Michael A. Joyce,^{a,†} Edward R. Brownie,^b Koto Hayakawa^b and Marie E. Fraser^{b*}^aDepartment of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, and ^bDepartment of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4, Canada

† Current address: Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada.

Correspondence e-mail: frasm@ucalgary.ca

Received 31 January 2007
Accepted 5 April 2007

Cloning, expression, purification, crystallization and preliminary X-ray analysis of *Thermus aquaticus* succinyl-CoA synthetase

Succinyl-CoA synthetase (SCS) is an enzyme of the citric acid cycle and is thus found in most species. To date, there are no structures available of SCS from a thermophilic organism. To investigate how the enzyme adapts to higher temperatures, SCS from *Thermus aquaticus* was cloned, overexpressed, purified and crystallized. Attempts to crystallize the enzyme were thwarted by proteolysis of the β -subunit and preferential crystallization of the truncated form. Crystals of full-length SCS were grown after the purification protocol was modified to include frequent additions of protease inhibitors. The resulting crystals, which diffract to 2.35 Å resolution, are of the protein in complex with Mn^{2+} -GDP.

1. Introduction

Succinyl-CoA synthetase (SCS; EC 6.2.1.3/4; reviewed in Bridger, 1974; Nishimura, 1986) is an enzyme of the citric acid cycle, where it catalyzes the only step that involves substrate-level phosphorylation. In the citric acid cycle, the enzyme acts not as a synthetase, but as a thiolase. It uses the energy of the succinyl-CoA thioester to phosphorylate itself on the active-site histidine residue and then transfers the phosphoryl group to nucleotide diphosphate to form nucleotide triphosphate. The reaction requires magnesium or other divalent cations.

The first structure of SCS to be determined was that of the *Escherichia coli* enzyme (Wolodko *et al.*, 1994). The *E. coli* enzyme is a heterotetramer of two α -subunits and two β -subunits (Bridger, 1971). The active-site histidine residue is residue 246 of the α -subunit, which was phosphorylated in the structure (Wolodko *et al.*, 1994). CoA, which is required for crystallization (Wolodko *et al.*, 1984), bound to the amino-terminal domain of the α -subunit, with the free thiol group near the phosphorylated histidine residue. Subsequent experiments proved that the nucleotide was bound to the amino-terminal domain of the β -subunit (Joyce *et al.*, 1999), 30 Å away from the location of the phosphohistidine in the crystal structure (Joyce *et al.*, 2000). Based on the crystal structures of SCS and of other members of the ATP-grasp family (Murzin, 1996), it was hypothesized that the phosphohistidine loop swings to shuttle the phosphoryl group from site I, where CoA and presumably succinate bind, to site II, where the nucleotide binds (Fraser *et al.*, 1999). *E. coli* SCS can use ADP or GDP, but in mammals there are two forms of SCS, one specific for ADP/ATP and the other specific for GDP/GTP (Johnson *et al.*, 1998). The structure of GTP-specific SCS from pig, which is active as an $\alpha\beta$ -dimer (Wolodko *et al.*, 1986), has been determined in both the phosphorylated and dephosphorylated forms (Fraser *et al.*, 2000), as well as in open conformations without nucleotide and closed conformations with nucleotide, GDP or GTP, bound (Fraser *et al.*, 2006).

To date, there are no structures available of a complete SCS heteromultimer from a thermophilic organism. The structure of the α -subunit of *Thermus thermophilus* SCS was determined as part of a structural genomics initiative (H. Takahashi, Y. Tokunaga, C. Kuroishi, N. Babayeba, S. Kuramitsu, S. Yokoyama, M. Miyano, M. & T. H. Tahirov, unpublished work) and the model and crystallographic data have been deposited in the Protein Data Bank (Berman *et al.*,

© 2007 International Union of Crystallography
All rights reserved

2000) with code 1oi7. The crystals diffracted to very high resolution, 1.23 Å, but no electron density was observed for the residues of the phosphohistidine loop (Kleywegt *et al.*, 2004). This is likely to be because the loop is disordered in the absence of the β -subunit, since in the structure of SCS the phosphohistidine loop interacts with the carboxy-terminal domain of the β -subunit (Wolodko *et al.*, 1994). It would be interesting to compare the structure of SCS from a thermophile with that from *E. coli* in order to gain a better understanding of how the enzyme protects its substrates, in particular succinyl-CoA, from hydrolysis at higher temperatures and to see what makes the thermophilic enzyme stable at higher temperatures. For this purpose, SCS from *T. aquaticus* was cloned, overexpressed, purified and crystallized.

