ELSEVIER

Journal of Chromatography A, 698 (1995) 341–349

JOURNAL OF CHROMATOGRAPHY A

Two-dimensional gel electrophoresis mapping of proteins isolated from the hyperthermophile *Pyrococcus furiosus*

Carol S. Giometti[a,*], Sandra L. Tollaksen[a], Swarnalatha Mukund[b], Zhi Hao Zhou[b], Keren Ma[b], Xuhong Mai[b], Michael W.W. Adams[b]

[a]*Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, Argonne, IL 60439, USA*
[b]*Department of Biochemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, GA 30602, USA*

Abstract

Two-dimensional gel electrophoresis (2DE) in polyacrylamide was used to map the proteins in lysates of the archaeon (formerly archaebacterium) *Pyrococcus furiosus* and to analyze enzymes purified from *P. furiosus*. The location of the enzymes in the 2DE maps was determined by comigration of lysate proteins with purified enzymes. A 2DE map of *P. furiosus* proteins with some identifications was produced, which will be useful for future studies of protein expression in this organism. In addition, the usefulness of 2DE for evaluating the purity of enzyme preparations and for characterizing their subunit structure under denaturing conditions was investigated.

1. Introduction

Hyperthermophilic microorganisms, grouped mainly within the domain of life termed Archaea, are unique in their ability to thrive in environments at temperatures near and even above 100°C [1–3]. The use of these organisms in industrial or commercial applications that require biological activity at high temperatures has prompted numerous laboratories to study their molecular biology and biochemistry, with particular attention to the thermostability of the enzymes they synthesize. However, the structural characteristics and cellular regulation mechanisms that account for the thermostability of proteins synthesized by hyperthermophilic archae have not been well studied. The primary structures of approximately a dozen proteins have been determined and the crystal structure of only one hyperthermophilic protein is known [3]. In addition, the regulation of protein synthesis at both the cellular and molecular level in these organisms is poorly understood. Further characterization of the structure, function, and regulation of proteins found in the hyperthermophiles will benefit from application of state-of-the-art protein biochemistry methods, including separation methods such as electrophoresis.

Two-dimensional electrophoresis (2DE) has been used extensively to study the expression of proteins in *Escherichia coli* [4–6]. Identification of specific *E. coli* proteins in 2DE patterns has been based on the criteria of comigration with purified proteins, induction or repression of specific genes by manipulation of growth con-

* Corresponding author.

SSDI 0021-9673(94)01262-8

ditions, and comparison of 2DE patterns of wild-type and mutant cell lysates [5]. This approach, equally applicable to the study of the proteins synthesized by hyperthermophilic microorganisms, has been used to generate a protein map that is linked to the genetic map for *E. coli*.

The hyperthermophilic archaeon *Pyrococcus furiosus* grows anaerobically with a temperature optimum of 100°C. *P. furiosus* has been the subject of many molecular biology and biochemical research projects and is rapidly becoming the "*E. coli*" of the hyperthermophiles [3]. Several enzymes, many unique to *P. furiosus*, have been isolated and are being characterized [3]. The regulation of expression of such proteins has not been studied in detail. In this paper, we describe the use of 2DE to produce a map of *P. furiosus* proteins that will be useful in future studies of regulation of protein expression in hyperthermophilic archaea. We describe the optimization of the 2DE gel parameters for studying proteins from this organism and show the 2DE patterns of eight enzymes (Table 1). The location of these enzymes within the 2DE protein map of *P. furiosus* was determined by comigration studies.

2. Materials and methods

2.1. Preparation of total P. furiosus protein samples

P. furiosus (DSM 3638) was grown in a medium containing maltose, yeast extract, and tryptone at 90°C in a 600-l fermentor as described previously [7]. Cells were rapidly frozen in liquid nitrogen and stored at −70°C. Prior to preparation for electrophoresis, the frozen cells were thawed anaerobically in five volumes of 10 m*M* Tris·HCl (Sigma, St. Louis, MO, USA), pH 7.5, containing 0.15 *M* NaCl (Mallinckrodt, Paris, KY, USA) (Tris–saline) and collected by centrifugation. The gelatinous material in the supernatant was removed from the cell pellet. The cell pellet was resuspended in a small volume of Tris–saline. Aliquots of the cell suspension were mixed with an equal volume of a solution containing 9 *M* urea (Bio-Rad, Her-

