × 150 mm). The gradient was (A = 0.1% formic acid in water, B = 0.08% formic acid in acetonitrile) 4−30% B from 0 to 105 min, 80% B from 105 to 110 min, 4% B from 110 to 125 min. A HCT ultra ETD II (Bruker Daltonics, Bremen, Germany) was used to record peptide spectra over the mass range of m/z 350−1500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100−2800. Repeatedly, MS spectra were recorded followed by three data-dependent CID MS/MS spectra and three ETD MS/MS spectra generated from three highest intensity precursor ions. An active exclusion of 0.4 min after two spectra was used to detect peptides of low abundance. The voltage between ion spray tip and spray shield was set to 1500 V. Drying nitrogen gas was heated to 150 °C and the flow rate was 10 L/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany). Searches were done by using the Mascot 2.3.02 (Matrix Science, London, U.K.) against latest UniprotKB

database for protein identification. Searching parameters were set as follows: enzyme selected as corresponding enzyme with two maximum missing cleavage sites, species limited to human (rhGH) or bacteria (L-ASNase), a mass tolerance of 0.2 Da for peptide tolerance, 0.2 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and variable modification of methionine oxidation and phosphorylation (Tyr, Thr, and Ser). Positive protein identifications were based on a significant MOWSE score. After protein identification, an error-tolerant search was done to detect unspecific cleavage and unassigned modifications. Protein identification and modification information returned were manually inspected and filtered to obtain confirmed protein identification and modification lists.

PTM searches were done using Modiro software with following parameters: enzyme selected as used with two maximum missing cleavage sites, a peptide mass tolerance of 0.2 Da for peptide tolerance, 0.2 Da for fragment mass tolerance, modification 1 of carbamidomethyl(C) and modification 2 of methionine oxidation. Searches for unknown mass shifts, for amino acid substitution and calculation of significance were selected on advanced PTM-explorer search strategies. A list of 172 common modifications including phosphorylation and hydroxylation was selected and added to virtually cleaved and fragmented peptides searched against experimentally obtained MS/MS spectra. Positive protein identification was first of all listed by spectra view and subsequently, the significance of each identified peptide was considered based on the ion-charge status of peptide, b- and y-ion fragmentation quality, ion score (>200) and significance scores (>80). Protein identification and modification information returned were manually inspected and filtered to obtain confirmed protein identification and modification lists.

Activity Assay for rhGH. GH activity measurements were based upon previous work.[40,41] N1E-115 mouse neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, CRL-2263) and were grown in Dulbecco's modified Eagle's medium (DMEM) at 4.5 g/L glucose, supplemented with 20% calf serum and antibiotics (30 mg/L penicillin, 50 mg/L streptomycin sulfate) at 37 °C and 5% CO_2. For assessing induction of mitogen-activated protein kinase (MAPK) signaling, cells were starved in serum-free medium for 16 h and treated with 2 μg/mL rhGH (pressurized and nonpressurized, in duplicate) or 100 ng/mL phorbol myristate acetate (PMA, Calbiochem, Darmstadt, Germany) for another 20 min.

For protein extraction and immunoblotting, extracts of cellular total protein were prepared by physical disruption of cell membranes by repeated freeze−thaw cycles. Briefly, cells were washed with PBS and harvested by trypsinization. Pellets were washed twice with ice-cold PBS and lysed in buffer A (20 mM HEPES (pH 7.9), 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.3 μg/mL benzamidine chloride, 10 μg/mL trypsin inhibitor) by freezing and thawing. Supernatants were collected by centrifugation at 20,000 × g for 20 min at 4 °C and stored at −80 °C. Denatured samples were resolved by 10% SDS-PAGE and transferred to nitrocellulose. For immunodetection, antibodies specific for the following proteins were used: p44/42 MAPK (Erk1/2) Thr202/Tyr204 (clone E10, Cell Signaling, #9106), p44/42 MAPK (Erk1/2) (Cell Signaling, #9102) and α-tubulin (DM1A, Calbiochem, #CP06). Rabbit polyclonal antibodies were detected using HRP-linked goat antirabbit IgG (A120-101P, Bethyl Laboratories), and mouse monoclonal antibodies were detected using HRP-linked goat antimouse IgG (A90−116P, Bethyl Laboratories). The signals were visualized with the enhanced chemiluminescence method (Pierce, Thermo Fisher Scientific, Rockford, IL, U.S.A.).