2. Materials and methods

2.1. Cloning and expression

The genes for the α - and β -subunits of *T. aquaticus* SCS were amplified from *T. aquaticus* genomic DNA using the polymerase chain reaction (PCR). Initial experiments were designed to produce an expression plasmid with the gene for the β -subunit upstream of that for the α -subunit, both under control of a single promoter as in *T. aquaticus*. However, the α -subunit was not well expressed and the expression plasmid was modified to have two T7 promoters and ribosome-binding sites, one for each gene. The primers for the 5'- and 3'-ends of the gene for the α -subunit were 5'-**AAT GAA AGG AGG TAA CAT ATG ATC CTA GTT AAT AAA GAG ACC CGC GTC CTG-3'** and 5'-**CCG GAT GAA TTC CAG CCC AGG GCC TTC TTG ACC AG-3'**, respectively. For the β -subunit, they were 5'-**CGA TGC CAT ATG AAC CTG CAC GAG TAT CAA GCG-3'** and 5'-**TTT ATT AAC TAG GAT CAT ATG TTA CCT CCT TTC ATT GCC ACG GTG ACC TTG-3'**. Since the sequence of the β -subunit was not available at the time, the sequence of the β -subunit of *T. flavus* SCS (Nishiyama *et al.*, 1991) was used to design the 5'-primer for the β -subunit. Bases in the primer sequences that do not match those of *T. flavus* or *T. aquaticus* are shown in bold and the restriction-endonuclease sites used in the cloning are shown in *italic*. The primers were designed to include these sites in order to change the start codon for the α -subunit to ATG and to make the sequences more AT-rich. The change to C from T after the restriction-enzyme site in the primer for the 3'-end of the α -subunit was an error, which led to a change from the stop codon to the codon for Trp. Using the Invitrogen TOPO TA Cloning Kit (Invitrogen), the PCR products were cloned individually into the cloning vector pCR2.1. Each gene was then subcloned into the expression vector pT7-7 (Tabor, 1990) after digestion with *NdeI* (New England Biolabs Inc.) and *EcoRI* (Bethesda Research Laboratories/Life Technologies Inc.) for the α -subunit and *NdeI* for the β -subunit. To create an expression plasmid containing both genes, the vector with the gene for the α -subunit was digested with *BglII*, while the vector with the gene for the β -subunit was digested with *BglII* and *BamHI* (New England Biolabs Inc.). The appropriate DNA fragments were purified, ligated with T4 DNA ligase (New England Biolabs Inc.) and transformed into chemically competent *E. coli* JM 103 cells (Sambrook *et al.*, 1989). Plasmid DNA was isolated using the Qiagen Plasmid Midi Kit (Qiagen Inc.). Seven of the eight colonies picked contained the gene for the β -subunit inserted upstream of the gene for the α -subunit and in the correct orientation.

For protein expression, the plasmid was transformed into *E. coli* BL21(DE3) cells (Novagen). The plasmid purified from this expression strain was sequenced by the DNA Sequencing Facility at the

