Molecular and Biochemical Characterization of Rat γ-Trimethylaminobutyraldehyde Dehydrogenase and Evidence for the Involvement of Human Aldehyde Dehydrogenase 9 in Carnitine Biosynthesis*

(Received for publication, July 30, 1999, and in revised form, December 6, 1999)

Frédéric M. Vaz, Sigrid W. Fouchier, Rob Ofman, Monica Sommer, and Ronald J. A. Wanders‡

From the Laboratory for Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, Emma Children’s Hospital, Academic Medical Center, University of Amsterdam, P. O. Box 22700, 1100 DE Amsterdam, The Netherlands

The penultimate step in carnitine biosynthesis is mediated by γ-trimethylaminobutyraldehyde dehydrogenase (EC 1.2.1.47), a cytosolic NAD⁺-dependent aldehyde dehydrogenase that converts γ-trimethylaminobutyraldehyde into γ-butyrobetaine. This enzyme was purified from rat liver, and two internal peptide fragments were sequenced by Edman degradation. The peptide sequences were used to search the Expressed Sequence Tag data base, which led to the identification of a rat cDNA containing an open reading frame of 1485 base pairs encoding a polypeptide of 494 amino acids with a calculated molecular mass of 55 kDa. Expression of the coding sequence in Escherichia coli confirmed that the cDNA encodes γ-trimethylaminobutyraldehyde dehydrogenase. The previously identified human aldehyde dehydrogenase 9 (EC 1.2.1.19) has 92% identity with rat trimethylaminobutyraldehyde dehydrogenase and has been reported to convert substrates that resemble γ-trimethylaminobutyraldehyde. When aldehyde dehydrogenase 9 was expressed in E. coli, it exhibited high trimethylaminobutyraldehyde dehydrogenase activity. Furthermore, comparison of the enzymatic characteristics of the heterologously expressed human and rat dehydrogenases with those of purified rat liver trimethylaminobutyraldehyde dehydrogenase revealed that the three enzymes have highly similar substrate specificities. In addition, the highest Vmax/Km values were obtained with γ-trimethylaminobutyraldehyde as substrate. This indicates that human aldehyde dehydrogenase 9 is the γ-trimethylaminobutyraldehyde dehydrogenase, which functions in carnitine biosynthesis.

Apart from the dietary intake of carnitine, most eukaryotes are able to synthesize this compound from trimethyllysine (5–7). This trimethyllysine is generated by the hydrolysis of proteins containing lysines that are trimethylated at their ε-amino group by a protein-dependent methyltransferase using S-adenosylmethionine as a methyl donor. In the carnitine synthetic pathway, trimethyllysine is first hydroxylated at the β-position by ε-trimethyllysine hydroxylase, after which the resulting β-hydroxytrimethyllysine is cleaved by a specific aldolase into γ-trimethylaminobutyraldehyde and glycine. γ-Trimethylaminobutyraldehyde is subsequently oxidized by γ-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH)¹ to form γ-butyrobetaine (8). In the last step, γ-butyrobetaine is hydroxylated at the β-position by a second hydroxylase, γ-butyrobetaine hydroxylase, yielding t-carnitine (5, 9, 10). In rat and mouse, γ-butyrobetaine hydroxylase is exclusively localized in the liver, whereas in man, the enzyme is present in kidney, liver, and brain. Although most tissues are capable of converting trimethyllysine into γ-butyrobetaine, liver and kidney are the main sites of carnitine biosynthesis in all animals (9–13).

Kaufman and Broquist (6) were the first to demonstrate that γ-trimethylaminobutyraldehyde is an intermediate in the carnitine biosynthesis of Neurospora crassa using isotope labeling experiments, and they suggested that an aldehyde dehydrogenase mediates its conversion to γ-butyrobetaine. Perfusion experiments showed that γ-trimethylaminobutyraldehyde is readily absorbed by rat liver and converted to carnitine via γ-butyrobetaine, demonstrating the conservation of the dehydrogenation step in higher eukaryotes (14). Subsequently, Rebouche and Engel (10) showed that TMABA-DH activity was present in the cytosolic fraction of human liver, kidney, brain, heart, and muscle homogenates. In the same year, Hulse and Henderson (8) purified a cytosolic NAD⁺-dependent aldehyde dehydrogenase from bovine liver showing maximum activity with γ-trimethylaminobutyraldehyde, converting it into γ-butyrobetaine.