Table 1
Proteins isolated from *Pyrococcus furiosus*

Protein	Molecular mass (M_r)[a]	Subunits (M_r)[b]	Ref.
Aldehyde ferredoxin oxidoreductase	80 000	α (80 000)[c]	7
Sulfide dehydrogenase	90 000	α (52 000), β (29 000)	8
Chaperonin	> 600 000	α (60 000)	9
Formaldehyde ferredoxin oxidoreductase	280 000	α_4 (70 000)	10
Hydrogenase	160 000	α (49 000), β (42 000), γ (33 000), δ (30 000)[c]	11
Indolepyruvate ferredoxin oxidoreductase	180 000	α_2 (66 000), β_2 (23 000)	12
2-Ketoglutarate ferredoxin oxidoreductase	115 000	α (44 000), β (34 000), γ (24 000), δ (13 000)	13
Pyruvate ferredoxin oxidoreductase	115 000	α (47 000), β (31 000), γ (24 000), δ (13 000)[c]	14

[a] By gel filtration.
[b] By SDS–gel analysis.
[c] Confirmed by gene sequence analysis.

cules, CA, USA), 4% (v/v) Nonidet P40 (NP40; Particle Data Labs., Elmhurst, IL, USA), 2% 2-mercaptoethanol (Sigma), and 2% ampholytes (Biolyte pH 8–10) (Bio-Rad) (NP40–urea mix). The samples were then centrifuged for 5 min at 435 000 *g* in a Beckman TL100 ultracentrifuge (Beckman, Fullerton, CA, USA). The pellets were discarded, and the supernatants were stored at −70°C until electrophoresis.

2.2. Purification of *P. furiosus* enzymes

The enzymes listed in Table 1 were isolated from *P. furiosus* according to the referenced methods [sulfide dehydrogenase (SuDH) at 6.0 mg/ml in 20 m*M* glycylglycine, pH 8.2, with 2 m*M* dithiothreitol; hydrogenase at 6.4 mg/ml in 50 m*M* Tris·HCl, pH 8.0 with 2 m*M* sodium dithionite; pyruvate ferredoxin oxidoreductase (POR) at 1.6 mg/ml in 10 m*M* Tris·HCl, pH 7.5, with 0.1 *M* NaCl; indolepyruvate ferredoxin oxidoreductase (IOR) at 9.6 mg/ml in 50 m*M* Tris·HCl, pH 8.0, with 2 m*M* dithiothreitol, 2 m*M* sodium dithionite and 10% (v/v) glycerol; chaperonin at 4.0 mg/ml in 50 m*M* Tris·HCl, pH 8.0, with 10% glycerol; aldehyde ferredoxin oxidoreductase (AOR) at 20 mg/ml in 50 m*M* Tris·HCl, pH 8.0, with 10% (v/v) glycerol; formaldehyde ferredoxin oxidoreductase (FOR) at 0.8 mg/ml in 50 m*M* Tris·HCl, pH 8.0, with 10% glycerol; 2-ketoglutarate ferredoxin oxidoreductase (KGOR) at 13.7 mg/ml in 50 m*M* Tris·HCl, pH 8.0 with 10% glycerol]. Solutions containing purified or enriched enzymes were diluted with NP40–urea mix to obtain a final protein concentration of 1 μg/μl. The samples were stored at −70°C until electrophoresis.

2.3. Two-dimensional electrophoresis

First-dimension isoelectric focusing (IEF) was done using 40-cm rod gels as previously described [15]. In the initial IEF run, gels contained 50% pH 3–10 and 50% pH 5–7 ampholytes (Bio-Rad). Subsequent IEF runs used 12% pH 3–10 and 88% pH 5–7 ampholytes (Bio-Rad). IEF gels were loaded with aliquots of cell lysate containing 25–50 μg of protein, enzyme preparations containing 1–5 μg of protein, or, for comigration studies, a mixture of cell lysate and enzyme containing 25 and 1 μg, respectively. After IEF, the tube gels were equilibrated in a buffer containing sodium dodecyl sulfate (SDS) (Bio-Rad) as described by O'Farrell [4]. Second-dimension SDS-polyacrylamide gel electrophoresis used the Dalt system [16] with slab gels that contained a linear gradient of 10–17% acrylamide. A molecular mass standard preparation (Sigma) containing albumin (66 000 M_r), ovalbumin (45 000 M_r), pepsin (34 700 M_r), trypsinogen (24 000 M_r), β-lactoglobulin (18 400 M_r) and lysozyme (14 300 M_r) was applied to one side of the slab gels prior to electrophoresis. After electrophoresis, the gels were fixed in 50% (v/v) ethanol (Aaper Alcohol, Shelbyville, KY, USA) with 0.1% formaldehyde (Mallinckrodt) and 1% acetic acid (J.T. Baker, Phillipsburg, NJ, USA) in preparation for silver staining or were placed directly into a solution containing 0.125% Coomassie Blue R250 (Bio-Rad), 2.5% phosphoric acid and 50% ethanol. Silver staining was as described previously, using a silver diamine protocol [17]. Coomassie blue-stained gels were destained in 20% (v/v) ethanol. The low protein concentration of the cell lysates necessitated the use of silver stain for protein detection, whereas the protein concentration of the enzyme preparations was high enough to be detected using Coomassie Blue R250.

