Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Suppl. I Vol. 83, November, 1988/Page 291

BIOCHEMICAL APPROACHES TO CHEMOTHERAPY OF TRYPANOSOMIASIS

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INTRODUCTION

Parasites are generally deficient in metabolic activities. It is possible to rely on biochemical knowledges and molecular biological techniques to explore these deficiencies as potential targets for antiparasitic chemotherapy. Recent biochemical investigations on African tryponosomes have accumulated a wealth of knowledge; some of which has already led to the identification of new therapeutic targets and, in a couple of cases, new antiparasitic agents. These positive experiences may provide some guidance for our future approaches to antiparasitic chemotherapy as well as our ability to recognize an opportunity in parasite metabolism for selective attack.

THE ORNITHINE DECARBOXYLASE

The formation of polyamines, required for cellular proliferation, is controlled by ornithine decarboxylase among eukaryotes (1). In the long-slender bloodstream trypomastigotes of African trypanosomes, putrescine and spermidine constitute the main pool of polyamines (2). They are synthesized mainly from ornithine in Trypanosoma brucei, and taken up from extracellular sources only slowly (3). DL-α-Difluoromethylornithine (DFMO), a catalytic, irreversible inhibitor of ornithine decarboxylase with certain antitumor activities (4), has good therapeutic activities against African trypanosomes both in vitro and in vivo (5). Because of the remarkable lack of toxicity of this compound, it is currently undergoing clinical trials with encouraging results especially on Trypanosoma gambiense infections (6).

DFMO has been shown to deplete the intracellular polyamine pool of T. brucei (7), and in its in vitro activity against T. brucei is reversible by putrescine (8). Its antitrypanosomal activity in the experimental murine model is also reversible by intraperitoneal injections of polyamines (9). It appears thus that DFMO acts on T. brucei by inhibiting the ornithine decarboxylase of the parasite. The physiological consequence of such enzyme inhibition turned out to be quite interesting. The long-slender form of T. brucei is simply transformed to the non-dividing short-stumpy form by

DFMO treatment either in vivo or in vitro (7). The non-dividing form is apparently incapable of changing its variant surface glycoprotein coat and is eventually caught up by the host immune response. The drug-induced transformation bears a close resemblance to the natural transformation of T. brucei from long-slender to short-stumpy forms, during which mitochondrial genes encoding cytochrome b, cytochrome oxydase and NADH dehydrogenase are transcribed at high levels in both cases (10). Preliminary studies in our laboratory indicated also that during the natural transformation of pleomorphic strains of T. brucei from long-slender to short-stumpy forms, the specific ornithine decarboxylase activity in parasite was decreased rapidly to a level below detection (unpublished observation), which was presumably accompanied by polyamine depletion in T. brucei.

Understanding the mechanism of antitrypanosomal action of DFMO cannot, however, explain why the trypanosomes are much more susceptible to DFMO than the mammalian host. Apparently, the trypanosomal ornithine decarboxylase has a higher K_I of 130 μM for DFMO (11) than does the mammalian enzyme (K_I = 39 μM) (12), and should be, in theory, less susceptible to DFMO inhibition. Detailed comparisons between the mammalian and the parasite ornithine decarboxylase are thus necessary for understanding the basis of therapeutic action of DFMO. This has been proven difficult due to the unavailability of T. brucei enzyme (~10 nmoles/hr/mg protein) for extensive purifications and characterizations. Fortunately, the close homology between the two enzymes allowed the use of a mouse lymphoma S49 cDNA of ornithine decarboxylase as a heterologous probe to identify and clone the T. brucei gene encoding ornithine decarboxylase (13). The sequence of the parasite enzyme gene revealed a 61.5% homology between the residues #21-#445 of T. brucei ornithine decarboxylase and the residues #1-#425 of Balb/c mouse ornithine decarboxylase. Clearly, these two enzymes are very similar to each other, which explains their similar susceptibilities toward DFMO, but fails to explain the selective susceptibility of trypanosomes toward DFMO.

There is, however, a major discrepancy between the primary structures of the two enzymes; T. brucei ornithine decarboxylase lacks the 3b amino acid peptide at the C-terminus of the mouse enzyme. This difference is of considerable interest because MaCrea and Coffino (14) were able to make C-terminal deletions of the mouse ornithine decarboxylase by cDNA excision, cloning and expression in Escherichia coli, and found that the mouse enzyme C-terminus could be deleted up to 38 amino acid residues without appreciable decrease in enzymatic activity. Thus the T. brucei enzyme must be a functional enzyme.

Furthermore, the mouse enzyme C-terminal #423-#449 is classified a PEST region of the protein which, by the PEST hypothesis (15), is the determining factor of the very short in vivo half life (20 min) of the mouse enzyme (16). It is thus likely that since T. brucei ornithine decarboxylase does not possess the PEST sequence, it should have a relatively long half life. This elucidation was confirmed by our recent observations that T. brucei procyclic forms, kept under an inhibited state of protein synthesis, maintained a constant level of ornithine decarboxylase for at least six hours (13). This lack of rapid turning over of the parasite enzyme, which may be necessary for well-controlled T. brucei differentiation, may have constituted the basis of therapeutic usefulness of DFMO as an antitrypanosomal agent.

