

Probing the active site of homoserine *trans*-succinylase

Ran Rosen^{a,b}, Dörte Becher^b, Knut Büttner^b, Dvora Biran^a, Michael Hecker^b, Eliora Z. Ron^{a,*}

^aDepartment of Molecular Microbiology and Biotechnology, Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel

^bInstitut für Mikrobiologie und Molekularbiologie, Ernst-Moritz-Arndt-Universität Greifswald, 17487 Greifswald, Germany

Received 20 July 2004; revised 20 September 2004; accepted 6 October 2004

Available online 27 October 2004

Edited by Lukas Huber

Abstract Homoserine *trans*-succinylase is the first enzyme in methionine biosynthesis of *Escherichia coli* and catalyzes the activation of homoserine via a succinylation reaction. The *in vivo* activity of this enzyme is subject to tight regulation by several mechanisms, including repression and activation of gene expression, feedback inhibition, temperature regulation and proteolysis. This complex regulation reflects the key role of this enzyme in bacterial metabolism. Here, we demonstrate – using proteomics and high-resolution mass spectrometry – that succinyl is covalently bound to one of the two adjacent lysine residues at positions 45 and 46. Replacing these lysine residues by alanine abolished the enzymatic activity. These findings position the lysine residues, one of which is conserved, at the active site. © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

Keywords: Methionine biosynthesis; Proteomics; Mass spectrometry; Succinyl homoserine; Two-dimensional gel electrophoresis; *Escherichia coli*

1. Introduction

Methionine is a central cellular compound; in addition to being a component of proteins, it is the first amino acid in every protein and is essential for initiation of translation. It is the precursor of *S*-adenosyl-methionine (SAM) that is involved in the transfer of C1 fragments for reactions such as methylation. SAM also participates in the biosynthesis of polyamines (spermidine and spermine). Recent findings indicate that methionine has yet another important physiological role – it is involved in the synthesis of *N*-acyl homoserine lactone autoinducer molecules that constitute quorum-sensing signals and act as cell density-dependent regulators of gene expression [1,2]. The synthesis of methionine from homoserine involves four enzymatic reactions [3]. The initial reaction serves as an activation step, which allows the subsequent condensation with cysteine to form the thioether-containing compound cystathionine, produced by acylation of the γ -hydroxyl of homoserine. Hydrolysis of cystathionine produces homocysteine, which is then methylated to form methionine [4].

In *Escherichia coli*, the initial step of methionine biosynthesis is catalyzed by homoserine *trans*-succinylase (HTS) encoded by the *metA* gene. HTS transfers a succinyl group from succinyl-CoA to homoserine, forming succinyl homoserine. As the

first specific enzyme in the methionine biosynthetic pathway, HTS activity affects major cellular processes and is, therefore, tightly regulated. So far, it has been shown that *metA* transcription is regulated by a specific repressor (MetJ), by a specific activator (MetR) and by the heat shock response [5–9]. The activity of the enzyme is feedback inhibited by the end-products of the pathway (methionine and *S*-adenosylmethionine; [10]) and is lost at elevated temperatures, above 32 °C [11–13]. This loss of activity at temperatures above 32 °C is due to protein unfolding followed by aggregation above 42 °C [14]. Recently, it was shown that HTS is an unstable protein, subject to energy-dependent, proteolytic degradation [15]. The control of HTS concentration by proteolysis constitutes a new level of regulating *metA* expression, in addition to the other regulatory mechanisms.

The enzymatic mechanism of HTS was previously described and the kinetic parameters for its natural substrates, as well as a number of alternative substrates were determined [4]. Born et al. suggested that the active site includes a sulfhydryl residue (which temporarily binds a succinyl group) because of the pH dependence of the enzyme activity and its inactivation by iodoacetamide. On the basis of solvent kinetic isotope effect studies, they proposed a model of the enzymatic chemical mechanism for this ping-pong reaction.

Here we show, using 2D gel electrophoresis and high-resolution mass spectrometry, a succinyl group covalently bound to a lysine residue in position 45 or 46. As succinate is the substrate of HTS, the lysines which covalently bind it are likely to be in the active site. This conclusion is supported by genetic experiments showing that replacement of these lysine residues abolishes the enzymatic activity and by the finding that lysine in position 46 is conserved in evolution.

2. Materials and methods

2.1. Bacteria, plasmids, PCR primers and growth conditions

Escherichia coli strain MG 1655 (wild type K12) harboring a pUC-*metA* plasmid (1.3 kb fragment containing the *metA* gene cloned into pUC18) was used for the 2D experiments; strain EG1 ($\Delta metA::kan^R$ – lab collection) was used for growth experiments. For amino acid replacement, pUC18 and pBAD24 were used. Bacteria were grown in Davis and Mingioli minimal medium [16] supplemented with 0.2% glucose at 30 °C with gentle agitation. Induction of expression from the arabinose promoter was accomplished using 0.1% arabinose. 50 μ g/ml methionine was added when needed.