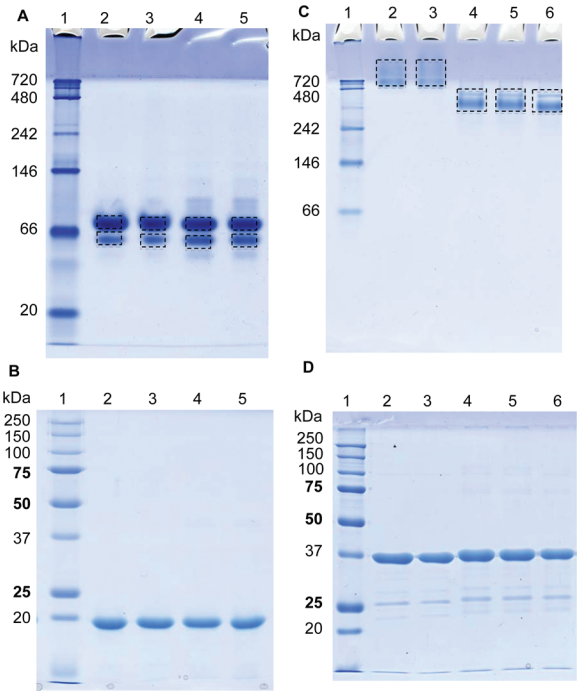

Figure 1. Native PAGE and SDS PAGE analysis of rhGH and L-ASNase before and after pressurization. (a) The 10% native PAGE was used for rhGH. Lane 1 is marker; lanes 2 and 3 are rhGH before pressurization; lanes 4 and 5 are rhGH after pressurization. The protein amount loaded in each lane is 4.5 μg. The bands at the position of the boxes are excised, enzymatic digested and analyzed on mass spectrometry. (b) The 12% SDS PAGE analysis of rhGH. Lane 1 is marker; lanes 2 and 3 are rhGH before pressurization; lanes 4 and 5 are rhGH after pressurization. The protein amount loaded in each lane is 4.5 μg. (c) The 8% native PAGE was used for L-ASNase. Lane 1 is marker; lanes 2 and 3 are L-ASNase before pressurization; lanes 4−6 are L-ASNase after pressurization. The protein amount loaded in each lane is 2 μg. The bands at the position of the boxes are excised, enzymatic digested and analyzed on mass spectrometry. (d) The 12% SDS PAGE analysis of L-ASNase. Lane 1 is marker; lanes 2 and 3 are L-ASNase before pressurization; lanes 4−6 are L-ASNase after pressurization. The protein amount loaded in each lane is 2 μg.

Activity Assay for Asparaginase. An asparaginase-activity test kit (medac Asparaginase-Aktivitäts-Test MAAT; Medac/Amomed Pharma, Vienna, Austria) was used to measure the activity of L-ASNase samples. Samples were diluted to around 10 ng/20 μL and added to 96-well plates. 20 μL of control and calibrator were also added to the corresponding wells. After addition of 20 μL substrate, the plate was gently mixed and incubated at 22 °C for 1 h. 100 μL Chromogen was added into each well and the plate was gently mixed and incubated at 22 °C for 1 h. The absorbance at 705 nm was recorded, and the activity of asparaginase (arbitrary units of asparaginase activity/mg) was calculated. For partial enzyme kinetics, a series of substrate from 5 to 40 μL were used.

RESULTS

On native PAGE, no differences in the electrophoretic mobility and patterns for untreated and pressurized rhGH were

Table 1. Sequence Conflicts of rhGH and L-ASNase before and after Pressurization Revealed by Mascot Error Tolerant Search and Modiro Search

