Kinetic properties and inhibition of *Trypanosoma cruzi* 3-hydroxy-3-methylglutaryl CoA reductase

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Abstract A detailed kinetic analysis of the recombinant soluble enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) from *Trypanosoma cruzi* has been performed. The enzyme catalyzes the normal anabolic reaction and the reductant is NADPH. It also catalyzes the oxidation of mevalonate but at a lower proportion compared to the anabolic reaction. We report that the catalytically active species of HMGR in solution is the tetrameric form. Fluvastatin inhibited competitively the enzyme while cerivastatin binds by a mechanism which is more accurately described by a biphasic process characteristic of a class of 'slow, tight-binding' inhibitors.

Key words: 3-Hydroxy-3-methylglutaryl CoA reductase, trypanosomatid; Cross-linking; Statin; Ergosterol

1. Introduction

Inhibitors of ergosterol biosynthesis interfere severely with the growth of protozoan parasites of the Trypanosomatidae family such as *Trypanosoma cruzi*, the causative agent of Chagas' disease, and various *Leishmania* species [1–3]. Urbina et al. [4] have shown with in vivo studies using a murine model of Chagas' disease that a combined treatment with mevinolin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), and azole drugs was able to essentially eliminate circulating parasites and produce complete protection against death. The combined administration of ergosterol biosynthesis inhibitors appears to be a promising strategy for the development of an effective treatment of Chagas' disease.

HMGR catalyzes the rate-limiting reaction in cholesterol biosynthesis, the NADPH-dependent $4e^-$ reduction of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate. This enzyme has been extensively studied as an example of a key biosynthetic enzyme under regulatory control by several mechanisms [5,6]. Recently, the crystal structure of the catalytic portion of human HMGR in complexes with HMG-CoA, and CoA, and with HMG, CoA and $NADP^+$ has been published [7]. The biosynthetic HMGRs of eukarya and archaea utilize NADPH to reduce HMG-CoA to mevalonate for ultimate synthesis of isoprenoids such as cholesterol. By contrast, the biodegradative HMGR of *Pseudomonas mevalonii* [8–10] utilizes NAD^+ in the oxidative acylation of mevalonate to HMG-CoA, the first reaction of mevalonate catabolism in this organism.

The HMGR of eukaryotes consists of an N-terminal membrane anchor domain joined to a C-terminal catalytic domain by a short linker region. Non-eukaryotic HMGRs [8–11] lack membrane anchor domains. In *T. cruzi* and *Leishmania major*, HMGR exists as a soluble form [12,13], and therefore trypanosomatids constitute the sole example of a soluble reductase in a eukaryotic organism.

In order to provide an appropriate source of enzyme for functional and structural characterization and to analyze the interaction of trypanosomatid reductase with inhibitors, we have expressed *T. cruzi* HMGR in *Escherichia coli*. Since it is soluble, we have purified and characterized it with respect to molecular mass, kinetic parameters and inhibitor binding.

2. Materials and methods

2.1. Materials

Inhibitors were kindly provided by Novartis (fluvastatin) and Bayer (cerivastatin). NaCl, dithiothreitol (DTT), dimethyl suberimidate, dimethyl adipimidate, diethyl pyrocarbonate, hydroxylamine, (*R*,*S*)-HMG-CoA, NADPH, mevalonate, coenzyme A and $NADP^+$ were from Sigma. Leupeptin and IPTG were purchased from Boehringer Mannheim. Molecular mass markers for SDS–PAGE, acrylamide, bisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine and ammonium persulfate were purchased from Bio-Rad.

2.2. Purification

Two buffers were used in different steps of the purification: buffer A (20 mM sodium phosphate, pH 6.75, 10% glycerol, 100 mM sucrose, 10 mM DTT) and buffer B (20 mM sodium phosphate, pH 7.4, 10% glycerol, 100 mM sucrose, 10 mM DTT). The expression system construction was reported previously [12]. The expression plasmid pETTCHMGR was used to transform the *E. coli* expression host BL21 (DE3).