3. Results

3.1. Two-dimensional electrophoresis patterns of *P. furiosus* total protein preparations

To produce an accurate map of the protein components present within intact cells, precautions must be taken against protein modification, including proteolysis, during preparation of samples for 2DE. In 2DE studies of proteins from mesophilic cells, sample preparation methods generally include the use of protease inhibitors in addition to chemical and temperature conditions that inhibit protease activity. Initially, we ap-

proached the preparation of protein samples from the hyperthermophile *P. furiosus* in a similar manner, lysing the samples in the cold to avoid the activation of proteases. We compared 2DE patterns from cells that were solubilized as a frozen pellet directly into 9 *M* urea, 2% 2-mercaptoethanol, 4% non-ionic detergent, and 2% pH 8–10 ampholytes with patterns from cells that were put through numerous freeze–thaw cycles prior to solubilization. We thawed frozen cells, solubilized proteins collected in supernatants and pellets from the thawed preparations by centrifugation, and compared the 2DE patterns. We compared the 2DE patterns of *P. furiosus* proteins isolated from frozen cells solubilized using non-ionic, cationic, and anionic detergents. In every case, the 2DE patterns were virtually identical, resembling that shown in Fig. 1A. The reproducibility of these results demonstrated that the *P. furiosus* cells lyse easily when frozen and thawed and that proteolysis under the conditions described for lysis did not occur.

Fig. 1 shows the 2DE patterns obtained using 25 μg of *P. furiosus* total protein extracted with NP40–urea mix. Fig. 1A was obtained using a 50:50 mixture of pH 3–10 and pH 5–7 ampholytes for the IEF first-dimension separation; Fig. 1B was obtained using 12% pH 3–10 and 88% pH 5–7. In the initial 2DE separation, the first-dimension IEF gels were broken due to high concentrations of protein in narrow regions of the pH gradient within the polyacrylamide gel matrix. By expanding the neutral pH range of the gradient, resolution of the *P. furiosus* proteins was optimized, and the IEF gels remained intact. Pattern reproducibility improved, so pattern comparison was possible.

