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A Proteomic Approach toward the Selection of Proteins with Enhanced Intrinsic Conformational Stability

Vesna Prosinecki,[†] Hugo M. Botelho,[†] Simona Francese,[‡] Guido Mastrobuoni,[‡] Gloriano Moneti,[‡] Tim Urich,[§] Arnulf Kletzin,[§] and Cláudio M. Gomes*,[†]

Instituto Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal, Mass Spectrometry Centre, University of Florence, Florence, Italy, and Institute of Microbiology and Genetics, Darmstadt, Germany

A detailed understanding of the molecular basis of protein folding and stability determinants partly relies on the study of proteins with enhanced conformational stability properties, such as those from thermophilic organisms. In this study, we set up a methodology aiming at identifying the subset of cytosolic hyperstable proteins using *Sulfurisphaea* sp., a hyperthermophilic archaeon, able to grow between 70 and 97 °C, as a model organism. We have thermally and chemically perturbed the cytosolic proteome as a function of time (up to 96 h incubation at 90 °C), and proceeded with analysis of the remaining proteins by combining one- and two-dimensional gel electrophoresis, liquid chromatography fractionation, and protein identification by N-terminal sequencing and mass spectrometry methods. In total, 14 proteins with enhanced stabilities which are involved in key cellular processes such as detoxification, nucleic acid processing, and energy metabolism were identified including a superoxide dismutase, a peroxiredoxin, and a ferredoxin. We demonstrate that these proteins are biologically active after extensive thermal treatment of the proteome. The relevance of these and other targets is discussed in terms of the organism's ecology. This work thus illustrates an experimental approach aimed at mining a proteome for hyperstable proteins, a valuable tool for target selection in protein stability and structural studies.

Keywords: Archaea • Thermophiles • Protein Folding and Stability • Superoxide dismutase • Ferredoxin

Introduction

Thermophilic organisms are a valuable source of proteins with very high stability, the so-called hyperstable proteins. At working temperatures above 80 °C, the stability of the proteome is kept due to the combined effect of several factors.[1,2] Among these, extrinsic factors like the compatible solutes, acting as chemical stabilizers, such as trehalose and β-mannosylglycerate are particularly relevant.[3] The concentration of these molecules in the cell can be very high, thus, resulting in a stabilizing effect which adds to intracellular protein crowding effects. However, intrinsic factors play a key role in accounting for the high thermal stability of thermophilic proteins. Protein stability relies essentially on the additive effect of noncovalent bonds of weak magnitude,[4] which result in an overall stabilization of the folded native conformation. Although there is not a general rule that accounts for the molecular determinants, a series of particularities have been outlined in proteins from thermophiles which could explain their increased stability.[1,2] Surface ion pairs, a

solvent-exposed hydrophobic surface, and efficient anchoring of terminal extensions and loop regions seem to be particularly relevant in enhancing protein stability. Thermophilic proteins have an emerging interest both from a fundamental and an applicative point of view. In particular, thermophilic enzymes are of interest due to their potential application in biotechnological or industrial processes where a biological catalyst is used.[5,6] Moreover thermophilic proteins constitute also excellent working models in more fundamental studies aiming at the elucidation of the molecular determinants of a particular fold[7–9] or protein structure; for example, see refs 6, 10. This knowledge is essential for the understanding of folding mechanisms and contributes to the design of better structure prediction algorithms.

Following our long standing interest in the study of the stability properties of thermophilic proteins (e.g., see refs 8, 11–18), we set up a methodology which aims at identifying a subset of the proteome of thermophilic organisms containing the most intrinsically stable proteins. The rationale for our approach is based on previous reports of thermophilic proteins, which at physiological pH conditions (pH 6–7) remain folded and functional even upon extensive incubation periods at high temperatures; for example, see ref 11. The thermoacidophilic archaea *Sulfurisphaera*[19] was selected as a model: it is a facultative anaerobe, living in acidic, hot, solfataric springs at

* To whom correspondence should be addressed. Cláudio M. Gomes, Instituto Tecnologia Química e Biológica. Universidade Nova de Lisboa, Av. República 127, 2780-756 Oeiras, Portugal. Tel: +351 214469332. Fax: +351 214411277. E-mail, gomes@itqb.unl.pt; URL, http://www.itqb.unl.pt/pbfs.
† Universidade Nova de Lisboa.
‡ University of Florence.
§ Institute of Microbiology and Genetics.

high temperatures with an optimal growth temperature of 85 °C, tolerating a broad pH range from 1 to 5.5, with an optimal pH at 2. Its genome, which is currently being sequenced, is more than 90% identical to those of the closely related organism *Sulfolobus tokodaii*,[20] for which there is inclusively proteomic data available.[21] Its broad growth temperature range (70−97 °C) allowed the study and analysis of the proteome obtained from cells grown close to physiological growth temperature extremes. We have outlined a protocol in which the cytosolic proteome of *Sulfurisphaera* was chemically and thermally perturbed as a function of time. The impact of the destabilization was assessed by combining several approaches including liquid chromatography, SDS-PAGE, two-dimensional gel electrophoresis (2DE), N-terminal sequencing, and mass spectrometry methods for the identification of purified proteins and spots of interest. A set of proteins with enhanced stabilities was identified, and their relevance on a thermophilic background is discussed.