A frozen pellet from 2 l of *E. coli* BL21 (DE3)/pETTCHMGR cells overproducing *T. cruzi* HMGR was thawed and resuspended in 30 ml of ice-cold buffer A containing 20 μg/ml leupeptin. Cells were sonicated and centrifuged at $10\,000 \times g$ for 30 min at 4°C. The supernatant was then treated with 30–50% ammonium sulfate. The 30–50% precipitate was dissolved in 5–10 ml of buffer and frozen at −80°C; this fraction retains full activity for several weeks.

The ammonium sulfate fraction was loaded onto a hydroxyapatite (HAP) chromatography column pre-equilibrated with buffer A at 1 ml/min. The HAP column was then washed with three volumes of buffer A and elution was performed with a 0–1.8 M NaCl gradient of 300 ml, with the active peak eluting at 0.9 M NaCl. Active fractions

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Abbreviations: HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA

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were pooled and concentrated with 50% ammonium sulfate. The precipitate was then applied to a blue-Sepharose column, equilibrated in buffer B at 1 ml/min. The column was washed with three volumes of buffer B, and elution was performed with a 0–1.8 M NaCl gradient of 120 ml, with the active peak eluting at 0.9 M NaCl. The major active fractions were pooled, concentrated and resuspended in buffer A for storage.

2.3. Assay for HMG-CoA reductase activity

(*R*,*S*)-HMG-CoA-dependent oxidation of NADPH (Sigma) was monitored at 340 nm in a Hewlett-Packard Model 8452 diode array spectrophotometer. Because of the instability of NADPH at pH 5.5 and elevated temperatures, we used an initial concentration of NADPH too great to be measured accurately at 340 nm ($e_{340} = 6220$ M^{-1} cm^{-1}). Disappearance of NADPH was therefore monitored at 366 nm ($e_{360} = 3300$ M^{-1} cm^{-1}). Standard assay mixtures contained, in a final volume of 200 μl, 270 mM (*R*,*S*)-HMG-CoA, 270 mM NADPH, 75 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 150 mM Na_xPO_4, pH 6.8. Reaction mixtures were first monitored to detect any (*R*,*S*)-HMG-CoA-independent oxidation of NADPH. Temperature for the measurements was set at 25°C.

The standard assay for oxidative acylation of mevalonate contained, in a final volume of 200 μl: 160 mM (*R*,*S*)-mevalonate, 16 mM CoA, 40 mM $NADP^+$, 0.1 M Tris–HCl, pH 8.3. Reactions were first monitored to detect any CoA-independent reduction of $NADP^+$.

One unit of HMG-CoA reductase is defined as the amount of enzyme which catalyzes the oxidation of 1 μmol of NADPH per min. Protein was determined by the method of Bradford [14] using bovine serum albumin as standard.

2.4. Gel filtration

A Superdex 200 HR 30/10 column attached to an ÄKTA system (Pharmacia) was used. Protein samples were dissolved in eluting buffer, 50 mM potassium phosphate pH 7.0, 150 mM NaCl. Standard proteins for molecular mass determination were from Pharmacia.

2.5. Cross-linking of HMGR

Cross-linking with dimethyl suberimidate was done by the method of Davies and Stark [15,16]. The conditions of the reaction were: 0.2 M triethanolamine HCl at pH 8.5, the bisimidoester HCl at a concentration of 5 mg/ml and the protein concentration was varied from 0.2 to 0.5 mg/ml. The reaction was allowed to proceed at room temperature for 3 h, stopped using trichloroacetic acid 25%, and washed with acetone. SDS–PAGE electrophoresis and Coomassie blue staining were used to visualize cross-linking.

3. Results and discussion

3.1. T. cruzi HMGR is a tetramer in solution

For production of recombinant protein, the purification protocol included three chromatographic steps which are summarized in Table 1. The yield was 7 mg of purified recombinant protein per 2 l of culture and enzyme purity was judged homogeneous by SDS–PAGE (Fig. 1).

To investigate the subunit structure of HMGR, we cross-linked the purified enzyme with dimethyl suberimidate according to the method of Davies and Stark [15,16]. When the products of the reaction were analyzed, four major Coomassie blue staining bands were apparent (Fig. 2). The bands presumably correspond to the monomeric (46 kDa), dimeric (92 kDa), trimeric (138 kDa) and tetrameric (184 kDa) forms.