Approximately 500 proteins ranging in molec-

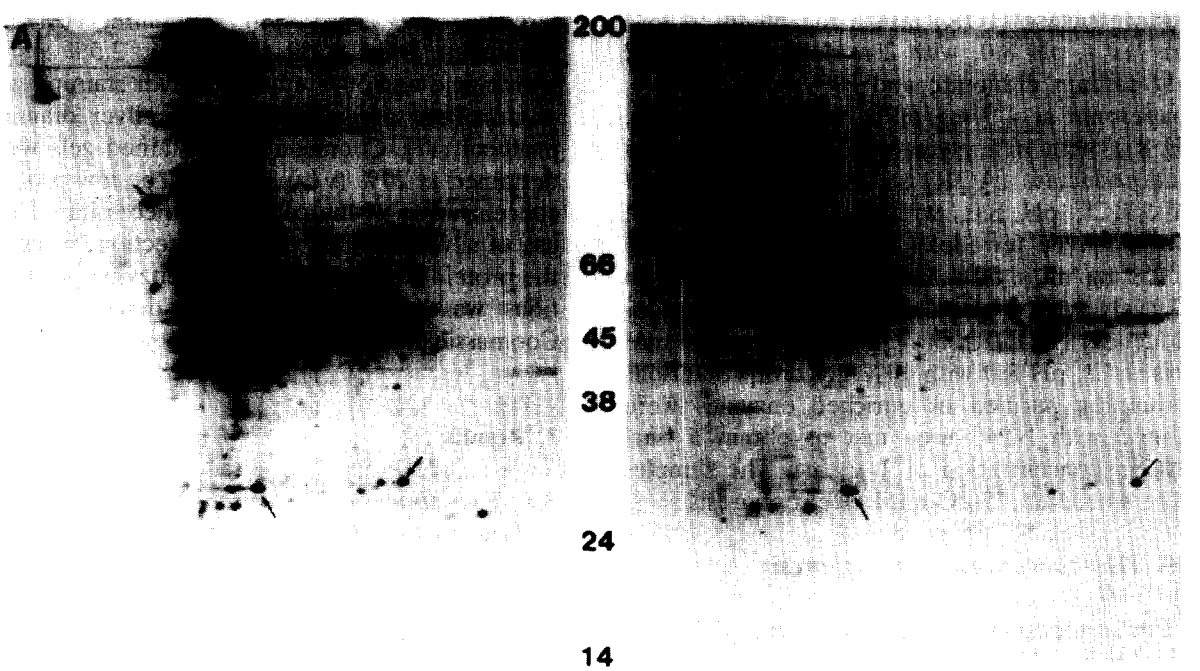

Fig. 1. Two-dimensional electrophoresis pattern of *P. furiosus* total proteins. Proteins isolated from washed *P. furiosus* cells were separated by two-dimensional gel electrophoresis using (A) 50% pH 3–10 and 50% pH 5–7 ampholytes or (B) 12% pH 3–10 and 88% pH 5–7 ampholytes in the first-dimension isoelectric focusing gels. The second-dimension separation was the same for both patterns. The arrows are provided as landmarks that indicate comparable proteins in each pattern. The patterns are oriented with the basic end to the right, acidic end to the left, high molecular mass at the top and low molecular mass at the bottom. Approximate relative molecular masses $\times 10^{-3}$, based on the migration position of the standard proteins discussed in Materials and methods and of myosin heavy chain (200 000 M_r [18]), are indicated.

ular mass from 14 000 up to 200 000 were detectable. A majority of the *P. furiosus* proteins were in the range of 30 000 to 50 000 M_r and had neutral isoelectric points (p*I* 4–6). Three major proteins with very high molecular masses were observed. The reproducibility of these patterns suggests that these are true molecular masses, although consistent incomplete disassociation of holoenzymes into subunits cannot be ruled out. When SDS, or "Zonyl" fluorosurfactant neutral (FSN) (DuPont, Wilmington, DE, USA) were substituted for the non-ionic detergent Nonidet P40 and urea in the preparation of *P. furiosus* lysates, patterns similar to those shown in Fig. 1 were obtained (data not shown). Some detergent-specific quantitative differences in specific proteins were observed, however, indicating differences in the denaturation properties of the proteins.

3.2. Two-dimensional electrophoresis patterns of proteins purified from P. furiosus

The availability of purified or partially purified proteins from *P. furiosus* makes identification of the major protein components in the *P. furiosus* 2DE pattern possible. First, however, the purity of the enzyme preparations must be assessed. Equal amounts of preparations of the enzymes listed in Table 1 were separated by 2DE and then detected using either the Coomassie Blue or silver staining method (Fig. 2). Silver stain is considerably more sensitive than Coomassie Blue in the detection of protein. Therefore, proteins in low abundance (i.e., less than 10 ng) that are undetectable in the Coomassie Blue-stained patterns are visible in the silver-stained patterns. For example, Coomassie Blue revealed only two polypeptides in the indolepyruvate ferredoxin oxidoreductase preparation (Fig. 2L), consistent with the α–β subunit structure reported previously. Silver stain, however, revealed a number of additional proteins present, albeit in lower abundance (Fig. 2K). Similar contamination was found in a majority of the enzyme preparations analyzed, with the exception of hydrogenase and pyruvate ferredoxin oxidoreductase.