Experimental Procedure

Cell Mass and Preparation of the Cytosolic Extract. *Sulfurisphaera* sp. cells were grown at 72 °C (SulfCP72) and 92 °C (SulfCP92) according to published procedures.[22] Preparation of the cellular fractions was essentially performed as described[23] using 40 mM phosphate buffer, pH 6.5, throughout all stages. All steps were carried out at 4 °C in the presence of the protease inhibitor PMSF (0.5 mM). The soluble extracts were divided in small portions and stored at −20 °C prior to use.

Thermal and Chemical Perturbation Protocols. The soluble extracts from SulfCP72and SulfCP92 were perturbed by two different protocols in 40 mM phosphate buffer, pH 6.5. The thermal perturbation included incubation at 90 °C for up to 96 h and sampling every 24 h. In the second perturbation protocol, 1 M of the chemical denaturant guanidinium hydrochloride (GuHCl) was added to the samples, with subsequent incubation periods at 90 °C. In both cases, after sampling, less stable precipitated proteins were removed by 10 min centrifugation at 14 000 rpm on a bench centrifuge at 4 °C.

Liquid Chromatography Analysis of Perturbed Proteomes. Soluble proteomes obtained by both perturbation protocols were chromatographically resolved using anionic exchange. A HiTrap Q-sepharose FF (5 mL) column equilibrated with 40 mM phosphate buffer, pH 6.5, was used, and fractions eluted with a linear gradient up to 1 M NaCl. Separated peaks were concentrated (Amicon, 5 kDa) and loaded into a gel filtration column (Superdex-75). Protein profiles were obtained monitoring absorption at 280 nm. Eluted fractions were checked by 12.5% SDS-PAGE and UV−visible spectrum.

2D Electrophoresis. For 2D-PAGE, samples were separated in the first dimension by isoelectrofocusing (IEF). Samples of thermally treated as well as not treated soluble extract were precipitated overnight with 10% trichloroacetic acid (TCA) in acetone, and the pellet was additionally washed with acetone and left to air-dry before solubilizing in immobilized pH gradient IPG rehydration buffer composed of 8 M urea, 2%(w/v) CHAPS, 60 mM DTT, and 0.5% IPG buffer (pH 3−11, Amersham Biosciences). Samples were rehydrated using 13 cm long pH 3−11 NL IPG strips (Amersham Biosciences). Isoelectric focusing was carried out using the following scheme: 30 V for 12 h, 250 V for 250 V/h, 500 V for 750 V/h, 1000 V for 1500 V/h, 2500 V for 2500 V/h, and 8000 V for 3237 V/h, and finally 8000 V for 24000 V/h. The strips were equilibrated for 15 min in 5 mL of solution per strip containing 50 mM Tris-HCl, pH

8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, with the addition of 0.1% (w/v) DTT and 0.25% (w/v) iodoacetamide, respectively, in the same solution composition. The equilibrated IPG strips were placed on the top of the 12.5% SDS gel, sealed with 0.5% (w/v) agarose, and run for 15 min on 30 mA, followed by 30 mA per gel until the end of the run. Silver staining was performed with "Silver staining kit−protein" (Amersham Biosciences), and gel images were analyzed using ImageMaster 2D Platinum (Amersham Biosciences).

In Situ Gel Digestion. Complete Coomassie destaining of excised gel bands/spots was performed by shrinking and rehydratating the gel pieces with acetonitrile and 50 mM ammonium bicarbonate, respectively. After vacuum drying in Speedvac (Thermo, San Jose, CA), the excised bands were rehydratated and submitted first to chemical reduction in a solution of 10 mM DTT in 50 mM ammonium bicarbonate left for 30 min at 56 °C, and then to alkylation with a iodoacetamide solution in 50 mM ammonium bicarbonate in the dark, for 30 min, at room temperature. In situ digestion was performed on both bands and spots by rehydrating the gel plugs with a solution of 12 ng/L and 6 ng/μL, respectively, of modified trypsin (Promega, Madison, WI) in 10 mM ammonium bicarbonate solution and left to incubate for 90 min at 4 °C. Supernatants were recovered, blocked by adding 10% TFA, and spotted on an AnchorChip (Bruker Daltonics, Bremen, Germany) target plate. One microliter of sample was deposited on the target plate and allowed to dry; 0.35 μL of matrix (5 g/L α-cyano-4-hydroxycinnamic acid in 50/50 acetonitrile/0.1% TFA) was then added and, again, allowed to dry. To the remaining gel plugs, 30 μL of 10 mM ammonium bicarbonate was added, and the reaction proceeded overnight at 37 °C. Supernatants were eventually withdrawn and analyzed by MALDI MS and MS/MS.