Except for the human γ-butyrobetaine hydroxylase, which has recently been identified in our laboratory (15), none of the enzymes of the carnitine biosynthetic route have been characterized at the molecular level. We therefore purified the aldehyde dehydrogenase responsible for the conversion of γ-trimethylaminobutyraldehyde to γ-butyrobetaine from rat liver

¹ The abbreviations used are: TMABA-DH, γ-trimethylaminobutyraldehyde dehydrogenase; ALDH9, aldehyde dehydrogenase 9, MBP, maltose-binding protein, GABA, γ-aminobutyric acid; MOPS, 4-morpholino-propanesulfonic acid; MES, 4-morpholinooethanesulfonic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; EST, Expressed Sequence Tag.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF170918, AF170919, and AF172093.

‡ To whom correspondence should be addressed: University of Amsterdam, Academic Medical Center, Depts. of Clinical Chemistry and Pediatrics, Laboratory for Genetic Metabolic Diseases (F0-224), P. O. Box 22700, 1100 DE Amsterdam, The Netherlands. Tel.: 31 20 5665958; Fax: 31 20 6962596; E-mail: wanders@amc.uva.nl.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 10, Issue of March 10, pp. 7390–7394, 2000
Printed in U.S.A.
and determined part of its amino acid sequence. Using this sequence information we identified the cDNAs encoding TMABA-DH from rat, human, and mouse. Finally, we expressed the cDNAs in Escherichia coli and compared the substrate specificities of the recombinant enzymes with those of the purified rat liver trimethylaminobutyraldehyde dehydrogenase.

**EXPERIMENTAL PROCEDURES**

**Materials—**4-Aminobutyraldehyde diethylacetal, 1.8-bis(dimethylamino)naphthalene, methyliodide, 2,4-dinitrophenylhydrazine, methanal, ethanol, propanol, butanol, pentanal, hexanal, octanal, and betanaphthalein chloride were from Sigma. ADH and NAD⁺ were from Roche Molecular Biochemicals. Hexadecan-1-ol and octadecan-1-ol were synthesized as described earlier (16). SP-Sepharose fast flow and red-Sepharose CL-6B were obtained from Amersham Pharmacia Biotech, and CHT-II hydroxylapatite was from Bio-Rad. All other reagents were of analytical grade. The pMAL-C2X vector was purchased from New England Biolabs (Herts, UK).

**Synthesis of γ-Trimethylaminobutyraldehyde—**4-Aminobutyraldehyde diethylacetal was trimethylated in ethyl acetate using methyl iodide in the presence of 1.8-bis(dimethylamino)naphthalene (proton sponge). The iodide salt of 4-N-Trimethylaminobutyraldehyde diethylacetal precipitated with the protonated proton sponge. This precipitate was subsequently dissolved in distilled water by heating the mixture to boil and cool to room temperature, only the protonated proton sponge crystallized, whereas 4-N-Trimethylaminobutyraldehyde diethylacetal remained in solution. After removal of the proton sponge by filtration, the process was repeated five times in smaller volumes of distilled water to completely remove the remainder of the proton sponge. Hydrolysis of the resulting acetal in 0.1 M HCl for 30 min at room temperature gave γ-trimethylaminobutyraldehyde. Water and HCl were evaporated in a rotavapor, and the γ-trimethylaminobutyraldehyde was taken up in distilled water. In solution, γ-trimethylaminobutyraldehyde was stable for at least three months at −20°C.

