Pyrimidines can be synthesized either through the \textit{de novo} or salvage pathways. Uridine phosphorylase (UP) is a pyrimidine nucleoside phosphorylase that is present in the pyrimidine salvage pathway and catalyzes the reversible phosphorolysis of uridine to uracil and ribose-1-phosphate. Although UP is present in most normal tissues and in tumors, its activity as well as its expression is elevated at the second. This enzyme regulates strictly the concentration of uridine in plasma and tissues and affects activation and catabolism of several base and nucleoside analogues used in chemotherapy of cancer, such as 5-fluorouracil (5-FU)\textsuperscript{1}. 5-FU is a uracil analogue with a fluorine atom at the C-5 position and was developed in 1957. Since then, 5-FU has been used in clinical practice against many types of solid tumors\textsuperscript{2}. Clinical studies have demonstrated that uridine can be used to reduce 5-FU toxicity, leading to an increased therapeutic index, and to selectively protect normal tissues from this host toxicity; high doses of exogenous uridine are not well tolerated in humans\textsuperscript{3}. In humans there are two UP isoforms, hUP\textsubscript{1}\textsuperscript{4} and hUP\textsubscript{2}\textsuperscript{1}, and there is a significant interest in the development of compounds that inhibit these isoforms.

In this study, we described the amplification and cloning of \textit{UPP1} encoding gene, and the expression, purification and kinetic studies of the recombinant hUP1. The \textit{UPP1} gene was PCR-amplified, the PCR-product was cloned into pCR-Blunt vector, subcloned into pET-23a(+) expression vector, and sequenced to ensure gene identity. The recombinant protein was expressed in \textit{Escherichia coli} Rosetta (DE3) cells, grown in TB medium, at 30°C, up to 36h without induction. The protein was purified through a single-step purification protocol, using a cation exchange column, and mass spectrometry analysis and N-terminal amino acid sequencing confirmed the identity of the recombinant hUP1. To determine the true steady-
state kinetic parameters, initial velocity studies were carried out at varying concentrations of one substrate and several fixed-varied concentrations of the co-substrate. Product inhibition patterns were determined at varying concentrations of one substrate, fixed concentrations of the co-substrate and fixed-varying concentrations of products [either ribose-1-phosphate (R1P) or uracil] to evaluate the order of substrate addition. The study of equilibrium binding by fluorescence was performed to confirm the order of substrate addition and determine the order of product dissociation. The intrinsic hUP1 fluorescence enhanced upon either P_i or R1P binding. The initial velocity, product inhibition, and equilibrium binding results are consistent with a steady-state ordered bi bi kinetic mechanism, where P_i binds first, followed by the binding of uridine, to form the catalytically competent ternary complex, and uracil is the first product to dissociate from the complex, followed by the dissociation of R1P. To determine the dependence of the kinetic parameters on pH, initial velocities were measured 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: 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0.

Amino acid residues involved in either catalysis or substrate binding were proposed based on apparent acid and base dissociation constant for ionization groups obtained by pH-rate profiles assays. The perspectives of this work are to complete the kinetic characterization of hUP1 and begin the studies with possible inhibitors. We also intend to determine the crystallographic structure of hUP1 at high resolution X-ray diffraction.

We also work with the other isoform, hUP2, and here we describe to the amplification and cloning of the UPP2 encoding gene, expression and purification of the recombinant protein hUP2. The open reading frame of the UPP2 gene was PCR-amplified with oligonucleotide primers, the PCR product was cloned into pCR-Blunt cloning vector and subcloned into pET-23(a)+ expression vector. The integrity and identity of the gene was confirmed by DNA automatic sequencing. Many strains of E.coli, different medium and temperatures were tested to define the expression protocol. The recombinant protein was obtained, in soluble form, in E.coli Rosetta (DE3) eletrocompetent host cells, grown in TB medium, at 30°C, up to 24h without induction. Protocol purification by FPLC is currently being determined using anion exchange, size exclusion and hydrophobic interaction columns. The perspectives of this work are establishing the purification protocol; determine the identity and experimental molecular weight of the recombinant protein and the kinetic parameters. Also, determine the crystallographic structure of hUP2 at high resolution X-ray diffraction.
Conclusions

All the data present here, referent to hUP1, were published on 2010 March on Archives Biochemistry and Biophysics (ABB)\(^5\). These data may be useful to the rational design of new inhibitors for this enzyme, and, thereby, contribute for chemotherapy of cancer. Besides, all the data produced to hUP2 will be important to make possible to compare the two isoforms and may permit the design of selective inhibitors.

References