Some of the additional proteins in the 2DE patterns of the enzymes, accounting for less than 10% of the total protein in the preparation, could be matched with other proteins in the *P. furiosus* lysate pattern and thus represent proteins that were co-purified with the enzyme of interest. Other protein spots in the enzyme preparations could not be matched to any spots present in the whole cell lysate 2DE pattern, suggesting they were products of proteolysis or chemical modifications generated during either enzyme purification or preparation for electrophoresis. Some degree of charge heterogeneity was observed in the 2DE patterns of all the enzymes analyzed, varying from two charge forms in the case of KGOR up to five charge forms for IOR. This heterogeneity also could be due to chemical modification of the proteins during purification or sample preparation. The presence of similar charge trains of proteins in the lysate pattern, independent of the sample preparation method (NP40–urea, SDS or FCN), suggests that the heterogeneity is present in the cell itself and is not generated by the sample preparation or enzyme purification procedures. Similar charge heterogeneity has been observed in 2DE patterns of proteins from numerous cell systems and is generally believed to represent post-translational protein modifications such as deamidation, glycosylation or phosphorylation.

3.3. The P. furiosus protein map

The most intensely stained spots in the Coomassie Blue-stained patterns shown in Fig. 2 were assumed to represent the 2DE migration position of the corresponding enzyme, assuming they had molecular masses consistent with the data shown in Table 1. Some interpretation of the data was required in several cases, however. For example, SuDH, predicted to have two distinct subunits that total approximately 90 000 M_r, produced a group of spots in the 2DE map that have a molecular mass of only approximately 60 000. The remaining 29 000 M_r subunit was not resolved under the IEF conditions used for these experiments. The position of hydrogenase is consistent with four subunits having identical

