Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 23 (2003) 367–372
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

A novel regulatory function of selenocysteine lyase, a unique catalyst to modulate major urinary protein

Mi-Sun Kwak, Hisaaki Mihara, Nobuyoshi Esaki*

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

Received 7 February 2003; received in revised form 8 April 2003; accepted 11 April 2003

Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

Mouse selenocysteine lyase (SCL) catalyzes the decomposition of L-selenocysteine into L-alanine and selenium with pyridoxal 5′-phosphate as a coenzyme. When using SCL as bait in a yeast two-hybrid screening method, major urinary protein I (MUP-I) was identified as a protein that interacts with SCL. This interaction was confirmed with an in vitro binding assay. MUP-I is known as a pheromone-binding protein that accommodates volatile effectors to affect the physiology and behavior of mice. We found that the binding of 2-naphthol to MUP-I was significantly inhibited by SCL, suggesting that SCL regulates the binding capacity of MUP-I.

Keywords: Selenium; Selenocysteine lyase; Major urinary protein; Yeast two-hybrid screening; Protein–protein interaction

1. Introduction

Selenocysteine lyase (SCL) is a pyridoxal 5′-phosphate-dependent enzyme that catalyzes decomposition of L-selenocysteine into L-alanine and selenium (Fig. 1) [1,2]. SCL has been purified from pig liver [3] and *Citrobacter freundii* [4] as the first enzyme that specifically acts on a selenium-containing compound and not on the sulfur analog. The mouse SCL gene was cloned, and the recombinant enzyme was characterized to be highly specific to selenocysteine [5]. SCL was proposed to cooperate with selenophosphate synthetase in selenoprotein biosynthesis: selenium produced by SCL is converted to selenophosphate by selenophosphate synthetase [6]. Then, selenophosphate is utilized by selenocysteine synthase to produce selenocysteyl-tRNA^{sec}, which is specifically directed to the in-frame UGA codons on mRNAs encoding selenoproteins [7]. Several attempts have been made to heterologously produce recombinant selenoproteins, which are expected to be useful for medicinal applications as well as the X-ray analysis of proteins [8,9]. However, no method has been established for the production of selenoproteins with a satisfactory yield due to the complex mechanism of the selenoprotein biosynthesis.

The amino acid sequence of SCL exhibits some similarities to that of cysteine desulfurase IscS from *Escherichia coli* [10]. Recent studies showed that IscS is involved in the biosynthesis of iron–sulfur clusters and thiamine via protein interactions with IscU [11–15] and ThiI [16–18], respectively. The enzymes incorporating sulfur/selenium atoms into sulfur/selenium-containing biomolecules are probably

* Corresponding author. Tel.: +81-774-38-3240; fax: +81-774-38-3248.
E-mail address: esaki@scl.kyoto-u.ac.jp (N. Esaki).

1381-1177/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1381-1177(03)00100-0

Fig. 1. Reaction catalyzed by selenocysteine lyase.

useful for the production of artificial but bioactive sulfur/selenium-containing compounds.

In order to clarify the regulatory mechanism of SCL, we looked for proteins that may interact with SCL by yeast two-hybrid screening and found mouse major urinary protein I (MUP-I) as a candidate. MUP-I is a pheromone-binding protein functioning as a carrier of volatile effectors to regulate the physiology and behavior of mice [19]. We found that SCL indeed interacts with MUP-I to affect its ligand-binding properties. Details of the mechanism of the interaction would provide the clues necessary to modulate the ligand specificity of MUP-I, and the construction of a modified MUP-I would probably be useful for pest control as well as for the food and cosmetic industries.

2. Materials and methods

2.1. Bacterial and yeast strains

Saccharomyces cerevisiae strains used in the yeast two-hybrid library screening were EGY48 (MATα *ura*3 *trp*1 *his*3 *leu*2::6LexAop-LEU2) and RFY206 (MATα *his*3Δ200 *leu*2–3 *lys*2Δ201 *ura*3-52 *trp*1Δ::*his*G) [20]. *E. coli* KC8 was used to isolate prey plasmids from yeast.