2.2. Protein extraction for 2DE

Twenty ml of exponentially growing cultures was collected and quickly cooled to 0 °C. The cells were centrifuged and washed twice

*Corresponding author.

E-mail address: eliora@post.tau.ac.il (E.Z. Ron).

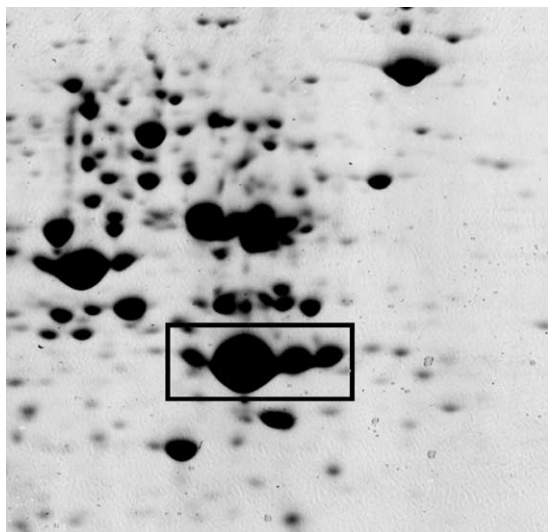


Fig. 1. Separation of HTS on a 2D-gel. A total cell extract of *E. coli* K12 harboring pUC $metA$ plasmid was separated on a 2D gel in pH range 4–7. The region of HTS is framed (left, alkaline; right, acidic).

with cold TE-PMSF [10 mM Tris, pH 7.5; 1 mM EDTA; and 1.4 mM PMSF (phenylmethylsulfonyl fluoride)]. The washed cells were resuspended in 0.5 ml of TE-PMSF and disrupted by sonication. Cell debris and protein aggregates were removed by centrifugation at $20\,000 \times g$ for 30 min at 4 °C. The protein concentration was determined using the Bradford method [17] with the Protein Assays kit (BioRad). The supernatants containing the proteins were lyophilized.

2.3. Two-dimensional gel electrophoresis

Isoelectric focusing was performed with commercially available IPG-strips (Amersham Biosciences, Uppsala, Sweden; 18 cm, pH 4–7). Samples, containing 800 µg of proteins, were loaded by rehydration for 24 h in a solution containing 8 M urea, 2 M thiourea, 1% w/v CHAPS, 20 mM DTT and 0.5% v/v Pharmalyte 3–10. The isoelectric focusing was performed with the MultiphorII unit (Amersham Biosciences) employing the following voltage profile [18]: linear increase from 0 to 500 V for 2500 Vh, 500 V for 2500 Vh, linear increase from 500 to 3500 V for 10000 Vh and a final phase of 3500 V for 35000 Vh. After consecutive equilibration of the gels in solutions containing DTT and iodoacetamide as suggested by Gorg et al. [19], the separation in the second dimension was performed in polyacrylamide gels of 12.5% T and 2.6% C on the Investigator 2D electrophoresis system (Genomic solutions, MD, USA) with approximately 2 W per gel. The gels were stained with Coomassie brilliant blue according to established protocols.

2.4. Mass spectrometric analysis of gel isolated proteins

Mass spectrometric analysis was performed as described by Bandow et al. [20]. Protein spots were excised from Coomassie stained 2D gels and washed in a 200 mM NH_4HCO_3 –50% acetonitrile solution for 30 min at 37 °C. The solution was discarded and the gels were dried in a vacuum centrifuge for 30 min. In gel digestion with Trypsin (Promega, Madison, WI, USA) was carried out for 16–18 h at 37 °C. Peptides were extracted from the gel by diffusion in 10% acetonitrile by incubation in an ultrasonic bath for 20 min. For desalting and concentration, home-made micro-columns containing a 1:1 mixture of POROS 50-R2 and Poros Oligo R3 (Applied Biosystems, Foster City, CA, USA) were prepared in GELoader tips, and washed with 100% CH_3CN following with 0.5% formic acid prior to sample application. The peptide-containing supernatant was loaded on the column, the column was washed with 0.5% formic acid, and the peptides were eluted with 0.9 µl of 60% methanol–0.5% formic acid, into a nanospray needle (Protana, Odense, Denmark). A quadrupole TOF (time-of-flight) mass spectrometer (Qstar Pulsar; Applied Biosystems) equipped with a nanospray ion source (Protana) was used for the measurements.

Analysis of mass spectra, including peptide sequencing, was performed with the Bioanalyst software version 1.1 (Applied Biosystems).

2.5. Methylation of carboxyl groups

Methylation of carboxyl groups was performed as described by Shevchenko et al. [21]. Briefly, the peptide mixture obtained from the above-described Trypsin digest was dried in a vacuum centrifuge prior to methylation. The methylation was performed by incubation for 45 min at room temperature in 15 µl freshly prepared methylation reagent [1 ml of freshly distilled methanol was incubated in a –20 °C freezer for 15 min. 150 µl of freshly distilled acetyl chloride was added (the mixture started to boil instantly) and left at room temperature for 10 min]. To finish, the reaction mixture was dried out in a vacuum centrifuge.

