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Characterization of the effect of alcohol on recombinant proteins derived from mammalian adenylyl cyclase

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CHARACTERIZATION OF THE EFFECT OF ALCOHOL ON RECOMBINANT PROTEINS DERIVED FROM MAMMALIAN ADENYLYL CYCLASE

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TABLE OF CONTENTS

ABSTRACT

Research suggests that the cyclic AMP (cAMP) signaling pathway is implicated in the development of alcoholism. Previous work in our laboratory has demonstrated that alcohol enhances the activity of adenylyl cyclase (AC) in an isoform specific manner; human type 7 AC (AC7) is most enhanced by ethanol as measured by cAMP accumulation assay in whole cells. We hypothesize that alcohol enhances AC activity by directly interacting with the protein and that alcohol effects on AC can be studied using recombinant AC expressed in bacteria. Our objectives include: 1) design and optimization of the conditions for protein expression and protein purity for recombinant AC proteins; 2) identification of the importance of each cytoplasmic domain to the alcohol effect through chimeric analysis; and 3) investigation of the interaction between alcohol and AC stimulators such as $MnCl_2$, forskolin, and Gsα through concentration-response experiments. To examine these objectives, we expressed in bacteria, and purified recombinant AC proteins carrying the C_{1a} and/or C_2 domains of AC2, AC7, and AC9. We present the optimal conditions for the expression and purification of multiple recombinant AC proteins and show that purified recombinant AC proteins retain enzymatic activity and alcohol responsiveness. Through chimeric analysis, we found that the C_{1a} and C_2 domains both contribute to the alcohol effect on AC7, however, AC7 C_{1a} may play a stronger role. We also find that recombinant AC responds to alcohol differently under varied conditions of AC activation by $MnCl_2$, forskolin, and Gsα. Through concentration-response experiments we found that there is some interaction between alcohol and forskolin or Gsα, but alcohol does not appear to be competing with forskolin or Gsα at an allosteric site. Overall the results suggest that alcohol interacts with AC proteins directly, independent of stimulants examined, and causes a

conformational change, which results in either enhancement or inhibition depending on stimulation conditions.

CHAPTER 1.
INTRODUCTION

Alcohol Use and Abuse

Alcohol use is common in the United States and in most countries around the world. The effects of alcohol use are wide ranging; many individuals can use alcohol with no development of problematic symptoms, many individuals develop long term problems and pathologies as a result of alcohol use, and there is even evidence to suggest that some alcohol use may be beneficial. The National Institute on Alcohol Abuse and Alcoholism (NIAAA) was established to address the health implications of alcohol use with a yearly budget of approximately $460 million dollars appropriated in 2012. According to the NIAAA, 51.5% of US adults age 18 and above are regular drinkers, defined as greater than or equal to 12 drinks per year. One drink is defined as 12 ounces of beer, 5 ounces of wine, or 1.5 ounces of 80-proof liquor. Per capita ethanol consumption was estimated at 2.26 gallons in 2010. Alcohol use is implicated in deaths both directly and indirectly. In 2010, 25,440 alcohol-induced deaths were reported that were not the result of traffic accidents or homicide (Murphy, Xu, & Kochanek, 2012). According to the Centers for Disease Control and Prevention, alcohol-impaired driving accounted for 10,228 deaths in 2010 which accounted for 31% of total traffic deaths that year. Furthermore, in 2010, more than 1.4 million drivers were arrested for impaired driving under the influence of alcohol or narcotics and approximately 112 million adults admit to alcohol-impaired driving. The overall costs of alcohol abuse to the US, accounting for health care costs, productivity impacts, motor vehicle accidents, crime, fire, and social welfare costs, equate to approximately $185 billion annually (National Institutes of Health, 2000).

The defining factors between alcohol use, alcohol abuse, and alcoholism are characterized by volume, frequency, and duration of use. Alcoholism is a general term used to

describe patterns of problematic alcohol drinking behaviors. Specifically, the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) classifies pathological alcohol use as alcohol abuse or alcohol dependence. Criteria for alcohol abuse requires one of the following in a 12-month period: recurrent alcohol use leading to the failure to fulfill obligations at work, home, or school; recurrent alcohol use leading to physically hazardous situations; alcohol-related legal problems; continued use of alcohol despite social or interpersonal problems caused by the effects of alcohol (American Psychiatric Association, 2000). Criteria for alcohol dependence requires three or more of the following in a 12-month period: the need for increased amounts of alcohol for desired effect; withdrawal syndrome; drinking larger amounts or for longer periods; persistent desire for alcohol or unsuccessful attempts to stop drinking; discontinued participation in social, occupational, or recreational activities due to alcohol use; large amount of time spent obtaining, using, or recovering from alcohol use; continued alcohol use despite physical or psychological problems caused by alcohol (American Psychiatric Association, 2000).

Alcohol and the Brain

The function of alcohol on the brain is diverse and complex. Alcohol affects many brain regions including the cerebral cortex, cerebellum, limbic system, ventral tegmental area, and nucleus accumbens (Zahr, Pitel, Chanraud, & Sullivan, 2010; Oscar-Berman & Marinković, 2007). The cerebral cortex innervates many other brain regions and is responsible for executive and cognitive functions as well as emotion. Loss of cortical neurons is commonly observed with chronic alcohol use (Oscar-Berman & Marinković, 2007). The cerebellum is mostly known for coordination of motor function. However, recent research suggests the existence of several cerebral-cerebellar circuits that implicate the cerebellum in cognitive functions such as memory

and attention (Oscar-Berman & Marinković, 2007). Like the cerebral cortex, neurodegeneration is also observed in the cerebellum following chronic alcohol use. Alcohol's effect on the cerebellum is thought to contribute to loss of impulse control, conflict processing, and disinhibition (Zahr et al., 2010). The limbic system includes several brain regions including the thalamus, hypothalamus, cingulate gyrus, amygdala, hippocampus, and basal ganglia and is responsible for the regulation of memory and emotion. The limbic system is also important for motivation. Activation of the amygdala, important for motivation and emotional processing, is attenuated in alcoholics (Oscar-Berman & Marinković, 2007). In addition to motivation and emotion, the hippocampus is implicated in the formation of memories. The hippocampus is one of the only brain regions that continue to produce new neurons throughout the life span; this neurogenesis is reduced in alcoholics (Oscar-Berman & Marinković, 2007). Chronic alcohol use can cause damage to the hypothalamus and is associated with memory loss, including both retrograde and anterograde amnesia (Oscar-Berman & Marinković, 2007). Additionally, the hypothalamic-pituitary-adrenocortical (HPA) axis, implicated in the response to stress, is suppressed in chronic alcoholics (Oscar-Berman & Marinković, 2007). The limbic system structures, along with the ventral tegmental area and the nucleus accumbens, make up the reward center in the brain. Acute exposure to alcohol modulates the neural transmission in this brain circuit and chronic exposure has been shown to reduce the volume of these structures (Zahr et al., 2008). What makes many of these particular brain regions susceptible to alcohol is the localization of the neurotransmitter systems within the groups of neurons.

Neurotransmitters and neuromodulators which are primarily involved in the response to alcohol include glutamate, gamma-aminobutyric acid (GABA), glycine, serotonin, dopamine, adenosine, and opioids (Lovinger, 2008; Gass & Olive, 2012). GABA is an inhibitory

neurotransmitter which works primarily through ligand-gated ion channels (LGIC), but can also act through G protein-coupled receptors (GPCR). Alcohol increases activity of the GABAergic system through numerous mechanisms and the GABAergic system is implicated in both acute and chronic alcohol effects. Acute effects of alcohol mediated by GABA include alcohol intoxication symptoms such as motor incoordination, anxiolytic effects, and sedation (Lovinger, 2008). With chronic and acute use, the increase in GABAergic transmission during alcohol exposure can lead to brain hyperexcitability during withdrawal from chronic alcohol use (Lovinger, 2008). Glutamate is an excitatory neurotransmitter and also functions primarily with LGICs; however, there are some glutamate-linked GPCRs. Acute alcohol use inhibits the glutamatergic system; this is thought to be partially responsible for the intoxicating effects of alcohol and associated memory impairment (Möykkynen & Korpi, 2012). Inhibition of the glutamatergic system by chronic alcohol use leads to an up regulation of some glutamate receptors that can contribute to the brain hyperexcitability that occurs during alcohol withdrawal (Lovinger, 2008). Glycine as a neurotransmitter has also been implicated in the response to alcohol. Glycine receptors are also LGICs and are inhibitory to neural transmission. Alcohol increases the effect of glycine at the glycine receptor (Perkins, Trudell, Crawford, Alkana, & Davies, 2010). The effect of alcohol on the glycinergic system has been linked to modulation of ethanol intake, ethanol-induced sedation, and incoordination (Perkins et al., 2010). Serotonin is mostly linked to GPCRs, but is also linked to one LGIC, both of these receptor types can be affected by alcohol. The serotonergic LGIC, 5-HT_3, is located on axon terminals responsible for the release of GABA. Activation of the 5-HT_3 results in the release of GABA which causes neural inhibition; alcohol potentiates the function of the 5-HT_3 receptor (Lovinger, 1999) thus increasing neural inhibition which is implicated in intoxication. Several other studies have

associated genetic polymorphisms in serotonin receptors with the development of alcoholism (Gass & Olive, 2012). The neurotransmitter dopamine is linked only to GPCRs and is implicated in the reward and learning aspects of alcoholism (Lovinger, 2008). Acute alcohol intake increases the release of dopamine in the brain and the rewarding effect of dopamine reinforces the consumption of alcohol (Lovinger, 2008). Dopaminergic activity is suppressed as a result of chronic alcohol use; in order to compensate, the expression of some dopamine receptors are up-regulated; a process which may be responsible for alcohol seeking behaviors and the development of addiction (Gass & Olive, 2012). Alcohol also affects adenosine, which is considered to be a neuromodulator and acts via GPCRs (Lovinger, 2008). Acute alcohol intake increases adenosine driven cell signaling in neurons (Nagy, Diamond, Casso, Franklin, & Gordon, 1990). Chronic alcohol intake down-regulates adenosine receptors in brain areas which are crucial for reward and addiction (Lovinger, 2008). Opioid peptides are also considered to be neuromodulators and act on GPCRs; they are a contributing factor to the development of addiction in many drugs of abuse including alcohol. Similar to the effect of alcohol on other neurotransmitters, acute alcohol use increases the release of opioid peptides and chronic alcohol use can modify opioid receptor expression (Olive, Koenig, Nannini, & Hodge, 2001). Naltrexone, a drug which is used in the treatment of alcoholism, is an opioid antagonist (Gass & Olive, 2012).

From a gross anatomical perspective, chronic alcohol use can result in brain atrophy (Harper & Kril, 1985; Chandrasekar, 2013). Postmortem comparisons of alcoholic brains with control brains show significant brain atrophy in the alcoholic brain (Harper & Kril, 1985). Imaging studies have also shown significant loss of brain matter volume in alcoholics; particularly in those areas most affected by alcohol abuse such as the cerebral cortex, cerebellum,

and limbic system (Oscar-Berman & Marinković, 2007). The cause of alcohol related brain atrophy is the loss of neurons by excitotoxicity (Chandrasekar, 2013). Excitotoxicity occurs when neurons are hyperactivated, leading to excess influx of calcium which is toxic to the cell. The upregulation of some glutamate receptors during chronic alcohol use may contribute to the hyperactivation of neurons and eventual cell death (Chandrasekar, 2013).

Finally, at the molecular level, there is evidence that alcohol can directly influence some proteins in the brain. Because ethanol is small in molecular structure, many traditional models for determining a "binding site" are not feasible. In fact, many researchers suggest that alcohol does not follow the typical high affinity binding style that is seen with many other drugs of abuse, but instead may have multiple sites of action (Perkins et al., 2010). Using alternative alcohols that are larger in molecular size, some groups have been able to analyze the structure of alcohol interacting with various ion channels which are present in the brain (Howard et al., 2011). LUSH proteins are odorant-binding proteins in the fruit fly Drosophila that are secreted into the fluid that bathes olfactory neurons. LUSH proteins are responsible for the detection of alcohol in Drosophila (Bucci, Kruse, Thode, Alvarado, & Jones, 2006). The structure of alcohol bound to LUSH was resolved by x-ray crystallography; a specific binding site was identified that consists of 3 amino acid residues which are also found in some ion channels that are known to be affected by alcohol (Kruse, Zhao, Smith, & Jones, 2003). Many other alcohol binding or action sites have been proposed based on extensive mutagenesis studies, however, definitive evidence remains elusive due to the low affinity of physiologically relevant alcohols, which limits the success of structural analysis.

Alcohol and the Body

While some moderate alcohol intake has been linked to beneficial effects on human health, alcohol abuse can lead to an abundance of health consequences. Alcohol has been shown to have both positive and negative effects on cardiovascular health. Furthermore, alcohol use and abuse is implicated in diseases of the liver and pancreas, it has also been linked to the development of several types of cancer, and is known to suppress immune function. Many studies have demonstrated that moderate alcohol intake is beneficial to cardiovascular health. A recent meta-analysis of such studies found that moderate alcohol drinkers had reduced incidence of cardiovascular disease, coronary heart disease, and stroke (Ronksley, Brien, Turner, Mukamal, & Ghali, 2011). Chronic alcohol abuse can have the opposite effect on the cardiovascular system. Heavy alcohol use is associated with cardiomyopathies, arrhythmias, stroke, and hypertension (Piano & Schwertz, 1994). Chronic alcohol use is also implicated in the development of liver disease including fatty liver disease, hepatitis, fibrosis, cirrhosis, and is linked to increased risk of liver cancer (Lee & Kowdley, 2012). Alcohol use may also be a risk factor for pancreatitis and the subsequent risk for the development of pancreatic cancer (Tramacere et al., 2010). Alcohol is also directly linked to the development of cancers of the mouth, pharynx, larynx, esophagus, colon, and rectum; and has been recently correlated with breast cancer in women (Boffetta & Hashibe, 2006). Finally, acute and chronic use of alcohol can suppress the immune system via both the innate and adaptive immune response (Molina, Happel, Zhang, Kolls, & Nelson, 2010).

Cyclic AMP signaling

Cell signaling is a means for communicating information about the environment between cells and propagating a response within individual cells to address the needs of the organism.

Most cell signaling cascades involve an extracellular first messenger, usually a hormone or neurotransmitter, which binds to a receptor located within the cell membrane. Depending on the type of cell, binding of the first messenger (also known as ligand) to the receptor will initiate an intracellular signaling cascade. Receptors can be ion channels, where binding of the ligand opens the ion channel leading to the influx or efflux of certain ions which change the electrical potential of the cell. Receptors can also be linked to other proteins in the membrane where binding of the ligand initiates various intracellular events; many of the signaling cascades involve second messengers as well. Cyclic adenosine monophosphate (cAMP) is a common second messenger and is involved in a diverse array of cellular functions.

cAMP is involved in many biological processes including learning and memory, glycolysis, fatty acid metabolism, myocardial contraction, oncogenesis, cytokine production, gene expression, and cell growth and survival to name a few. cAMP signaling pathways consist of a GPCR linked to heterotrimeric G proteins (guanine nucleotide-binding regulatory proteins). Binding of a receptor ligand to the GPCR causes a conformational change in the receptor leading to interaction with the heterotrimeric G protein; this interaction causes the displacement of GDP by GTP. The GTP-bound G protein is in the active state which causes the dissociation of the protein into the subunits α and βγ; both of these subunits are implicated in activating effector proteins and thus modifying second messenger levels within the cell (Figure 1.1) (Strader, Fong, Tota, Underwood, & Dixon, 1994). The effector protein responsible for the production of cAMP is adenylyl cyclase (AC).