MALDI Peptide Mass Fingerprinting. Mass spectrometry analysis was performed on a matrix-assisted laser desorption ionization tandem mass spectrometer having time-of-flight/time-of-flight optics (Ultraflex MALDI-TOF/TOF, Bruker Daltonics) by using the data acquisition software FlexControl 2.4. Mass spectra were acquired in reflectron mode over the m/z range 800−3500. The instrumental parameters were chosen by setting the ion source 1 at 25 kV, the reflector at 26.30 kV, and the delay time at 20 ns. The instrument was externally calibrated prior to analysis by using the Bruker peptide calibrant kit (1000−3000 Da), and the sample spectra were internally recalibrated with trypsin autolysis signals. Mass spectra were elaborated by using FlexAnalysis 2.4. The peptide masses present in each mass spectrum, through the integrated software Biotools2.2., are used to search the NCBI nonredundant database by using MASCOT software available online (http://www.matrixscience.com/cgi/nph-mascot.exe?1), which compares the experimentally determined tryptic peptide masses with theoretical ones calculated for proteins contained in the protein database. When NCBInr is used as protein databank to search against, Mascot significance threshold is 64. The taxonomy was set on Archea; carbamidomethylation and methionine oxidation were selected as complete and partial modifications, respectively; two missed cleavages were allowed for trypsin chosen as cleaving agent. Searches were performed setting a mass tolerance ranging from 20 to 40 ppm. Quick confirmation of our results was made by searching also against the Swiss-Prot (http://expasy.org/sprot) database by using the softwares Profound (http://65.219.84.5/service/prowl/profound/profound_E_adv.html) and MS-FIT (http://prospector.ucsf.edu/

ucsfhtml4.0/msfit.htm) which use different searching algorithms to identify proteins. The accession numbers were retrieved from the NCBI (http://www.ncbi.nlm.nih.gov).

MALDI MS/MS Peptide Sequencing. The best S/N peptides signals present in each MS spectrum and those identifying the protein were submitted to MS/MS analysis on the Ultraflex MALDI-TOF/TOF by using LIFT technology (Bruker Daltonics).

Miscellaneous Biochemical and Spectroscopic Methods. Protein purity was monitored by 12.5% SDS-PAGE. Gels were stained with Coomassie brilliant blue G or silver (Silver staining kit-protein, Amersham Biosciences), and images were obtained on ImageScanner (Amersham Biosciences). Protein content was determined by the Bradford[24] quantification method. UV and visible spectra were recorded on UV-1700 Shimadzu spectrophotometer. Folded structure of ferredoxin purified from the thermally treated proteome was tested with fluorescence spectroscopy measurements (Cary Varian Eclipse spectrofluorimeter), monitoring tryptophan fluorescence emission between 300−450 nm, with excitation at 280 nm. Peroxidase activity of the Bacterioferritin comigratory protein (BCP) was tested by its ability to remove added H_2O_2 from a reaction mixture containing 50 mM HEPES, pH 7, and 10 mM DTT as electron donor, at 25 °C using an amperometric H_2O_2 detection (WPI Apollo 4000 system). Assay of SOD activity was done at 25 °C by the xanthine/xanthine oxidase method[25] in 50 mM phosphate buffer, pH 7.8, with 0.1 mM EDTA; one activity unit was defined as the amount of enzyme that caused 50% inhibition of the cytochrome c reduction by superoxide (O_2^-).

Results and Discussion

High Temperature and Chemical Denaturants Induce Proteome Perturbation. The cytosolic proteomes obtained from *Sulfurisphaera* cells grown at 72 °C (SulfCP72) and 92 °C (SulfCP92) were perturbed by two different protocols. The thermal perturbation protocol consisted in incubation at 90 °C for up to 96 h, sampling every 24 h. After 96 h incubation, the protein yield was 5%. To detail the enrichment of the thermostable proteome, a combination of small-scale ionic exchange and SDS-PAGE was used. Aliquots of the SulfCP72 proteome, which had been sampled during different periods of the thermal perturbation protocol, were applied into a HiTrap Q-sepharose FF column for chromatographic separation. From the obtained protein chromatograms, it became clear that the extract is being selectively enriched in thermostable proteins, which eluted at identical ionic strengths despite the longer incubation at 96 °C (Figure 1). This provided a good indication that the tertiary fold of the proteins in solution was kept; in fact, protein unfolding results in the exposure of a higher number of side chains thus changing the charged surface with respect to the native state, resulting in a modification of the elution profile which is not the case. The global impact of the thermal perturbation on the proteome was also investigated by SDS-PAGE analysis (Figure 2). An increasingly lower number of bands were observed as the incubation time increases, evidencing protein selection. Identical results were obtained for cells grown at 92 °C. The second perturbation protocol involved the combined use of high temperature (90 °C) and a moderate concentration (1 M) of the chemical denaturant guanidinium hydrochloride (GuHCl). SDS-PAGE analysis shows that the presence of denaturant is sufficient to significantly reduce the time necessary to produce a subset of the proteome (24 and 48 h) (Figure 2). Interestingly, a comparable profile was obtained from both perturbation protocols on SDS-PAGE,
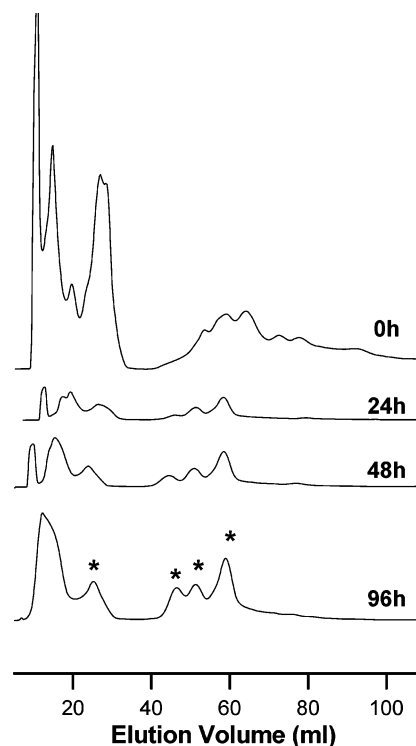