2.6. Sequence analysis

DNA sequences were aligned by neighbor-joining method, with 1000 bootstrap trail, using Clustal X [22].

3. Results

3.1. Homoserine trans-succinylase yields four spots on 2D gels

In a strain of *E. coli* K12 (MG1655, pUC $metA$) overexpressing the *metA* gene, four proteins appeared on a 2D gel, which could not be detected in the wild-type strain, not carrying the *metA* plasmid. These spots corresponded to HTS, as could be determined by Western blotting with anti-HTS antibodies and mass spectrometry. The four spots had a similar apparent molecular weight and differed in their *pI* (Fig. 1). Such variations in the *pI* of a protein suggest the existence of post-translational modifications that result in addition or neutralization of charged groups. Therefore, further mass spectrometric analysis was carried out for characterization of the differences between the four protein populations.

3.2. The two acidic spots contain modified lysine residues

Comparison of the peptide pattern obtained by mass spectrometry after in-gel trypsin digestion of the proteins showed 3 major different peaks that were found, consistently, in the two acidic spots and were absent from the two alkaline spots. These three peaks, 634.89 and 642.89 and 689.18, which carried a charge of 2⁺, were sequenced by mass spectrometry. Peaks 634.89 and 642.89 (Fig. 2A) were found to consist of the T5-T6 fragment (trypsin fragment 5–6) of HTS with a modification of lysine 45 that resulted in a mass shift of approximately 100 Da (Fig. 2B and C). The mass difference of 16 Da between the two peptides is a result of an oxidation of methionine 43 in the peptide represented by the 642.89 peak. The third peak (689.18) was found to be the T6-T7 fragment of HTS with a modification of lysine 46, which also resulted in a mass shift of approximately 100 Da (Fig. 2D).

The appearance of the modified and non-modified fragments results from the fact that the modification of lysine occurs on either lysine 45 or lysine 46, and that trypsin cleaves between the two lysines. (Thus, the precursor T5 peptide has a mass of 1039.66 and yields a peak with a mass to charge value of 520.83, and the precursor T7 peptide, mass 1148.58, and yields a peak with a mass to charge value of 575.29. Fragment T6 consists of a single lysine residue, therefore, it could not be differentiated from non-specific fragmented lysine residues in the spectra. The non-modified T5–T6 and T6–T7 fragments were not detected in the spectra, as they are a result of mis-cleavage of the trypsin which is probably due to the lysine modification). It should be noted