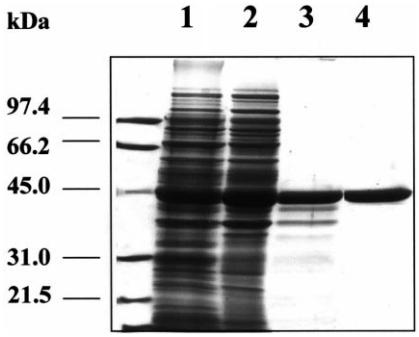

Fig. 1. Purification of *T. cruzi* HMGR. An SDS–PAGE gel was stained with Coomassie brilliant blue. Lane 1, soluble crude extract; lane 2, 30–50% ammonium sulfate precipitate; lane 3, HAP fraction (10 μg); lane 4, blue-Sepharose fraction (10 μg).

Similar results were obtained in cross-linking experiments with dimethyl adipimidate. In addition size exclusion chromatography on a Superdex 200 HR 30/10 column was used to study the molecular mass of the native protein and subunit arrangement. HMGR elutes as a single peak with a retention time that indicates a molecular mass of 184 kDa. Regarding the native structure of class I enzymes, the catalytically active soluble form of human HMGR [17] has been reported to be a tetramer of approximate M_r 200 000 Da [7]. Likewise, HMGR from radish seedlings is a tetramer of 45 000 Da subunits [18]. The catalytically active form of the class II *P. mevalonii* HMGR in solution, based on gel permeation chromatographic experiments, was reported to be a hexamer [19]. In the present case the data taken together suggest a tetrameric conformation for the enzyme. Moreover, structure-based sequence alignments of human and *T. cruzi* HMGR (not shown) indicate that elements important for the formation of the tetramer in the human enzyme are all present in the protozoan structure. Sequence elements important for dimerization are also conserved [7].

3.2. Kinetic characterization

Recombinant *T. cruzi* HMGR was analyzed for its kinetic properties. The enzyme catalyzes the reaction with NADPH, but not with NADH as reductant. NADH oxidation was assessed using two different cofactor concentrations (350 and 700 μM) and two different enzyme concentrations (14 nM and 54 nM). No activity was detectable even when high concentrations of both cofactor and enzyme were used. Initial velocity studies regarding NADPH oxidation were determined at 28°C. HMGR exhibited optimal activity for the conversion of HMG-CoA to mevalonate in the pH range 5.7–6.3 (data not shown). pK_1 and pK_2 values for the enzyme–substrate complex obtained from Dixon–Webb log plots were 5.21 and 7.4 respectively. K_m values for the overall reaction were 13 ± 2 μM for (*R*,*S*)-HMG-CoA and 43 ± 3 μM for NADPH; V_{max} was 28 ± 3 U/mg.

Table 1
Summary of a typical purification of *T. cruzi* HMGR[a]

Fraction	Activity (μmol/min)	Total protein (mg)	Specific activity (U/mg)	Enrichment (fold)	Yield (%)
Soluble extract	2409	325	7.4	1	100
Ammonium sulfate 30–50%	1650	110	15	2.02	68
Hydroxyapatite	506	22	23	3.1	21
Blue-Sepharose	196	7	28	3.8	8

[a]The data are for the purification from 2 l of culture.