**TMABA-DH Assay—**TMABA-DH activity was determined either spectrophotometrically or fluorometrically at 37°C by monitoring the formation of NADH using a centrifugal analyzer (CORAS FARO, Roche Molecular Biochemicals). The assay mixture used in both methods contained 0.1 mM sodium pyrophosphate buffer at pH 9.0, 0.5 mM NAD⁺, and the enzyme sample in a final volume of 250 μl. The reaction was started by adding γ-trimethylaminobutyraldehyde to a final concentration of 100 μM, unless otherwise indicated. In the spectrophotometric assay, for in vivo work, the absorbance at 340 nm was measured, and the activity was calculated using 6220 M⁻¹ cm⁻¹ as the molar extinction coefficient of NADH. In the fluorometric method, NADH detection was formed by measuring the fluorescence at 450 nm after excitation at 340 nm. Standard solutions of NADH were used for calibration.

**Purification of TMABA-DH—**Livers were taken from Wistar rats and homogenized by five strokes of a Teflon pestle in a Potter-Elvehjem glass homogenizer at 500 rpm in a 5 mM MOPS buffer, pH 6.0, containing 0.25 mM sucrose and 2 mM EDTA. The crude homogenate was centrifuged for 10 min at 8000 × g at 4°C to remove nuclei and whole cells. The resulting post-nuclear supernatant was centrifuged for 3 h at 20,000 × g at 4°C to obtain the cytosolic fraction. The cytosolic fraction was applied to an SP-Sepharose fast flow column (inner diameter = 2.8 cm, length = 10 cm), which was pre-equilibrated with a 10 mM MOPS buffer, pH 6.0, containing 200 μl/g liter glycerol and 1 mM dithiothreitol (DTT). Bound proteins were eluted with a linear gradient from 0 to 100 mM NaCl in the same buffer. Fracctions containing high TMABA-DH activity were pooled and loaded onto a red Sepharose CL-6B column (inner diameter = 0.8 cm, length = 7.5 cm), that was pre-equilibrated with a 10 mM MOPS buffer, pH 6.0, containing 200 μl/g liter glycerol, 1 mM DTT, and 25 mM NaCl. Bound proteins were eluted with a linear gradient from 25 to 500 mM NaCl in the same fraction. Fractions containing TMABA-DH activity were pooled and dialyzed against a 10 mM MOPS buffer, pH 6.0, containing 200 μl/g liter glycerol, 1 mM DTT, and 20 mM potassium phosphate. This dialysate was loaded onto an Econo-Pac 5/2-Hydroxylapatite column (inner diameter = 1 cm, length = 5 cm) equilibrated with the same buffer. Bound proteins were eluted with a linear gradient from 25 to 250 mM potassium phosphate. Fractions were tested for TMABA-DH activity and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) followed by silver staining. SDS-PAGE and silver staining were performed as described by Laemmli (17) and Rabilloud et al. (18), respectively. Protein concentrations were determined by the method of Bradford (19), using bovine serum albumin as standard.

**Protein Digestion, Western Blotting, and Automated Edman Degradation—**10 μg of the purified TMABA-DH was digested for 1 h at 37°C with 0.05 μg of endoprotease Glu-C (Roche Molecular Biochemicals) in a 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% SDS. Protein fragments were resolved on a 15% SDS-PAGE gel, and a multiphoton II Nova Biotechnology confocal laser scanning microscope was used to transfer proteins onto a polyvinylidene difluoride-sequecing membrane (Millipore, Bedford, MA) as described by the manufacturer of the transfer unit. Proteins were visualized with Coomassie Brilliant Blue. N-terminal amino acid sequencing was performed using a Procise 494 protein sequencer.