Figure 1. Protein chromatograms of purification of SulfCP72 thermally treated proteome. The extract is being selectively enriched in thermostable proteins (marked with an asterisk), which elute at identical ionic strengths despite the longer incubation at 90 °C. In agreement, a decreasing number of bands were observed in denaturing electrophoresis (see Figure 2).
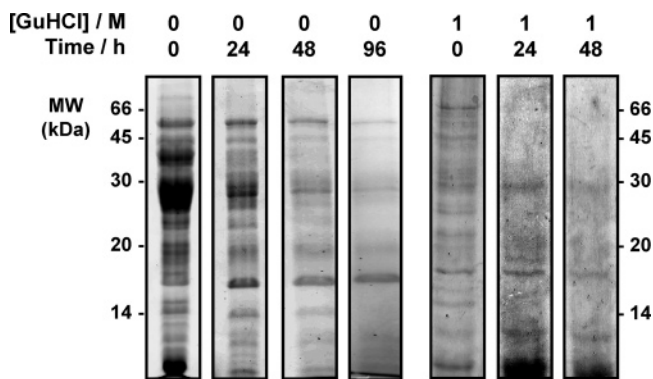
Figure 2. SDS-PAGE analysis of the *Sulfurisphaera* cytosolic proteome. The SulfCP72 cytosolic extract was incubated at 90 °C up to 96 h either in the absence or in the presence of GuHCl. Each lane was loaded with ≈1 μg of protein. Gels were Silver-stained.

suggesting a common selection toward proteins with enhanced stability properties.

To further investigate and identify some of these proteins, 13 cm gels were prepared from SulfCP72 and SulfCP92 which had been incubated for 24 h at 90 °C in presence of 1 M GuHCl as well as in its absence (Figure 3). From these gels, relevant bands were excised and subjected to MALDI-TOF/TOF analysis, leading to the identification of five proteins: a DNA binding protein 7e (DNA-BP, ≈14 kDa), the bacterioferritin comigratory protein (BCP, ≈21 kDa), a superoxide dismutase (SOD, ≈28 kDa), the large subunit of an hypothetical CO dehydrogenase (CO DH, ≈80 kDa), and a hypothetical Acyl-CoA dehydrogenase
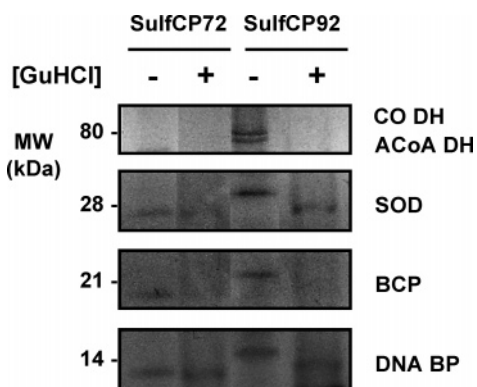
Figure 3. Detail of a 13 cm resolving 12.5% SDS-PAGE Silver-stained gel highlighting proteins whose excision led to their identification by mass spectrometry. The SulfCP72 and SulfCP92 cytosolic proteomes were incubated during 24 h in the presence (+) or absence (-) of 1 M GuHCl. The chemical denaturant was removed by extensive dialysis prior to electrophoresis. DNA BP, DNA binding protein Alba-2; BCP, bacterioferritin comigratory protein; SOD, superoxide dismutase; CO DH, hypothetical CO dehydrogenase large subunit; and ACoA DH, hypothetical acyl-CoA dehydrogenase. See also Table 1 for details.

(ACoA DH, ≈78 kDa) (Figure 3 and Table 1). These proteins have enhanced stabilities with respect to the total proteome, but they are not all equally stable; for example, whereas the DNA binding protein and the SOD are found in thermally and chemically perturbed extracts, the BCP and the dehydrogenases are more susceptible to simultaneous chemical degradation. In fact, the slight differences observed in the electrophoretic mobility of these proteins may be suggestive of moderately perturbed conformational states.