	Mascot		Modiro	
protein	untreated	pressurized	untreated	pressurized
rhGH[a]	Y61S, K64E, S69G, D173N, R193G, D195G	M40T, M40L, R45T, R45I, T53A, K64N, E65 V, K67N, K67E, S69I, D133H, E145K, M151 V, K171I, K194E	D52H, A60E, N73I, Y169F, N185I, F192Y	M40I, I62N, D142N, D142H, E144Q, M151 V, K194E, R209P
L-ASNase[a]	I37N, G44D, V64G, A71G, E76G, S79P, L94Q, R104K, V111M, D117G, M174 V, E202G, G303R, D308A, E310K	K24N, L25W, A42D, M42T, G49C, V67G, L92P, S95W, D106N, D108N, I112N, F125 V, K131E, A150G, M174L, G207R, V208D, K240T, R296T, S318G, A331S, P338A	S83N, M154 V, M174 V M271I, A324E, M329 V, Y344F	F78 V, E84K, I102N, S149A, D208H, I259N, Y268D, M271T, I304M, L311M, L328R
rhGH[b]	A26G, R45G, Y61S, K64E, S69G, C79G, C79S, W112G, R160H, D173N, S176P, R193G, D195G, R204G	A26G, M40T, M40L, R45T, R45I, R45G, T53A, K64N, E65 V, K67N, K67E, S69I, C79S, C79G, W112G, D133H, E145K, M151 V, R160H, K171I, S176P, K194E, R204G	L41R, D52H, A60E, N73I, I84N, D133H, E155G, Y169F, K171E, D173N, A181E, K184E, N185I, F192Y	M40I, L41R, I62N, S69L, I84N, D133H, D142N, D142H, E144Q, M151 V, E155G, D173N, A181E, K184E, K194E, R209P
L-ASNase[b]	V29E, I37N, G44D, V64G, A71G, E76G, S79P, D89G, L94Q, R104K, V111M, D117G, M142T, V173D, M174 V, E202G, Y268D, M271T, R285M, G303R, D308A, E310K, M329 V, M329T, T333I	K24N, L25W, V29E, A42D, M42T, G49C, V67G, D89G, L92P, S95W, D106N, D108N, I112N, F125 V, K131E, M142T, A150G, V173D, M174L, G207R, V208D, K240T, Y268D, M271T, R285M, R296T, S318G, M329 V, M329T, A331S, T333I, P338A	I30N, I37N, D58H, I61N, K69E, S79C, S83N, I112N, M154 V, M174 V, D196H, I266R, M271I, D308H, A324E, M329 V, Y344F	I30N, I37N, D58H, I61N, K69E, F78 V, S79C, E84K, I102N, I112N, S149A, D196H, D208H, I259N, I266R, Y268D, M271T, I304M, D308H, L311M, L328R, Y344F

[a] The same conflicts found in untreated and pressurized proteins are omitted. [b] All sequence conflicts revealed by Mascot error tolerant search and Modiro search.

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Table 2. Modifications of rhGH and L-ASNase before and after Pressurization Revealed by Mascot Error Tolerant Search and Modiro Search

	Mascot		Modiro	
protein	untreated	pressurized	untreated	pressurized
rhGH[a]	Acetylation: E91	Amidation: Y61, F165, L183	Dihydroxylation: Y190, R193	Deamidation: R45, N73, Q75, Q148, N185, R204, R209
	Amidation: L41, F57	Carbamidomethylation: R42, D52, K67, E114, D142, E155, K184, D197, K198	Hydroxylation: D156, D197, K198	Dihydroxylation: M40
	Biotinylation: K96	Carbamylation: M196		Hexosamine: N89
	Carbamidomethylation: E82, D133, D180, D195	Carboxymethylation: R45, E65, K67, C79		Hydroxylation: K67, K194
	Carbamylation: D195	Deamidatioan: Q48, Q55, Q66, N73, N89, N98, N135, Q207		
	Deamidation: R45	Dimethylation: N98		
	Dioxidation: F192	Ethanedithiolation: S170		
	Gln→pyro-Glu: Q55, Q167	Formylation: K141		
	Piperidine: M196	HexN: K96		
	SMA: E114	Maleimide: C79, K171		
		Oxidation: M40, Y68, D142		
		Pro→pyro-Glu: P63		
L-ASNase[a]	Amidation: R104, Q167, M271	Acetylation: V119	2-amino-3-oxo-butanoic_acid: Y268	Alpha-amino adipic acid: K339
	Biotinylation: T188	Amidation: Y50, K51, T116, L195, Y215, Y216, Y344	Acetylation: S79, S83	Carboxyethyl: K69
	Carbamidomethylation: K51, K74, K109, A147	Amino: Y205	Amino: Y215	Deamidation: R97, R161, N201, Q248, Q260, N320, R325
	Carbamylation: R143, R325	Biotinylation: K189	Deamidation: Q46, R334	Dihydroxylation: Y268
	Deamidation: N80, R180, N201	Carbamidomethylation: E66, D89, D179, E202, D249, K264, G265, I266, A273, D317, H323	Dihydroxylation: M271	GlyGly: K51
	Dioxidation: F345	Carbamylation: A269, M271, M329	Dimethylation: P144, P153, P306, P307, *P*312, *P*321	Hexose: K51, T59, N62, T129, K131, T188, K189, Y215, K222, K240, K264
	Glu: E66	Deamidation: N27, Q77, N191, R213, N218, Q248, N320, R325,	GlyGly: K68	HexNAc2: N201
	GlyGly: K68, K339	Decanoyl: T33, T36	Hydroxymethyl: N80	Hydroxylation: D89, P144,*P*312
	Hexose: L70, K198	Dioxidation: Y205	Nitro: Y268	Palmitoylation: S40
	Menodione: K199	Ethylation: E66		
	Oxidation: P144, Y216, R325	Formylation: T33		
		GlyGly: T188, T194, T300, S318		
		HexNAc(1)dHex(1): N201		