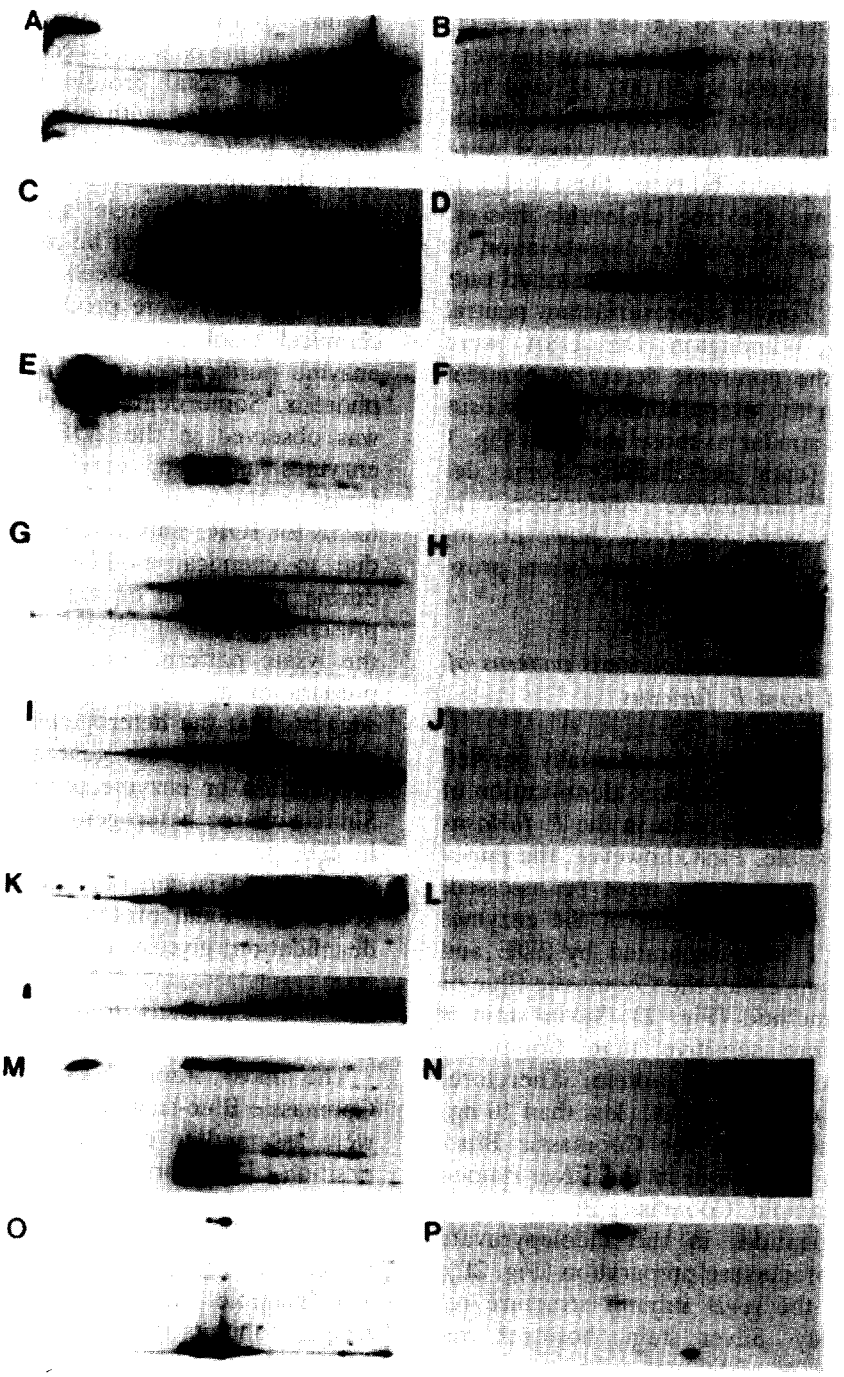

Fig. 2. Two-dimensional electrophoresis patterns of enzymes purified from *P. furiosus*. Two-dimensional electrophoresis was used to analyze the purity of enzyme preparations from *P. furiosus*. Panels on the left show silver-stained patterns; panels on the right show Coomassie Blue R250 patterns of the same proteins. (A, B) Aldehyde ferredoxin oxidoreductase; (C, D) sulfide dehydrogenase; (E, F) chaperonin; (G, H) formaldehyde ferredoxin oxidoreductase; (I, J) hydrogenase; (K, L) indolepyruvate ferredoxin oxidoreductase; (M, N) 2-ketoglutarate ferredoxin oxidoreductase; (O, P) pyruvate ferredoxin oxidoreductase. Patterns are oriented as in Fig. 1.

molecular masses rather than the four non-identical subunits predicted by SDS gel analysis and recently confirmed by gene sequence data [19]. The IOR pattern can be explained as representing two identical high-molecular-mass (approximately 70 000 M_r each) subunits coupled with two identical low-molecular-mass subunits (approximately 25 000 M_r each), totaling 190 000 M_r, which is slightly higher than the expected 180 000. Only one subunit of KGOR and POR appeared in the preparations analyzed, at molecular masses of approximately 45 000 and 50 000, respectively. The remaining three non-identical subunits of each of these enzymes, identified by SDS analysis and confirmed by gene sequence analysis [20], were not resolved using the IEF parameters described for these experiments.

Comigration experiments were done to determine which of the proteins in the 2DE patterns of *P. furiosus* lysate corresponded to the enzymes analyzed. Fig. 3 shows a 2DE pattern of *P. furiosus* lysate proteins with identifications based on such comigration experiments. These results indicate the relative abundance of each of these proteins within *P. furiosus* grown under the conditions described in Materials and methods.

4. Discussion

The purification of thermostable enzymes synthesized by hyperthermophilic organisms such as *P. furiosus* provides material for more detailed structural and biochemical studies designed to reveal the unique molecular features of these proteins. Characterizing the reactivity, reaction sites, and molecular regulation of these proteins improves the potential for their usefulness in biotechnology applications that can benefit from heat-resistant catalysts.

We show here that two-dimensional gel electrophoresis can be used to evaluate the purity of enzyme preparations although discrepancies observed between the 2DE patterns of some enzyme preparations and their known subunit structures remain to be resolved. Since 2DE is generally done under denaturing conditions (i.e., urea, mercaptoethanol and detergent), the subunit structure of purified proteins can usually be described. However, if the extreme stability of the *P. furiosus* proteins presents a problem in denaturation, dissociation of an holoenzyme into its subunits may not take place under conditions typically used for denaturation of mesophilic proteins. For example, *P. furiosus* pyruvate oxdoreductase requires heating at 105°C for 30 min in 1% SDS to achieve denaturation [21]. Sample preparation methods to ensure total dissociation of holoenzymes into their subunits should, therefore, be more thoroughly examined. The possibility that some of the enzyme subunits not seen in these 2DE studies lie outside the p*I* range of the IEF gels used should also be explored, using the non-equilibrium pH gradient gel electrophoresis technique to resolve very basic proteins [22].