2.2. Plasmid construction for the yeast two-hybrid system

Oligonucleotide primers 5′-CCGGAATTCATGGACGCGGCGCGAAATGGC-3′ and 5′-ACGCGTCGACCTAGTTCTAGAGCCGCCCTT-3′ were used in PCR to amplify the 1.3 kbp coding sequence of the mouse SCL gene, *Scly* [5]. The PCR product was cut with *Eco*RI and *Sal*I restriction enzymes (Takara) and subsequently cloned into pEG202 to produce pEGScl as the bait. The cloning junction was sequenced to confirm the fusion.

2.3. Yeast two-hybrid library screening

The interaction trap assay [21] was performed using a DupLEX-A Yeast Two-Hybrid System (OriGene Technologies, Rockville, MD, USA). *S. cerevisiae* EGY48(pJK103) was transformed with pEGScl using the lithium acetate procedure and grown with a minimal synthetic dropout medium (SD medium) (Ura^-, His^-) at 30 °C for 3–5 days. A mouse liver cDNA library (OriGene Technologies) with 9.5×10^6 independent inserts cloned in pJG4–5 was then introduced into the yeast strain containing the pEGScl bait plasmid, and the transformants were plated on an SD medium (Ura^-, His^-, Trp^-). A total of 3.2×10^8 primary yeast cotransformants were scraped, pooled, and stored at −80 °C. The amplified yeast cotransformants were plated onto an SD induction medium (SD/Gal/Raf, an SD medium containing galactose, raffinose, and X-gal) (Ura^-, His^-, Trp^-, Leu^-). After 2 days, the plasmids were isolated from blue clones that grew on an SD induction medium as described by Hoffman and Winston [22] and introduced into *E. coli* strain KC8 cells by electroporation. Library plasmids were selected by plating the transformation mixture onto an M9 plate containing ampicillin, uracil, histidine, and leucine but lacking tryptophan. cDNAs were analyzed by PCR and restriction mapping using *Eco*RI and *Xho*I and sorted into groups depending on their restriction map patterns. Putative true interactors were sequenced using a 310 Genetic Analyzer (Applied Biosystems, Foster, CA, USA). Sequence homology searches were done using the BLAST search programs of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

2.4. Expression and purification of MUP-I

The expression vector for MUP-I was constructed according to a previous method [23] with some modifications. The coding sequence of MUP-I was amplified from clone 74-1, which was obtained from the yeast two-hybrid screening, by PCR using the primers 5′-GCAATACC**CATATG**CATCATCATCATCATCATAGCAGCGGCCATATCGAAGGTCGTGAAGAAGCTAGTTCTACG-3′ and 5′-CGC**AAGCTT**TTCTCGGGCCTGGAGGCAGCG-3′. The former primer was designed to introduce an *Nde*I site (bold) and a hexahistidine (His_6) tag-coding sequence (single

underline) terminated by a factor Xa protease recognition site (double underline). The latter contained a *Hin*dIII site (bold) and a sequence to produce an in-frame fusion with a His_6 tag [24] at the C-terminus of MUP-I. The amplified fragment was inserted into a pT7Blue vector (Novagen, Madison, WI, USA) and digested with *Nde*I and *Hin*dIII. The insert was purified and ligated into the *Nde*I and *Hin*dIII sites of pET21a(+) (Novagen). *E. coli* NovaBlue (Novagen) was used for cloning. The recombinant plasmid was introduced into *E. coli* BL21(DE3) (Novagen) for protein expression. One liter of a 2 × YTA medium containing 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl, and 100 mg of ampicillin was inoculated with an overnight culture from a single colony of the BL21(DE3) transformant and incubated with shaking at 37 °C. When the absorbance of the culture at 600 nm reached 0.6, expression was induced by addition of 10 ml of 0.1 M isopropyl-β-D-thiogalactoside. After 7 h, the cells were harvested by centrifugation for 10 min at 5000 × *g*. The cell pellet was resuspended in 40 ml of a binding buffer (5 mM imidazole, 500 mM sodium chloride, 20 mM Tris–HCl, pH 7.9), and the cells were lysed by sonication. The lysate was centrifuged at 30,000 × *g* for 40 min. The supernatant was filtered through a sterile 0.2 μm filter and loaded onto a His-Bind column (5 ml) (Novagen). After washing the column with 100 ml of the binding buffer, the His_6-tagged protein was eluted with the same buffer containing 1 M imidazole. The buffer was replaced with 50 mM Tris–HCl (pH 7.9) containing 100 mM sodium chloride and 1 mM calcium chloride during concentration with Centriprep-10 (Millipore, Bedford, MA, USA). The His_6 tag at N-terminus of MUP-I (approximately 8 mg) was cleaved off with 5 units of factor Xa protease in 3.5 ml of the above solution at 21 °C for 2.5 days. The MUP-I was purified by anion-exchange chromatography with a Resource Q column (Amersham Biosciences, Piscataway, NJ, USA).