University of Calgary through the region containing the two genes and their promoters. For the α -subunit, the cloning led to the conservative mutation of R to K near the amino-terminus and the addition of eight residues at the carboxy-terminus: WNSRPGIL. These changes resulted from the primers. For the β -subunit, the cloning led to the change of residues 369 and 370 from AI to VT, 374 from V to K, 377 and 378 from NI to AA and the addition of 19 residues at the carboxy-terminus: WLEFAPGDLPVHSQYNLL. The changes resulted from the primers, as well as from the fact that insertion of the *NdeI* site left the codon for Trp rather than a stop codon at the end of the gene for the β -subunit. Bases in the pT7-7 plasmid coded for the residues at the carboxy-terminus of the β -subunit. Despite these changes in the sequence, the protein was active as a succinyl-CoA synthetase and was stable at high temperatures. For protein expression, an overnight culture of transformed BL21(DE3) cells was grown in 400 ml Luria-Bertani broth and used to inoculate 1.5 l fresh medium. The culture was grown at 310 K to mid-log phase ($A_{600\text{ nm}} = 0.5$) prior to induction of protein expression with 0.5 mM IPTG. Growth was continued at 303 K for 12 h.

2.2. Purification and crystallization

Crystallization of full-length *T. aquaticus* SCS required frequent additions of protease inhibitors during purification of the protein. During the initial purifications, only 1.0 mM benzamidine-HCl was used. Subsequently, a protease-inhibitor cocktail was added to bring the concentrations of the inhibitors to 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM benzamidine-HCl, 1 $\mu\text{g ml}^{-1}$ leupeptin and 0.7 $\mu\text{g ml}^{-1}$ pepstatin A. Cells were harvested by centrifugation at 277 K, resuspended in 0.1 M potassium phosphate, 0.1 M KCl, 10 mM 2-mercaptoethanol, 1.0 mM benzamidine-HCl pH 7.4 and frozen at 193 K. After thawing, the protease-inhibitor cocktail was added and the cells were lysed by sonication. Cell debris was removed by centrifugation and the supernatant was heated at 343 K with occasional stirring for 30 min to precipitate proteins and other macromolecules that were not thermostable. The subsequent steps, up to the crystallization experiments, were performed at 277 K. After cooling, centrifugation was repeated, retaining the supernatant. The protease-inhibitor cocktail was added, followed by 20 g ammonium sulfate per 100 ml initial solution to precipitate the protein. The precipitant was left to settle overnight and collected by centrifugation. The pellet was dissolved in 10 mM Tris-HCl, 10 mM 2-mercaptoethanol and 1 mM benzamidine-HCl pH 8.0 (buffer A) and then phosphorylated with 1 mM ATP and 10 mM MgCl_2 . The solution was clarified by centrifugation and desalted on a Sephadex G-50 coarse column equilibrated with buffer A. Proteins were separated by elution from a Q Sepharose FF column using a linear gradient of increasing KCl concentration in buffer A. *T. aquaticus* SCS eluted when the concentration of KCl was between 0.15 and 0.25 M. The best fractions, as judged by SDS-PAGE, were pooled and concentrated using an Amicon ultrafiltration cell with a YM30 membrane (nominal molecular-weight cutoff 30 kDa). The sample was loaded onto a Sephacryl S200 SF column and eluted with 0.1 M potassium phosphate, 0.1 M KCl, 10 mM 2-mercaptoethanol, 1 mM benzamidine-HCl, 1% glycerol pH 7.4. The best fractions were pooled and the protease-inhibitor cocktail was added. The sample was concentrated and loaded onto a hydroxyapatite FF column. The protein eluted with an increasing concentration of potassium phosphate in a buffer originally containing 10 mM potassium phosphate, 10 mM 2-mercaptoethanol, 1 mM benzamidine-HCl and 10%

Table 1Information on the three crystal forms of *T. aquaticus* SCS.

Values in parentheses are for the highest resolution shell.