**Cloning, Expression, and Purification of the Rat TMABA-DH and Aldehyde Dehydrogenase 9 (ALDH9) in E. coli—**The complete open reading frame (ORF) of TMABA-DH was amplified by the polymerase chain reaction from rat liver cDNA using Advantage cDNA polymerase (CLONTECH, Palo Alto, CA) and the following primers: an EcoRI-tagged forward primer 5′-tatagattaATGAGCAGCTGCACCTCTGG-3′ and a SalI-tagged reverse primer 5′-tatagtagacTITTCAAAAGCWC-GAYTCAC-5′. The degenerate nature of the second primer also allowed the amplification of the ALDH9 ORF from human liver cDNA using the same primer set. The polymerase chain reaction products were cloned downstream of the isopropyl-1-thio-β-galactopyranoside-inducible P_{lac} promoter into the EcoRI and SalI sites of the bacterial expression vector pMAl-C2X, to express the TMABA-DH and ALDH9 as a fusion protein with maltose-binding protein (MBP). The ORFs were excised with EcoRI and SalI and cloned into the polymerase chain reaction after, which the constructs were transformed to the E. coli strain BL21. Transformed cells were grown on LB medium to an A_{600} of 0.7, and isopropyl-1-thio-β-galactopyranoside was added to a final concentration of 1 mM to induce expression of the fusion protein. After 2 h, cells were pelleted and lysed in one-tenth of the culture volume in a 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100 for 30 min at 8 W. The bacterial lysate was centrifuged for 10 min at 14,000 × g, and the pellet was discarded. Fusion proteins were purified from the supernatant following the specifications of the manufacturer of the expression system (New England Biolabs) and stored at −80°C in a 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl, 200 μM 1,4-dithiothreitol, 1 mM DTT, and 3 mg/ml bovine serum albumin.

**Characterization of TMABA-DH—**The Michaelis-Menten constant (K_{m}) and maximal velocity (V_{max}) for γ-trimethylaminobutyraldehyde and several other aldehydes were determined for the purified rat liver enzyme and the purified recombinant fusion proteins using the assay described above. The concentration of γ-trimethylaminobutyraldehyde was determined with 2,4-dinitrophenylhydrazine as described by Arigita and Fukumoto (21). Its concentration was determined with o-aminobenzaldehyde as reported by Jakoby and Fredrick (22). Because of the instability of γ-aminobutyraldehyde at alkaline pH, activity measurements with this compound as substrate were performed at pH 7.4 using a 0.1 mM sodium phosphate buffer. For the determination of the K_{m} of NAD⁺ and NADP⁺, γ-trimethylaminobutyraldehyde was used at a fixed concentration of 100 μM.

**RESULTS**

**Purification of TMABA-DH from Rat Liver—**In initial experiments, high TMABA-DH activity (~3 nmol/min/mg) could be measured in crude rat liver homogenates. Subsequent measurement of TMABA-DH activity in subcellular fractions of rat liver showed that the activity was only present in the cytosolic fraction (results not shown). Therefore, rat liver cytosol was used as source of enzyme for the purification of TMABA-DH using liquid chromatography. An overview of the purification scheme is given in Table I. TMABA-DH activity was completely retained by all columns used and eluted as a single peak during all purification steps. Samples obtained after each purification step were analyzed by SDS-PAGE followed by silver-staining (see legend of Fig. 1). A single protein band with an apparent molecular mass of 55 kDa was observed after the last purification step. The purified enzyme was highly unstable, except when stored at −80°C in the presence of 1 mM DTT and 200 μl/g liter glycerol. Even after several months of storage, no loss of activity could be measured.

**Identification of the cDNA Encoding TMABA-DH—**Attempts
ESTs all corresponded to the ALDH9 cDNA (GenBank homology to the peptide sequences. The homologous human identified several rat, mouse, and human EST clones with high human ALDH9 (EC 1.2.1.19, Swiss-Prot P49189). Subsequent with these sequences, the only homology found was with the unambiguously). When the Swiss-Prot data base was screened from the Swiss-Prot data base, accession number AF172093. The MBP-ALDH9 fusion protein was affinity-purified from E. coli lysate to determine whether ALDH9 was also active toward γ-trimethylaminobutyraldehyde. The fusion protein exhibited high TMABA-DH activity, which indicates that ALDH9 is the human orthologue of rat TMABA-DH.