There are nine membrane-bound isoforms and one soluble isoform of mammalian AC. AC is found in many cell types in the body, various isoforms are expressed differentially among tissue type and organ systems (Sunahara & Taussig, 2002). AC isoforms are classified based on

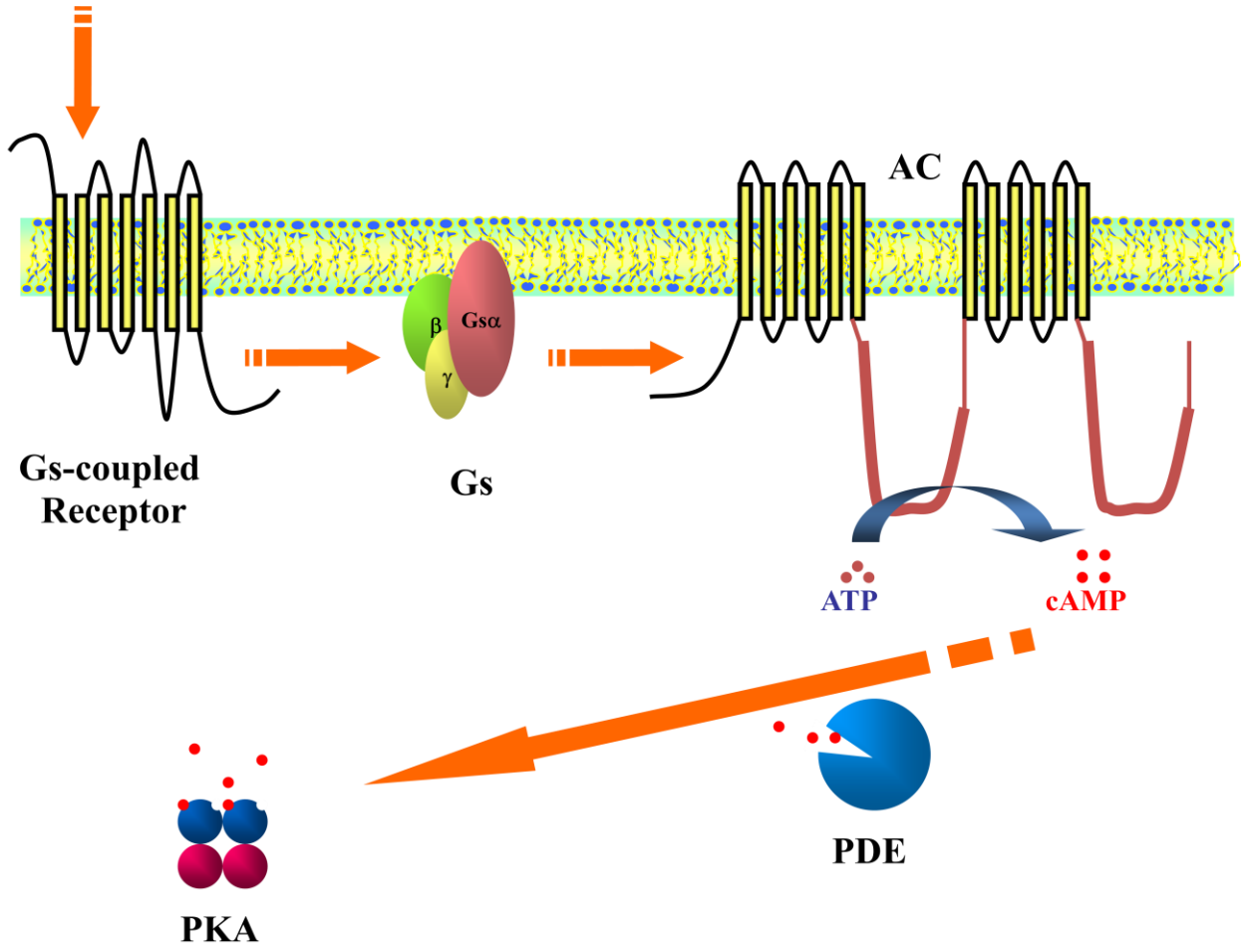

Figure 1.1. Schematic representation of cAMP signaling cascade. The binding of an extracellular ligand to the GPCR activates the G protein which activates AC. AC converts ATP to cAMP. cAMP has many effectors including protein kinase A (PKA). Excess cAMP is degraded by phosphodiesterase (PDE).

the variation in regulatory mechanisms; primary regulators of AC activity include G proteins, calcium, calmodulin, protein kinase C (PKC), and small molecules such as forskolin and magnesium (Sunahara & Taussig, 2002; Cooper, 2003). The structure for membrane-bound AC consists of a cytoplasmic N-terminal domain (N), followed by a membrane-spanning domain (M_1), a cytoplasmic domain (C_{1a} and C_{1b}), a second membrane-spanning domain (M_2), and a second cytoplasmic domain (C_{2a} and C_{2b}) (Tesmer & Sprang, 1998). Adenylyl cyclase catalyzes the reaction that converts ATP into cAMP. The C_{1a} and C_{2a} domain are homologous and

constitute the catalytic core (Tesmer & Sprang, 1998). The cytoplasmic domains are also where key regulators of AC interact with the protein, including G protein subunits as well as small molecule activators of AC such as magnesium and forskolin (Sunahara & Taussig, 2002). The G protein subunits Gα and Gβγ have multiple isoforms which can stimulate or inhibit AC depending on the characteristics of the AC isoform. $G\alpha_s$ always stimulates AC activity, $G\alpha_i$ is inhibitory to susceptible isoforms, $G\alpha_o$ is typically inhibitory to affected AC isoforms, and recent research implicates $G\alpha_{12/13}$ as stimulatory to some AC isoforms, especially Type 7 AC isoform (AC7) (Sunahara & Taussig, 2002; Jiang, Collins, Davis, Fraser, & Sternweis, 2008). The subunit Gβγ can either stimulate or inhibit AC activity depending on the AC isoform; furthermore, the effect of Gβγ often requires concurrent activation by Gα (Sunahara & Taussig, 2002). G protein subunits bind to the catalytic complex along the perimeter of the C_1/C_2 interface; binding sites for the ATP substrate along with AC regulators such as magnesium and forskolin are located within the catalytic cleft (Sunahara & Taussig, 2002).

Effect of Alcohol on cAMP Signaling

The cAMP signaling pathway has been implicated in the development of and predisposition to alcoholism. Abnormalities in cAMP signaling and changes in the activity of adenylyl cyclase have been observed in clinical populations of alcoholics. Furthermore, experimental models have been designed that implicate the cAMP signaling system in the response to alcohol and particularly establish adenylyl cyclase as a major contributor to the alcohol response.

Clinical Evidence of Alcohol Effect on Adenylyl Cyclase

Early clinical research established a link between cAMP signaling and alcoholism. Diamond and colleagues found that basal and stimulated levels of cAMP were lower in the

lymphocytes of alcoholics (Diamond, Wrubel, Estrin, & Gordon, 1987). Lymphocytes were extracted from blood samples from alcoholic patients and age matched controls. Mixed lymphocyte preparation and isolated T cells were assayed for cAMP accumulation alone or in the presence of an adenosine receptor agonist or 80mM ethanol. The results indicated that basal, receptor-stimulated, and ethanol-stimulated cAMP levels were reduced significantly in both the mixed lymphocytes and isolated T cells from alcoholic patients (Diamond et al., 1987). Further research demonstrated a reduction of ethanol-stimulated cAMP in cultured lymphocytes of alcoholic patients. Again, lymphocytes were extracted from blood samples from alcoholics and controls; lymphocytes were propagated using cell culture for at least 4 generations of growth in medium not containing alcohol. Despite the multiple rounds of cell division in the absence of alcohol, ethanol-stimulated cAMP levels were still suppressed in the lymphocytes of alcoholics when compared to controls (Nagy, Diamond, & Gordon, 1988). Platelets from abstaining alcoholics and controls with family history positive or negative for alcoholism were analyzed for AC activity. Controls which were complete abstainers from alcohol but with a family history positive for alcoholism had lower basal and stimulated AC activity levels than complete abstainers with no family history of alcoholism, suggesting AC activity as a possible trait marker for genetic predisposition for the development of alcoholism (Menninger, Baron, & Tabakoff, 1998). Patients with a history of alcoholism had reduced stimulated AC activity in the platelets when compared to individuals with no history of alcoholism; these results were taken after the patient had abstained from alcohol for at least 4 days (Menninger et al., 2000), suggesting a biochemical marker for the diagnosis of alcohol dependence.

Experimental Evidence of Alcohol Effect on Adenylyl Cyclase

Alcohol has been shown to modulate cAMP signaling in experimental animal models. Several alcohol-related parameters have been associated with modulated cAMP signaling. Initial sensitivity to ethanol, as measured by test of balance on a dowel, was shown to correlate with cAMP accumulation in the cerebellum and basal ganglia of mice (Kirstein & Tabakoff, 2001). Several studies have also been carried out which focus on the AC component of cAMP signaling in the alcohol response. Double knock-out (DKO) mice lacking both AC1 and AC8 had increased sensitivity to alcohol-induced sedation, further, DKO mice and AC8 KO mice exhibit decreased voluntary ethanol consumption compared to wild type mice (Maas, Jr. et al., 2005). AC5 KO mice have also been examined for alcohol response. AC5 KO mice show higher preference for ethanol as well as increased total consumption of ethanol compared to wild type mice (Kim, Kim, Baek, Lee, & Han, 2011). Additionally, AC5 KO mice had reduced sensitivity to ethanol under the measures of ethanol-induced hypothermia and sedation by high-dose ethanol (Kim et al., 2011). AC5 has also been implicated in the anxiolytic effects of alcohol and is highly expressed in the nucleus accumbens, a component of the reward center in the brain. Decreased AC5 mRNA expression was found in the nucleus accumbens of rats as early as 1 hour following ethanol treatment (Morales-Mulia et al., 2012). Another study found that overexpression of AC7 in mice increased basal and ethanol-induced DARPP-32, a downstream element of the cAMP pathway implicated in motivation for ethanol intake (Donohue, Hoffman, & Tabakoff, 2005).

From a basic science viewpoint, our laboratory has focused on the interaction of adenylyl cyclase with alcohol. Early research found an isoform specificity of the alcohol effect (Yoshimura & Tabakoff, 1995). The following study found that receptor-stimulated cAMP

accumulation to be increased over 50% in response to treatment with 50mM ethanol in cells overexpressing AC7 (Yoshimura & Tabakoff, 1999). Subsequent analysis identified the ethanol responsive domains of AC, i.e. the coding regions required for an ethanol effect to occur (Yoshimura, Pearson, Kadota, & Gonzalez, 2006). Full length chimeric mutants were designed from the sequences of AC2, AC3, and AC7. This study showed that regions of the cytoplasmic domains, C_{1a} and C_2, were the only elements required for the alcohol effect; neither the membrane-spanning regions nor the N-terminal tail were necessary (Yoshimura et al., 2006). The work on isoform-specificity was extended by demonstrating that different AC isoforms have different responses to a series of *n*-alkanols (Kou & Yoshimura, 2007). Alcohol cut-off point on the function of a protein is defined as the length of the carbon chain of an *n*-alkanol beyond which increasing the length of the carbon chain does not further increase alcohol effect on the protein. AC6, AC7, and AC9 were found to have different alcohol cut-off points, which are proposed to be the result of specific alcohol binding pockets on the AC protein (Kou & Yoshimura, 2007). Additional studies showed that these isoforms also responded differently to the number and position of hydroxyl groups in a straight chain alcohol (Hasanuzzaman & Yoshimura, 2010). Using a FRET based cAMP sensor, cAMP was monitored in real-time and the results confirmed biochemical assays showing increased cAMP in the presence of alcohol, that the alcohol effect is isoform specific, and presented detailed time course of intracellular cAMP change (Gupta, Qualls-Creekmore, & Yoshimura, 2013).

Because this research was executed in mammalian cells, the role of AC is not definitive; endogenous ACs or other proteins may also contribute to the observations described. Therefore, a recombinant AC protein was designed to contain the regions of AC previously identified as ethanol responsive domains. This recombinant protein, AC7sol, was expressed in *E. coli*

containing no endogenous AC activity (Dokphrom, Qualls-Creekmore, & Yoshimura, 2011). This study found that the C_{1a} and C_2 domains of AC7 were sufficient to obtain the enhancing effect of alcohol and demonstrated that no other mammalian proteins are required for the alcohol enhancing effect on AC. The alcohol cutoff point for AC7sol was determined to be pentanol, which is in agreement with the alcohol cutoff of the native AC7 expressed in mammalian cell culture (Kou & Yoshimura, 2007; Dokphrom et al., 2011). Additionally, kinetic analysis of AC7sol found that ethanol increases both the K_m and V_{max} values associated with the enzymatic activity of AC7sol indicating that substrate turnover number and rate are increased in the presence of ethanol (Dokphrom et al., 2011).

Statement of the Problem and Specific Aims

In light of this most recent research, we have determined that the bacterial expression of recombinant AC is a very good model for investigating the specific interactions between alcohol and AC. It is necessary to affirm that no other proteins are contributing to the alcohol effect on AC; this requires obtaining purified protein for further analysis. Because native adenylyl cyclase is a membrane-bound protein, it is not feasible to express and purify the protein in full length form. Previous research in our lab has narrowed down the interaction of alcohol and AC to the cytoplasmic domains. Therefore, recombinant DNA design for the expression of soluble constructs of the AC cytoplasmic domains is sufficient for the study of alcohol's effect on AC.

Although the structures have been resolved for some segments of AC isoforms, the structure of AC7 has not been resolved. Based on the resolved AC structures, the binding sites for catalytic substrates including ATP and magnesium, along with the binding sites for AC regulators such as G proteins and forskolin, are already established. This knowledge permits us to interpret the mechanism of alcohol on AC through manipulation of the AC substrates and

regulators and subsequent kinetic analysis. We can also manipulate the sequence of AC and combination of AC regions in order to further investigate the alcohol effect. Identifying the specific AC regions required for the alcohol effect can help reveal a mechanism for how alcohol affects protein function and potentially open the door for future drug development. We hypothesize that alcohol directly interacts with the adenylyl cyclase protein, leading to the observed alteration in cAMP accumulation. Our objective is to determine the mechanism by which alcohol exerts its effect on adenylyl cyclase. We have addressed these research problems with the following aims:

Aim 1: Design, express, and purify recombinant AC proteins for AC2, AC7, and AC9.

Aim 2: Characterize AC activity and isoform specificity for the effect of alcohol on AC.

Aim 3: Use pharmacological analysis to identify potential regulatory mechanism for alcohol on AC.

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CHAPTER 2.
DESIGN AND OPTIMIZATION OF CONDITIONS FOR THE EXPRESSION AND PURIFICATION OF RECOMBINANT MAMMALIAN ADENYLYL CYCLASE PROTEINS

Introduction

One of the most significant challenges in protein research is the availability of sufficient quantities of the protein under investigation. The development of recombinant DNA technology has broadened the possibilities within this field of research. Researchers now have the capability to engineer DNA for encoding the RNA sequence of partial or complete proteins in the laboratory. Various expression and purification systems can then be used to express and extract proteins for further use and analysis.