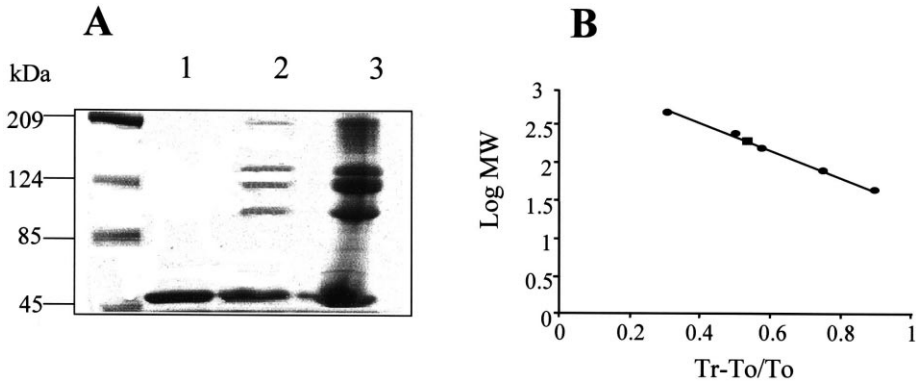

Fig. 2. A: SDS–PAGE gel of cross-linked HMGR. Purified HMGR was cross-linked with dimethyl suberimidate (5 mg/ml) for 3 h at room temperature. The cross-linked enzyme was electrophoresed on 8% SDS gels. Uncross-linked HMGR is shown in lane 1. The concentration of HMGR utilized was 0.2 mg/ml (2) or 0.5 mg/ml (3). The molecular mass (M_r) standards used were: myosin (209 kDa), galactosidase (124 kDa), albumin (85 kDa) and ovalbumin (45 kDa). B: Chromatography of HMGR on a Superdex 200 HR 30/10 column. Ferritin (439 kDa), catalase (231 kDa), aldolase (154 kDa), bovine serum albumin (69 kDa) and ovalbumin (43 kDa) were used as relative molecular mass standards. Data are expressed as the ratio $T_r - T_o/T_o$, where T_r is the retention time and T_o is the void time estimated using blue dextran.

For the conversion of mevalonate to HMG-CoA, HMGR exhibited optimal activity in the pH range 8.7–9.6 (data not shown) and the pK_2 for the enzyme–substrate complex was 7.4. At pH 8.3, K_m values for mevalonate, CoA and $NADP^+$ were 252 ± 50, 74 ± 15 and 220 ± 20 μM respectively and the V_{max} was 0.86 ± 0.04 U/mg.

The study of the kinetic behavior of the enzyme demonstrates its equivalence in function with previous reported HMGRs of class I. In addition, the K_m values for HMG-CoA and NADPH parallel those obtained for other characterized biosynthetic HMGRs. The K_ms for $NADP^+$, mevalonate and CoA were comparable to those obtained for *P. mevalonii* HMGR although the V_{max} was considerably lower [20]. In agreement with this observation the nucleotide and deduced amino acid sequences determined for *T. cruzi* and *L. major* HMGRs are homologous to the reported sequences of