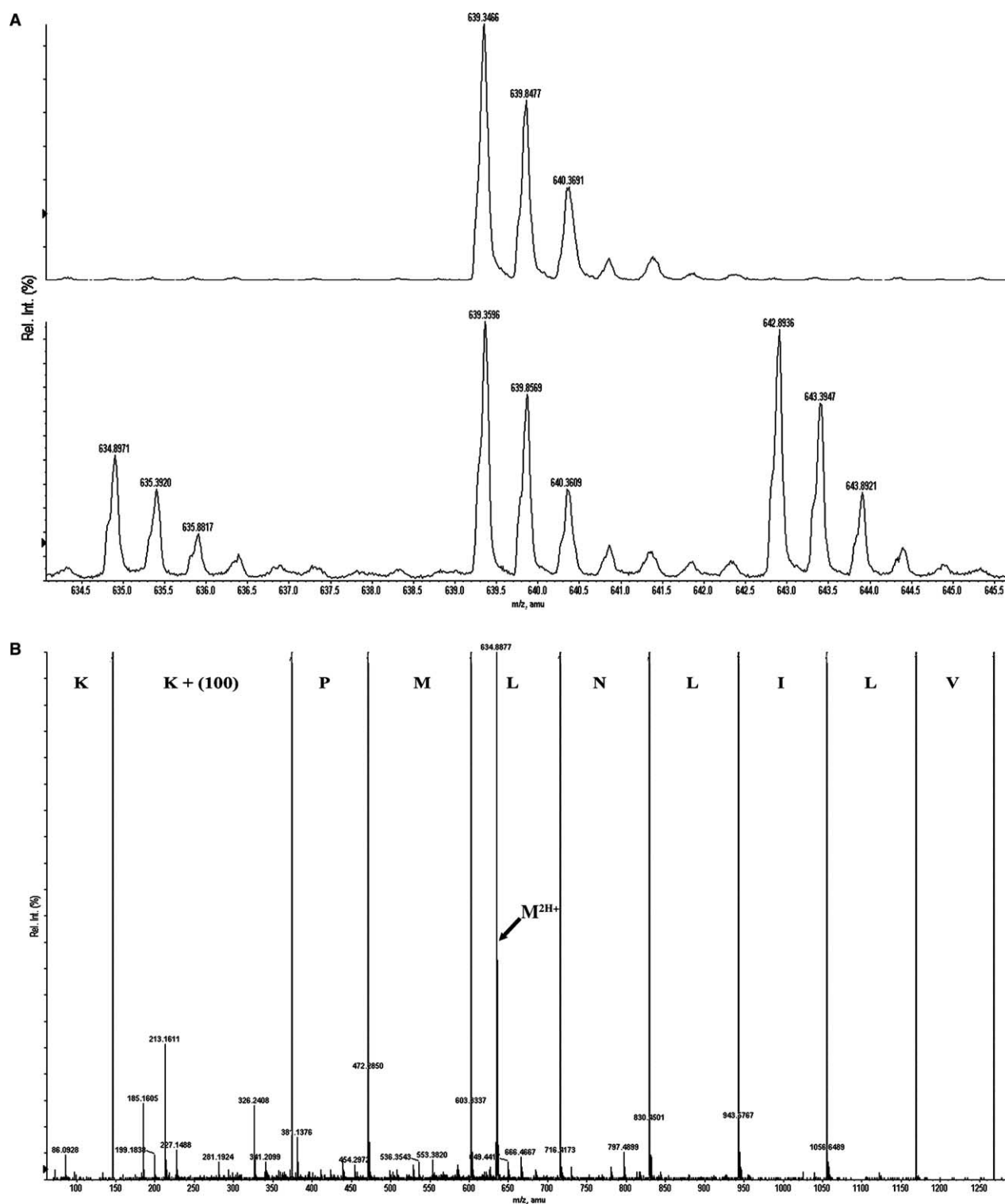


Fig. 2. Mass spectrometric comparison of the alkaline spot and acidic spot pairs. Trypsin digests of HTS from each of the four spots were investigated by Q-TOF mass spectrometry and the spectra were compared. Panel A shows a region of the spectrum from the most alkaline spot (upper chart) and the most acidic spot (lower chart), the spectrum of the second acidic spot was similar to the presented acidic spectrum and the spectrum of the second alkaline spot was similar to the presented alkaline spectrum. The peptides that were unique in the acidic spots were sequenced by mass spectrometry and the MS/MS spectra of peaks 634.89, 642.89 and 689.18 are presented in panels B, C and D, respectively, with sequence interpretation of the data.

that we never obtained a spot that contained two modified lysines and have to conclude that the modification is either on lysine 45 or on lysine 46.

The mass of the modification was calculated by determining the mass deviation between the modified and unmodified peptides and found to be 100.019 Da, using high