Methodologies for expressing recombinant protein include expression in mammalian cells, insect cells, yeast, bacterial cells, and even cell-free protein synthesis. Each method has benefits and limitations; choice of expression system will depend upon the needs of the specific protein to be expressed (post-translational modifications), desired protein yield, and cost/time limitations. In the current study, we chose the bacterial expression system for its ability to yield large amounts of protein, relatively fast production rate, and because our protein, adenylyl cyclase, does not require any post-translational modification for the purpose of our research. Furthermore, numerous strains of *Escherichia coli* (*E. coli*) have been engineered carrying plasmids with features specifically designed to control the process of protein expression and improve the final protein product.

Protein purification methods include the extraction of proteins from E. coli, separation of soluble and insoluble cell matter, followed by the separation of proteins based on a selected feature using chromatography. Several methods of chromatography are available which separate proteins by characteristics such as size, hydrophobicity, or ionic charge. In affinity

chromatography, a column is loaded with beads engineered to bind a specific compound. The DNA plasmid encoding the target protein can also be designed to include a tag for the purpose of purification. In immobilized metal affinity chromatography (IMAC), a sequence of multiple histidine residues is included at the N-terminal or C-terminal of the target protein sequence. Histidine binds to metal ions including cobalt and nickel, which can be attached to column beads for binding the histidine tag. For elution of proteins, the columns are flushed with a solution that competes with the target protein for binding to the column; in the example of IMAC, imidazole, a chemical similar in structure to histidine, is added in increasing concentration to displace the bound histidine residues.

Optimization of protein expression and purification can occur at each stage of the process. Selection of the plasmid for carrying the target protein coding sequence is the first crucial step. Many plasmid features, including plasmid copy number and choice of RNA polymerase, can be manipulated to maximize protein expression. Variables of cell culture can be modified such as *E. coli* strain, culture medium, incubation time, incubation temperature, incubator rotation speed, and concentration of induction reagent. Cell lysis is the first step in protein purification and can be performed by physical means such as homogenization, sonication, or freeze-thaw cycling, or by chemical means such as a detergent-based solution. Again, determining the method for lysis depends on the specific needs of the protein; some methods may be too harsh and damage the functionality of the protein. Many of the variables associated with protein purification involve maintaining the integrity of the protein, these include appropriate buffer selection, maintenance of an appropriate and consistent temperature throughout purification protocols, and the addition of protease inhibitors, chelators, reducing agents such as beta-mercaptoethanol and dithiothreitol (DTT), or glycerol to working buffers to

prevent protein degradation and improper protein folding. Method of chromatography is also important for recovering maximum protein yield. Chromatography selection or the use of sequential columns is important for protein yield and protein purity. For some proteins, affinity chromatography may be sufficient. Other proteins may require a second or third chromatography in addition to the affinity column, such as an ion exchange column or gel filtration column for achieving maximum protein purity.

The adenylyl cyclase protein is a functional enzyme, and thus must not only maintain structural integrity, but functional integrity as well. This remains the most significant challenge in the production of purified AC. Because AC is a membrane-bound protein, it is difficult to express and purify the full sequence of the protein and must be done using insect or yeast cells. Therefore, research has focused on the cytoplasmic domains of AC, especially the regions responsible for catalytic activity. Still, many attempts by researchers to express and purify the cytoplasmic (C_1 and C_2) regions of AC have resulted in low levels of expression and insolubility. From the nine membrane-bound AC isoforms, successful expression and purification has been reported for AC1 C_1, AC2 C_2, AC5 C_1, AC5 C_2, AC7 C_1, AC7 C_2, AC9 C_1, and AC9 C_2 (Tang & Gilman, 1991; Dessauer & Gilman, 1996; Yan, Huang, Rao, Hurley, & Tang, 1997; Zhang, Liu, Ruoho, & Hurley, 1997; Tesmer, Sunahara, Gilman, & Sprang, 1997; Tesmer, Sunahara, Fancy, Gilman, & Sprang, 2002; Scholich, Barbier, Mullenix, & Patel, 1997; Yan & Tang, 2002; Yan, Huang, Andrews, & Tang, 1998).

In order to study the effects of alcohol on AC, our lab has generated recombinant protein constructs for multiple AC isoforms. Research suggests that the cyclic AMP (cAMP) pathway is implicated in the development of and/or predisposition to alcoholism. Previously, our laboratory has demonstrated that alcohol enhances the activity of AC, that the alcohol effect is isoform

specific, and that the isoform AC7 is most enhanced by alcohol. We hypothesize that alcohol enhances AC activity by directly interacting with the protein. Testing our hypothesis will be most definitive with the use of purified AC protein. In order to achieve purified protein, we designed recombinant DNA plasmids encoding protein sequences for C_{1a} and C_2 regions of AC joined by an amino acid linker; AC2sol and AC7sol. We also designed recombinant DNA plasmids for expressing the C_{1a} and C_2 regions separately for AC2, AC7, and AC9. Here we present the results of optimization conditioning for both the expression and purification stages of recombinant AC protein production.

Materials and Methods

Materials

Restriction enzymes were from New England Biolabs (Ipswich, MA). Phusion Hot Start DNA polymerase and Quick Ligation Kit were from New England Biolabs (Ipswich, MA). Bacto Tryptone and Bacto Yeast were from BD (Franklin Lakes, NJ). NiNTA superflow nickel chromatography cartridges were from Qiagen (Germantown, MD). HisPur Cobalt chromatography cartridges were from Pierce Biotechnology (Rockford, IL). HiTrap SP HP and HiTrap Q HP ion exchange chromatography cartridges were from GE Healthcare (Piscataway, NJ). BCA protein assay kit was from Pierce Biotechnology (Rockford, IL). His-Tag monoclonal antibody was from Novagen by EMD Millipore (Darmstadt, Germany). Anti-mouse secondary antibody conjugated horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). ECL western blotting detection reagents were from GE Healthcare (Piscataway, NJ). Calbiochem Protease Inhibitor Cocktail II was from EMD Millipore (Darmstadt, Germany). [^{3}H] cAMP was from Moravek Biochemicals (Brea, CA). [α-

^{32}P] ATP was from Perkin Elmer (Boston, MA). All other chemicals and reagents used were from Sigma-Aldrich (St. Louis, MO).

Construction of Recombinant AC

Recombinant proteins were designed to include the ethanol responsive domains already identified in the native AC7 protein (Yoshimura, Pearson, Kadota, & Gonzalez, 2006). AC2 and AC9 recombinants were designed to include the regions corresponding in sequence homology to the ethanol responsive domains of AC7 (Figure 2.1.).

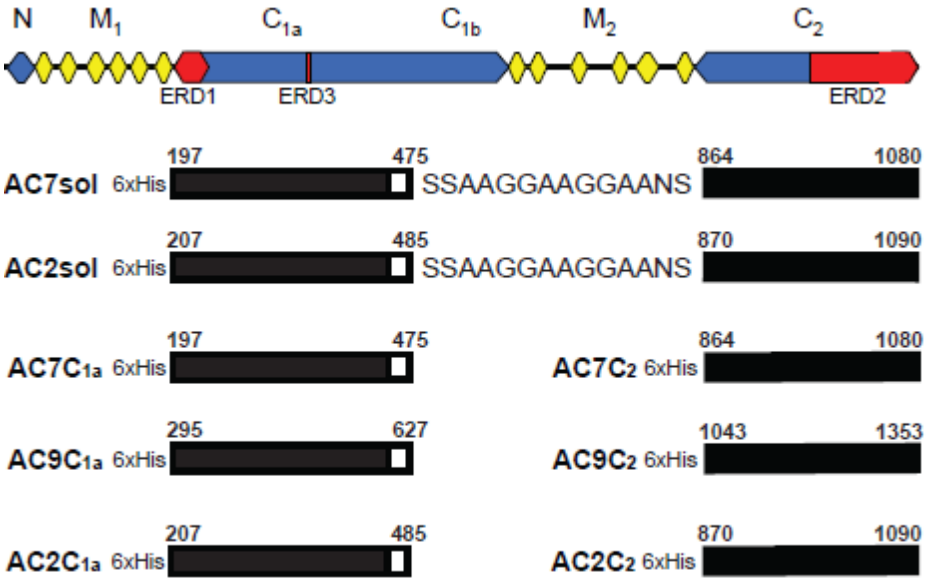

Figure 2.1. Schematic representation of recombinant AC constructs. The top figure represents the structure of the native form of AC7; ethanol responsive domains (ERD) have been indicated in red. The numbers indicate the positions of the residues in the respective native isoform. 6xHis indicates a hexahistidine tag.

AC2sol and AC7sol included both C_{1a} and C_2 regions linked by a 14-amino acid linker; C_{1a} and C_2 were also expressed separately for isoforms AC2, AC7, and AC9. The N-terminal tail, transmembrane-spanning domains, and C1b regions were excluded from the recombinant proteins. All recombinant proteins included a hexahistidine tag for purification purposes. DNA

fragments were amplified by polymerase chain reaction (PCR) from their respective template DNA (Table 2.1.), DNA polymerase, and two oligonucleotide primers (Table 2.2.).

Table 2.1. Gen Bank identification numbers for template DNA used in the design and construction of recombinant AC proteins.

Template DNA	Gen Bank Identification
Rat AC2	M80550
Human AC7	D25538
Mouse AC9	MN009624

Table 2.2. Oligonucleotide primers used for the construction of recombinant AC proteins.

Plasmid	Primer
AC2sol – C_{1a}	Forward: 5’ – ATCGGATCCACAAGCACCTCATGGAGCT Reverse: 5’ – GAACTCGAGGCTCCGTCCAGAGTGTGTC
AC2sol – C_2	Forward: 5’ – CACGAATTCTAGGAGCCTGAAAAATGAGG Reverse: 5’ – CAGAAGCTTCAGGATGCCAAGTTGCTCT
AC2sol linker	Forward: 5’ – TCGAGTGCAGCAGGAGGAGCAGCAGGAGGAGCAGCG Reverse: 5’ – AATTCGCTGCTCCTCCTGCTGCTCCTCCTGCTGCAC
AC7sol – C_{1a}	Forward: 5’ – ATCGGATCCACAAGCACCAAATGCAGGA Reverse: 5’ – GAACTCGAGGCCGCGTCCCCCTTGGGCC
AC7sol – C_2	Forward: 5’ – ATCGGATCCGACAAGTTAAACGAGGACTG Reverse: 5’ – CAGAAGCTTCAGTTCAGCCCCAGCCCCTGAAA
AC7sol linker	Forward: 5’ – TCGAGTGCAGCAGGAGGAGCAGCAGGAGGAGCAGCG Reverse: 5’ – AATTCGCTGCTCCTCCTGCTGCTCCTCCTGCTGCAC
AC2 C_{1a}	Forward: 5’ – ATCGGATCCACAAGCACCTCATGGAGCT Reverse: 5’ – GAAAAGCTTCAGGCTCCGTCCAGAGTGTGTC
AC2 C_2	Forward: 5’ – CCAGGATCCTCAGGAGCCTGAAAAATGAGGAGC Reverse: 5’ –CAGAAGCTTCAGGATGCCAAGTTGCTCT
AC7 C_{1a}	Forward: 5’–ATCGGATCCACAAGCACCAAATGCAGGA Reverse: 5’ –GAAAAGCTTCAGGCCGCGTCCCCCTTGGGCC
AC7 C_2	Forward: 5’ – ATCGGATCCGACAAGTTAAACGAGGACTG Reverse: 5’ – CAGAAGCTTCAGTTCAGCCCCAGCCCCTGAAA
AC9 C_{1a}	Forward: 5’ – ATGGGATCCATCTGTTTGTCATG Reverse: 5’ – CAGAAGCTTCAAAGGTTATCAAAGGTTTTGA
AC9 C_2	Forward: 5’ – ATCGGATCCAGCTCAAGGTCTCTCAGAC Reverse: 5’– CAGAAGCTTCACACACTCTTTGAGACAT

For separate C_{1a} and C_2 constructs, the resulting PCR product was digested with BamHI and HindIII and ligated into pQE82L or pET30c also digested by Bam HI and HindIII. For AC2sol and AC7sol, the PCR product for the C_{1a} domain was digested with BamHI and XhoI and ligated into pQE82L digested with the same enzymes. The PCR product for the C_2 domain was digested with EcoRI and HindIII and ligated into pQE82L containing the C_{1a} coding sequence digested with the same enzymes. The 14-amino acid linker was inserted into the plasmid containing the C_{1a} and C_2 coding sequences digested by XhoI and EcoRI. Resulting plasmids included pET30c AC7C_{1a}, pQE82L AC2sol, pQE82L AC7sol, pQE82L AC7 C_{1a}, pQE82L AC7 C_2, pQE82L AC2 C_{1a}, pQE82L AC2 C_2, pQE82L AC9 C_{1a}, and pQE82L AC9 C_2 (Fig. 2.2.). The coding sequence of each plasmid was confirmed by DNA sequencing analysis.

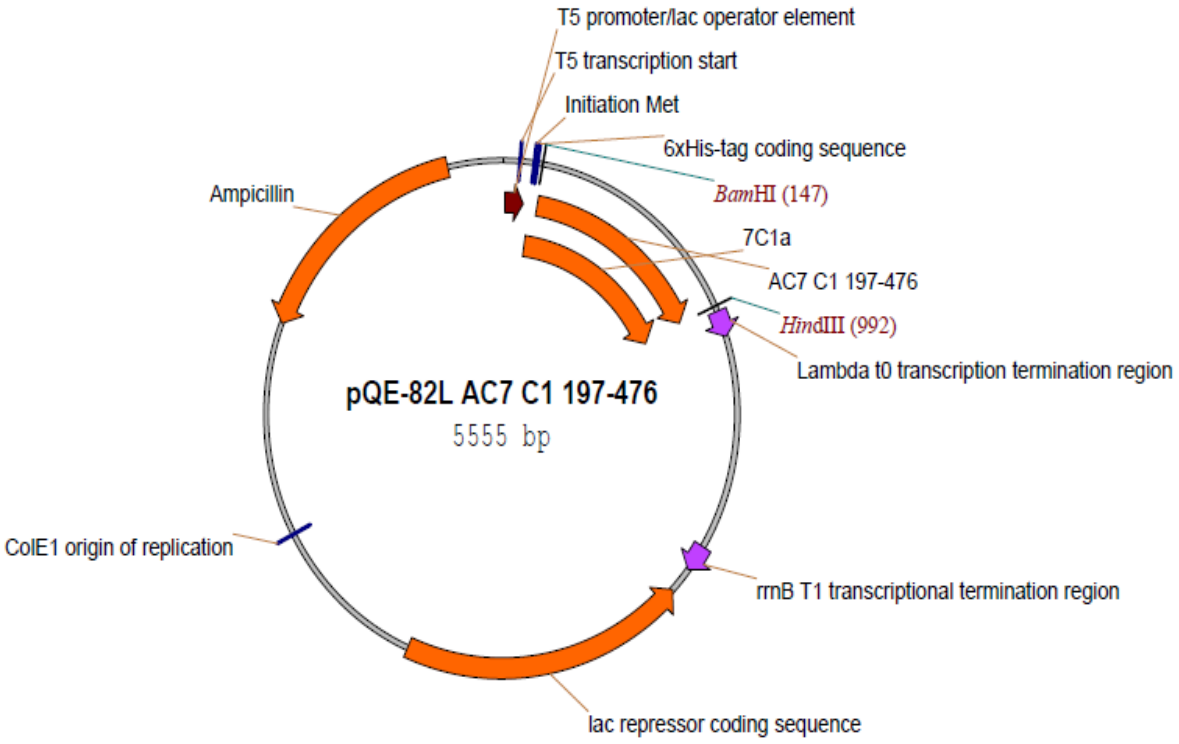

Figure 2.2. Diagram representing the design and features of the plasmid with vector pQE82L and AC7 C_{1a} insert.