The solubility properties of the proteins from *P. furiosus* cells were similar to those of proteins from mesophilic cells we have studied previously, going into solution readily in the presence of concentrated urea, a reducing agent, and non-ionic detergent. Total dissociation of all proteins may not have been optimized, based on our results with the enzyme preparations, but patterns consistent with those of the enzyme preparations allowed identification of some subunits to be made. There was no evidence of proteolytic activity under the sample preparation conditions used, as shown by the reproducibility of the 2DE patterns obtained. The proteins synthesized by *P. furiosus* produce a unique 2DE pattern, with a large number of proteins having neutral isoelectric points. This observation suggests selective pressure on this organism to favor synthesis of neutral proteins rather than basic or acid proteins, necessitating development of purification protocols that optimize separation of neutral proteins. The improved resolution of the *P. furiosus* proteins obtained by using narrow-range ampholytes in the neutral pH range showed that separation of these proteins by electrophoresis is a viable approach.

The 2DE map of *P. furiosus* proteins generated from these studies, as well as more refined maps to come, make it possible to study changes in the expression of enzymes within cells in

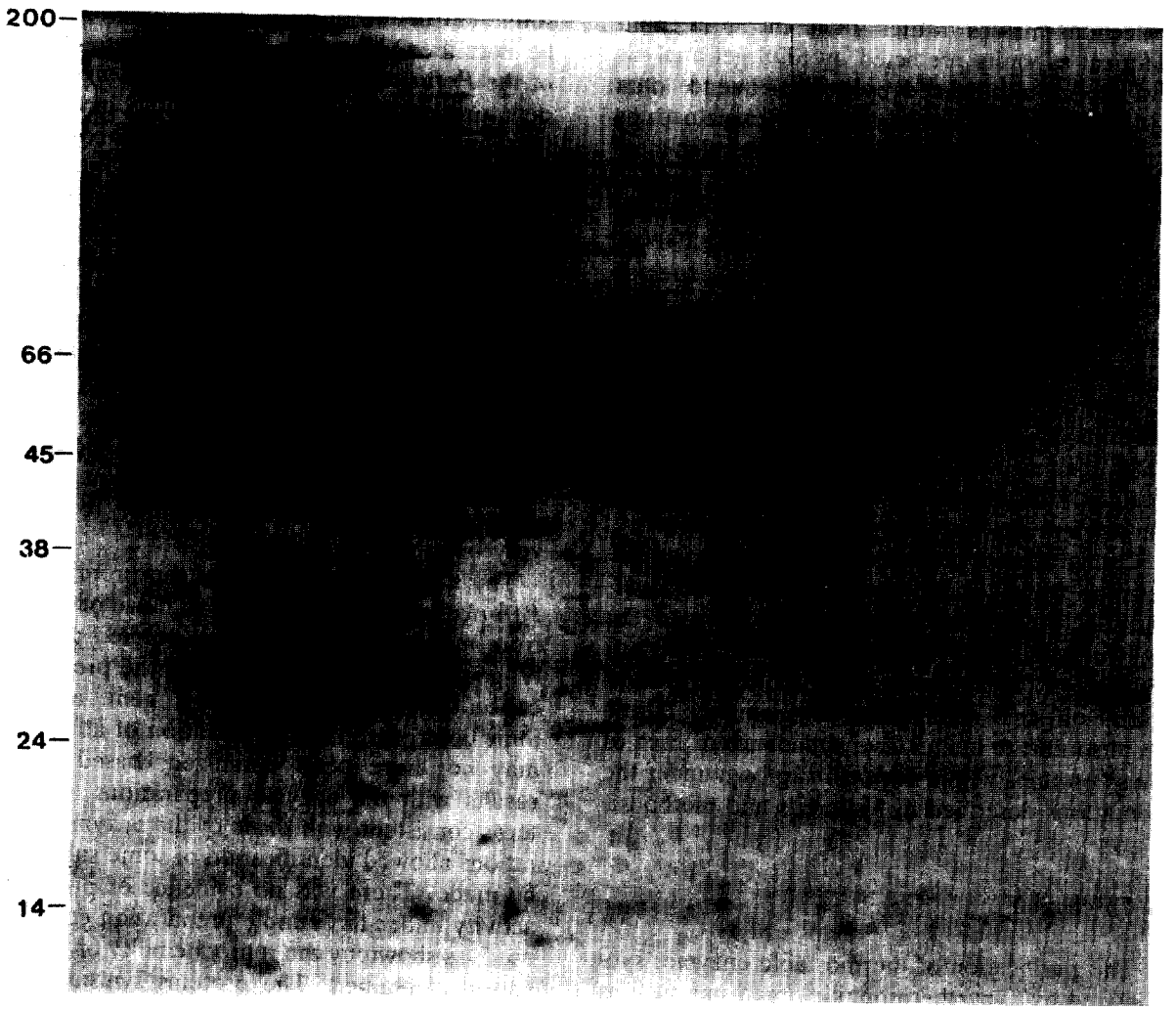

Fig. 3. Master pattern of *P. furiosus* proteins. The location of the proteins shown in Fig. 2 within the pattern of total lysate proteins from *P. furiosus* was determined by comigration experiments. AOR = Aldehyde ferredoxin oxidoreductase; SuDH = sulfide dehydrogenase; CHAP = chaperonin; FOR = formaldehyde ferredoxin oxidoreductase; HYD = hydrogenase; IOR, IOR′ = subunits of indolepyruvate ferredoxin oxidoreductase; KGOR = 2-ketoglutarate ferredoxin oxidoreductase; POR = pyruvate ferredoxin oxidoreductase. The pattern is oriented as in Fig. 1.

response to different growth conditions or genetic alterations. The generation of a map of proteins normally expressed by a hyperthermophilic organism such as *P. furiosus* and the identification of the proteins within that map provide a foundation for future studies of biochemical manipulations used to optimize enzyme production.