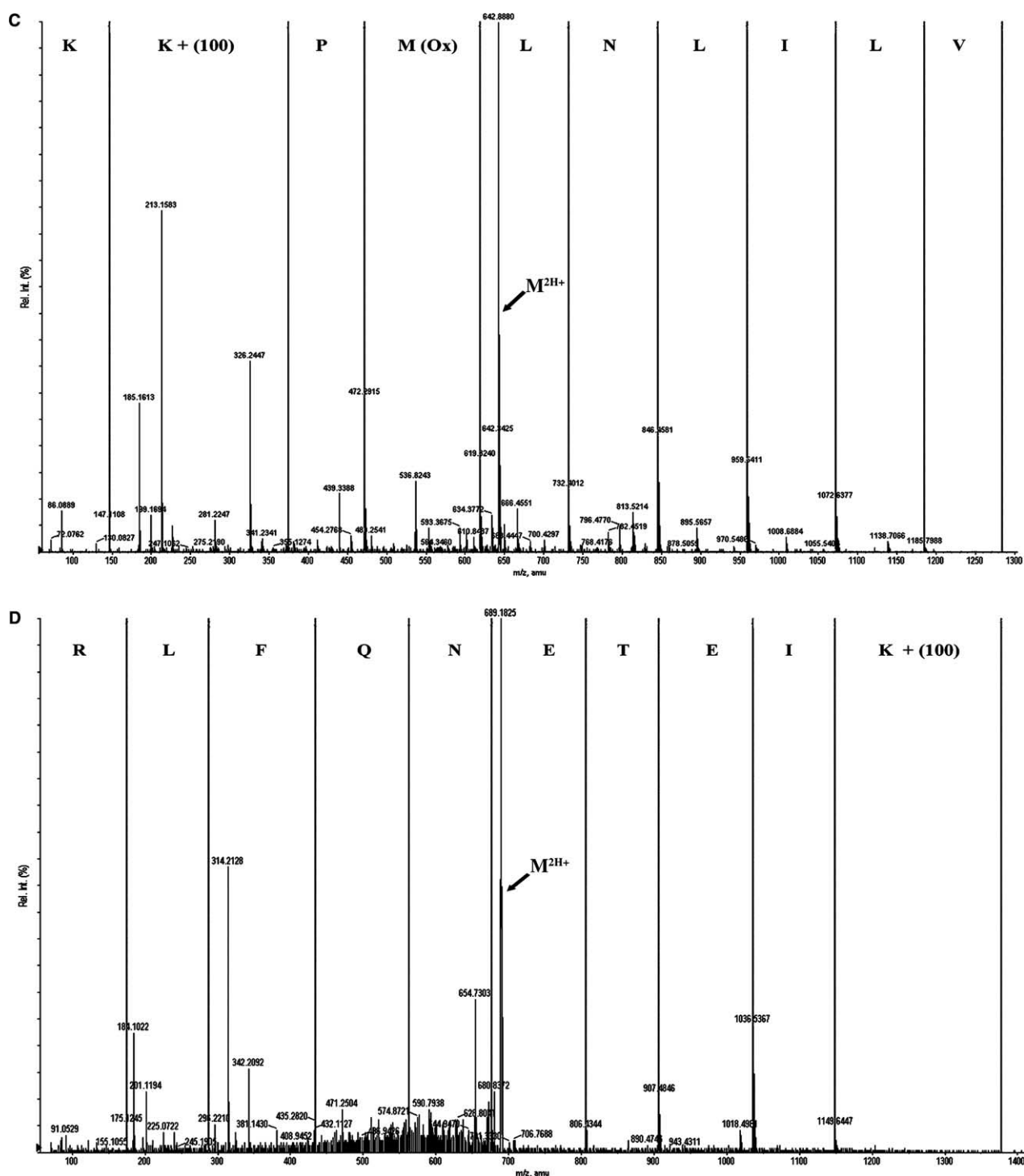


Fig. 2 (continued)

accuracy calculation by internal calibration with non-modified HTS peptides. This mass value of approximately 100 Da does not correspond to any common natural post-translational modifications. Therefore, the possibility arose that the apparent modification is actually the substrate-bound enzyme.

The HTS catalyzed-reaction of transfer of a succinyl group from succinyl-CoA to homoserine, forming succinyl

homoserine, is shown in Fig. 3. The succinyl group ($C_4H_5O_3$) has an exact mass of 101.024 Da, which will cause a mass shift of 100.016 Da, if bound to a lysine, as a result of a hydrogen-loss from the ϵ -amine of the lysine. The delta mass between the measured deviation and the calculated shift by succinyl is 2.4 ppm on a 1283.8 precursor. Such a delta mass is well within the acceptable error range.

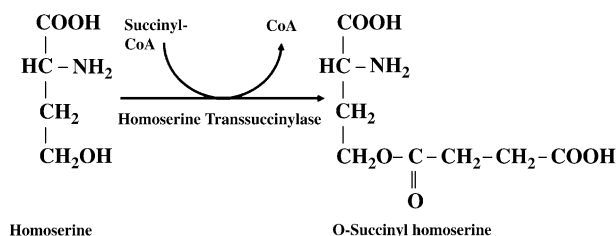


Fig. 3. HTS catalyzes the transfer of succinyl from succinyl-CoA to homoserine, forming succinyl-homoserine.

3.3. HTS lysines 45 and 46 are covalently bound to succinyl

In order to prove that the modification of lysine 45 and lysine 46 is a succinyl group, the peptides were methylated by methanol/acetyl chloride, a process which enables to determine the number of free carboxyl groups. A peptide with a mass to charge value of 648.87 (precursor mass 1295.74 Da, +2 charge) was sequenced by MS/MS (Fig. 4). The resulting sequence indicated that the modified lysine in position 45 changed its mass from 228 Da (lysine + 100 Da) to 242 Da (lysine + 100 Da + 14 Da), suggesting the methylation of a single carboxyl group. As succinyl-lysine has one free carboxyl group, the result strongly supports that the 100 Da shift in the lysine mass is a result of succinylation. In addition, as expected, the free carboxyl group of the C' terminal lysine 46 was methylated, forming a residue with a mass of 161 Da (C' terminal lysine + 14 Da).

3.4. Conservation of lysines and cysteines in HTS

The amino acid sequences of HTS from 12 bacterial species of the Proteobacteria, Firmicutes and Thermotogae were aligned by Clustal X (Fig. 5). The lysine 46 residue of the *E. coli* HTS was found to be conserved in all the aligned sequences, while lysine 45 was found to be conserved only in five of the 12 sequences.

It was suggested [4] that the active site in the *E. coli* HTS includes a cysteine. The protein has two cysteines, at positions 89 and 141. The cysteine in position 89 is not conserved as was found only in the closely related *Yersinia pestis* of the γ -Proteobacteria. On the other hand, cysteine 141 is conserved, but only in 11 out of the 12 sequences (in the Gram-positive *Bacillus halodurans* it is replaced by histidine). It, therefore, appears unlikely that the cysteine is in the active site of the molecule. Rather, we assume that its function is in the formation of dimers, probably required for enzyme activity and allosteric response [10]. This assumption is supported by experiments showing that addition of β -mercaptoethanol prevented formation of HTS dimers.