Expression of Recombinant AC

Plasmids were transformed in an *E. coli* expression system using cell lines TP2000 carrying plasmid pUSB520, Rosetta Gami B, BL21 (DE3) carrying plasmid pUSB520, or C41, and grown overnight on LB plates treated with appropriate antibiotics. The next day, colonies were harvested and inoculated in LB, T7, or TB medium with antibiotics required for the plasmid and/or cell line and grown at various temperatures and rotational speeds until the A600 reached 2. This seed culture was then inoculated in 4L medium plus antibiotics and continued to grow at various temperatures and speeds. Protein expression was induced at various A600 values with variable concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG). Following IPTG induction, the culture was grown for multiple durations at various temperatures and speeds. The culture was then harvested by centrifugation and cell pellets were stored at -80°C.

Purification of Recombinant AC

The purification procedure was carried out either on ice or at 4°C. Cells were suspended in cold lysis buffer solution (20mM Tris pH 7.4, 5mM βmercaptoethanol, 100μM phenylmethylsulfonyl fluoride (PMSF), 22μg/ml N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 22μg/ml N-$_{\alpha}$-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), trypsin inhibitor, and Sigma Protease Inhibitor Cocktail 1 tablet/100ml (4-(2-Aminoethyl) benzenesu lfonyl fluoride hydrochloride (AEBSF), Bestatin hydrochloride, Leupeptin, E-64, Aprotinin, Pepstatin A, and Phosphoramidon disodium salt) and lysed by sonification in the presence of lysozyme (1mg/ml) on ice. Soluble proteins were separated by ultracentrifugation at 45,000rpm and loaded onto an immobilized metal affinity column (IMAC), washed with a Tris pH 7.4 buffer containing 500mM NaCl, followed by the same buffer containing 100mM NaCl and

10mM Imidazole until A280 decreased to a plateau. Proteins were eluted into 20 fractions using an imidazole gradient 10mM-500mM. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fractions containing the target protein were pooled for subsequent purification by ion exchange chromatography. The pooled fractions were diluted by 5 volumes in loading buffer (20mM Tris pH 7.4, 1mM DTT, 500μM ethylenediaminetetraacetic acid (EDTA), Calbiochem Protease Inhibitor Cocktail, and 100μM PMSF) and loaded to the HiTrap Q-HP or HiTrap SP-HP 5ml column depending on the charge of the target protein. The column was washed with the loading buffer and proteins were eluted into 50 fractions using a NaCl gradient 0-1M. Eluted fractions were again analyzed by SDS-PAGE and fractions containing the target protein were pooled. Pooled fractions were concentrated using Amicon Ultra 10K filters under centrifugation at 4°C, 4000rpm and stored at -80°C.

Protein Analysis

Purified proteins were separated by SDS-PAGE and stained with Bio-safe coomassie stain (BioRad). SDS-PAGE results were also transferred to nitrocellulose membranes for western blot analysis. Western-blotting used Anti-His monoclonal antibody to identify our proteins containing a 6 histidine tag at the N-terminus of the protein construct. Briefly, membranes were blocked with 1% milk in tris-buffered saline (20mM Tris HCl pH 7.5, 150mM NaCl) with 0.2% tween 20 (TBST) for 1hr at room temperature. Following washing, membranes were incubated with Anti-His antibody 1:1000 dilution overnight at 4°C. Membranes were washed again and incubated with anti-mouse secondary antibody conjugated horseradish peroxidase, 1:5000 dilution. Blots were developed using ECL chemiluminescent

immunodetection. Finally, protein quantity was estimated using the BCA protein assay kit or based on their molecular extinction coefficient and A280.

Enzymatic Activity Assay

Our AC activity assay protocol uses [α-^{32}P] ATP as a substrate. The total reaction contains 10-15μg of total purified protein (ACsol or one C_{1a} protein and one C_2 protein combined), 250μM ATP, 25mM Tris-HCl pH 7.4, 100μM 3-isobutyl-1-methylxanthine (IBMX), 10mM phosphocreatine, 5 U creatine phosphokinase, and [α-^{32}P] ATP (50-80 cpm/pmol). AC activity is stimulated with $MnCl_2$. Reaction mixtures are prepared on ice and the assay is initiated by temperature-shift to 30°C for 15min. The reaction is terminated by the addition of a solution containing 2% SDS, 1mM ATP, and 1mM cAMP. Sequential column chromatography is used to separate [α-^{32}P] ATP and [α-^{32}P] cAMP by dowex 50 and neutral alumina columns. ^{3}H cAMP was added to estimate recovery of cAMP. A liquid scintillation counter is used to detect radioactivity in the final sample (Salomon, Londos, & Rodbell, 1974). All assays are carried out in triplicate and repeated for analysis of accuracy.

Results

AC2Sol & AC7sol

AC2sol was expressed under manipulations of multiple conditions including cell line, culture medium, growth temperature, cell density at induction (A600), and IPTG induction concentration. Cell lines included TP2000 pUSB520 and Rosetta Gami B; neither cell line produced a detectable improvement in protein expression or AC activity. Culture mediums included T7 and TB; while T7 is standard use, TB includes more nutrients and is considered a richer growth medium. However, change in culture medium did not improve protein expression. We tested two time and temperature conditions following the induction of protein expression

with IPTG; 6hr at 30°C and 19hr at 25°C. No detectable difference in expression was found between conditions. Finally, we varied conditions of protein induction including cell culture density (A600) at time of induction ranging from A600 value of 0.4 – 2 and IPTG concentration ranging from 100μM-1mM IPTG. Again, no significant improvements resulted from any of these induction conditions. Low protein expression and very low enzymatic activity did not encourage the purification of AC2sol and therefore further analysis of AC2sol was abandoned.

Similar optimization of AC7sol was performed and optimal conditions were found to be the following: transformation in TP2000 pUSB520 cell line induction with 100μM IPTG when cell culture growth reached A600 value of 1 and continued growth of culture for 19hr at 25°C with shaking at 250rpm. AC7sol expression and enzymatic activity was higher than AC2sol, therefore, we continued to protein purification. AC7sol was purified using a Ni-NTA nickel affinity column eluted with imidazole, followed by an ion exchange column HiTrap SP-HP eluted with NaCl. Initial purification of AC7sol did not result in the recovery of a large protein yield. Therefore, in the subsequent purification, samples were collected from the pass through for each stage of purification including the unbound lysate sample and washes and analyzed by SDS-PAGE (Figure 2.3.) and enzymatic assay for AC activity (Table 2.3.).

Table 2.3. Estimated recovery of AC7sol during purification steps as measured by AC enzymatic activity assay.

Purification Step	Quantity (mg)	Total Activity (pmol/min)	% recovery
Lysate	1012.7	85557	100
Ni-NTA Unbound	962.2	77822	91
Wash I	57.6	10380	12.1
Wash II	10.8	395	0.46
Ni-NTA Elution	0.7	33.50	0.04

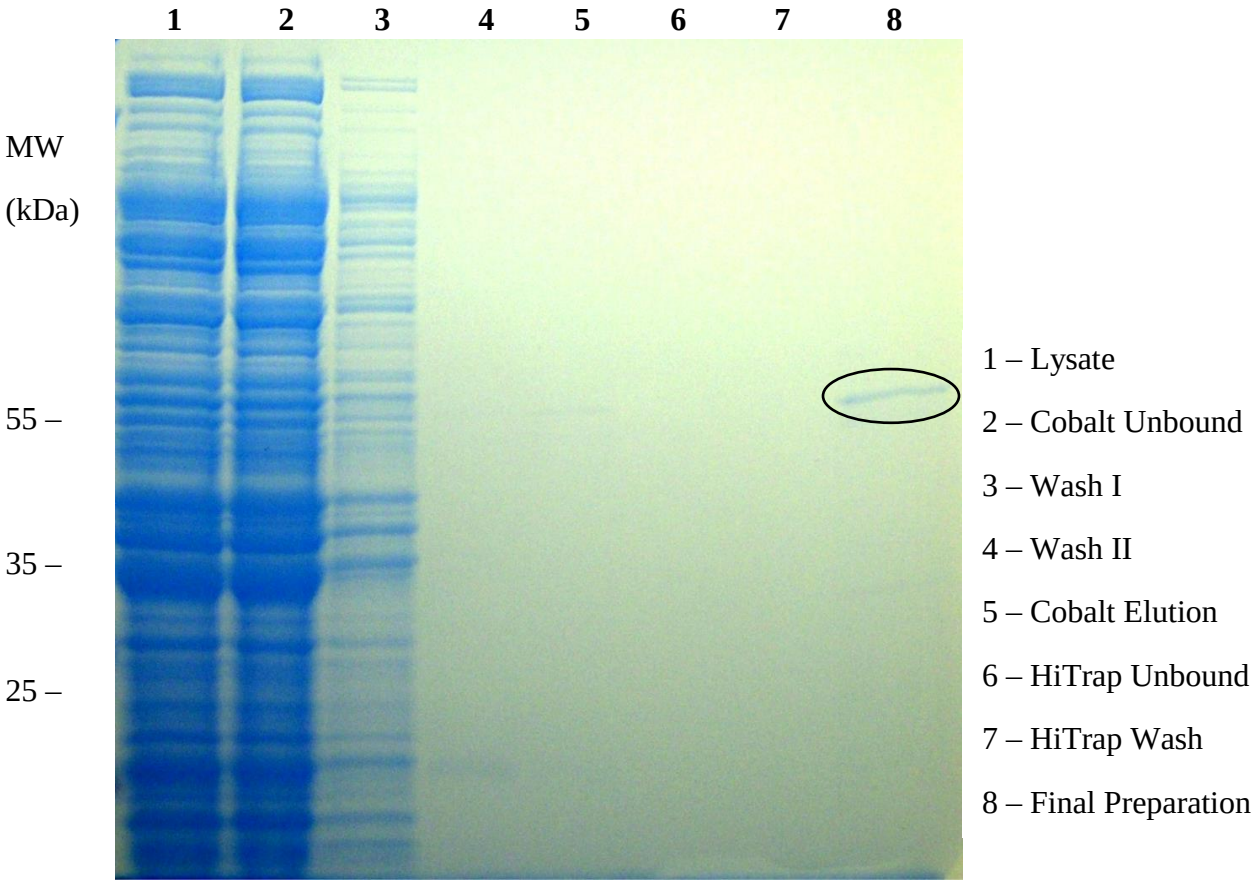

Figure 2.3. SDS-PAGE analysis of each stage in the purification process of AC7sol (60.9 kDa molecular weight).

This data revealed that much of the active AC7sol was lost during the purification steps and not recovered in the final sample. Attempting to improve the recovery of AC7sol during purification, we replaced the nickel affinity column with a cobalt affinity column. Cobalt affinity chromatography did not result in greater recovery in the case of AC7sol. Based on the difficulty encountered in the expression and purification of AC2sol and AC7sol and because the low level of enzymatic activity was not sufficient for study of the alcohol effect on AC, we decided to express and purify the C_{1a} and C_2 regions separately.

AC7 C_{1a} & AC7 C_2

Optimization of protein expression was first carried out for AC7 C_{1a}. One theory in the field of protein production is that "low and slow", using low temperatures, and slow shaking speeds, and less rich culture medium such as LB, results in slower cell replication, and will yield the highest quality protein expression. This theory is based upon the notion that slower growth will allow the bacterial machinery time to properly translate and fold the protein. Our results did not find protein expression to be greater, however, we did notice a slight increase in the resulting enzymatic activity of the protein; suggesting that the protein may be more likely to fold correctly under the "low and slow" conditions maintaining a functional enzyme. Optimal conditions for AC7 C_{1a} expression were transformation in BL21 (DE3) competent cells, culture growth in T7 medium with induction at A600 = 0.6 with 100μM IPTG and continued growing at 25°C, 100rpm, for 19hr. AC7 C_2 was easy to express, conditions identified in the optimization of AC7 C_{1a} were used for AC7 C_2 as well.

Optimizing purification first required determining the best choice of column for chromatography. We first determined that the cobalt affinity column recovered more of the target protein than the nickel affinity column. Cobalt affinity resins were available in prepacked columns from several companies; we chose to compare columns from three companies including Pierce, Clontech, and Novagen. Initial expression and purification procedures had determined that AC7 C_2 consistently demonstrated high protein expression and high protein recovery during purification. Therefore, we used AC7 C_2 for comparison of cobalt column quality. Surprisingly, columns from different manufacturers did affect both the recovery of protein as well as the elution profile for AC7 C_2. Based on the SDS-PAGE analysis of chromatography fractions from

each column (Figure 2.4.) we selected the HisPur cobalt affinity column by Pierce which was used for purification of all recombinant constructs thereafter.

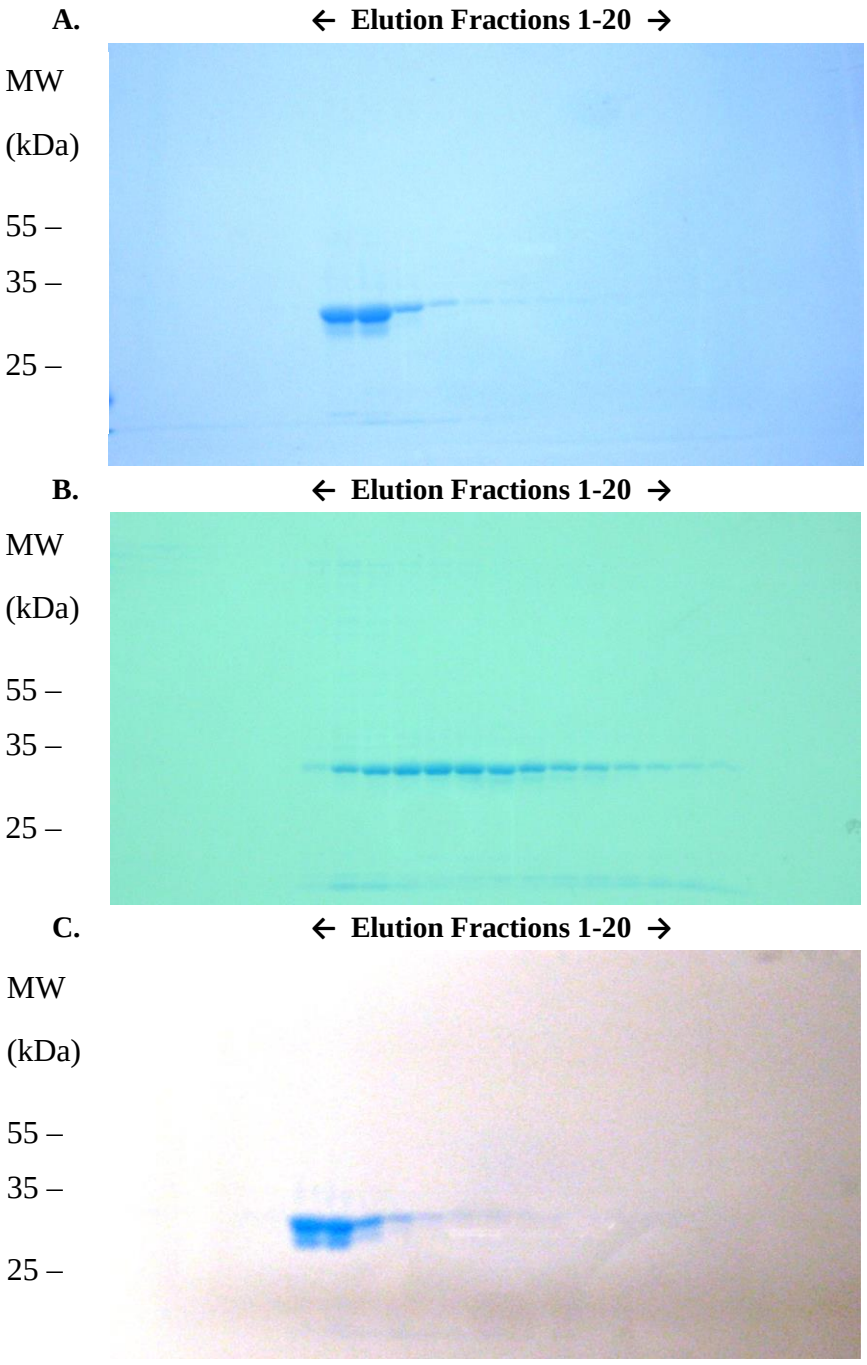

Figure 2.4. Comparison of affinity chromatography cobalt columns Pierce (A), Novagen (B), and Clontech (C) by purification of AC7 C_2 (25.6 kDa molecular weight), elution in 20 fractions.