**Characterization of the Purified Rat Liver TMABA-DH and Comparison with MBP Fusion Proteins**—To investigate if the purified rat liver TMABA-DH and the rat MBP-TMABA-DH could also handle the substrates reported for ALDH9 and to further characterize the substrate specificity of the three enzymes, their kinetic properties were determined. Table II shows the kinetic parameters of the purified rat liver TMABA-DH, rat MBP-TMABA-DH, and MBP-ALDH9 with NAD⁺, NADP⁺, γ-trimethylaminobutyraldehyde, γ-aminobutyraldehyde, betaine aldehyde, and a range of aliphatic aldehydes as substrates. The Kᵣ and relative Vᵣ max values of the purified MBP fusion proteins for the different substrates show a similar profile as the purified rat liver TMABA-DH. NAD⁺ was by far the preferred oxidant for all substrates, although NADP⁺ could also be used. The three enzymes have the lowest Kᵣ for γ-trimethylaminobutyraldehyde in combination with a high V max value. As a consequence, the V max/Kᵣ ratio is highest for γ-trimethylaminobutyraldehyde when compared with the other substrates. The presence of a free amino group in γ-aminobutyraldehyde instead of the trimethylated amino group in γ-trimethylaminobutyraldehyde results in considerably lower efficiency. Betaine aldehyde, the carbon backbone of which is two atoms shorter than γ-trimethylaminobutyraldehyde but that contains the trimethylated amino group, is readily oxidized to betaine as reflected in the high V max values. The three enzymes have a high Kᵣ for betaine aldehyde, however, which results in a substantially lower efficiency when compared with γ-trimethylaminobutyraldehyde. For the aliphatic aldehydes in the C₇-C₂₀ range, the decrease in the Kᵣ values is accompanied by a steady increase of V max showing that the efficiency of the enzymes is higher when the chain length of the aliphatic aldehyde increases. The efficiency of the enzymes with the longer aldehydes, hexadecanal and octadecanal, is very low if not undetectable.

**DISCUSSION**

To identify the enzymes of the carnitine biosynthetic pathway at the molecular level, we previously purified rat liver

| Purification step | Protein | Specific activity (nmol/min/mg) | Activity (nmol/min) | Yield (%) | Purification *-fold |
|------------------|---------|--------------------------------|-------------------|----------|-------------------|
| Post-nuclear supernatant | 1760 | 2.9 | 6867 | 100 | 1.4 |
| 20,000 × g supernatant | 751 | 5.6 | 4206 | 61 | |
| SP-Sepharose fast flow | 8.3 | 288 | 2390 | 35 | |
| Red Sepharose CL-6B | 1.1 | 483 | 531 | 8 | |
| Hydroxyapatite CHT-II | 0.6 | 772 | 463 | 7 | |

Fig. 1. Overview of TMABA-DH purification. Protein samples of the various purification steps were analyzed by 12% SDS-PAGE followed by silver staining. Lane 1, molecular weight marker; lane 2, 20,000 × g rat liver supernatant and pooled fractions of SP-Sepharose (lane 3), red Sepharose (lane 4), hydroxyapatite CHT-II (lane 5).
γ-butyrobetaine hydroxylase, the last enzyme in carnitine biosynthesis and used protein sequence data in combination with the EST data base to identify the corresponding human cDNA (15). In this study the same approach was used to identify TMABA-DH, which mediates the penultimate step in carnitine biosynthesis. The enzyme was purified from rat liver to apparent homogeneity and used for peptide sequencing. The resulting peptide sequences were subsequently used to search the EST data base, and two ORFs were identified from rat and mouse encoding proteins with high homology to the previously reported human ALDH9. The following observations demonstrated that the identified rat cDNA truly encodes TMABA-DH. First, the peptide sequence obtained by sequencing of the purified rat TMABA-DH exactly matched a 19-amino acid stretch in the translated coding region of the rat cDNA. Second, the cDNA encodes a protein with a calculated molecular mass of 55 kDa, which is in accordance with the apparent molecular mass of the purified rat liver TMABA-DH. Third, heterologously expressed rat cDNA exhibited high TMABA-DH activity. Finally, the kinetic properties of the recombinant rat MBP fusion protein are highly similar to those of TMABA-DH purified from rat liver. Although we did not express the mouse ORF in E. coli, it has 96% positional identity with the rat TMABA-DH and, therefore, most likely represents the mouse orthologue of rat TMABA-DH.

