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Chemical properties and active site characterization of bovine pancreatic asparagine synthetase

Mehlhaff, Paige Marie, Ph.D.

The University of Nebraska - Lincoln, 1987

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CHEMICAL PROPERTIES AND ACTIVE SITE CHARACTERIZATION OF BOVINE PANCREATIC ASPARAGINE SYNTHETASE

by

Paige Marie Mehlhaff

A DISSERTATION

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Chemical Properties and Active Site Characterization of

Bovine Pancreatic Asparagine Synthetase

BY

Paige Marie Mehlhaff

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CHEMICAL PROPERTIES AND ACTIVE SITE CHARACTERIZATION OF BOVINE PANCREATIC ASPARAGINE SYNTHETASE

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University of Nebraska, 1987

Advisor: Dr. Sheldon M. Schuster

Bovine pancreatic asparagine synthetase was studied with regard to the ammonia-dependent production of asparagine. Asparagine was found to be a partial competitive inhibitor with respect of ammonia, except when MgATP was nonsaturating, then it became linear competitive. This is consistent with a kinetic mechanism where ammonia is bound followed by MgATP which causes asparagine release.

Monoclonal antibodies were produced to asparagine synthetase and used in immunoaffinity chromatography to purify the enzyme 1159-fold. Two of the antibodies inhibited glutamine and ammonia dependent activities but not glutaminase, and another inhibited glutamine dependent and glutaminase activities but not ammonia dependent. This is discussed in terms of multiple substrate binding domains in asparagine synthetase.

The monoclonal antibodies which inhibited asparagine synthetase were mapped to two topographically separate regions of the enzyme surface using competitive binding assays. The results of these assays suggest a topographical separation between an asparatyl-AMP and glutamine binding sites. The non-inhibitory antibodies could be mapped to a third distinct site. Binding studies showed extensive cross-reaction of these antibodies

with enzyme from bovine liver and sheep pancreas, and to a lesser degree with rat liver or pancreas, a human tumor cell line, and a mouse tumor cell line. Only one of the four antibodies inhibitory toward the ruminants was inhibitory toward rodent, suggesting significant structural differences between the enzymes.

The presence of dimer and monomer forms of asparagine synthetase and factors affecting the relative amounts of each was studied. The inhibition of the enzyme by substrate analogs DON and CONV and their effect on subunit composition was also studied. Among other things the analogs were found to shift the enzyme to the dimer form and CONV caused it to become uv invisible as well. When Mabs were allowed to bind to enzyme previously inactivated by the analogs the binding of Mab 5A6 was prevented, giving further proof that 5A6 recognizes the glutamine binding site of the enzyme.

This dissertation is dedicated to my parents, Ray and Pat Mehlhaff, whose love, encouragement and constant support seems to make all things possible.

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I also want to thank Margaret for her advice and support. Although she may never know what a difference she made, I will always be deeply grateful for her help.

PREFACE

A portion of this dissertation has been published, therefore the chapters have been written to be complete within themselves, each with its own introduction, experimental section, discussion etc. The references for the published chapters are as follows:

Chapter Two: Mehlhaff, P. M., Luehr, C. A. and Schuster, S. M. "Studies of the Ammonia-dependent Reaction of Beef Pancreatic Asparagine Synthetase" *Biochemistry* **24**, 1104-1110 (1985).

Chapter Three: Pfeiffer, N. E., Mehlhaff, P. M., Wylie, D. W. and Schuster, S. M. "Monoclonal Antibodies Specific for Bovine Pancreatic Asparagine Synthetase" *J. Biol. Chem.* **261**, 1914-1919 (1986).

Chapter Four: Pfeiffer, N. E., Mehlhaff, P. M., Wylie, D. W. and Schuster, S. M. "Topographical Separation of the Catalytic Sites of Asparagine Synthetase Explored with Monoclonal Antibodies" *J. Biol. Chem.* September (1987).

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Chapter One

Introduction and Literature Review

More than thirty years ago it was first noted that a factor in guinea pig serum demonstrated antitumor activity against two strains of murine lymphoma (1). This antitumor activity was subsequently shown to be associated with L-asparaginase activity, which is in particularly high concentration in guinea pig serum (2). Since that time L-Asparaginase has been used in many clinical studies (3-9). Tumors that respond to L-asparaginase therapy include melanomas, some lymphomas, and leukemias, especially acute lymphoblastic leukemia (10). When *E. Coli* asparaginase was used as a single chemotherapeutic agent for treatment of acute lymphoblastic leukemia, complete remission was observed in 40% of the cases (6). In combination with vincristine and prednisone, L-asparaginase therapy produced a 95% complete remission rate in previously untreated acute lymphoblastic leukemia (6). Although L-asparaginase therapy can be very effective there are some drawbacks to its use. There are numerous side effects associated with its use, including immunological responses (8, 9), hypolipidemia, hypoproteinemia (11), clotting disorders (12), central nervous system abnormalities (13), hyperglycemia due to insulin suppression (12), and others (14-16). In addition, the tumor cells often develop resistance to the therapy (17, 10) and treatment can result in metastasis of the resistant tumors (18).