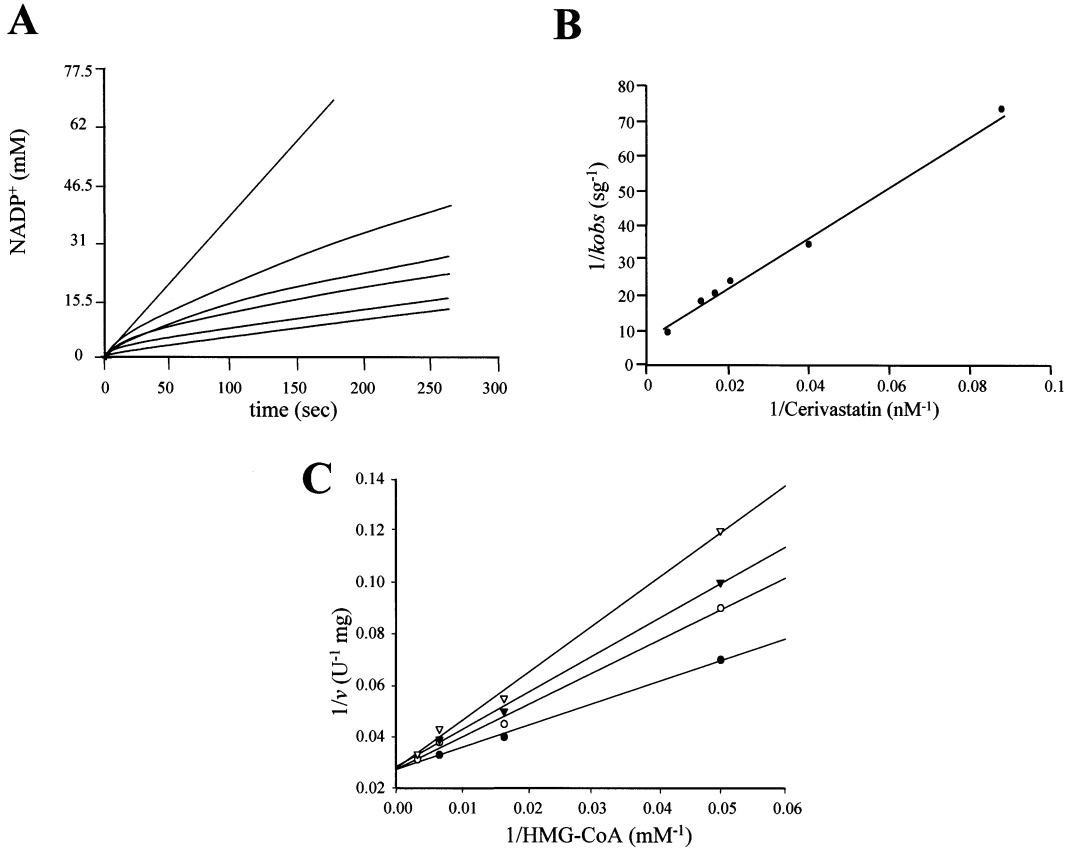

Fig. 3. A: Progress curves for the binding of cerivastatin to HMGR at 200 μM HMG-CoA. The top line is a control curve with no inhibitor. The remaining curves, from top to bottom, are for inhibitor concentrations of 25, 50, 75, 112.5 and 250 nM, respectively. B: Plot of the reciprocal k_{obs} vs. the reciprocal of the cerivastatin concentration. C: Double reciprocal plots of the inhibition of HMGR by fluvastatin at 10, 30 and 60 nM.

class I enzymes suggesting a common ancestral origin of these genes although a distinct feature is that they lack the membrane domain characteristic of eukaryotic reductases.

3.3. Inhibition by fluvastatin and cerivastatin

Statins are potent competitive inhibitors of HMGR. All share an HMG moiety and rigid hydrophobic groups [21]. We assayed the susceptibility of homogeneous *T. cruzi* HMGR to inhibition by fluvastatin and cerivastatin. These compounds are known to inhibit HMGR from mammals [21,22]. Fluvastatin was shown to be a classic competitive inhibitor with respect to HMG-CoA (Fig. 3), with a K_i of 27 ± 3 nM, while cerivastatin behaved as a slow-binding inhibitor (Fig. 3). The data was analyzed assuming a two-step mechanism for binding and the equilibrium constant for the formation of both the initial (K_i) and final (K_i*) complexes were calculated. The slow-binding nature of this inhibitor was observable when the reaction was initiated with enzyme and the cerivastatin concentration was varied from 0 to 250 nM; a time-dependent decrease in the rate was seen that varied as a function of inhibitor concentration. The kinetics were characteristic of enzyme–inhibitor interactions where the initial step involves rapid formation of a weak complex, followed by a slow conversion to the tight-binding complex. We obtained a value for the rate constant of this slow-binding process by assuming it was analogous to enzyme inactivation by a 'slow, tight-binding inhibitor' [23,24]. First, the progress curves were analyzed by assuming that the rates of inactivation reflected a pseudo-first order process; we computer-fitted the data to:

$$[\mathrm{NADP}^+] = v_f t - (v_f - v_i)\,(1 - e^{-k_{obs} t})/k_{obs} \quad (1)$$

where v_i and v_f are the initial and final HMGR steady-state rates, and k_{obs} is the pseudo-first order rate constant [23,24]. The reciprocals of the observed pseudo-first order rate constants were plotted vs. the reciprocal of the cerivastatin concentration, employing (Fig. 3):

$$1/k_{obs} = (1/k_{slowbind}) + (K_i/k_{slowbind}\,[\mathrm{cerivastatin}]) \quad (2)$$

where K_i is the equilibrium constant for the initial inhibition complex, and $k_{slowbind}$ (k_3) is the rate constant for the slow-binding process of inhibition. The $k_{slowbind}$ for the wild-type enzyme was 8.73 min^{-1}, K_i was 14.9 nM and K_i* 0.3 ± 0.04 nM. Slow-binding inhibition for this kind of compounds has been previously documented [25] and the values obtained were comparable to results obtained with inhibition of the human enzyme by potent lovastatin-related inhibitors suggesting a similar binding mechanism.