The rat TMABA-DH has high positional identity (92%) with human ALDH9. ALDH9 is a cytosolic NAD⁺-dependent dehydrogenase belonging to the human aldehyde dehydrogenase gene family (26). It has been extensively investigated because of its proposed function in the alternative synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (23, 27–29). In this pathway, diamine oxidase oxidatively deaminates putrescine (1,4-diaminobutane) to γ-aminobutyraldehyde, which is subsequently oxidized to GABA by ALDH9. The majority of the GABA in rat adrenal gland is produced via this alternative pathway, whereas the GABA in brain is predominantly synthesized from glutamate by glutamate decarboxylase (30). Both the physiological importance of the conversion of putrescine to γ-aminobutyric acid and the function of GABA outside the central nervous system is not well understood and remains to be established.

More recently, ALDH9 has also been implicated in the synthesis of betaine. Betaine can serve as a methyl donor in the biosynthesis of methionine and has also been proposed to be involved in the regulation of the osmolarity in the kidney during antidiuresis (31–33). For the synthesis of betaine, choline is oxidized by choline dehydrogenase to betaine aldehyde, which is subsequently converted to betaine by ALDH9 (25, 34). In human tissues, betaine aldehyde dehydrogenase activity is predominantly found in liver, adrenal gland, and kidney. Northern blot analysis has shown the presence of the ALDH9 mRNA in liver, kidney, skeletal muscle, heart, brain, pancreas, lung, and placenta (23, 27, 34).

The high homology with rat TMABA-DH and the structural resemblance of the substrates of ALDH9 with γ-trimethylamino- butyraldehyde prompted us to study whether human ALDH9 is in fact the human TMABA-DH. The finding that the recombinant human MBP-ALDH9 fusion protein exhibits high TMABA-DH activity suggests that human ALDH9 is, indeed, the human TMABA-DH.

Since ALDH9 has been reported to oxidize betaine aldehyde and γ-aminobutyraldehyde, the kinetic properties of the two recombinant MBP fusion proteins and the purified rat liver

![Fig. 2. Structure of γ-aminobutyraldehyde (A), γ-trimethylamino- butyraldehyde (B), and betaine aldehyde (C).](image-url)
TMABA-DH were also determined for these and other substrates. Like the ALDH9 MBP fusion protein, both rat liver TMABA-DH and the rat TMABA-DH MBP fusion protein oxidized γ-aminobutyraldehyde and betaine aldehyde, which is considered to be the cod orthologue of human ALDH9. The structural information revealed that the active site of cod ALDH9 is capable of handling larger aldehydes than betaine aldehyde, which is in accordance with our results, which reveal that the three forms of ALDH9 studied in this paper show the highest $V_{\text{max}}/K_m$ ratio for straight-chain aldehydes with a length of 7/8 carbon atoms. The preference for longer aldehydes also explains the relatively low $K_m$ values for γ-trimethylaminobutyraldehyde and γ-aminobutyraldehyde opposed to the high $K_m$ value for betaine aldehyde. The high $V_{\text{max}}$ value of the enzymes for betaine aldehyde is difficult to explain on the basis of the data presented here. Additional research is needed to understand this phenomenon.

Further investigation of the active site of cod ALDH9 showed that there is no negatively charged residue in the substrate pocket that interacts with the trimethylated amino group of betaine aldehyde. Instead, a hydrophobic interaction has been proposed between a tryptophan residue and the trimethylated amino group of betaine aldehyde. This suggests that there is no negatively charged residue in the substrate pocket that interacts with the trimethylated amino group of betaine aldehyde.

The high activity of the heterologously expressed human ALDH9 with γ-trimethylaminobutyraldehyde and the highly similar substrate specificity of ALDH9 and rat TMABA-DH strongly suggest that the human ALDH9 is the human TMABA-DH. This is supported by the presence of high betaine aldehyde dehydrogenase activity in human kidney and liver and the ALDH9 mRNA in tissues that contain high TMABA-DH activity (10, 23, 34). Although our data do not exclude an additional function of ALDH9 in GABA and/or betaine synthesis, the results presented in this paper indicate that ALDH9 is the predominant, if not exclusive aldehyde dehydrogenase that functions in carnitine biosynthesis.

Acknowledgments—We thank M. Q. Slagt and J. Ruiter for technical assistance, A. O. Muellers for peptide sequencing, and Dr. H. R. Waterham for critical comments on the manuscript.

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