3.5. Replacement of lysine residues 45 and 46 to alanine abolished HTS activity

In order to validate lysines 45 and 46 as part of the active site of HTS, these two residues were replaced by alanine residues using PCR [15]. The mutated *metA* and its wild type isogen were cloned as a *EcoRI/HindIII* fragment into a pBAD24 plasmid under the regulation of the arabinose promoter generating pBADKK and pBADHTS, respectively. The plasmids

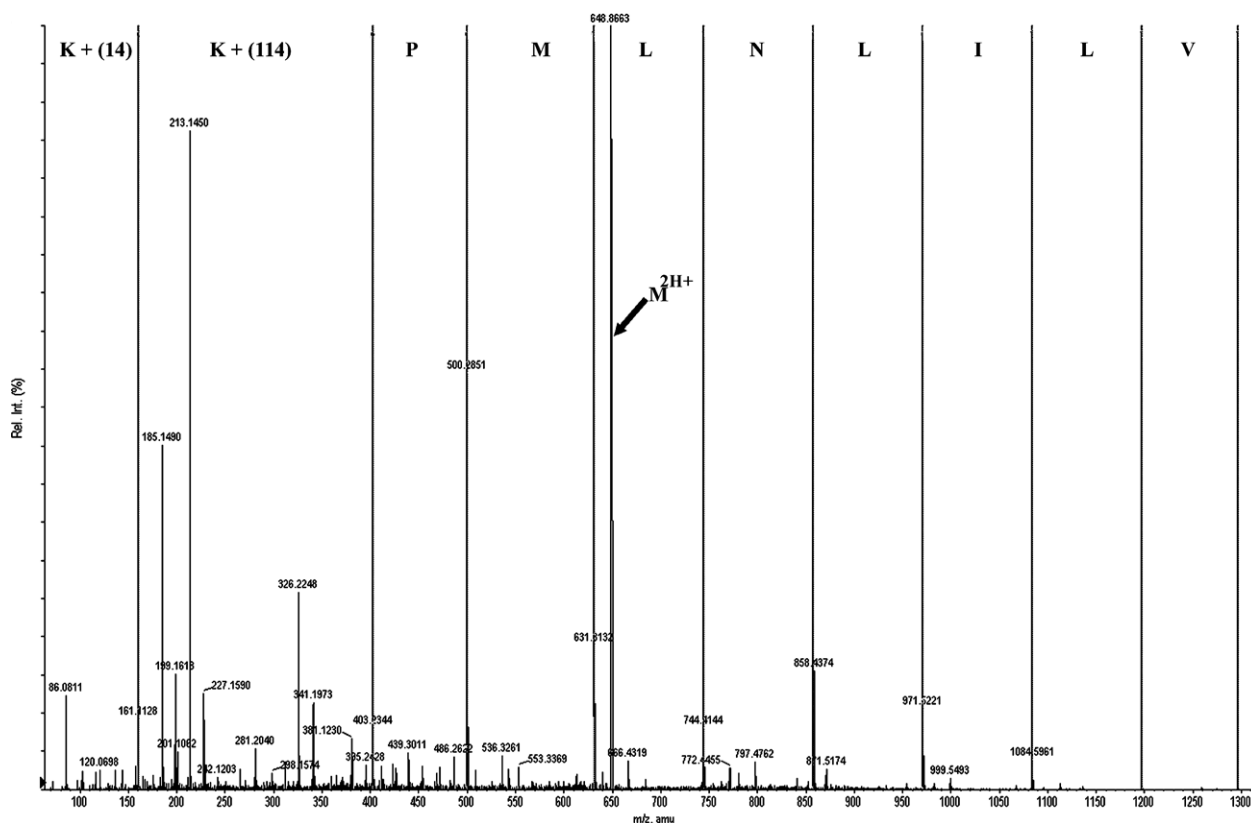


Fig. 4. Mass spectrum of methylated peptide. The carboxyl groups in the peptide mixture were methylated by methanol/acetyl chloride. The 648.87 (precursor mass 1295.74, +2 charge) was sequenced by MS/MS and the mass spectrum with its sequence interpretation is presented.