Once we determined the most efficient chromatography column, we then focused on the quality of purified protein for each construct. AC7 C_2 eluted in a narrow peak with minor contamination by other proteins, however, we did observe some extent of protein precipitation. The addition of glycerol to the elution buffer was able to reduce protein precipitation of AC7 C_2. The elution profile for AC7 C_{1a} showed a wider peak and more contamination by other proteins. We assumed that the lower expression of AC7 C_{1a} could be contributing to the contamination because the lower quantity of AC7 C_{1a} binding to the column allowed for more non-specific binding of non-target proteins leading to the contamination in the final sample.

Although we had already established an optimal method for the expression of AC7 C_{1a} in our current vector system, pQE82L, we constructed a new plasmid by inserting the AC7 C_{1a} into vector pET30. The pET vector expression system is known for high levels of target protein expression and tight control over basal protein expression; however, the high expression levels can also increase protein precipitation and the pET vector includes a larger histidine tag that may interfere with enzymatic analysis. Our results indicated that pET may slightly increase the expression of AC7 C_{1a}; however, there is also more contamination. Furthermore, analysis of enzymatic activity found slightly higher enzymatic activity but the alcohol effect on AC7 disappeared (Figure 2.5.); confirming our fear that the use of the pET vector could interfere with enzymatic analysis.

<u>AC2 C_{1a}, AC2 C_2, AC9 C_{1a}, & AC9 C_2</u>

Expression and purification of AC2 C_{1a}, AC2 C_2, AC9 C_{1a}, & AC9 C_2 was carried out using the same methods outlined for AC7 C_{1a}. Despite multiple efforts, expression of AC2 C_{1a} never resulted in a detectable amount of protein by western blot analysis or enzymatic assay. Expression of AC2 C_2 and AC9 C_2 followed a similar pattern as AC7 C_2 with high levels of

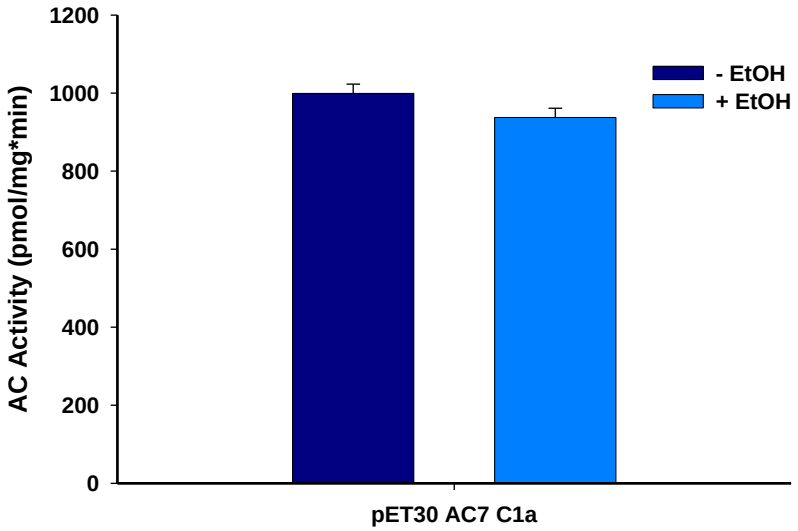

Figure 2.5. Enzymatic assay for comparison of AC7 C1a in pET30 paired with pQE82L AC7 C2. Enzymatic activity stimulated with 2.5mM $MnCl_2$ in the presence and absence of 200mM ethanol.

expression and only minor contamination following purification. AC9 C_{1a} followed a similar pattern as AC7 C_{1a} with modest expression, though slightly higher than AC7 C_{1a}, and purification resulting in a wide peak in the elution profile with more non-specific protein contamination. Figure 2.6. shows the final quality of purified AC7 C_{1a}, AC7 C_2, AC2 C_2, AC9 C_{1a}, & AC9 C_2 as demonstrated by SDS-PAGE analysis.

Discussion

AC2sol and AC7sol are considered large proteins (~60 kDa) relative to the bacterial expression system. Expression of these constructs may have been stretching the capability of the system. In the bacterial expression system, the larger proteins result in less successful expression. AC2sol and AC7sol are both over 50kDa molecular weight. Similar to our AC2sol and AC7sol, other researchers have expressed C_1 and C_2 regions in one intact recombinant

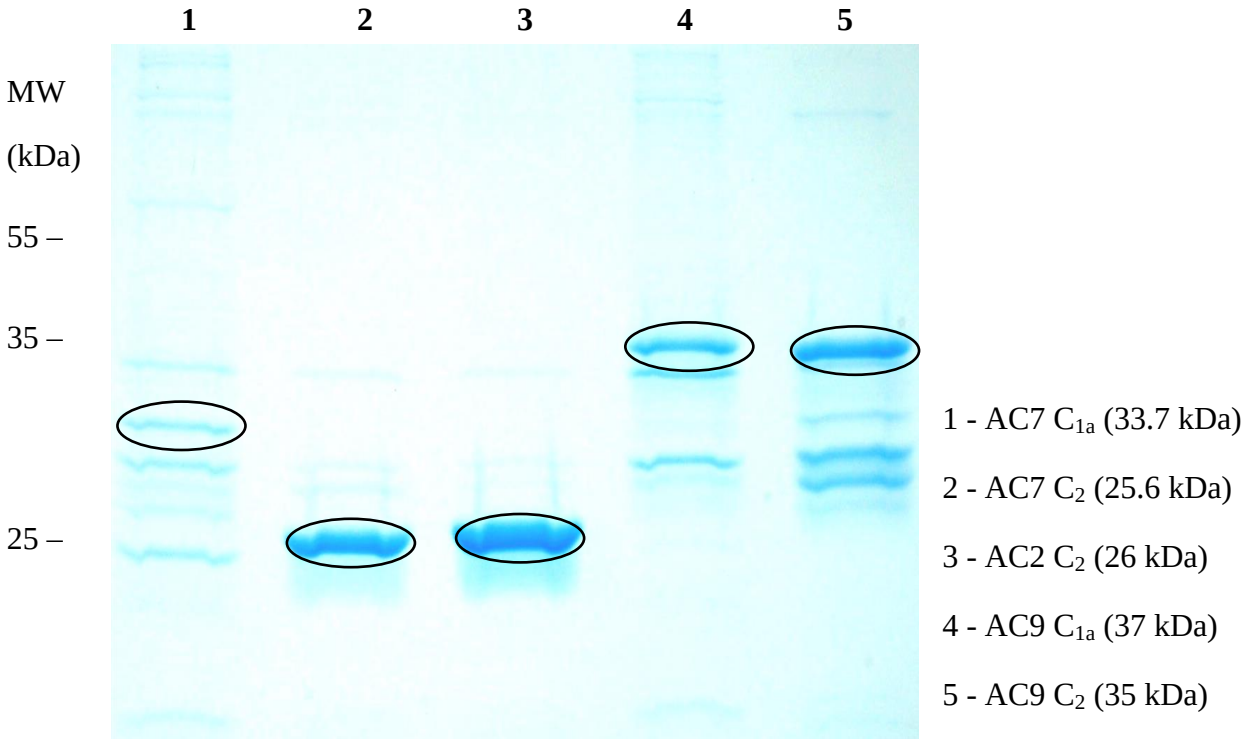

Figure 2.6. SDS-PAGE analysis of purified recombinant AC constructs.

protein by linking the two regions with an artificial amino acid sequence (Tesmer et al., 1997; Scholich et al., 1999). However, these studies used isoforms AC5 and a chimera, AC5 C_1+AC2 C_2. The isoform AC7 in purified form has only been studied by expressing and purifying the C_1 and C_2 regions separately (Yan & Tang, 2002). The sequence of AC2 is highly homologous to the sequence of AC7 and this could be one reason why we have had more difficulty with the expression of these isoforms compared to other research with AC1 and AC5. While AC2 C_2 was included in a recombinant chimera of AC1 C_1 + AC2 C_2 (Yan, Hahn, Huang, & Tang, 1996), our own data demonstrates the robust quality of AC2 C_2, therefore our limiting factor is most likely AC2 C_{1a}. Although our intention with ACsol was to create one soluble intact construct for the isoforms under investigation, the designed proteins did not exhibit the expression or enzymatic activity necessary for further study of the alcohol effect on AC. Fortunately, with the exception

of AC2 C_{1a}, the expression of the C_{1a} and C_2 regions separately resulted in proteins with sufficient expression and activity, allowing us to examine the alcohol effect on the purified proteins.

Recombinant AC7 C_{1a} and AC7 C_2 have been separately expressed and purified by other researchers in the past. Following separate protein production, investigators found that these proteins could be recombined and form an enzymatically active protein complex (Yan & Tang, 2002). We used the protocols established by this group as a guide for our own expression and purification of AC7 C_{1a} and AC7 C_2. However, when designing our recombinant AC7 proteins, we needed to include the regions previously identified as ethanol responsive domains (Yoshimura, 2006). The coding sequence for AC7 C_2 used by Yan and Tang included the sequence noted as ethanol responsive domain 2; therefore we included the same sequence for our own AC7 C_2 construct. Our AC7 C_{1a} construct needed to include two ethanol responsive domains, requiring us to code for amino acid sequence 197-475, encompassing the entire N-terminal end of the AC7 C_{1a} region, resulting in a larger AC7 C_{1a} than had been expressed by the earlier group. The larger size of AC7 C_{1a} could be the cause of our difficulty with the expression levels and purification quality of AC7 C_{1a}, however, this was unavoidable considering our knowledge of the ethanol responsive domains.

We had high hopes for the expression of AC7 C_{1a} in the pET vector. While the protein was active, improvement of expression and purity was minor and we were not able to detect the effect of alcohol on enzymatic activity. As mentioned, the histidine tag was much larger with the pET vector and could have interfered with the conformation of AC7 C_{1a} or blocked the site of alcohol interaction. Removal of the histidine tag from proteins expressed by the pET vector is possible because the tag sequence encoded by pET30 vector carries an enterokinase cleavage

site. The enterokinase cleavage reaction requires incubation at room temperature or higher for at least 16 hours. Following the enterokinase cleavage, the recombinant protein must be isolated from the excised histidine tag to achieve a pure protein sample. This requires an additional pass through his-tag affinity resin. All of these additional steps greatly increase the exposure of the recombinant protein to fluctuations in temperature and buffer conditions, and increase the probability for protein precipitation and degradation of catalytic integrity of the enzyme. Considering the already fragile state of our AC7 C_{1a} recombinant, any benefits of the pET vector would be overshadowed by the added exposure of his-tag removal.

All of our C_2 constructs express and purify much cleaner than C_{1a}. While our C_{1a} constructs are somewhat larger in molecular weight (C_{1a} = ~35kDa & C_2 = ~26kDa), this is likely not the only factor leading to the resulting differences in expression and purity. One possibility is that the protein folding of C_{1a} is more complex resulting in more difficulty during expression. The folding of the protein could also affect purification results if the conformation of the protein is folded in a way that may obstruct the his-tag making it less likely to bind the cobalt resin during purification. While we acknowledge that our various AC C_{1a} preparations are not completely homogenous, we will demonstrate in chapter 3 that our recombinant proteins retain enzymatic activity and demonstrate a response to alcohol similar to which we have observed in mammalian cell culture studies and in bacterial lysate preparations.

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CHAPTER 3.
CHARACTERIZATION OF THE EFFECT OF ALCOHOL ON RECOMBINANT ADENYLYL CYCLASE

Introduction

Many biological processes are implicated in the development of alcoholism and the behavioral response to alcohol. Both human and model animal studies have implicated the cAMP signaling system in the development of and predisposition to alcoholism. Specifically, many of these studies have implicated adenylyl cyclase as a crucial component of the ethanol effect on cAMP signaling.

Enzymatic activity of AC in the platelets of alcoholics was shown to be reduced in patients after abstaining from alcohol for 4 days, and individuals who had consumed alcohol in the 3-4 days prior to platelet extraction, regardless of alcoholism history, showed higher basal and stimulated AC activity (Menninger et al., 2000). Furthermore, complete abstainers from alcohol but with a family history positive for alcoholism had lower basal and stimulated AC activity levels than complete abstainers with no family history of alcoholism (Menninger, Baron, & Tabakoff, 1998), suggesting AC activity as a possible trait marker for genetic predisposition for the development of alcoholism. Animal models of alcoholism have also explored the role of the cAMP signaling system. Double knock-out (DKO) mice lacking both AC1 and AC8 had increased sensitivity to alcohol-induced sedation, further, DKO mice and AC8 KO mice exhibit decreased voluntary ethanol consumption compared to wild type mice (Maas, Jr. et al., 2005). AC5 KO mice show higher preference for ethanol as well as increased total consumption of ethanol compared to wild type mice (Kim, Kim, Baek, Lee, & Han, 2011). Another transgenic study found that overexpression of AC7 in mice increased basal and ethanol-induced DARPP-

32, a downstream element of the cAMP pathway implicated in motivation for ethanol intake (Donohue, Hoffman, & Tabakoff, 2005).

Our laboratory has demonstrated that alcohol enhances the activity of AC in an isoform specific manner and that the isoform AC7 is most enhanced by ethanol as measured by cAMP accumulation assay in intact mammalian cells (Yoshimura & Tabakoff, 1995). Additionally, ethanol responsive domains of AC7 were shown to be localized in the cytoplasmic regions of the protein (Yoshimura, Pearson, Kadota, & Gonzalez, 2006). Our lab found that different AC isoforms have different responses to a series of *n*-alkanols; AC6, AC7, and AC9 were found to have different alcohol cut-off points (Kou & Yoshimura, 2007). We also generated a recombinant AC7 protein consisting of the C_{1a} and C_2 regions linked together which included the ethanol responsive domains. This protein, named AC7sol, was expressed in a strain of *E. coli* lacking AC activity and lysate preparation was assayed for enzymatic activity devoid of any other mammalian proteins. We found that AC activity was enhanced by alcohols in a manner consistent with our previous mammalian studies and demonstrated that the C_{1a} and C_2 regions are sufficient to produce the alcohol effect (Dokphrom, Qualls-Creekmore, & Yoshimura, 2011).

In order to demonstrate that only the C_{1a} and C_2 regions are necessary for the alcohol effect, we used purified recombinant AC proteins to determine the effect of alcohol on AC in the absence of any other proteins. Here we will confirm that our purified recombinant AC proteins for isoforms AC2, AC7, and AC9 are enzymatically functional through assays to characterize the response of our proteins to known AC stimulators. Furthermore, we will confirm isoform specificity of the alcohol effect and perform chimeric analysis by combining C_{1a} and C_2 regions from different isoforms in order to test the importance of each region to the alcohol effect.