2.5. In vitro binding assay

Approximately 0.5 mg of purified MUP-I was incubated with 1.1 mg of purified SCL in a 20 mM Tris–HCl (pH 7.9) buffer at 4 °C for 2 h. The mixture was loaded onto a column containing 1 ml of a Novagen His-Bind resin, and the column was washed with 20 ml of the binding buffer. The His_6-tagged protein was eluted with the binding buffer containing 1 M imidazole. The bound proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride membranes, and probed with the anti-SCL antibody. The immunoreactive proteins were detected with an ECL western blotting system (Pierce Biotechnology, Rockford, IL, USA) and a hyper ECL film (Amersham Biosciences).

2.6. Ligand binding to MUP-I

A 10 mM phosphate buffer (pH 7.2) containing 25 μM 2-naphthol and various concentrations of MUP-I was placed in a cuvette in the presence or absence of 0.14 mg/ml SCL. Fluorescence spectra were recorded with an RF-5300PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) under the following conditions: excitation, 330 nm; emission, 290–500 nm; excitation slit, 5 nm band pass; emission slit, 3 nm band pass; fluorescence cell path length, 1 cm.

3. Results and discussion

3.1. Isolation and identification of proteins that interact with mouse SCL in yeast two-hybrid screening

Yeast two-hybrid screening was used to identify proteins that may interact with SCL. The 280 Leu^+-positive clones demonstrated galactose-dependent activation of the LexA-driven reporters, indicating that they contained cDNA-encoded proteins that interacted with the Lex–SCL fusion protein. In order to sort out the clones, PCRs were carried out with a primer flanking the cDNA insertion site in pJG4–5 (5′-CTGAGTGGAGATGCCTCC-3′) and a primer encoding the C-terminus region of SCL (5′-CCGTAGAACTTATGTCCCAC-3′). We found that 210 out of the 280 positive clones encoded SCL. This is due to the SCL–SCL interaction between the subunits and is consistent with the fact that SCL is a homodimeric enzyme. We chose the 70 clones encoding non-SCL proteins for further analysis. Plasmids were isolated from the 70 yeast clones by transformation of *E. coli* KC8 with the plasmids followed by

Table 1
Putative true interactor clones obtained from the yeast two-hybrid screening with SCL

Clone	BLAST search hit	Accession no.
5-3	Major urinary protein 2	gb: MUSMUPII
6-1	Aldehyde reductase	gb: AF225564
18-1	Major urinary protein I	gb: AK013972
30-3	Glutathione *S*-transferase	gb: AK002213
32-3	Lysosomal pepstatin-insensitive protease	gb: AK002418
33-1	Major urinary protein 6	sp: MUP6
50-1	None[a]	–
57-1	Major urinary protein	gb: M28649
58-1	NADH dehydrogenase subunit 4L	gb: AB042432
70-2	Major urinary protein 2	sp: MUP2_MOUSE
74-1	Major urinary protein	prf: 1009257A
85	Vitronectin precursor	gb: AK01736
98-1	None[a]	–
117-1	Contraspin	prf: 2808243A
125-1	Apolipoprotein A-II	sp: APA2_MOUSE
125-2	Albumin	gb: AK050248
133-1	RNA helicas	sp: PL10_MOUSE
133-2	None[a]	—
147-1	ATP synthase coupling factor 6	sp: ATPR_MOUSE
148-1	None[a]	—
155-2	Hypothetical protein weakly similar to intermediate filament-associated protein	gb: AK011170
159-1	ATP synthase A	sp: ATP6_MOUSE
172-1	Homo sapiens molybdopterin cofactor sulfurase	gb: BC012079
174-1	Vitamin D-binding protein precursor	gb: BC010762
183-2	Major urinary protein 8 and 11	sp: MUP8_MOUSE