Proteome Analysis by 2DE and MS: Identification of Hyperstable Proteins. The cytosolic fractions perturbed during 96 h at 90 °C were further resolved by two-dimensional electrophoresis. The SulfCP92 proteome was analyzed before and after the thermal perturbation (Figure 4): it became clear that the number of proteins decreases substantially and that even after such extensive incubation there is still a large number of proteins detected. Proteins present in the native (Figure 4A) and thermally perturbed (Figure 4B) SulfCP92 proteome were selected for further investigation by MALDI

peptide mass fingerprinting. Excised gel spots were digested and submitted to MALDI peptide mass fingerprinting analysis. To confirm protein identifications, the lists of the identifying peptide masses deriving from each spot were carefully inspected searching for the best S/N peptides signal to sequence. The structural information deriving from both the peptide mass fingerprinting and the MS/MS-derived peptide sequences greatly enhanced the protein identification confidence. The subset of proteins identified from analysis of the 2DE gel (Table 1) can be grouped into three distinct functional categories: proteins involved in cellular processes and detoxification; DNA binding, translation, and protein modification; and energy metabolism. Two of the identified proteins, superoxide dismutase and Bacterioferritin comigratory protein (BCP, a peroxiredoxin) had already been identified from the 1D gel analysis. Four identified proteins (DNA binding protein Alba-2, hypothetical DNA-binding protein 7e, hypothetical elongation factor 1-alpha, Thermosome β subunit) are involved in nucleic acid processing and protein modification processes. In particular the DNA binding protein Alba-2 was previously identified by analyzing 1D gel of perturbed proteome. Finally, eight proteins involved in energy metabolism processes were identified, among which are several hypothetical dehydrogenases and the iron-sulfur protein ferredoxin (Table 1). MALDI MS and MS/MS spectra of superoxide dismutase, DNA binding protein Alba-2, and ferredoxin, each belonging to one of the three different biological classes, have been selected as an example and reported in Figure 5.

The type of cellular processes in which the identified proteins are involved is very interesting and noteworthy from the point of view of the *Sulfurisphaera*'s optimal growth conditions at high temperatures. The identified proteins participate in cellular processes (e.g., defense against reactive oxygen species, nucleic acid protection, and energy production) in which some key proteins have enhanced thermal stabilities, which may relate to their importance on the metabolism of a thermophile. For example, at temperatures above 70 °C, DNA is particularly susceptible to chemical modifications, mainly depurination followed by cleavage of the nearby phosphodiesteric bond, thus, making DNA binding proteins particularly important as these wind and compact DNA, protecting it.[1] In agreement, several DNA-binding proteins were identified in this study

Table 1. Selected Proteins Identified from the Cytosolic *Sulfurisphaera* Proteome after Perturbation

spot	protein annotation	MW (kDa)	p*I*	homologue accession number
	Celular Processes/Detoxification			
1	Superoxide dismutase[a]	24.3	3.2	15922615
2	Bacterioferritin comigratory protein (Peroxiredoxin)*	17.7	7.5	15922097
	DNA Binding/Translation/Protein Modification			
3	DNA binding protein Alba-2[a]	10.5	n.d.	68567728
4	hypothetical DNA-binding protein 7e	7.3	10	15621644
5	hypothetical elongation factor 1-alpha	48.3	10	15920458
6	Thermosome β subunit	60.7	5.4	15920519
	Energy Metabolism			
7	hypothetical CO dehydrogenase large subunit[a]	81.8	n.d.	15922093
8	hypothetical CO dehydrogenase middle subunit	31.2	10	15922095
9	hypothetical acetil-CoA synthetase	74.5	n.d.	BAB65737
10	hypothetical glutamate dehydrogenase (hyp)	45.7	6.1	15922573
11	hypothetical sulphide dehydrogenase	43.0	6.8	15920835
12	hypothetical L-lactate dehydrogenase	34.7	6.3	15622912
13	Ferredoxin [3Fe4S][4Fe4S]	11.3	5.5	2554684
14	hypothetical metal-dependent hydrolase	25.5	5.8	22096255