Acknowledgements

This work was supported by the US Department of Energy Office of Health and Environmental Research, under contract No. W-31-109-ENG-38 (C.S.G. and S.L.T.) and FG09-88ER13901 (M.W.W.A.), the Office of Naval Research under contract N00014-90-J-1894

(M.W.W.A.), and the National Science Foundation under Contract BCS-9320069 (M.W.W.A.). The authors thank Rebecca Brown for her assistance with 2DE analysis of the proteins purified from *P. furiosus* as part of her participation in the Argonne National Laboratory SERS program funded by the US Department of Energy.

References

[1] C.R. Woese and G.E. Fox, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 5088.
[2] C.R. Woese, O. Kandler and M.L. Wheelis, *Proc. Natl. Acad. Sci. U.S.A.*, 87 (1990) 4576.
[3] M.W.W. Adams, *Ann. Rev. Microbiol.*, 47 (1993) 627.
[4] P.H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007.
[5] P.L. Bloch, T.A. Phillips and F.C. Neidhardt, *J. Bacter.*, 141 (1980) 1409
[6] R.A. Van Bogelen, P. Sankar, R.L. Clark, J.A. Bogan and F.C. Neidhardt, *Electrophoresis*, 13 (1992) 1014.
[7] S. Mukund and M.W.W. Adams, *J. Biol. Chem.*, 266 (1991) 14208.
[8] K. Ma and M.W.W. Adams, *J. Bacter*, submitted for publication.
[9] X. Mai, J. Trent, A. Joachimiak and M.W.W. Adams, unpublished results.
[10] S. Mukund and M.W.W. Adams, *J. Biol. Chem.*, 268 (1992) 13592.
[11] F.O. Bryant and M.W.W. Adams, *J. Biol. Chem.*, 264 (1989) 5070.
[12] X. Mai and M.W.W. Adams, *J. Biol. Chem.*, 269 (1994) 16726.
[13] X. Mai and M.W.W. Adams, unpublished results.
[14] J.M. Blamey and M.W.W. Adams, *Biochemistry*, 33 (1994) 1000.
[15] N.G. Anderson and N.L. Anderson, *Anal. Biochem.*, 85 (1978) 331.
[16] N.L. Anderson and N.G. Anderson, *Anal. Biochem.*, 85 (1978) 341.
[17] C.S. Giometti, M.A. Gemmell, S.L. Tollaksen and J. Taylor, *Electrophoresis*, 12 (1991) 536.
[18] C.S. Giometti, N.G. Anderson, S.L. Tollaksen, J.J. Edwards and N.L. Anderson, *Anal. Biochem.*, 102 (1980) 47.
[19] G. Grandi, personal communication.
[20] A. Kletzin and M.W.W. Adams, unpublished results.
[21] J.M. Blamey and M.W.W. Adams, *Biochim. Biophys. Acta.*, 1161 (1993) 5070.
[22] P.Z. O'Farrell, H.M. Goodman and P.H. O'Farrell, *Cell*, 12 (1977) 1133.