Databases used were GenBank (gb), PRF (prf), and SwissProt (sp).
[a] A sequence with significant similarity was not obtained for these clones.

selection on a minimal medium lacking tryptophan. We obtained 25 representative plasmids encoding proteins that interact with SCL. These 25 cDNAs were partially sequenced and analyzed by the BLAST program at http://blast.genome.ad.jp/ (Table 1). We found that seven clones (clones 5-3, 18-1, 33-1, 57-1, 70-2, 74-1, and 183-2) encoded major urinary proteins. One of the representative clones, 74-1, was used as a template for PCR to construct a MUP-I expression vector and fully sequenced. We found that the coding sequence of the clone 74-1 was different from that deposited in GenBank (accession number NM_031188) at 220 (T → G), 249 (G → C), and 481 (A → G) nucleotide residues, corresponding to the substitutions of amino acid residues at 74 (Phe → Val) and 161 (Ile → Val). However, the recombinant MUP-I from the 74-1 clone bound 2-naphthol in the same manner as previously described [25], and we used it throughout our study.

3.2. MUP-I interacts with SCL in vitro

MUPs belong to a highly homologous family of pheromone-binding proteins [26,27]. MUPs bind a variety of volatile pheromones [28,29] that affect various aspects of mouse physiology and behavior, including estrus, puberty, and inter-male aggression. Volatile pheromones interact with receptors in the vomeronasal organ of the female mice, inducing hormonal and physiological responses by an as yet unknown mechanism. In order to confirm the interaction between MUP-I and SCL observed in the yeast two-hybrid system, we examined the interaction in vitro by using a His-Bind column. His_6-tagged MUP-I was incubated with SCL, loaded on the column, and eluted with the buffer containing 1 M imidazole. The eluted fractions were analyzed by Western blotting with an anti-SCL antibody. We found that SCL was co-eluted with His_6-tagged MUP-I (Fig. 2). The small amount of SCL seen in the first elution fraction in the control experiment without MUP-I is probably due to non-specific interactions between SCL and a His-Bind resin. These results suggest that SCL directly interacts with MUP-I in vitro.

3.3. Effect of SCL on the binding of MUP-I to 2-naphthol

MUP-I belongs to the lipocalin superfamily, whose conserved structure is a β-barrel made of eight antiparallel β-strands forming a hydrophobic pocket that accommodates small hydrophobic pheromones including 2-*sec*-butyl-4,5-dihydrothiazole and 6-hydroxy-6-methyl-3-heptanone. The fluorescent molecule 2-naphthol was shown to bind to the natural ligand-binding site of MUP-I with high affinity [25]. When 2-naphthol binds to MUP-I, its fluorescence is blue-shifted, and the quantum yield is increased, providing a convenient and quick method for testing MUP-I binding capacity. We examined the effect of SCL on the MUP-I binding capacity. 2-Naphthol shows two emission bands at 350 and