Since the effectiveness of L-asparaginase treatment ultimately depends on decreasing the circulating level of asparagine, an alternate

approach to L-asparaginase therapy is to inhibit the biosynthesis of asparagine. It is concievable that this could destroy the sensitive tumor cells without the side effects inherent in L-asparaginase treatment. In order to attempt such an approach, extensive knowledge of asparagine metabolism and of the enzyme responsible for the production of asparagine is required.

Background. A pathway for L-asparagine biosynthesis was first reported in 1961 (19, 20). The enzyme, asparagine synthetase, was reported to produce asparagine from aspartic acid and ammonia with the concurrent hydrolysis of ATP to ADP and inorganic phosphate. A short time later another investigator reported the formation of asparagine from aspartate and ammonia in *Lactobacillus arabinosus* but showed that the hydrolysis of ATP formed AMP and inorganic pyrophosphate, instead of ADP and P_i (21). Since that time asparagine synthetase has been looked at in a variety of plants and animals including mouse leukemia cells (22), rat liver (23, 24), chinese hamster ovary cells (25), mouse pancreas (26), lupine seedlings (27), guinea pig (28), *Streptococcus bovis* (29), *E. coli* (30), *Sacchuromyces cerevisiae* (31), *Neurospora* (32), corn roots (33), soybean leaves (34), and bovine pancreas (35). A study of the distribution of asparagine synthetase among several species, showed that the guinea pig had the highest activity of asparagine synthetase, and that in all mammals tested the pancreas was the organ of greatest synthetase levels (36). Another study using fetal rats showed high levels of asparagine synthetase in the fetal livers until birth when the levels decreased to that of adults (37). In addition to organ specificity and age dependence the level of asparagine synthetase also appears to depend on diet. Patterson and Orr found that a lack of dietary

asparagine produced a seven-fold increase in rat liver asparagine synthetase per mg of protein (23). Hongo et al was able to repeat this by feeding rats a low protein diets for up to two months, at which time the asparagine synthetase levels again dropped (24).

Asparagine synthetase in bovine pancreas and other mammalian systems have three distinct reactions; a glutamine dependent syntheatse [1], an ammonia dependent synthetase [2], and a glutaminase reaction [3].

[1] glutamine + aspartate + Mg-ATP → asn + glu + AMP + PP_i + Mg^{2+}

[2] NH_3 + aspartate + Mg-ATP → asparagine + AMP + PP_i + Mg^{2+}

[3] glutamine → glutamate + NH_3

Most bacterial asparagine synthetase can utilize only ammonia in the synthesis of asparagine, but in 1980 Humbert and Simoni reported that *E. coli* contained two separate genes that coded for two distinct asparagine synthetases. The Asn A gene codes for an enzyme similar to other bacterial asparagine synthetases that can utilize only ammonia as the nitrogen source. The other gene, Asn B, codes for an enzyme that can use either glutamine or ammonia in the synthesis of asparagine, as do the mammalian enzymes (38).

Most of the work on asparagine synthetase involves either the glutamine dependent or ammonia dependent synthesis of asparagine or both, but very little work has been done on the glutaminase reaction alone, with the exception that Horowitz and Meister have measured the rates of the reactions and note that the glutaminase is much faster than the synthetase reactions and suggest that this involves an uncoupling between the two reactions (39).

Properties. The asparagine synthetases from several sources have been purified and some of their physical properties have been determined. Patterson and Orr (40) purified asparagine synthetase 108-fold from Novikoff hepatoma, Horowitz and Meister (39) obtained a 173-fold purification from RADA1 mouse leukemia, Holcenberg (28) got only a 13-18-fold purification from normal guinea pig liver, spleen and small intestine. Cedar and Schwartz (41) obtained a 370-fold purification from an E. coli mutant, Gantt and Arfin (25) got 420-fold from bovine pancreas, and Leuhr and Schuster (42) obtained a 1400-fold purification of bovine pancreatic asparagine synthetase. Hongo and Sato (43) reported a 2500-fold purification from rat liver using a reactive blue 2-agarose affinity column. The ability of asparagine synthetase to bind to such an affinity resin appears to be unique to the rat liver form, since bovine pancreatic asparagine synthetase binds only weakly to the resin (44) and no other reports using this affinity resin have appeared.

Subunit composition and molecular weight of asparagine synthetase from several sources have been reported. The ammonia dependent E. coli asparagine synthetase has a weight of 82,000 determined by size exclusion chromatography (41). The asparagine synthetase from Lupinus luteus has a molecular weight of 160,000 but associates to a 320,000 molecular weight dimer in the presence of $MgCl_2$ and ATP (27). *Saccharomyces cerevisiae* has a molecular weight of 152,000 (31) and mouse leukemia cell asparagine synthetase of 105,000 (40). In a mouse lymphoma Davies and Marshall found three different peaks showing asparagine synthetase activity using Sephadex G-100 chromatography. These peaks correspond to weights of 70,000, 11,600 and a late eluting peak of smaller molecular weight. When