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Table 2. Continued

protein	Mascot		Modiro	
	untreated	pressurized	untreated	pressurized
		Hexose: K24, G44, K51, K68, K74, K93, K134, R143, K166, K189, K199, K222, K286, K290 Pro→pyro-Glu: P65, P135 Propargylamine: T118, A257 Propionyl: P153 Thioacyl: L311 TMAB: I30		
rhGH[b]	Acetylation: E91	Amidation: Y61, L78, F165, L183	Biotinylation: K194	Biotinylation: K194
	Amidation: L41, F57, L78	Ammonia-loss: C191, C208	Deamidation: Q48, Q55, R90, R103, N135, N175, N178, R193	Deamidation: R45, Q48, Q55, N73, Q75, R90, R103, N135, Q148, N175, N178, N185, R193, R204, R209
	Ammonia-loss: C191, C208	BHTOH: K184	Dihydroxylation: M151, S176, Y190, R193	Dihydroxylation: M40, Y61, P63, K64, M151,S176
	BHTOH: K184	Carbamidomethylation: R42, R45, L46, H47, D52, Q55, E56, E58, E59, K64, E65, K67, K96, E100, E114, D142, E144, E155, K166, K171, D173, H177, D179, K184, K194, D197, K198	GlyGly: K64, K67, K96, K171, K184	GlyGly: K64, K67, K96, K166, K171, K184
	Biotinylation: K96	CarbamidomethylDTT: C191	Hydroxylation: D156, N178, D179, D197, K198	Hexosamine: N89
	Carbamidomethylation: R45, L46, H47, Q55, E56, E58, E59, K64, E65, E82, K96, E100, D133, E144, K166, K171, D173, H177, D179, D180, K194, D195,	Carbamylation: M40, M151, M196	Monoglutamyl: E58, E59	Hydroxylation: K67, D138, K141, N178, D179, K194
	CarbamidomethylDTT: C191	Carboxymethylation: R45, E65, K67, C79	Oxidation: M40, M151, M196	Monoglutamyl: E58, E59
	Carbamylation: M40, M151, D195	Deamidatioan: Q48, Q55, Q66, N73, N89, Q95, N98, N125, N135, Q163, N175, N178, N185, Q207		Oxidation: M40, M151, M196
	Deamidation: R45, Q95, N125, Q163, N175, N178, N185	Dimethylation: N98		
	Dioxidation: M151, F192	Dioxidation: M151		
	Gln→pyro-Glu: Q55, E56, E91, Q167	Ethanedithiolation: S170		
	Hexose: K96	Formylation: K141		
	Octanoylation: S81	Glu→pyro-Glu: E56, E91		