[a] Denotes proteins identified also from 1D SDS-PAGE gel bands. N.d., not determined.
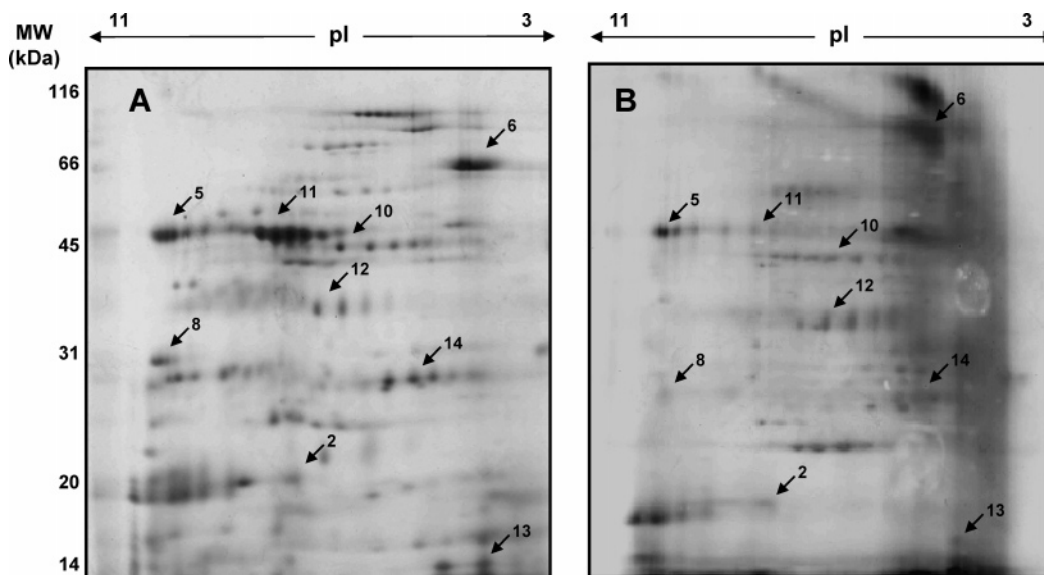
Figure 4. 2DE Silver-stained gel (12.5% SDS-PAGE) using a 13 cm, 3-11NL IPG strip, resulting from the application of 500 μg of total protein. (A) Native SulfCP92 without any heat treatment. (B) Subset of the perturbed proteome obtained after 96 h incubation at 90 °C. Numbered arrows indicate spots which were selected for identification (see Table 1 for details).

(Table 1). Concerning the energy metabolism proteins, the hypothetical dehydrogenases identified suggest that some electron-transfer pathways involved in energy production processes involve particularly stable proteins. A common feature among these proteins is that they comprise a NAD(P)-binding Rossman fold; however, since this is one of the most populated folds in proteins, typical among dehydrogenases, a direct correlation with enhanced protein stability cannot be postulated. Finding proteins involved in cellular detoxification processes to be hyperstable may be rationalized with the fact that high temperatures and aerobic growth conditions are prone to an increased cellular oxidative stress. In these circumstances, the amount of reactive oxygen species (ROS) formed during electron-transfer processes is likely to be increased as a result of electron leakage from redox proteins and reduced cofactors. This calls for a particularly stable set of ROS defense proteins such as peroxiredoxin and superoxide dismutase (Table 1). Altogether, these results suggest that some metabolic processes in thermophiles may require a 'thermostable character' in order to cope with the specificities of life at high temperatures.

Proteins from the Pool of Selected Hyperstable Proteins Are Biologically Active. Thermophilic proteins can be intrinsically very stable and exhibit a very high kinetic stability toward thermal degradation. We have validated our proteome perturbation method by verifying the extent to which the subset of proteins obtained after the perturbation corresponds to biologically active molecules. Three distinct proteins were selected from the perturbed subset: the iron-sulfur protein ferredoxin (Fd), the peroxiredoxin Bacterioferritin comigratory protein (Prx-BCP), and superoxide dismutase (SOD). Whereas Fd is an electron carrier present in most thermoacidophilic Archaea, Prx-BCP and SOD are enzymes that are, respectively, involved in the detoxification of peroxides and superoxide. For this characterization, we have set up a small-scale two-step chromatographic procedure for partly resolving the thermally perturbed SulfCP92 proteome. Starting from the 96 h incubated SulfCP92 proteome, and using a combination of anionic exchange (Q-sepharose HiPrep) and gel filtration (S-75), a

protein fraction corresponding to a molecular weight of $\approx$12 kDa, as determined from SDS-PAGE, was obtained. Sequencing of its N-terminus (1-GIDPNYRTNRQVVGEHSK-G) clearly showed that this protein corresponds to ferredoxin.

A preliminary biophysical characterization was carried out on this purified band, and its assignment as a Fd was corroborated by obtaining the typical UV−visible spectrum, with the characteristic band at 410 nm corresponding to native Fd with intact Fe−S clusters (not shown). The *Sulfurisphaera* ferredoxin has orthologues in other thermophilic Sulfolobales,[11,16] and these proteins are characterized by a very high thermal stability; they have very high midpoint melting transitions ($T_m = 110$ °C at pH 7 for the *Acidianus ambivalens* Fd) as well as a very high kinetic stability.[11] The protein core harboring the two iron-sulfur centers is likely to play an important role in protein thermostability, as well as a Zn-containing N-terminal extension.[11,26]

The SOD[27] and Prx-BCP[28] activities were measured in the native and perturbed SulfCP72 proteome. Not only were both activities present, but also a specific activity increase of the enzymes was observed (Figure 6). This observation is compatible with a significant enrichment of these two enzymes in the thermally treated extract, as a result of their enhanced thermostability. Whereas little is yet known concerning the molecular origins of Prx-BCP stability, there are several studies on thermophilic SODs.[27] The available data on Archaeal Fe-SODs corroborates our finding that these proteins are among the most thermostable in the cell. For example, the *Sulfolobus acidocaldarius* SOD, which is a functional tetramer, has a very high stability undergoing structural thermal melting at 125 °C, a temperature which is 40 °C above the organism optimal growth temperature.[27] Structural analysis suggests that the increase protein stability may result from its quaternary structure, a tight packing of buried hydrophobic residues, and an increased number of ion pairs.[27]