Crystal form	I	II	III
Resolution limit (Å)	2.2 (2.38–2.20)	3.3 (3.36–3.30)	2.35 (2.39–2.35)
Crystal system	Orthorhombic	Monoclinic	Monoclinic
Space group	<i>I</i> 222	<i>C</i> 2	<i>C</i> 2
Unit-cell parameters (Å, °)	$a = 100.90, b = 114.24, c = 121.63,$ $\alpha = \beta = \gamma = 90$	$a = 247.76, b = 127.20, c = 112.50,$ $\alpha = \gamma = 90, \beta = 117.0$	$a = 261.73, b = 126.80, c = 110.60,$ $\alpha = \gamma = 90, \beta = 112.76$
Unit-cell volume (Å ³)	1401170	3158250	3385809
No. of measurements	242452	184934	310767
No. of unique reflections	36307	48749	120106
$\langle I \rangle / \langle \sigma(I) \rangle^\dagger$	29.0 (2.4)	11.9 (4.4)	9.5 (1.2)
$R_{\text{merge}}^\ddagger$ (%)	5.6 (35.6)	12.3 (32.4)	12.9 (41.4)
Completeness (%)	99.0 (97.2)	99.9 (100)	86.7 (49.3)

$^\dagger \langle I \rangle$ is the mean intensity for all reflections and $\langle \sigma(I) \rangle$ is the mean sigma for these reflections. $^\ddagger R_{\text{merge}} = (\sum \sum |I_i - \langle I \rangle|) / \sum \sum \langle I \rangle$, where I_i is the intensity of an individual measurement of a reflection and $\langle I \rangle$ is the mean value for all equivalent measurements of this reflection.

glycerol pH 7.4. SDS–PAGE and the specific activity were used to judge which fractions should be pooled. Activity was measured using the same spectrophotometric assay as for *E. coli* SCS (Bridger *et al.*, 1969) and the amount of protein was quantified using the Bio-Rad protein assay (Bradford, 1976). The maximal specific activity was 12.4 U mg^{−1}. The yield of purified *T. aquaticus* SCS from 3 l culture was approximately 20 mg.

Crystals were grown in hanging drops using vapour diffusion at 294 K. For crystallization, the protein was concentrated to approximately 10 mg ml^{−1} and the buffer was exchanged for 10 mM Tris–HCl pH 8.0. As well as attempting to crystallize the free protein, CoA, ADP, ATP, GDP, GTP, magnesium, manganese and phosphate were added to the protein solution in various combinations to form complexes for crystallization trials. A typical experiment used 1 µl protein solution containing 7 mg ml^{−1} protein, 5 mM nucleotide and 10 mM divalent cation as the chloride salt and 1 µl precipitant solution equilibrated over 1 ml precipitant solution. The first crystal form (I) was grown using 5 mM GDP and 10 mM MgCl₂ in the protein solution and 12% polyethylene glycol monomethylether 5000 (P5Kmm) and 100 mM Tris–HCl (pH 7.4 for 1 M solution) as the precipitant solution. Crystal form II grew with 5 mM GDP and 10 mM MnCl₂ in the protein solution and a precipitant solution containing 10% polyethylene glycol 4000 (P4K) and 100 mM Tris–HCl (pH 7.3 for 1 M solution). The crystals of full-length *T. aquaticus* SCS (crystal form III) were grown using a precipitant solution containing 10% polyethylene glycol 3350 (P3350), 100 mM 4-morpholinoethanesulfonic acid (MES; pH 6.4 for 1 M solution) and 200 mM KCl and a protein solution containing 5 mM GDP and 10 mM MnCl₂.

2.3. X-ray crystallography

X-ray diffraction data were collected at beamline 8.3.1 of the Advanced Light Source. The crystals were transferred through solutions containing increasing amounts of P5Kmm, P4K or P3350 (up to 20%) and glycerol as the cryoprotectant (up to 25%) before being mounted in a cryoloop and vitrified in a stream of nitrogen at 110 K. Vitrified crystals were stored in liquid nitrogen until shipment to the synchrotron in a dry dewar. Beamline 8.3.1 is equipped with an ADSC Quantum-210 2 × 2 CCD detector, which was used to collect the X-ray diffraction data. The wavelength used for the data collection was 1.1159 Å. The data were processed using the *HKL* program package (Otwinowski & Minor, 1997). Programs from the *CCP4* suite were used to perform most of the calculations (Collaborative Computational Project, Number 4, 1994). For molecular replacement, the program *AMoRe* (Navaza, 1994) from the *CCP4* suite was used,

with search models that included the structure of *E. coli* SCS (Fraser *et al.*, 2002; PDB code 1jkj) and the structure of the α -subunit of *T. thermophilus* SCS (H. Takahashi, Y. Tokunaga, C. Kuroishi, N. Babayeba, S. Kuramitsu, S. Yokoyama, M. Miyano, M. & T. H. Tahirov, unpublished work; PDB code 1oi7). Partial models were refined using the program *CNS* (Brünger *et al.*, 1998) and the models and electron density were visualized using the program *XFIT* (McRee, 1999).