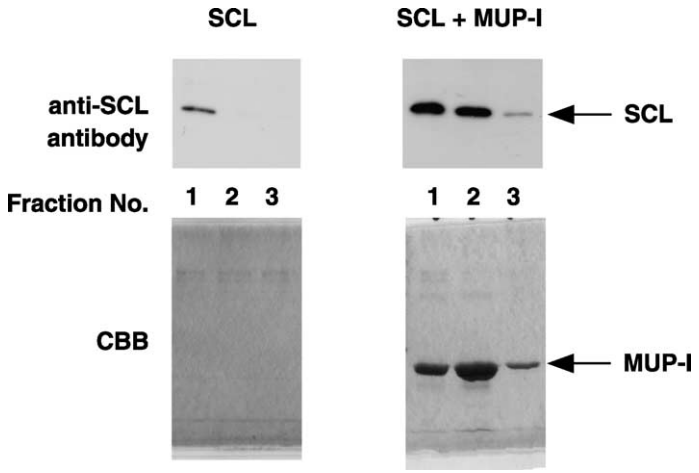

Fig. 2. Interaction of SCL with MUP-I in vitro. SCL (1.1 mg) alone (left panels) or SCL (1.1 mg) plus His_6-tagged MUP-I (0.5 mg) (right panels) was incubated at 4 °C for 2 h and loaded onto a His-Bind column. After the column was washed with 20 ml of a binding buffer, proteins were eluted with a buffer containing 1 M imidazole. The eluted fractions (lanes 1–3) were analyzed by 12% SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Brilliant Blue staining (lower panels) and Western blotting using an anti-SCL antibody (upper panels).

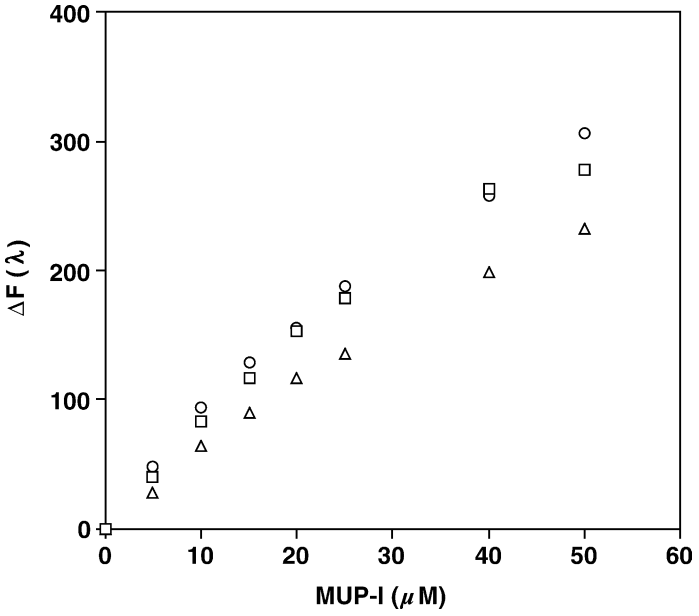

Fig. 3. Inhibition of binding of 2-naphthol to MUP-I by SCL. $\Delta F(\lambda)$ [$\Delta F(\lambda) = F_{MUP}(\lambda) - F_{Buffer}(\lambda)$] at 350 nm as a function of MUP-I concentration. $F_{MUP}(\lambda)$ is the fluorescence of 25 μM 2-naphthol in the presence of MUP-I, and $F_{Buffer}(\lambda)$ is the fluorescence of 25 μM 2-naphthol in the absence of MUP-I. Fluorescence spectra were measured in a 10 mM phosphate buffer containing 25 μM 2-naphthol, 2.5 mM dithiothreitol, and MUP-I alone (circles), MUP-I plus 0.28 mg/ml bovine serum albumin (squares), or MUP-I plus 0.14 mg/ml SCL (triangles).

420 nm in a 10 mM phosphate buffer due to undissociated (NaphOH) and dissociated ($NaphO^-$) forms of 2-naphthol, respectively [29]. An increase in the emission band at 350 nm by addition of MUP-I indicates the binding of 2-naphthol into the hydrophobic site of MUP-I. We found that the binding of 2-naphthol to MUP-I was significantly inhibited by SCL (Fig. 3). In contrast, bovine serum albumin (BSA) has little effect on the binding of 2-naphthol to MUP-I. Our results suggest that SCL regulates the affinity of MUP-I to physiological volatile effectors.

MUPs are produced and secreted by the liver and filtered by the kidneys into the urine of adult male mice and rats. MUPs carry small hydrophobic pheromones through an aqueous environment and probably protect pheromones from decomposition and control excretion of volatile pheromones from urine. SCL is also highly expressed in the liver and kidney [5]. Therefore, SCL probably interacts with MUP-I and prevents undesired small hydrophobic molecules from binding to the pheromone-binding site of MUP-I in vivo.