Since the successful elucidation of the mechanism of DFMO action, we have further pursued the investigation on T. brucei ornithine decarboxylase. The genomic DNA encoding this enzyme was engineered by site-directed mutagenesis techniques, and ligated in frame to an expression vector pCQV2 (14) at its 5'-end downstream from a strong lambda p_R promoter and a mutant lambda cI gene encoding a thermolabile repressor of the p_R promoter. This recombinant construct, termed PQTODC, was used to transform the E. coli HT289 ornithine decarboxylase deletion mutant. The E. coli transformants were cultivated and shocked at a higher temperature of 42 °C to inactivate the lambda repressor. Enzyme activity assays and gel electrophoretic analysis of the [^{14}C]-DFMO labeled protein indicated that about 0.2% of the total E. coli protein was T.

brucei ornithine decarboxylase (17). This protein was purified from E. coli to total homogeneity by ammonium sulfate fractionation (25-55%), pyridoxal phosphate agarose affinity column chromatography and fast performance liquid chromatography on a Mono Q column. The purified recombinant enzyme has been characterized and found identical to the native T. brucei ornithine decarboxylase in all aspects examined. This success may be the first time a therapeutic target enzyme in a parasite is produced in pure form in significant quantities via genetic engineering means. Future efforts will be concentrated on enzyme crystallization, X-ray crystallography and inhibitor design by computer graphics.

THE GLYCOSOMES

In the long-slender bloodsream forms of T. brucei, each trypomastigote consists of a single mitochondrion devoid of a functioning tricarboxylic acid cycle or a cytochrome chain of electron transport (18). The parasite is entirely dependent on glycolysis for energy production, which generates, from every glucose molecule, two ATP molecules and two pyruvate molecules under aerobic conditions. (19). The relatively inefficient energy production is compensated by making the glycolysis proceeding at an exceedingly high rate in order to support the rapid cell divisions of T. brucei every 6-8 hours (20). This is apparently made possible by aggregating the first seven glycolytic enzymes and two glycerol-metabolizing enzymes in membrane-bound, microbody-like organelles, termed the glycosomes (21). The glycosome, having a protein concentration of 340 mg/ml and steady concentrations of glycolytic intermediates in the millimolar range (22), regenerates NAD^+ from NADH by dihidroxyacetone phosphate: glycerol-3-phosphate shuttle plus a glycerol-3-phosphate oxidase in the mitochondrion. During anaerobiosis, the oxidase cannot function. Glycerol-3-phosphate becomes accumulated in the glycosome, and is accompanied by the accumulation of NADH and ADP. Glycerol-3-phosphate and ADP eventually revert the glycerol kinase catalyzed reaction to generate glycerol and ATP to enable the parasite to survive (23).

This delicate and indispensable glycolitic system and the mechanisms leading to the biogenesis of glycosomes are obvious and attractive targets for antitrypanosomal chemotherapy. It has been assumed that the genes encoding glycosomal proteins are located in the nucleus, and the products of these genes synthesized in the cytoplasm on free polysomes (24). Thus, glycosomal assembly would require import of proteins accross the organelle membrane post-translationally. Thus far, in vitro translation of mRNA yields glycosomal proteins with the same molecular weights as the mature products inside the glycosome (24), thus suggesting that the import may not involve proteolytic processing. Recent studies on the genomic sequences of four T. brucei glycosomal enzymes, aldolase (25), glyceraldehyde-3-phophate dehydrogenase (26), 3-phosphoglycerate kinase (27) and triosephosphate isomerase (28) indicated that none of them have a leader sequence, but all have very high isoelectric points (29). The high pI's are the results of basic amino acids interspersed along the molecules. Three dimensional structural analyses of these enzymes by superimposing their deduced sequences on the crystalline structures of the same mammalian enzymes suggested that some of the extra basic amino acids are clustered in two areas on the surface of the molecule, 40A part. It was postulated that these "hot spots" may be involved in the import of glycosomal proteins into the glycosome (30). We have recently established an in vitro protein import assay with the purified intact glycosomes and [^{35}S]-methionine labeled in vitro translational products (31). Specificity of the protein import was verified using translational products derived from cloned genes encoding T. brucei glycosomal 3-phosphoglycerate kinase (PGK) and its 95% homologous cytosolic isozyme. Glycosomal PGK was inserted into the glycosome with a 27.6% efficiency, but no imported cytosolic PGK was detectable. Preliminary data obtained from the import of deletion mutant and chimeric mutant proteins of glycosomal PGK suggest that the peptide sequence (#66-#79), which consists of one of the "hot spots" and is not present in yeast, human or horse PGK, may be the signal peptide for import of glycosomal PGK. Future studies with the potential use of substrate proteins altered by site-directed mutagenesis may

reveal the detailed mechanism and the recognition systems involved in glycosome biogenesis. They can be potential targets for future antitrypanosomal chemotherapy.

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