3. Results

Three crystal forms were grown from samples of *T. aquaticus* SCS. The unit-cell parameters and space-group information and the statistics for the best data set collected from each crystal form are given in Table 1.

Crystal form I grew over a period of weeks from the first batches of protein purified for crystallization. The symmetry and unit-cell parameters suggested that the asymmetric unit contained one $\alpha\beta$ -dimer, implying that the dimer would pack with a second dimer at one of the crystallographic twofold axes to form the biologically relevant tetramer. The Matthews coefficient and the solvent content were calculated to be 2.4 Å³ Da^{−1} and 48%, respectively, using the website <http://ruppweb.dyndns.org> (Kantardjiev & Rupp, 2003; Matthews, 1968). Only after the structure had been solved did we question the quality of the protein. The structure determination confirmed that the asymmetric unit contains a heterodimer, but there was no electron density for the carboxy-terminal domain of the

Figure 1 Photomicrograph of crystals of full-length *T. aquaticus* SCS, crystal form III.

β -subunit beyond residue 224 or for the phosphohistidine loop. Despite requiring nucleotide for crystallization, these crystals showed no electron density for the nucleotide. Subsequent SDS-PAGE analysis of the crystals confirmed that what had crystallized was a protein with a truncated β -subunit. If the β -subunit were cleaved after residue 224, its molecular weight would be 24 517.47 Da, leading to an increase in V_M and the solvent content of the crystals to $3.2 \text{ \AA}^3 \text{ Da}^{-1}$ and to 61%, respectively. Cleavage of the β -subunit of *T. aquaticus* SCS was reduced during subsequent purifications by frequent additions of protease inhibitors.

The second crystal form showed poorer diffraction than the first, but data collected to $3.3 \text{ \AA}$ resolution were sufficient for a molecular-replacement solution. The Matthews coefficient and the solvent content were calculated to be $2.7 \text{ \AA}^3 \text{ Da}^{-1}$ and 54%, respectively, assuming the presence of full-length protein. The electron density showed two full-length $\alpha\beta$ -dimers and two truncated ones, again lacking electron density for residues 237–256 of the α -subunits and paired with β -subunits that had electron density only for residues 1–224. Each biological tetramer contained one β -subunit that was full length and one that was truncated. It was apparent that the protease inhibitors had reduced the amount of cleavage and that the new crystallization conditions did not favour the crystallization of a cleaved form of the enzyme, but favoured the crystallization of octamers in which two of the β -subunits were cleaved. The V_M and solvent content calculated for these crystals with half of the β -subunits truncated are $3.1 \text{ \AA}^3 \text{ Da}^{-1}$ and 60%, respectively.

Crystal form III belonged to the same space group as crystal form II, but one unit-cell edge was significantly longer. The volume of this third crystal form was 7% greater than that of crystal form II. A photomicrograph of these crystals is shown in Fig. 1. The crystals grew as thin plates to maximum dimensions of $0.2 \times 0.2 \times 0.04 \text{ mm}$. The Matthews coefficient and the solvent content were calculated to be $2.9 \text{ \AA}^3 \text{ Da}^{-1}$ and 57%, respectively. Initial electron-density maps showed electron density for 289 residues of the α -subunits and 373 residues of the β -subunits as well as for the nucleotide. This crystal form will be used for the refinement of the structure of *T. aquaticus* SCS in complex with Mn^{2+} -GDP.