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Table 2. Continued

protein	Mascot		Modiro	
	untreated	pressurized	untreated	pressurized
	Oxidation: R42, M151, R153, D195, M196, D197	HexN: K96		
	Piperidine: M196	Hexose: K96		
	Propargylamine: R90	Maleimide: C79, K171		
	SMA: E114	Octanoylation: S81		
		Oxidation: M40, R42, Y68, D142, M151, R153, D195, M196, D197		
		Pro→pyro-Glu: P63		
		Propargylamine: R90		
L-ASNase[b]	Amidation: R104, Q167, F198, M271	Acetylation: V119	2-amino-3-oxo-butanoic_acid: Y268	Acetylation: S149
	Biotinylation: T188	Amidation: Y50, K51, T116, L195, F198, Y215, Y216, Y344	Acetylation: S79, S83	Alpha-amino adipic acid: K339
	Carbamidomethylation: A22, K51, D58, K68, K69, K74, E76, S79, K93, K109, H114, H127, K131, D133, A147, D151, K189, A200, K222, A269, I304, K339, E343, H346,	Amino: Y205	Amino: Y205, Y215, Y268	Amino: Y205, Y268
	Carbamylation: M142, R143, M174, R325	Biotinylation: K189	Deamidation: Q46, N62, R143, N302, R334	Carbamylation: K189
	Deamidation: Q46, N62, N80, Q96, N99, R180, N201, N212, N302	Carbamidomethylation: A22, D58, E66, K68, K69 E76, S79, D89, K93, H114, H127, K131, D133, D151, D179, K189, A200, E202, K222, D249, K264, G265, I266, A269, A273, I304, D317, H323, K339, E343, H346	Dihydroxylation: Y205, M271	Carboxyethyl: K69
	Dioxidation: F345	Carbamylation: M142, M174, A269, M271, M329	Dimethylation: P144, P153, P306, P307, *P*312, *P*321	Deamidation: N62, R97, R143, R161, N191, N201 Q248, Q260, N302, N320, R325
	Glu→pyro-Glu: E121	Deamidation: N27, Q46, N62, Q77, Q96, N99, N191, N212, R213, N218, Q248, N302, N320, R325,	GlyGly: K68, K189, K339	Dihydroxylation: Y205, Y268
	Glu: E66	Decanoyl: T33, T36	Hexose: K68, K69, K74, N80, K339	GlyGly: K51, K189, K339
	GlyGly: T36, K68, K189, K339	Dioxidation: Y205	Hydroxylation: N80, N85, P153, N201	Hexose: K51, T59, N62, K68, K69, N72, K74, T87, K93, R104, T129, K131, T188, K189, T194, K199, N201, Y215, K222, T236, K239, K240, K264, K286, K290, T335, K339
	Hexose: A22, K69, L70, K131, K198, R213, K240, K339,	Ethylation: E66	Hydroxymethyl: N80	HexNAc2: N201

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Table 2. Continued

protein	Mascot		Modiro	
	untreated	pressurized	untreated	pressurized
	Menodione: K199	Formylation: T33	Nitro: Y268	Hydroxylation: N80, N85, D89, P144, D151, P153, N201, K286, *P*312
	Oxidation: N80, M81, N85, M86, M142, R143, P144, D151, P153, M154, N155, Y205, Y216, M271, R325, M329	Glu→pyro-Glu: E121	N-Acetylhexosamine: N80	Oxidation: M81, M86, M142, M154, M174, M271, M288, M329
		GlyGly: T36, T188, K189, T194, T300, S318	Oxidation: M81, M86, M142, M154, M174, M271, M329	Palmitoylation: T33, T36, S40
		HexNAc(1)dHex(1): N201	Pamitoylation: T33, T36	
		Hexose: A22, K24, G44, K51, K68, K69, K74, K93, K131, K134, R143, K166, K189, K199, R213, K222, K240, K286, K290, K339		
		Oxidation: R25, M42, R43, P53, M54, N80, M81, N85, M86, M142, R143, D151, P153, M154, N155, M174, N201, Y205, M271, M288, *P*321, M329,		
		Pro→pyro-Glu: P65, P135		
		Propargylamine: T118, A257		
		Propionyl: P153		
		Thioacyl: L311		
		TMAB: I30		

[a] The same modifications found in untreated and pressurized proteins are omitted. [b] All modifications revealed by Mascot error tolerant search and Modiro search.