Conclusions

Here we have outlined an experimental strategy which allows mining proteomes for proteins having enhanced stability
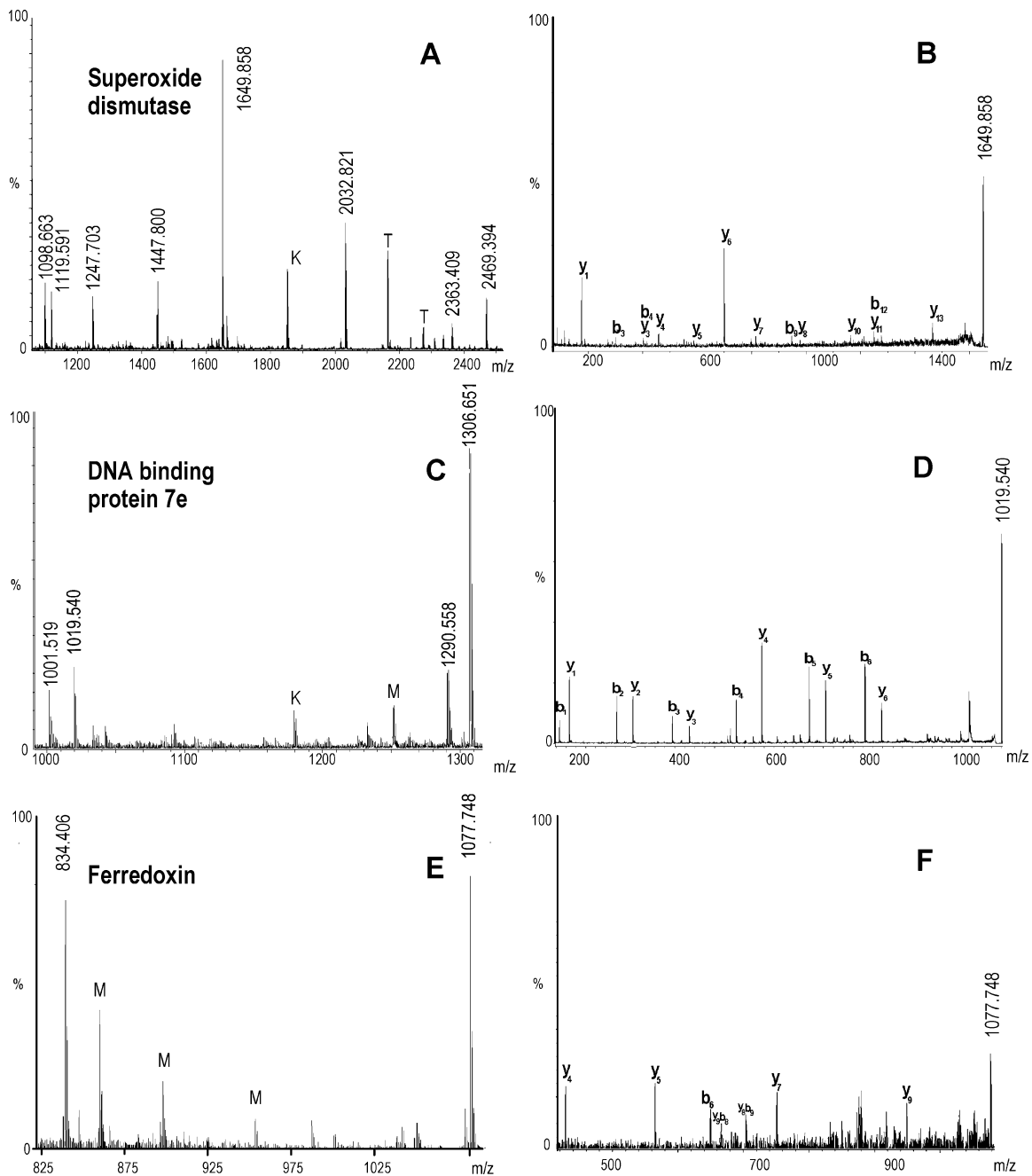
Figure 5. MALDI MS and MALDI MS/MS spectra of 3 out of 14 identified proteins from *Sulfurisphaera*. (A, C, and E) Panels show the MALDI MS spectra of superoxide dismutase, hypothetical DNA-binding protein, and ferredoxin, respectively. K, M, and T stand for keratin, matrix, and trypsin mass signals, respectively. (B, D, and F) Panels show the corresponding MALDI MS/MS spectra of the above proteins where a peptide mass signal was chosen for each protein (1649.858, 1019.540, and 1077.748, respectively) and submitted to tandem MS in order to confirm protein identifications. See table in Supporting Information for assignments.