Funding for the production and purification of *T. aquaticus* SCS was provided by the Canadian Institutes of Health Research, while funding for the crystallization was provided by the Natural Sciences and Engineering Research Council of Canada. X-ray diffraction data were collected at beamline 8.3.1 of the Advanced Light Source (ALS) at Lawrence Berkeley Laboratory, under an agreement with the Alberta Synchrotron Institute (ASI). The ALS is operated by the Department of Energy and supported by the National Institutes of Health. Beamline 8.3.1 was funded by the National Science Foundation, the University of California and Henry Wheeler. The ASI

synchrotron-access program is supported by grants from the Alberta Science and Research Authority (ASRA) and the Alberta Heritage Foundation for Medical Research (AHFMR). MEF is a Biomedical Scholar supported by the AHFMR.

References

- Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. & Bourne, P. E. (2000). *Nucleic Acids Res.* **28**, 235–242.
- Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
- Bridger, W. A. (1971). *Biochem. Biophys. Res. Commun.* **42**, 948–954.
- Bridger, W. A. (1974). *The Enzymes*, edited by P. D. Boyer, Vol. 10, pp. 581–606. New York: Academic Press.
- Bridger, W. A., Ramaley, R. F. & Boyer, P. D. (1969). *Methods Enzymol.* **13**, 70–75.
- Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, N., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* **D54**, 905–921.
- Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* **D50**, 760–763.
- Fraser, M. E., Hayakawa, K., Hume, M. S., Ryan, D. G. & Brownie, E. R. (2006). *J. Biol. Chem.* **281**, 11058–11065.
- Fraser, M. E., James, M. N. G., Bridger, W. A. & Wolodko, W. T. (1999). *J. Mol. Biol.* **285**, 1633–1653.
- Fraser, M. E., James, M. N. G., Bridger, W. A. & Wolodko, W. T. (2000). *J. Mol. Biol.* **299**, 1325–1339.
- Fraser, M. E., Joyce, M. A., Ryan, D. G. & Wolodko, W. T. (2002). *Biochemistry*, **41**, 537–546.
- Johnson, J. D., Mehus, J. G., Tews, K., Milabetz, B. I. & Lambeth, D. O. (1998). *J. Biol. Chem.* **273**, 27580–27586.
- Joyce, M. A., Fraser, M. E., Brownie, E. R., James, M. N. G., Bridger, W. A. & Wolodko, W. T. (1999). *Biochemistry*, **38**, 7273–7283.
- Joyce, M. A., Fraser, M. E., James, M. N. G., Bridger, W. A. & Wolodko, W. T. (2000). *Biochemistry*, **39**, 17–25.
- Kantardjieff, K. A. & Rupp, B. (2003). *Protein Sci.* **12**, 1865–1871.
- Kleywegt, G. J., Harris, M. R., Zou, J., Taylor, T. C., Wahlby, A. & Jones, T. A. (2004). *Acta Cryst.* **D60**, 2240–2249.
- McRee, D. E. (1999). *J. Struct. Biol.* **125**, 156–165.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Murzin, A. G. (1996). *Curr. Opin. Struct. Biol.* **6**, 386–394.
- Navaza, J. (1994). *Acta Cryst.* **A50**, 157–163.
- Nishimura, J. S. (1986). *Adv. Enzymol. Relat. Areas Mol. Biol.* **58**, 141–172.
- Nishiyama, M., Horinouchi, S. & Beppu, T. (1991). *Mol. Gen. Genet.* **226**, 1–9.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
- Tabor, S. (1990). *Current Protocols in Molecular Biology*, edited by F. A. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl, pp. 16.12.11–16.12.11. New York: Greene Publishing/Wiley Interscience.
- Wolodko, W. T., Fraser, M. E., James, M. N. G. & Bridger, W. A. (1994). *J. Biol. Chem.* **269**, 10883–10890.
- Wolodko, W. T., James, M. N. G. & Bridger, W. A. (1984). *J. Biol. Chem.* **259**, 5316–5320.
- Wolodko, W. T., Kay, C. M. & Bridger, W. A. (1986). *Biochemistry*, **25**, 5420–5425.