<i>Escherichia coli</i>	MPIRVPDELPVNFLEENVFVMTTSRASGQEIRPLKVLILNLMPK K IETENQFLRLLSN 60
<i>Bacillus halodurans</i>	MPIKIPDNLPAGEILTKENIFVMAESRAYSQDIRPLKIVILNLMPI K QTETQQLRLLSN 60
<i>Clostridium acetobutylicum</i>	MPIKIPDNLPAAKTLEENIFFMDEDRAYHQDIRPLNIVIVNLMPT K IETETQILRLLSN 60
<i>Salmonella typhimurium</i>	MPIRVLDELPVNFLEENVFVMTTSRASGQEIRPLKVLILNLMPK K IETENQFLRLLSN 60
<i>Yersinia pestis</i>	MPIRVPDELPVNFLEENVFVMASSRAKTQEIRPLKVLILNLMPK K IETENQFLRLLSN 60
<i>Vibrio cholerae</i>	MPIRIPDQLPASDVLRLNENIFVMSERASTQEIRPLKVLILNLMPK K IETETQFLRLLSN 60
<i>Campylobacter jejunii</i>	MPLIIPENIPAYELLK-EHAFIMGLRRAKHQDIRPQEILIVNLMPK K IETENQILSLLSN 60
<i>Streptococcus pneumoniae</i>	MPIRIDKKLPVNEILRTENIFVMDQRAAHQDIRPLKILILNLMPO K IMVTETQQLRLLSN 60
<i>Lactobacillus lactis</i>	MPVKVIEGLPAIDDLRADNIFVMDERAKNQIRPLNLLVNVNLMPO K LITERQILRLLSN 60
<i>Thermotoga maritima</i>	MPINVPSPGLPAVKVLAKEGIFVMTKRAIHQDIRPLEILILNLMPO K IKTEIQQLRLLSN 60
<i>Sinorhizobium meliloti</i>	MPIKIPDTLPVAFETLVHEGVRMLTETETAIRQDIRPLQIGLLNLMPO K IKTEIQMARLGA 60
<i>Brucella abortus</i>	MPIKIPDDLPAVLEAGVVMREADAVRQDIRPLRIGLLNLMPO K VTETETQIARLLGA 60
	***: : . : ** . * : . * * * : : : : : * * * : :

Fig. 5. CLUSTAL X (1.82) multiple sequence alignment of bacterial HTS sequences. The first 60 amino acids are shown.

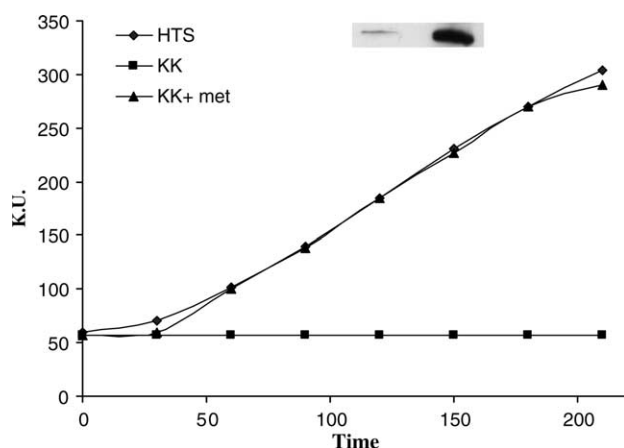


Fig. 6. Replacement of HTS lysine residues 45 and 46 to alanines results in auxotrophy for methionine. EG1 harboring either pBADHTS or pBADKK were grown on minimal media supplemented with arabinose and methionine to mid exponential growth phase. The cultures were washed and transferred to minimal media with arabinose or minimal media with arabinose and methionine. The growth curves are presented. Western blot analysis [15] confirmed the expression of the mutated HTS from the pBADKK before (left) and after (right) arabinose induction.

were transformed into *E. coli* EG1, deleted for the *metA* gene, and the cultures were grown with and without methionine. The growth curves (Fig. 6) indicate that replacement of the lysine residues by alanine abolished HTS activity and stopped growth on minimal medium, while addition of methionine to the medium resumed the growth. The inhibition of growth was not transient, as indicated by the finding that the bacteria did not grow with arabinose even after prolonged incubation.

4. Discussion

Here, we probed the active site of HTS by 2D gel electrophoresis, mass spectrometry and site-directed mutagenesis. We demonstrated that one of the substrates, succinyl, is covalently bound to a highly conserved lysine in position 46. It can also bind to a lysine in position 45, however, this lysine is not conserved in HTS from other bacteria. Replacement of the lysines by alanine abolished the enzymatic activity and results in auxotrophic mutants, which require methionine for growth.

In a comprehensive study of *E. coli* HTS [4], a chemical mechanism was suggested for the succinyl transferase reaction. The model assumed that succinyl-CoA binds to the enzyme and is attacked by an active site thiolate to form the first tetrahedral intermediate. This model is based on the finding that the enzyme is inactivated by 5,5'-dithiobis(2-nitrobenzoate) and by iodoacetamide, implying the participation of cysteine in the active site. Additional support for the involvement of cysteine was obtained by kinetic pH studies, showing a pK value of ca. 6.4, implying that the cysteine is primarily present as the thiolate anion.

This assumption is challenged by the finding that none of the two cysteines in HTS is present in all the tested bacteria. Our results also appear in conflict with the previous model, as we show that the succinyl is bound to a lysine residue. If so, the results demonstrating the essentiality of cysteine could be due to its involvement in maintaining the correct conformation of the molecule, or its binding to the other monomer. It is assumed that HTS is active as a dimer, since it is subject to allosteric inhibition by the end products of the pathway – methionine and SAM [10]. It is likely that the cysteine in position 141, which is present in 11 out of 12 bacteria examined, is the one required for dimer formation. As predicted, the addition of β -mercaptoethanol, which reduces S–S bonds, prevented the formation of dimers.