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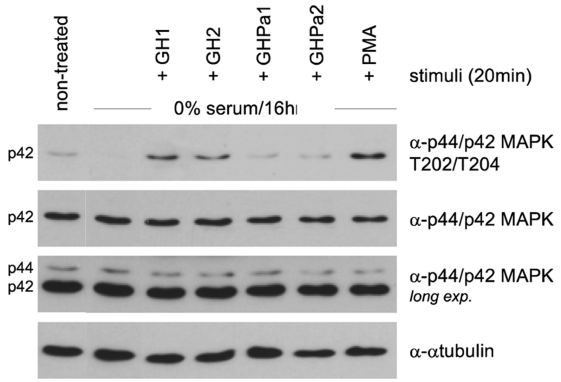

Figure 2. Activity assay of rhGH before and after pressurization. Induction of MAPK signaling pathway upon treatment with pressurized and nonpressurized forms of rhGH. N1E-115 neuroblastoma cells were starved overnight (0% serum for 16 h) and stimulated with 2 μg/mL growth hormone (GH, nonpressurized or GHPa, pressurized) for 20 min. GH1 represents original GH obtained from the pharmacy, GH2 is GH1 changed to phosphate buffer, pH 7.0, lyophilized and dissolved again. GHPa1 and GHPa2 represent pressurized forms of GH2 and are duplicates. Protein extracts were prepared and analyzed for phosphorylated and total p44/42 (Erk1/2) via immunoblotting. Cells treated with the MAPK inducer PMA (100 ng/mL) or cells that were left untreated were analyzed in parallel.

observed (Figure 1a), while untreated L-ASNase presented with several bands at an apparent molecular weight of about 720 kDa and higher. Following pressurization at 8 GPa, a different pattern was observed; bands between 480 and 242 kDa of apparent molecular weight are present (Figure 1c).

Neither rhGH nor L-ASNase showed changes of pattern and electrophoretic mobility on SDS-PAGE (Figure 1b,d), indicating no cleavage of the polypeptide chain occurred during pressurization.

Mass spectrometrical analysis from both proteins was carried out from in-gel digested bands with trypsin, chymotrypsin and AspN and proteins were unambiguously identified.

The total sequence coverages for untreated and treated samples were higher than 90% and detailed information for trypsin, chymotrypsin and AspN peptides, peptide sequences, mass error, ion score are provided in supplemental Table 1.

All peptides were assigned to rhGH or L-ASNase.

Mascot error tolerant search as well as Modiro searches revealed the presence of a series of amino acid exchanges (Table 1).

Searches were conflicting due to different algorithms used. The modified peptides are listed in the Supporting Information, Tables 2 and 3.

A series of protein modifications or post-translational modifications were observed in untreated samples and additional modifications showed up in pressurized samples that are shown in Table 2. These were also different when the two individual search tools were applied. Modifications of peptides are listed in the Supporting Information, Tables 2 and 3.

Differences between untreated and pressurized proteins showed deamidation, dihydroxylation, hydroxylation, methylation, trimethylation, hexosaminidation in rhGH (Table 2). Differential modifications for L-ASNase were deamidation, dihydroxylation, hydroxymethylation, methylation, dimethylation, 2-amino-3-oxo-butanoic acid, alpha-amino adipic acid, carboxyethylation, acetylation, amination, nitration, glycyl−glycyl (non-ubiquitination-related) formation, hexosylation, HexNAc2 and palmitoylation.

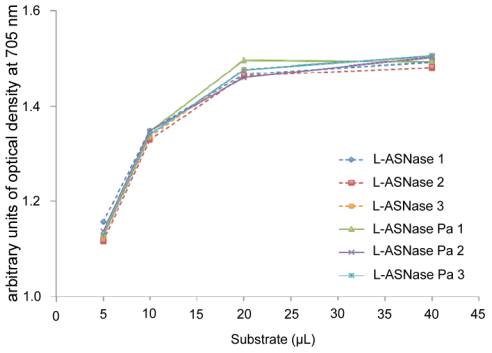

Figure 3. Enzyme kinetics of asparaginase before and after pressurization. There are no visible changes in the enzyme kinetics before and after pressurization. The activity of L-ASNase samples were measured using 10 ng of substrate in 20 μL. Triplicates were carried out.

Representative spectra of HexNAc2, palmitoylation, hexosamine are provided in the Supporting Information, Figure 1.

