Biochemical Studies of GMP reductase from *Escherichia coli*

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Guanosine monophosphate (GMP) reductase (NADPH: GMP oxireductase; EC 1.6.6.8) catalyzes the irreversible reductive deamination of GMP to inosine monophosphate (IMP) (Deng, 2002), and is encoded by the *guaC* structural gene, playing an important role in the interconversion of purine nucleotides, particularly adenine and guanine (Andrews and Guest, 1988). Thereby, this enzyme represents the only known metabolic step by which guanine nucleotides can be converted to the pivotal precursor of both adenine and guanine nucleotides (Spector, Jones and Miller, 1979). GMP reductase is not normally required for the efficient growth of *Escherichia coli*, since the lack of its activity leaves the *de novo* synthesis of both GMP and adenosine monophosphate (AMP) unimpaired; however, when the *de novo* synthesis of IMP is blocked, GMP reductase activity becomes necessary to supply AMP when guanine and its derivatives are the solely purine sources (Andrews and Guest, 1988). The objectives of this work were to amplify and clone the *guaC* gene, overexpress, purify and characterize the recombinant GMP reductase enzyme.

The *guaC* structural gene (1038 bp) was PCR-amplified from *E. coli* K12 genomic DNA. The PCR product was cloned into pCR-Blunt® vector and subcloned into pET-23a(+) expression vector, with *NdeI* and *BamHI* restriction sites. The resulting pET-23a(+):*guaC* plasmid was sequenced to ensure gene integrity. The recombinant protein was expressed in the soluble form when the plasmid was transformed into *E. coli* BL21(DE3) host cells and grown in LB medium during 24hs. Three chromatographic steps (anion exchange, size exclusion, anion exchange) were used to obtain GMP reductase in its homogenous form. The homogeneous sample was submitted to electrospray ionization mass spectrometry (ESI-MS) and the identity of *E. coli* GMP reductase was confirmed. The molecular mass of GMP...
reductase recombinant protein was determined by gel filtration chromatography and reveals a tetrameric quaternary structure.

GMP reductase activity was determined spectrophotometrically by measuring the conversion of NADPH into NADP⁺ at 340 nm in order to determine the apparent and true steady-state constants (K_M, V_max, k_cat) for the catalyzed reaction. The apparent K_M and V_max values for 5’GMP are respectively 6.9±0.3µM and 0.065±9x10⁻⁴U mg⁻¹, while the apparent K_M and V_max values for NADPH are respectively 11.1±1.2µM and 0.402±0.011U mg⁻¹. True steady-state kinetic constants are 5.5±1.0µM as the K_M value for the 5’GMP, 14.7±2.5µM as the K_M value for the NADPH, and 0.28±0.02 s⁻¹ as the catalytic constant, k_cat, for the catalyzed reaction.

The dependence of kinetics parameters on pH was determined by measuring initial velocities in the presence of varying concentrations of one substrate and a saturating level of the other, in a buffer mixture over the following pH values: 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5. Fluorescence titration was carried out to understand the interaction at equilibrium between GMP reductase and either substrate(s) or product(s) at 25°C. The results showed that GMP reductase intrinsic fluorescence changed only upon binding of NADPH.

In order to determine the energy of activation of the GMP reductase-catalyzed reaction, initial velocities were measured in the presence of varying concentrations of one substrate at a saturating level of the other, at temperatures ranging from 15 to 35°C, and the results showed a value of 4.4 kcal mol⁻¹ to initiate the GMP reductase reaction. Solvent kinetic isotope effects were determined by measuring initial velocities using a saturating level of one substrate and varying concentrations of the other on either H₂O or 90 atom % D₂O. The proton inventory was determined using saturating concentrations of both substrates at various mole fractions of D₂O. No solvent isotope effect was observed, which indicate that the solvent proton-transfer does not contribute to the rate-limiting step of the catalyzed reaction.
References