properties. This approach is particularly valuable for the identification of proteins which can be used in subsequent studies aimed at the understanding of protein stability, function, or structural features. Following our interest in the study of conformational properties of thermophilic proteins, we have selected *Sulfurisphaera* sp. as a model organism to implement this methodology; the identification of hyperstable proteins in a thermophilic background required drastic perturbation protocols (e.g., 4 days at 90 °C), which nevertheless resulted in the identification of a subset of proteins which remained folded after the perturbation and provided an insightful perspective into the type of essential cellular processes requiring particu-

larly resistant proteins. In fact, many of the identified proteins are involved in stress response mechanisms that aim at protecting nucleic acids and proteins from aggression elements such as thermal stress and reactive oxygen species. In this respect, this approach highlighted not only proteins interesting for subsequent stability studies, but also key metabolic processes which may have themselves a 'thermophilic character'. Recently, efficient expression systems have been developed[29] using the closely related hyperthermophile *Sulfolobus solfataricus* as an efficient expression system of tagged proteins, thus, greatly facilitating the production of proteins of interest such as those identified in this work for subsequent studies.
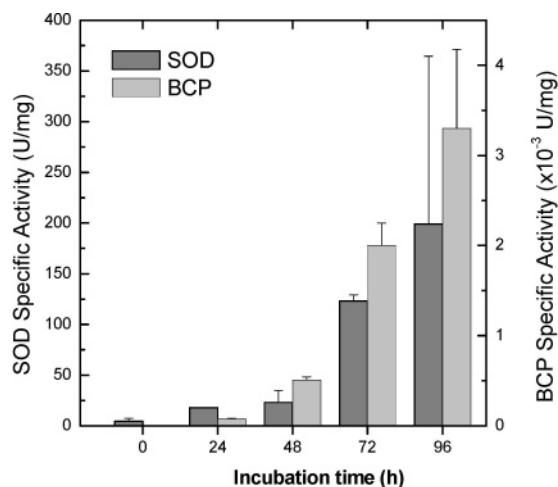
Figure 6. Variation of the BCP and SOD specific activity as a function of incubation time of the SulfCP92 proteome. Error bars are from the standard deviation of activity assays ($n = 2$).

Supporting Information Available: MALDI MS and MS/MS signal assignments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supplementary Table. MALDI MS and MS/MS signal assignments. The table shows the peptide masses and the corresponding sequences identifying the Superoxide Dismutase, the Hypothetical DNA-binding protein 7e and the Ferredoxin reported in figure 5. The last three columns show the MS/MS assignments (a, b and y series) for the above proteins.

Protein	MALDI TOF spectrum		MALDI TOF/TOF spectrum		
	m/z	Peptide sequence	Fragment type	m/z	sequence
Superoxide Dismutase	1098.663	(74-83) GLVFNINGHK			
	1119.591	(10-18) YELPPLPYK			
	1247.703	(9-18) KYELPPLPYK			
	1447.800	(29-40) DIIDVHYNGHHK			
	1649.858	(59-73) GEITSGQYDIQGLLR	b3	300.155	GEI
			b4	401.203	GEIT
			b5	488.235	GEITS
			b7	673.315	GEITSGQ
			b8	836.378	GEITSGQY
			b9	951.405	GEITSGQYD
			b10	1064.489	GEITSGQYDI
			b14	1249.570	GEITSGQYDIQGLLR
			y1	175.118	R
			y3	401.287	RLL
			y4	458.287	RLLG
			y5	586.367	RLLGQ
			y6	699.451	RLLGQI
			y7	814.478	RLLGQID
			y8	977.541	RLLGQIDY
			y10	1162.621	RLLGQIDYQG
			y11	1249.653	RLLGQIDYQGS
			y13	1463.785	RLLGQIDYQGSTI
			y15	1649.849	RLLGQIDYQGSTIEG
	2032.821	(56-73) IIRGEITSGQYDIQGLLR			
	2363.409	(9-28) KYELPPLPYKVDALEPYISK			
	2469.394	(98-101) GGKPGGALADLIDKQYGSFDKFK			
Hypothethical DNA-binding protein	1001.519	(43-52) GAVSEKDAPK			
	1019.540	(53-60) ELLQMLEK + Met ox	b1	130.050	E
			b2	243.134	EL
			b3	356.218	ELL
			b4	484.277	ELLQ
			b5	631.312	ELLQM
			b6	744.396	ELLQM
			y1	147.112	K
			y2	276.155	KE
			y3	389.239	KEL
			y4	536.274	KELM
			y5	664.333	KELMQ
			y6	777.417	KELMQL
			y8	1019.540	KELMQLLE
	1290.558	(29-39) MISFITYDDNGK			
	1306.651	(29-39) + Met ox			
Ferredoxin	834.406	GPDINYR			
	1077.748	QVVGEHSGHK	b6	650.093	QVVGEH
			y4	428.167	KHGS
			y5	565.188	KHGSH
			y7	751.279	KHGSHEG
			y10	1077.587	KHGSHEGVVQ
			y9b8	665.861	VVGEHSG
			y8b9	703.831	VGEHSGH