The finding of an active lysine residue at the active site is not unique to HTS. In hydroxynitrile lyases from *Hevea brasiliensis*, structural and site directed mutagenesis experiments demonstrated that lysine 236 is part of the substrate binding site [23]. Another example is the glycerol-3-phosphate cytidyltransferase enzyme of *Bacillus subtilis* in which structural studies found that lysines 44 and 46 interact with the glycerol and cytosine phosphates of CDP-glycerol, a role that was confirmed by site directed mutagenesis of lysine to alanine [24].

In this work, we take advantage of electrophoresis and mass spectrometric results. These technologies are of increasing importance in proteomic studies and we demonstrate that they can also be used for mechanistic studies of enzyme activity. The advantage of these technologies is that they constitute a direct analysis at the molecular level, which can be an important addition to biochemical reactions, which are often indirect. In the case of HTS, the biochemical studies were essential for understanding the kinetic parameters, as well as for showing the critical role of a cysteine. However, mass spectrometry provided direct evidence for the role of lysine in binding the succinyl. This method was already used in similar

studies of characterization of the active site of 2-deoxy-scylo-inosose synthase, which found a lysine residue at the active site covalently bound to the enzyme inhibitor carbagluco-6-phosphate [25]. We expect that this sort of studies will become more important for biochemical analysis, as the use of mass spectrometry combined with electrophoresis of proteins becomes widely used.

Acknowledgements: This work was partially supported by the Manja and Moris Leigh Chair (E.Z.R.) for Biophysics and Biotechnology.

References

- [1] Hanzelka, B.L. and Greenberg, E.P. (1996) *J. Bacteriol.* 178, 5291–5294.
- [2] Val, D.L. and Cronan Jr., J.E. (1998) *J. Bacteriol.* 180, 2644–2651.
- [3] Saint Girons, I., Parsot, C., Zakin, M.M., Barzu, O. and Cohen, G.N. (1988) *CRC Crit. Rev. Biochem.* 23, S1–S42.
- [4] Born, T.L. and Blanchard, J.S. (1999) *Biochemistry* 38, 14416–14423.
- [5] Davidson, B.E. and Saint Girons, I. (1989) *Mol. Microbiol.* 3, 1639–1648.
- [6] Biran, D., Brot, N., Weissbach, H. and Ron, E.Z. (1995) *J. Bacteriol.* 177, 1374–1379.
- [7] Ron, E.Z., Alajem, S., Biran, D. and Grossman, N. (1990) *Antonie Van Leeuwenhoek* 58, 169–174.
- [8] Maxon, M.E. et al. (1989) *Proc. Natl. Acad. Sci. USA* 86, 85–89.
- [9] Shoeman, R., Coleman, T., Redfield, B., Greene, R.C., Smith, A.A., Saint-Girons, I., Brot, N. and Weissbach, H. (1985) *Biochem. Biophys. Res. Commun.* 133, 731–739.
- [10] Lee, L.W., Ravel, J.M. and Shive, W. (1966) *J. Biol. Chem.* 241, 5479–5480.
- [11] Ron, E.Z. and Shani, M. (1971) *J. Bacteriol.* 107, 397–400.
- [12] Ron, E.Z. and Davis, B.D. (1971) *J. Bacteriol.* 107, 391–396.
- [13] Ron, E.Z. (1975) *J. Bacteriol.* 124, 243–246.
- [14] Gur, E., Biran, D., Gazit, E. and Ron, E.Z. (2002) *Mol. Microbiol.* 46, 1391–1397.
- [15] Biran, D., Gur, E., Gollan, L. and Ron, E.Z. (2000) *Mol. Microbiol.* 37, 1436–1443.
- [16] Davis, B.D. and Mingioli, E.S. (1950) *J. Bacteriol.* 60, 17–28.
- [17] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [18] Rosen, R., Biran, D., Gur, E., Becher, D., Hecker, M. and Ron, E.Z. (2002) *FEMS Microbiol. Lett.* 207, 9–12.
- [19] Gorg, A., Postel, W., Domscheit, A. and Gunther, S. (1988) *Electrophoresis* 9, 681–692.
- [20] Bandow, J.E., Becher, D., Buttner, K., Hochgrafe, F., Freiberg, C., Brotz, H. and Hecker, M. (2003) *Proteomics* 3, 299–306.
- [21] Shevchenko, A., Chernushevich, I., Wilm, M. and Mann, M. (2000) *Methods Mol. Biol.* 146, 1–16.
- [22] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) *Nucleic Acids Res.* 25, 4876–4882.
- [23] Gruber, K., Gartler, G., Krammer, B., Schwab, H. and Kratky, C. (2004) *J. Biol. Chem.*
- [24] Patridge, K.A., Weber, C.H., Friesen, J.A., Sanker, S., Kent, C. and Ludwig, M.L. (2003) *J. Biol. Chem.* 278, 51863–51871.
- [25] Nango, E., Eguchi, T. and Kakinuma, K. (2004) *J. Org. Chem.* 69, 593–600.