Materials and Methods

Materials

Bacto Tryptone and Bacto Yeast were from BD (Franklin Lakes, NJ). NiNTA superflow nickel chromatography cartridges were from Qiagen (Germantown, MD). HisPur Cobalt chromatography cartridges were from Pierce Biotechnology (Rockford, IL). HiTrap SP HP and HiTrap Q HP ion exchange chromatography cartridges were from GE Healthcare (Piscataway, NJ). BCA protein assay kit was from Pierce Biotechnology (Rockford, IL). His-Tag monoclonal antibody was from Novagen by EMD Millipore (Darmstadt, Germany). Anti-mouse secondary antibody conjugated horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). ECL chemiluminescent immunodetection was from GE Healthcare (Piscataway, NJ). Calbiochem Protease Inhibitor Cocktail II was from EMD Millipore (Darmstadt, Germany). [^{3}H] cAMP was from Moravek Biochemicals (Brea, CA). [α-^{32}P] ATP was from Perkin Elmer (Boston, MA). All other chemicals and reagents used were from Sigma-Aldrich (St. Louis, MO).

Recombinant AC Production

Protein production is described in detail in Chapter 2. Briefly, recombinant protein genes including hexahistidine tag for AC2 C_2, AC7 C_{1a}, AC7 C_2, AC9 C_{1a}, and AC9 C_2 were constructed and inserted in vector pQE82L. Resulting plasmids were transformed in *E. coli* cell line BL21 (DE3) and protein expression was induced with IPTG. Cells were harvested by centrifugation, lysed by sonication, and the soluble protein fraction (lysate) was separated by ultracentrifugation. The lysate preparation was loaded onto a cobalt metal affinity column, HisPur, washed to remove contaminants, and eluted in 20 fractions with an imidazole concentration gradient. Fractions containing the target protein were pooled, and loaded onto an

ion exchange column, HiTrap Q-HP or HiTrap SP-HP, depending on the isoelectric point for each individual protein, washed to remove contaminants, and eluted in 50 fractions with a NaCl gradient. Finally, fractions containing the target proteins were pooled and concentrated using an Amicon Ultra 10K filter by centrifugation at 4ºC, 4000rpm. Protein quantity was measured by BCA protein assay kit or based on their molecular extinction coefficient and A280.

Enzymatic Activity Assay

Our AC activity assay protocol used [α-^{32}P] ATP as a substrate. The total reaction contained 10-15μg of total purified protein (one C_{1a} protein and one C_2 protein combined), 250μM ATP, 25mM Tris-HCl pH 7.4, 100μM IBMX, 10mM phosphocreatine, 5 U creatine phosphokinase, and [α-^{32}P] ATP (50-80 cpm/pmol). AC activity was stimulated with $MnCl_2$. Reaction mixtures were prepared on ice and the assay was initiated by temperature-shift to 30°C for 15min. The reaction was terminated by the addition of a solution containing 2% SDS, 1mM ATP, and 1mM cAMP. ^{3}H cAMP was added as an internal control to estimate the recovery of cAMP. Sequential column chromatography by dowex 50 and neutral alumina columns was used to separate ATP and cAMP. A liquid scintillation counter was used to detect radioactivity in the final sample (Salomon, Londos, & Rodbell, 1974).

Data Analysis

All assay data were acquired in triplicate and each experiment performed at least twice to ensure reliability of measures. Statistical analysis was carried out by t-test. Sigma Plot and Sigma Stat (Systat Software, Point Richmond, CA) were used to plot and analyze data.

Results

Enzymatic Activity of Recombinant AC

Before investigating the effect of alcohol on recombinant AC, it was first necessary to establish that our protein constructs form catalytically active complexes *in vitro*. We performed the enzymatic activity assay for preparations of AC7 C_{1a} alone, AC7 C_2 alone, and AC7 C_{1a} and AC7 C_2 combined. For each preparation, AC activity was stimulated with 2.5mM $MnCl_2$. We found that when assayed alone, C_{1a} or C_2 had activity levels not significantly different from background. However, the combined preparation of C_{1a} and C_2 showed high levels of AC activity (Figure 3.1.). These results demonstrated that even though the catalytic components, C_{1a} and C_2, are expressed and purified separately, they can be combined *in vitro* and enzymatic activity is restored.

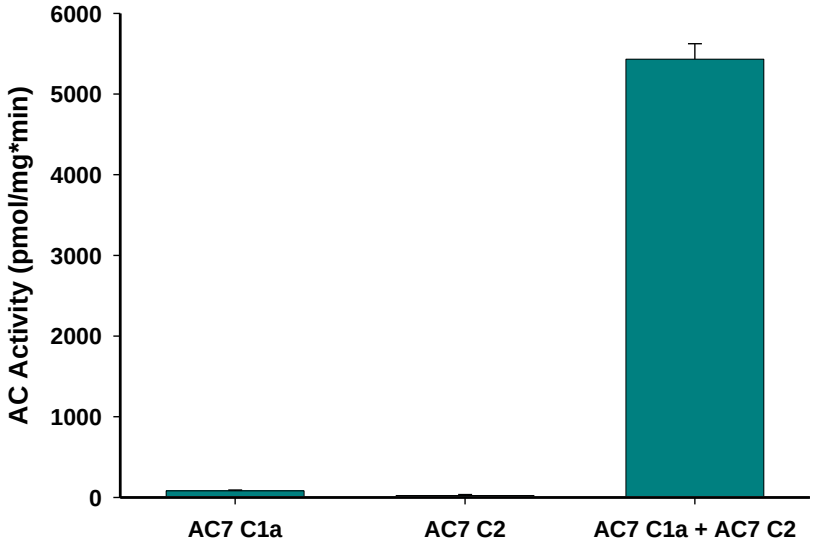

Figure 3.1. AC activity of recombinant AC7 C_{1a} and AC7 C_2 alone, and AC7 C_{1a} + AC7 C_2 combined preparation. AC activity is stimulated by 2.5mM $MnCl_2$.

In our previous study using recombinant protein AC7sol, enzymatic activity assay was performed on AC7sol in a bacterial lysate preparation. It was necessary to include an ATP

regeneration mechanism in our assay system to regenerate any ATP decomposed by potential ATPase in the crude protein sample. Phosphocreatine and creatine kinase were used to regenerate ATP. In the current study, we assumed the use of the ATP regeneration system would be unnecessary due to the purity of our protein samples. We tested this assumption by assaying the combined preparation of AC7 C_{1a} and AC7 C_2 in the presence or absence of the ATP regeneration system. As predicted, the ATP regeneration system was not necessary for our purified proteins; in fact, AC activity was actually suppressed by the use of the ATP regeneration system (Figure 3.2.).

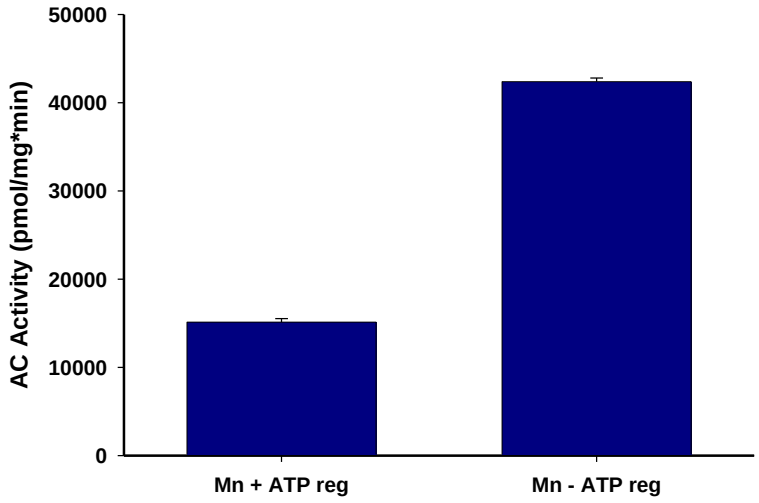

Figure 3.2. AC7 C_{1a} + AC7 C_2 activity in the presence or absence of ATP regeneration system. AC activity stimulated by 2.5mM $MnCl_2$.

Isoform Specific Alcohol Effect

We first examined the effect of alcohol on isoforms AC7 and AC9 homologous combinations. Previous work in our lab established that the isoform AC7 is most responsive to ethanol while AC9 has a very weak response to ethanol. AC7 C_{1a} + AC7 C_2 and AC9 C_{1a} + AC9 C_2 combinations were assayed using manganese plus or minus 200mM ethanol. As a control,

basal enzymatic activity was also measured. No significant basal activity was observed for either AC7 or AC9 with or without ethanol (Figure 3.3.). Both protein combinations were active when stimulated by $MnCl_2$ alone (Figure 3.3.). We examined the condition of $MnCl_2$ + forskolin because AC9 is known to be less responsive to forskolin. However, in this experiment, there was no significant difference between AC activity under conditions of $MnCl_2$ alone or $MnCl_2$ + forskolin for either AC7 or AC9. In the presence of ethanol, we observed a significant enhancement of AC activity for the AC7 combination (Figure 3.3.A), while the AC9 combination was significantly inhibited by ethanol (Figure 3.3.B). These results are consistent with previous work in our lab indicating that the alcohol effect is isoform specific and AC7 is the most enhanced by alcohol.

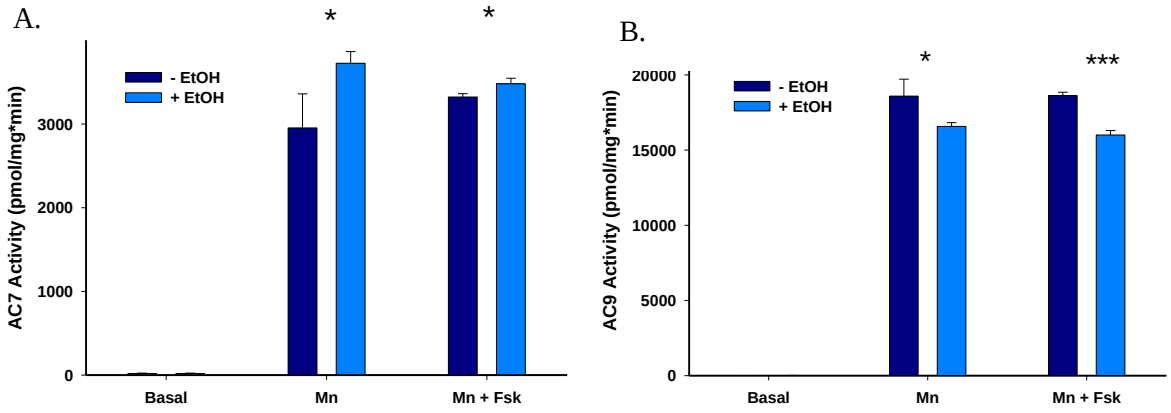

Figure 3.3. Basal AC activity, 2.5mM $MnCl_2$ stimulated activity, and 2.5mM $MnCl_2$ + 10 μM forskolin stimulated activity in the absence or presence of 200mM ethanol for AC7 C_{1a} + AC7 C_2 (A) and AC9 C_{1a} + AC9C_2 (B). * $p < 0.05$, *** $p < 0.001$

Alcohol Effect on AC Chimeras

In order to determine if one of the cytoplasmic domains has a dominant role in the ethanol effect on enzyme activity, we combined cytoplasmic regions from different AC isoforms. These chimeras included AC7 C_{1a} + AC2 C_2, AC7 C_{1a} +AC9 C_2, AC9 C_{1a} + AC2 C_2, and AC9

C_{1a} + AC7 C_2. First we needed to ensure that the chimeric protein combinations maintained catalytic activity. All of the chimeras were active, however, the specific activity for each of these chimeras varied (Figure 3.4.). Furthermore, most of the chimeras were responsive to 200mM ethanol. Permutations containing AC7 C_{1a} were enhanced by ethanol; permutations not containing AC7 C_{1a} were inhibited by ethanol or showed no effect (Figure 3.5.). This data would suggest that AC7 C_{1a} plays a more vital role in the alcohol effect than does AC7 C_2.

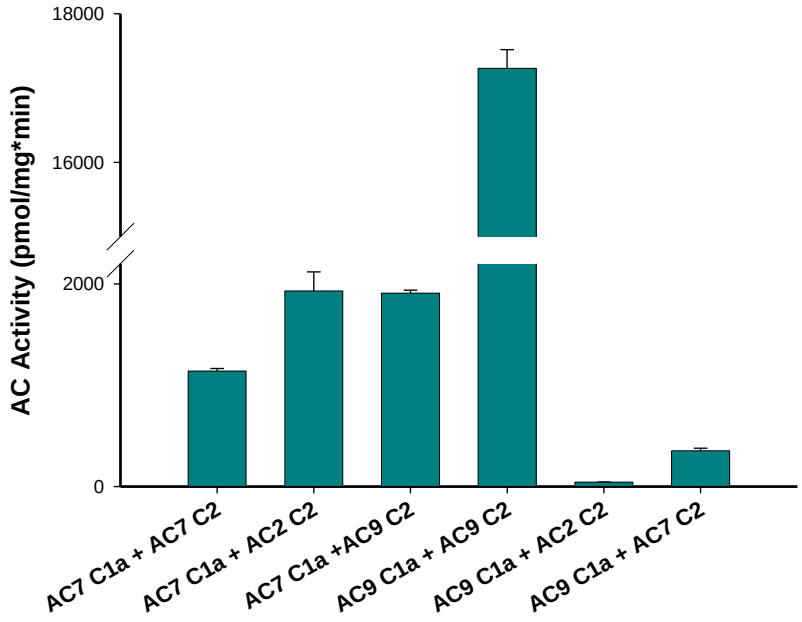

Figure 3.4. AC activity for chimeric recombinant proteins. AC activity stimulated with 2.5mM $MnCl_2$.

Discussion

To establish a direct effect of alcohol on adenylyl cyclase and that the alcohol effect has isoform specificity, we designed, expressed, and purified recombinant proteins for AC7, AC2, and AC9. Cytoplasmic domains for each of these isoforms were expressed and purified separately. Each of these recombinant proteins yielded acceptable levels of protein expression

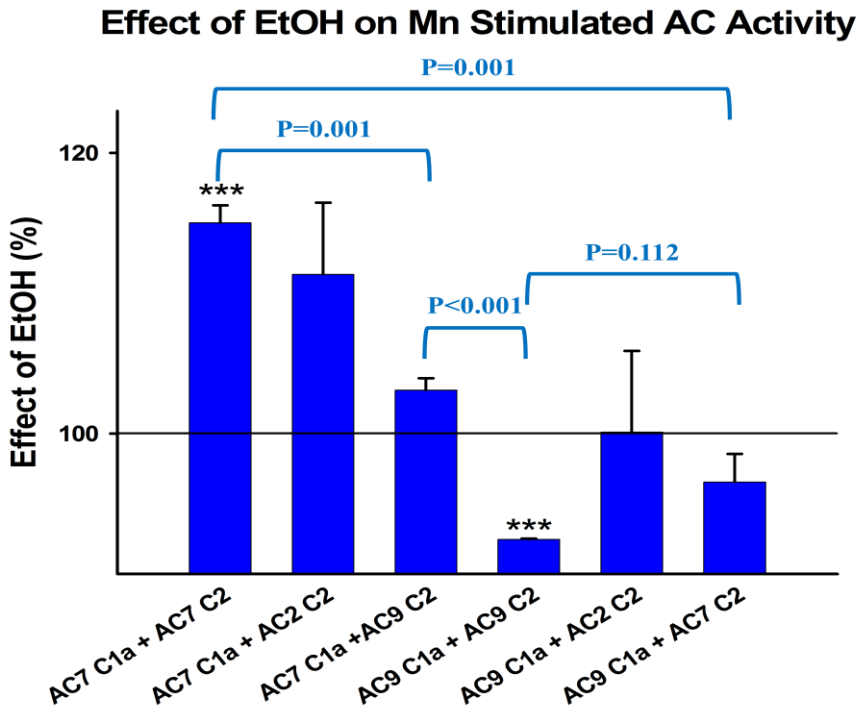

Figure 3.5. Effect of ethanol on $MnCl_2$ stimulated AC activity in AC recombinant chimeras. *** $p < 0.001$. Blue brackets represent the result of t-test in alcohol effect between chimeric conditions.

and purification resulted in a highly pure protein product, with the exception of AC2 C_{1a}. The recombinant proteins were enzymatically active in both homologous and chimeric combinations of C_{1a} and C_2 domains.