References

[1] N. Esaki, K. Soda, Methods Enzymol. 143 (1987) 415.
[2] N. Esaki, K. Soda, Methods Enzymol. 143 (1987) 493.
[3] N. Esaki, T. Nakamura, H. Tanaka, K. Soda, J. Biol. Chem. 257 (1982) 4386.
[4] P. Chocat, N. Esaki, K. Tanizawa, K. Nakamura, H. Tanaka, K. Soda, J. Bacteriol. 163 (1985) 669.
[5] H. Mihara, T. Kurihara, T. Watanabe, T. Yoshimura, N. Esaki, J. Biol. Chem. 275 (2000) 6195.
[6] G.M. Lacourciere, T.C. Stadtman, Biofactors 14 (2001) 69.
[7] T. Stadtman, J. Nutr. Sci. Vitaminol. (Tokyo) (1992) 58.
[8] E.S.J. Arner, H. Sarioglu, F. Lottspeich, A. Holmgren, A. Böck, J. Mol. Biol. 292 (1999) 1003.
[9] S. Bar-Noy, S.N. Gorlatov, T.C. Stadtman, Free Radic. Biol. Med. 30 (2001) 51.
[10] L. Zheng, V.L. Cash, D.H. Flint, D.R. Dean, J. Biol. Chem. 273 (1998) 13264.
[11] S. Kato, H. Mihara, T. Kurihara, Y. Takahashi, U. Tokumoto, T. Yoshimura, N. Esaki, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 5948.
[12] H.D. Urbina, J.J. Silberg, K.G. Hoff, L.E. Vickery, J. Biol. Chem. 276 (2001) 44521.
[13] A.D. Smith, J.N. Agar, K.A. Johnson, J. Frazzon, I.J. Amster, D.R. Dean, M.K. Johnson, J. Am. Chem. Soc. 123 (2001) 11103.
[14] J.N. Agar, C. Krebs, J. Frazzon, B.H. Huynh, D.R. Dean, M.K. Johnson, Biochemistry 39 (2000) 7856.
[15] J.N. Agar, L.M. Zheng, V.L. Cash, D.R. Dean, M.K. Johnson, J. Am. Chem. Soc. 122 (2000) 2136.
[16] R. Kambampati, C.T. Lauhon, J. Biol. Chem. 275 (2000) 10727.
[17] C.T. Lauhon, R. Kambampati, J. Biol. Chem. 275 (2000) 20096.
[18] J. Xi, Y. Ge, C. Kinsland, F.W. McLafferty, T.P. Begley, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 8513.
[19] D.E. Timm, L.J. Baker, H. Mueller, L. Zidek, M.V. Novotny, Protein Sci. 10 (2001) 997.
[20] R.L. Finley Jr., R. Brent, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 12980.
[21] A.R. Mendelsohn, R. Brent, Curr. Opin. Biotechnol. 5 (1994) 482.
[22] C.S. Hoffman, F. Winston, Gene 57 (1987) 267.
[23] L. Zidek, M.J. Stone, S.M. Lato, M.D. Pagel, Z. Miao, A.D. Ellington, M.V. Novotny, Biochemistry 38 (1999) 9850.
[24] T. Nakaishi, M. Ishizuka, K. Iio, T. Yomo, Y. Inokuchi, M. Kajitani, K. Yamamoto, Y. Shima, I. Urabe, J. Mol. Catal. B: Enzyme 10 (2000) 351.
[25] G. Sartor, R. Pagani, E. Ferrari, R.T. Sorbi, A. Cavaggioni, P. Cavatorta, A. Spisni, Anal. Biochem. 292 (2001) 69.
[26] A. Cavaggioni, C. Mucignat-Caretta, Biochim. Biophys. Acta 1482 (2000) 218.
[27] D.H.L. Robertson, R.J. Beynon, R.P. Evershed, J. Chem. Ecol. 19 (1993) 1405.
[28] A. Bacchini, E. Gaetani, A. Cavaggioni, Experientia 48 (1992) 419.
[29] M.V. Novotny, W.D. Ma, D. Wiesler, L. Zidek, Proc. R. Soc. Lond. B Biol. 266 (1999) 2017.