Since inhibitors of HMGR, such as lovastatin, increase the antiproliferative effects of ketoconazole and terbinafine against *T. cruzi* in both in vitro and in vivo studies [4], future studies on the inhibition of the enzyme may be of interest for its development as a drug target in trypanosomatid protozoans.

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References

[1] Florin-Christensen, M., Florin-Christensen, J., Garin, C., Isola, E., Brenner, R. and Rasmussen, L. (1990) Biochem. Biophys. Res. Commun. 166, 1441–1445.
[2] Haughan, P.A., Chance, M.L. and Goad, L.J. (1992) Biochem. Pharmacol. 44, 2199–2206.
[3] Urbina, J.A. (1997) Parasitology 114, S91–S99.
[4] Urbina, J.A., Lazardi, K., Marchan, E., Visbal, G., Aguirre, T., Piras, M.M. and Piras, R. (1993) Agents Chemother. 37, 580–591.
[5] Brown, M.S. and Goldstein, J.L. (1980) J. Lipid Res. 21, 505–515.
[6] Brown, M.S. and Goldstein, J.L. (1985) Curr. Top. Cell Regul. 26, 3–15.
[7] Istvan, E.S., Palnitkar, M., Buchanan, S.K. and Deisenhofer, J. (2000) EMBO J. 19, 819–830.
[8] Rodwell, V.W. (1997) Biochemistry 36, 2173–2177.
[9] Lawrence, C.M., Rodwell, V.W. and Stauffacher, C.V. (1995) Science 268, 1758–1762.
[10] Tabernero, L., Bochar, D.A., Rodwell, V.W. and Stauffacher, C.V. (1999) Proc. Natl. Acad. Sci. USA 96, 7167–7171.
[11] Lam, W.L. and Doolittle, W.F. (1992) J. Biol. Chem. 267, 5829–5834.
[12] Peña-Díaz, J., Montalvetti, A., Camacho, A., Gallego, C., Ruiz-Pérez, L.M. and González-Pacanowska, D. (1997) Biochem. J. 324, 619–626.
[13] Montalvetti, A., Peña-Díaz, J., Hurtado, R., Ruiz-Pérez, L.M. and González-Pacanowska, D. (2000) Biochem. J. 349, 27–34.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Davies, G.E. and Stark, G.R. (1970) Proc. Natl. Acad. Sci. USA 66, 651–656.
[16] Lundblad, R. and Noyes, C.M. (1984) Chemical Reagents for Protein Modification, Vol. 1, pp. 105–125, CRC Press, Boca Raton, FL.
[17] Chin, D.J., Luskey, K.L., Faust, J.R., MacDonald, R.J., Brown, M.S. and Goldstein, J.L. (1982) Proc. Natl. Acad. Sci. USA 79, 7704–7708.
[18] Geelen, M.J.H., Gibson, D.M. and Rodwell, V.W. (1986) FEBS Lett. 10, 183–186.
[19] Rogers, K.S., Rodwell, V.W. and Geiger, P. (1997) Biochem. Mol. Med. 61, 114–120.
[20] Tuajuanda, C., Jordan-Starck, T.C. and Rodwell, V.W. (1989) J. Biol. Chem. 264, 17913–17918.
[21] Endo, A. (1985) J. Med. Chem. 28, 401–405.
[22] Endo, A., Kuroda, M. and Tanzawa, K. (1976) FEBS Lett. 72, 323–326.
[23] Morrison, J.F. and Walsh, C.T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 201–203.
[24] Schloss, J.V. (1988) Acc. Chem. Res. 21, 348–353.
[25] Louis-Flamberg, P., Peishoff, C.E., Bryan, D.L., Leber, J., Elliott, J.D., Metcalf, B.W. and Mayer, R.J. (1990) Biochemistry 29, 4115–4120.