Consistent with our studies of the native AC protein expressed in a mammalian cell culture model system, the alcohol effect on AC appears to be isoform specific. Also through our mammalian cell culture system, we found that AC9 is weakly stimulated by alcohols, but has a much different alcohol cutoff than AC7. This is why we chose AC9 to investigate the isoform specificity of the alcohol effect. We chose to include AC2 in the chimera study because among all of the AC isoforms, AC2 shares the most sequence homology with AC7 and both proteins are similarly regulated by components of the cAMP signaling cascade. The response of purified

AC9 to alcohol was somewhat different than the alcohol response observed in native AC9 expressed in mammalian cell culture. When exposed to 200mM ethanol, the full length native AC9 expressed in HEK293 cells showed ~ 20% increase in activity; comparatively, under the same conditions AC7 showed ~40% increase in activity (Kou & Yoshimura, 2007). The effect of alcohol on purified recombinant AC9 is an inhibition of enzymatic activity. It is not unreasonable to find that the purified recombinants respond somewhat different than the native proteins for a number of reasons. Because the purified proteins are assayed *in vitro*, the activity of AC must be stimulated differently than in the mammalian cell culture system; mammalian cells are stimulated through the activation of GPCRs, while the pure proteins are activated by small molecules that can bind within the catalytic cleft such as manganese or forskolin. Furthermore, in absence of the membrane-spanning domains, there is a potential for differences in protein conformation that may affect AC activity. These results do not weaken our conclusions because our recombinant protein system still maintains the ability to produce an alcohol effect in an isoform specific manner.

Previous research has shown that recombinant proteins representing the cytoplasmic domains from different AC isoforms can join together to form an active catalytic complex (Whisnant, Gilman, & Dessauer, 1996; Yan, Hahn, Huang, & Tang, 1996). In fact, this is the manner by which much of the structural research on AC has been carried out. Therefore, we decided to combine different AC cytoplasmic domains in order to test the importance of the C_{1a} versus C_2 domain to the observed isoform specific effect of alcohol on AC. All of our chimeras had enzymatic activity when stimulated with manganese. The activity of the chimera AC9 C_{1a} + AC2 C_2 had the lowest activity, but is still significantly higher in activity than the control reactions containing no protein.

Each AC chimera responded differently in the presence of 200mM ethanol. The AC activity of chimeras containing AC7 C_{1a} was enhanced by ethanol. The AC activity of chimeras containing AC9 C_{1a} was inhibited by ethanol. Based on chimeric mutational studies carried out in the native AC structures, we expected that both cytoplasmic domains were necessary for the alcohol effect. The study used components of isoforms AC2, AC3, and AC7 to create chimeras through exchange of all of partial sequences for all regions of the native AC, N-terminal tail, two membrane-spanning domains, and two cytoplasmic domains. Following analysis of 30 chimeric mutants, ethanol responsive domains (ERD) were identified as the N-terminal 28 amino acid sequence of AC7 C_{1a} and the C-terminal 140 amino acid sequence of AC7 C_2 (Yoshimura, 2006). Our lab also found a third ERD, a short amino acid sequence located in the middle of the AC7 C_{1a} region (unpublished data). Our data supports the existence of ERDs in the AC7 C_{1a} region but the contribution of ERD2 in AC7 C_2 may be small since without AC7 C_{1a} the alcohol effect is lost. However, our data for AC7 C_2 chimeras is somewhat limited. Because we were not able to successfully express AC2 C_{1a}, we only have one chimera including AC7 C_2, AC9 C_{1a} + AC7 C_2. This limits any conclusions we might be able to formulate regarding ERD2.

In conclusion, we have generated a series of recombinant AC proteins for the cytoplasmic regions of AC2 (C_2), AC7, and AC9. Although C_{1a} and C_2 regions are expressed separately, when joined together in homomeric or chimeric permutations, these constructs are enzymatically active and respond to ethanol. We have confirmed that the alcohol effect is isoform specific and that AC7 is more enhanced by ethanol. Finally, we have indicated the importance of AC7 C_{1a} for the effect of alcohol on AC.

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CHAPTER 4.
ANALYSIS OF THE EFFECT OF ALCOHOL ON ADENYLYL CYCLASE: ALCOHOL CUTOFF AND ENZYME KINETICS

Introduction

Adenylyl cyclase (AC) has been identified as a potential target for the effect of alcohol on cellular function. As reviewed in Chapter 3, initial research established an effect of alcohol on the cAMP signaling system. Studies in clinical populations of alcohol users as well as experimental laboratory models demonstrated changes to cAMP signaling during acute and chronic alcohol exposure. There was also evidence that within the cAMP signaling cascade, it was the activity of adenylyl cyclase that was modified by alcohol exposure. The primary objective in our laboratory has been to define the mechanism by which alcohol affects AC. In review, through *in vivo* experimentation in a mammalian cell culture system our laboratory has demonstrated that alcohol enhances the activity of AC in an isoform specific manner with AC7 most enhanced by ethanol (Yoshimura & Tabakoff, 1995; Yoshimura & Tabakoff, 1999). Furthermore, ethanol responsive domains of AC7 are in the cytoplasmic regions of AC (Yoshimura, Pearson, Kadota, & Gonzalez, 2006), alcohol cutoff points are isoform specific (Kou & Yoshimura, 2007), and AC response to alcohol stereoisomers is isoform specific (Hasanuzzaman & Yoshimura, 2010). Additionally, the design and production of recombinant proteins for several AC isoforms allowed us to demonstrate that the effect of alcohol on AC persists in the absence of any other mammalian proteins while confirming the findings from the mammalian cell culture system indicating that the alcohol effect is specific to the cytoplasmic regions and that the alcohol effect is isoform specific (Dokphrom, Qualls-Creekmore, & Yoshimura, 2011).

The first recombinant AC protein designed in our lab was AC7sol, which carried the C1a and C2 domains designed to include the ethanol responsive domains previously identified (Yoshimura, Pearson, Kadota, & Gonzalez, 2006). AC7sol was expressed in *E. coli*, cells were lysed by sonication, crude bacterial lysate were prepared by ultracentrifugation, and AC activity analysis was carried out on the crude bacterial lysate. Given that AC7sol is outside of the constraints of the cell membrane, we were able to examine the effects of several AC stimulators and multiple *n*-alkanols, and to perform kinetic analysis. AC7sol retains the ethanol response and alcohol cutoff of native AC7 observed in intact cells (Yoshimura & Tabakoff, 1995; Kou & Yoshimura, 2007). Furthermore, ethanol and butanol increased both V_{max} and K_m values for AC7sol indicating that ethanol increases the turnover rate for enzymatic activity of AC (Dokphrom et al., 2011). Although AC7sol was expressed in TP2000, an *E. coli* strain which has no endogenous AC activity, because these experiments are carried out with crude bacterial lysate, we could not definitively conclude that the alcohol effect on AC does not involve additional proteins. Purification of recombinant ACs was necessary to further research the alcohol effect on AC. As presented in Chapter 3, purified recombinant proteins for AC2, AC7, and AC9 were enzymatically active and demonstrated isoform specific responses to alcohol. Further experiments were warranted to determine if the purified AC7 C_{1a} and AC7 C_2 combination responded similarly to recombinant AC7sol and the native AC7 expressed in mammalian cells. However, the alcohol effect observed in the analysis of our AC recombinant proteins was less dramatic than the alcohol effect seen on the native protein in mammalian cells or on AC7sol. Because longer chain alcohols have more potent effects on AC, we decided to use longer chain alcohols such as butanol in further experiments on purified recombinant AC7.

Here were present data to demonstrate the effect of AC stimulators manganese, forskolin, and purified Gsα protein on AC7 C_{1a} + AC7 C_2. During the first experiment we found that the use of all three of these AC stimulators synergistically increased the activity of AC. Furthermore, under this condition as well as conditions that included Gsα, the effect of alcohol on AC activity was inhibition rather than enhancement. Somewhat similar observations were made for AC7sol when stimulated with Gsα, however, this is the first time to our knowledge that recombinant AC has been assayed in the presence of all three of these AC stimulators. As a test of the validity of this unexpected inhibition, we designed an experiment to test if the inhibition of AC activity by alcohol when activated by manganese, forskolin, and Gsα followed the same alcohol cutoff point observed in the native AC7 and the recombinant AC7sol. Alcohol cutoff is the point at which increasing the chain length of alcohol does not further increase the potency of the alcohol effect on AC. Furthermore, based on these unexpected results, we determined that we could conduct concentration response analysis of AC7 C_{1a} + AC7 C_2 through the manipulation of concentration of AC stimulators and alcohols. We expected that this analysis might further elucidate the mechanism of alcohol action on AC.

Materials and Methods

Materials

Bacto Tryptone and Bacto Yeast were from BD (Franklin Lakes, NJ). HisPur Cobalt chromatography cartridges were from Pierce Biotechnology (Rockford, IL). HiTrap SP HP and HiTrap Q HP ion exchange chromatography cartridges were from GE Healthcare (Piscataway, NJ). BCA protein assay kit was from Pierce Biotechnology (Rockford, IL). His-Tag monoclonal antibody was from Novagen by EMD Millipore (Darmstadt, Germany). ECL chemiluminescent immunodetection was from GE Healthcare (Piscataway, NJ). Calbiochem

Protease Inhibitor Cocktail II was from EMD Millipore (Darmstadt, Germany). [^{3}H] cAMP was from Moravek Biochemicals (Brea, CA). [α-^{32}P] ATP was from Perkin Elmer (Boston, MA). Gsα was produced using plasmid pQE60 Gsα including a hexahistidine tag, expressed in *E. coli* cell line BL21 (DE3) containing pUBS520, and purified by affinity chromatography using the HisPur cobalt column and HiTrap Q HP ion exchange chromatography cartridge (Yan & Tang, 2002). Forskolin analogue NKH477 was from Tocris, R&D systems (Minneapolis, MN). All other chemicals and reagents used were from Sigma-Aldrich (St. Louis, MO).

Recombinant AC Production

Protein production is described in detail in Chapter 2. Briefly, recombinant protein genes including hexahistidine tag for AC7 C_{1a} and AC7 C_2 were constructed and inserted in vector pQE82L. Resulting plasmids were transformed in *E. coli* cell line BL21 (DE3) containing pUBS520 and protein expression was induced with IPTG. Cells were harvested by centrifugation, lysed by sonication, and the soluble protein fraction (lysate) was separated by ultracentrifugation. The lysate preparation was loaded onto a cobalt metal affinity column, HisPur, washed to remove contaminants, and eluted in 20 fractions with an imidazole concentration gradient. Fractions containing the target protein were pooled and loaded onto an ion exchange column, HiTrap Q-HP for AC7 C_2 or HiTrap SP-HP for AC7 C_{1a}, washed to remove contaminants, and eluted in 50 fractions with a NaCl gradient. Finally, fractions containing the target proteins were pooled and concentrated using an Amicon Ultra 10K filter by centrifugation at 4ºC, 4000rpm. Protein quantity was measured by BCA protein assay kit or based on their molecular extinction coefficient and A280.

Enzymatic Activity Assay

Our AC activity assay protocol used [α-^{32}P] ATP as a substrate. The total reaction contained 10-15μg of total purified protein (one C_{1a} protein and one C_2 protein combined), 250μM ATP, 25mM Tris-HCl pH 7.4, 100μM IBMX, 10mM phosphocreatine, 5 U creatine phosphokinase, and [α-^{32}P] ATP (50-80 cpm/pmol). AC activity was stimulated with one or more of the following: $MnCl_2$, forskolin, purified Gsα protein, or forskolin analogue NKH477. Reaction mixtures were prepared on ice and the assay was initiated by temperature-shift to 30°C for 15min. The reaction was terminated by the addition of a solution containing 2% SDS, 1mM ATP, and 1mM cAMP. ^{3}H cAMP was added as an internal control to estimate the recovery of cAMP. Sequential column chromatography by dowex 50 and neutral alumina columns was used to separate ATP and cAMP. A liquid scintillation counter was used to quantify radioactivity in the final sample (Salomon, Londos, & Rodbell, 1974).

Data Analysis

All assay data were acquired in triplicate and each experiment performed at least twice to ensure reliability of measures. Concentration response curve fitting was carried out by non-linear regression analysis using either 3 or 4 parameter logistic equation:

$$y = \frac{a}{1 + e^{-(\frac{x-x0}{b})}} \; or \; y = y0 + \frac{a}{1 + e^{-(\frac{x-x0}{b})}}$$

a: maximal value, b: Hill's coefficient, x0: EC50, y0: minimal value. Statistical analysis was carried out by t-test. Sigma Plot and Sigma Stat (Systat Software, Point Richmond, CA) were used to plot and analyze data.

Results

Our first objective was to test the effect of AC activators on AC7 C_{1a} + AC7 C_2 using several conditions of activators alone, or in combinations of two or all three. Basal activity of AC7 C_{1a} + AC7 C_2 was also assayed for comparison. As expected the AC activity under the basal condition was not significantly different from background levels and the forskolin condition resulted in very weak AC activation. AC activation by forskolin generally requires co-activation by another activator such as manganese or Gsα. Gsα alone was a weak activator of AC activity and $MnCl_2$ alone, $MnCl_2$ + forskolin, and Gsα + forskolin showed intermediate activation. $MnCl_2$ + Gsα activation provided a high level of AC activity. Finally, the combination of all three AC stimulators, $MnCl_2$ + forskolin + Gsα appeared to have a synergistic effect on AC activity. The presence of all three AC activators stimulated AC activity far greater than the sum of any of the other conditions. Figure 4.1 presents the degree of variability between these conditions of AC activation; $MnCl_2$ concentration is 2.5mM, forskolin concentration is 10μM, and 8.5μg purified proteins per reaction.

Using the various combinations for AC activation, we next examined the effect of 100mM butanol on AC activity. The conditions for AC activation not only affected the magnitude of the alcohol effect, but also determined in the effect of alcohol was positive or negative on AC activity (Figure 4.2.). The only conditions resulting in enhancement of AC activity were $MnCl_2$ alone + 100mM BuOH or the combination of $MnCl_2$ + forskolin + 100 mM BuOH. All other combinations of AC activators resulted in AC inhibition when in the presence of 100mM BuOH, and the extent of inhibition varied depending on the combination of AC activators in the condition.