A series of miscleavages were observed in untreated and pressurized samples and these are listed in the Supporting Information, Table 4.

Activity Assays. *Determination of rhGH Activity.* As shown in Figure 2, alpha-p44/p42 MAPK was not activated in the control without rhGH and in two pressurized rhGH samples. Untreated rhGH and the known inducer of phosphorylation (PMA) generated alpha-p44/p42 MAPK threonine phosphorylation at T202 and T204. Thus, pressurization renders growth hormone inactive in this assay.

Determination of L-ASNase Activity. The Supporting Information, Table 5, shows that there were no differences between untreated and pressurized L-ASNase samples. The activity of L-ASNase before pressurization (10657 ± 407 asparaginase activity units/μg) and after pressurization (10607 ± 956 asparaginase activity units/μg) are essentially identical. At different substrate volumes, the measured activity of untreated and pressurized L-ASNase was similar, indicating preserved enzyme activity following VHP treatment (Figure 3).

DISCUSSION

Life in deep sea is compatible with enormous pressure loads that are in the order of 0.1 GPa.[42] Furthermore, some bacterial spores can survive pressure treatments of 1.4 GPa,[43] and *E. coli* can rapidly evolve to stand 2 GPa.[44] Here, we studied VHP-induced protein changes at the molecular and functional level in two pharmaceutical model proteins: human growth hormone and *E. chrysanthemi* L-asparaginase. Following application of 8 GPa to the two proteins, sequence conflicts representing amino acid exchanges, miscleavages against trypsin activity, protein modifications, as well as functional changes were unambiguously observed.

The pressurization of rhGH deteriorated its activity in a cell-based assay, while electrophoretic analysis indicated no significant differences to the nontreated samples. Protein modifications and amino acid exchanges may also contribute to the inhibition of rhGH function because they are in important functional domains of the protein. The functional role of protein modifications may be also reflected by the finding of several miscleavages, a fact that has been already shown to occur following HP treatment and may be due to steric hindrance of proteolytic cleavage by modifications.[45] A series of protein modifications

may be responsible for impaired function of rhGH: Amino acids of the growth hormone receptor binding site 1 (Supporting Information, Table 6) showed hydroxylation and this may cause impaired rhGH binding to its receptor. Other modifications may also affect rhGH function either directly or by allosteric effects.

L-ASNase mobility on native electrophoresis was altered by pressurization, probably resulting into overall charge or complex formation. Also pressurized L-ASNase was presenting with protein modifications and amino acid exchanges that could lead to impaired function of L-ASNase: L-ASNase enzymatic activity was preserved, however, indicating the majority of the pressurized domains and molecules remained functional. The active site at T36 was not modified and although the substrate binding site at S83 was acetylated, this may not have had any functional consequence in the used assay (Supporting Information, Table 6). Acetylation, theoretically, could have been removed, however, during the assay. Miscleavages were observed for L-ASNase as well, and can be interpreted as given above.

CONCLUSIONS

Herein, a pressure of 8 GPa (VHP) was applied and induced irreversible changes including amino acid exchanges, miscleavages, a series of protein modifications and in the case of rhGH also functional changes. This is the first report to show the effect of such a high pressure on protein samples and its chemical and functional consequences. The current work herein also confirms previous work reviewed by Hemley[36] that the free-energy is high enough to cleave strongest molecular bonds as expressed by amino acid changes. The findings per se introduce a technical rather than a chemical tool to modify proteins that may be of relevance in biotechnology.

ASSOCIATED CONTENT

Supporting Information. Table 1: Mascot identification result of rhGH and L-ASNase bands from native PAGE. Table 2: Mascot error tolerant search result of rhGH and L-ASNase bands from native PAGE. Table 3: Modiro result of rhGH and L-ASNase bands from native PAGE. Table 4: Miss cleaved site of trypsin digestion of rhGH and L-ASNase before and after pressurization. Supplemental Table 5: Activity result of L-ASNase before and after pressurization. Table 6: gravy value and active binding site of rhGH and L-ASNase. Figure 1: MS/MS spectra of modified peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +43-1-404003215. Fax: +43-1-404006065. E-mail: gert.lubec@meduniwien.ac.at.

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