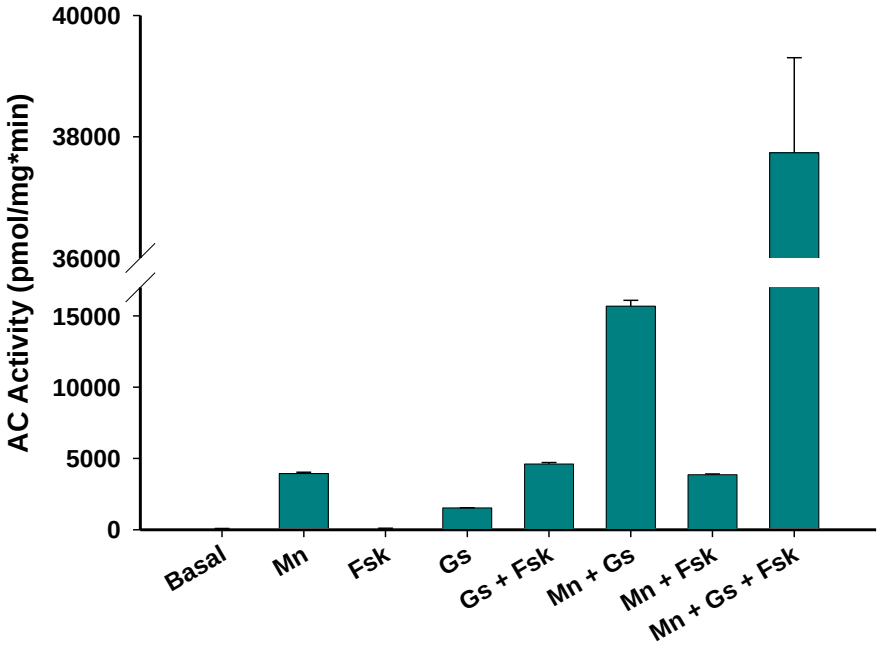

Figure 4.1. AC7 C_{1a} + AC7 C_2 activity under various conditions of AC activation (Mn = $MnCl_2$, Fsk = forskolin, Gs = Gsα).

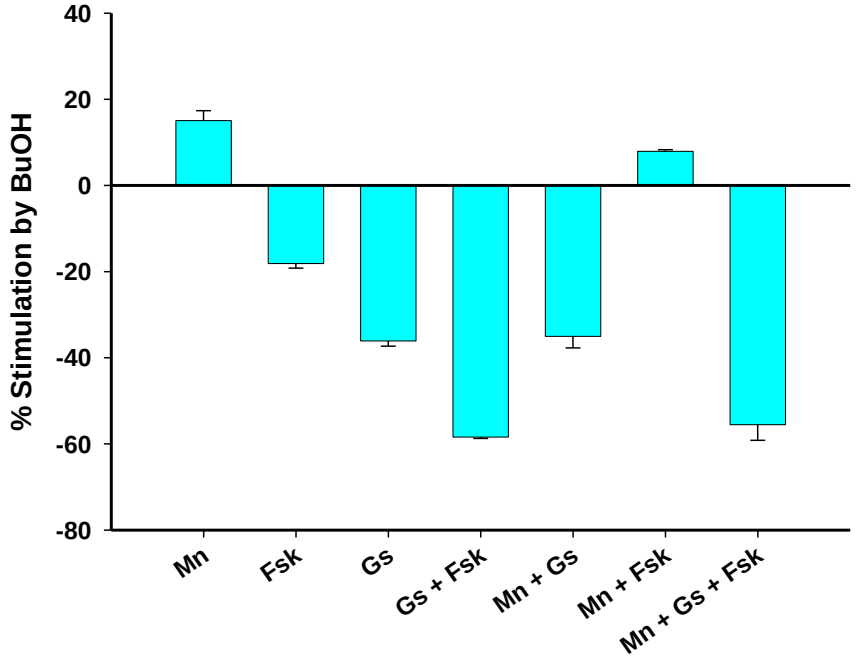

Figure 4.2. Effect of 100mM butanol on AC7 C_{1a} + AC7 C_2 activity under various conditions of AC activation.

Because many of these conditions resulted in the inhibition of AC activity when exposed to butanol, we designed an experiment to test the alcohol cutoff point for AC7 when activated by $MnCl_2$, forskolin, and Gsα combined. The observed inhibition was unexpected, and to test the validity of this observation, we needed to see if the inhibition followed the same alcohol cutoff seen in native AC7 and recombinant AC7sol. AC activity was activated using $MnCl_2$, forskolin, and Gsα; activity was assayed in the presence of various concentrations of ethanol, butanol, pentanol, or hexanol. Under these conditions, the potency of alcohol to inhibit AC activity increased until hexanol. Inhibition of AC activity by hexanol was no greater than inhibition by pentanol, suggesting that the alcohol cutoff was pentanol (Figure 4.3.). This is consistent with our previous observations in native AC7 and AC7sol and provides rationale for use of these AC activation conditions in concentration response studies of recombinant AC7.

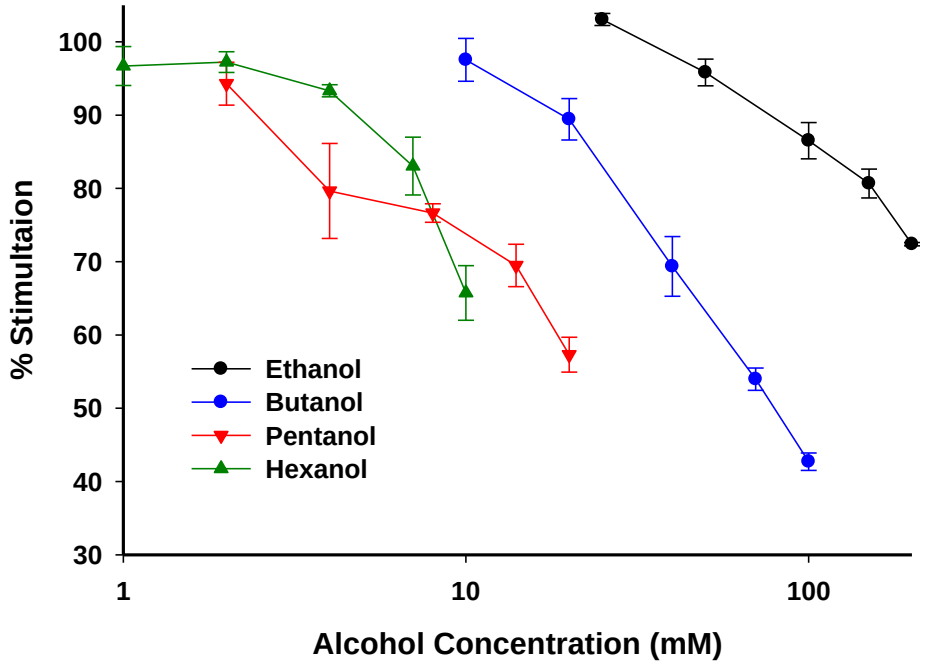

Figure 4.3. Determination of alcohol cutoff point for AC7 C_{1a} + AC7 C_2 activated by $MnCl_2$, forskolin, and Gsα. Alcohol cutoff point was pentanol.

All studies were carried out under AC activation by $MnCl_2$, forskolin, and Gsα. The parameter determined was EC50, which stands for half maximal effective concentration. EC50 is derived from a concentration response curve and indicates the concentration of a drug at which the response (AC activity) is midway between the baseline and maximum response. The concentration of $MnCl_2$ remained constant at 2.5mM; concentrations of forskolin and Gsα varied by experiment. All experiments used butanol at concentrations ranging from 0-400mM. Our first set of experiments explored the relationship between alcohol and forskolin and the resulting changes in EC50 of AC7 activity.

A forskolin concentration-response experiment was performed using forskolin concentrations of 1, 10, 25, 100, and 200μM; concentrations of 2.5mM $MnCl_2$, 4μg Gsα remained constant, and experiments were carried out in the absence or presence of 100mM BuOH. The solubility of forskolin is limited in water or aqueous solution and the forskolin concentration-response regression curve did not reach a plateau (Figure 4.4.), and without knowing the effects of higher concentrations of forskolin we were not able to determine EC50 accurately.

In order to address the solubility constraints of forskolin, we acquired a forskolin analogue, NKH 477, which is soluble in water at a maximum concentration of 40mM. Using NKH 477, a concentration-response experiment was performed using concentrations of 10, 30, 100, and 300μM, 1mM, and 3mM; concentrations of 2.5mM $MnCl_2$, 4μg Gsα remained constant, and experiments were carried out in the absence or presence of 100mM BuOH. EC50 was significantly shifted right by butanol, meaning the potency of forskolin decreased (Figure 4.5.). To test the effect of forskolin on the butanol effect, we performed a concentration-response experiment using concentrations of butanol ranging from 0-400mM while keeping the forskolin

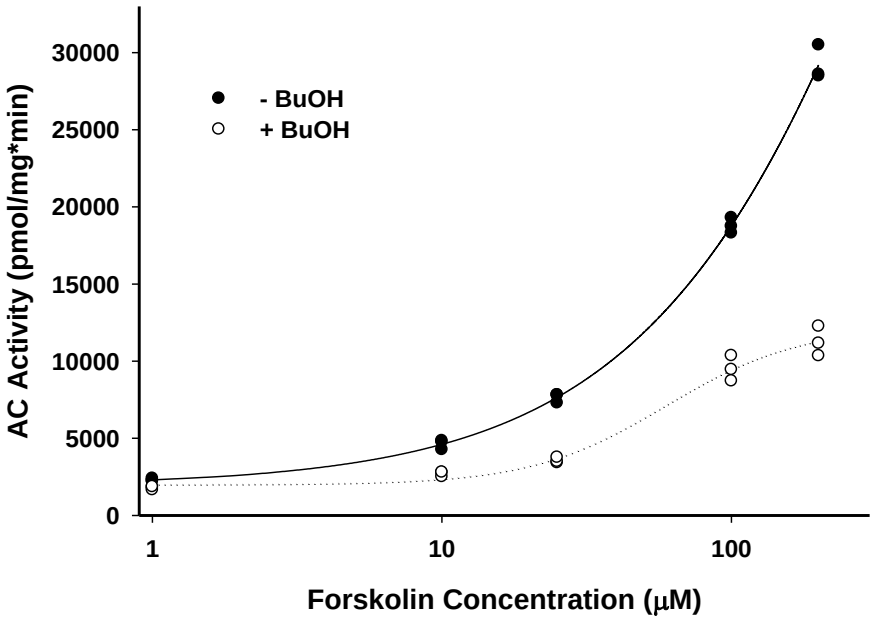

Figure 4.4. Forskolin concentration response curve for AC7 C_{1a} + AC7 C_2 in the presence or absence of 100mM butanol. Because of forskolin solubility restraints, maximum response was not achievable and the EC50 value was not valid. EC50 (-) BuOH 786.5 ± 854.5 mM, EC50 (+) BuOH 59.1 ± 9.3 mM, P = 0.405.

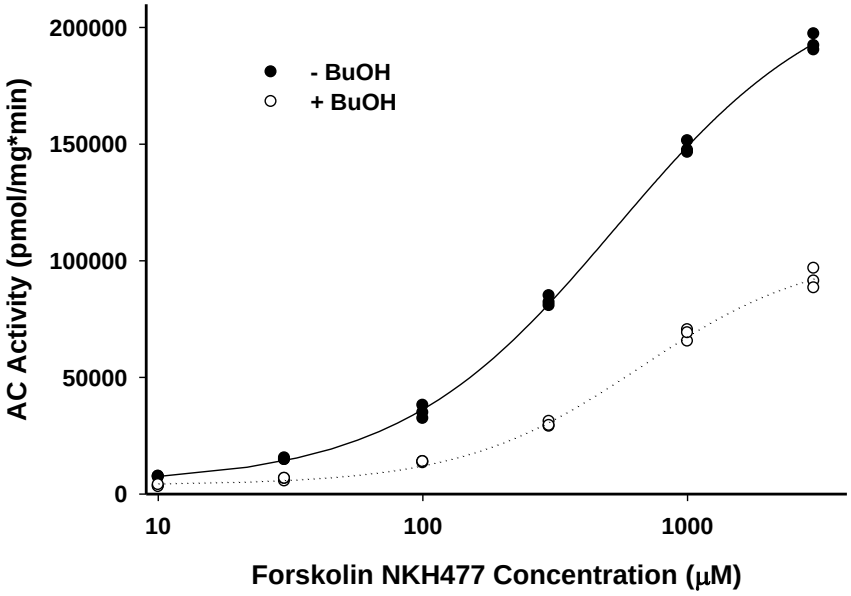

Figure 4.5. Forskolin analogue, NKH477 concentration response curve for AC7 C_{1a} + AC7 C_2 in the presence or absence of 100mM butanol. EC50 value was significantly different in the presence of butanol. EC50 (-) BuOH 533.6 ± 26.8 μM, EC50 (+) BuOH 659.1 ± 52.9 μM, P = 0.044

concentration constant at 25µM. In this experiment, the addition of forskolin appeared to enhance the potency of BuOH (Figure 4.6.) and EC50 was significantly shifted left by forskolin.

The effect of butanol on AC activation by Gsα was examined next. We performed a concentration-response experiment essentially modifying the extent to which Gsα is activated in the reaction. Concentrations of GppNHp 0, 0.5, 3, 5, 10, and 50µM were used in the activation of Gsα; the actual quantity of Gsα protein remained constant at 4µg per reaction, along with 2.5mM $MnCl_2$ and 25µM forskolin. The presence of butanol did decrease AC activity (Figure 4.7.), however, EC50 of GppNHp was not significantly affected by butanol.

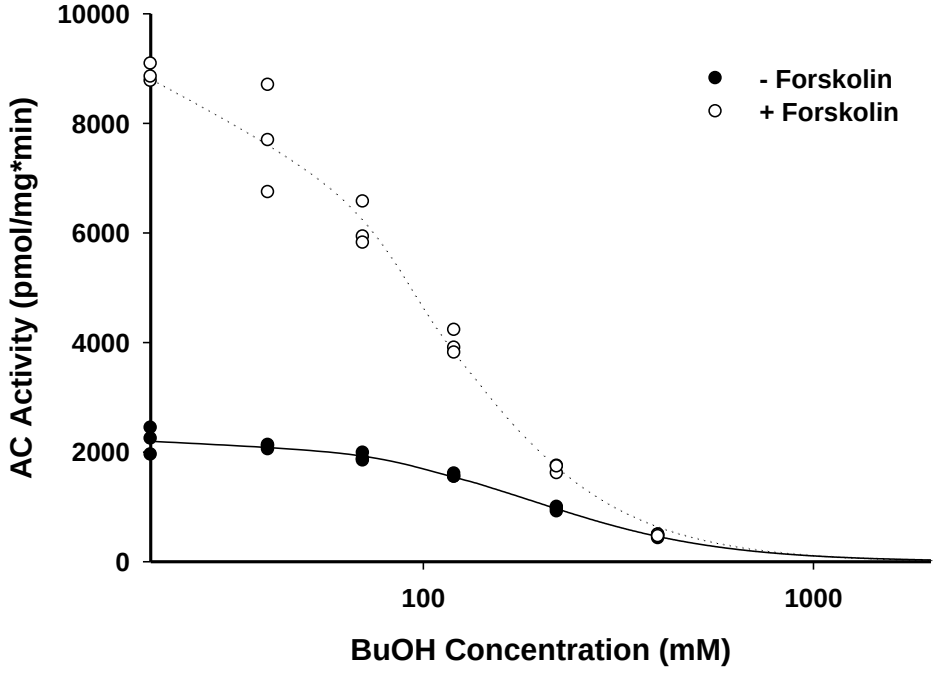

Figure 4.6. Butanol concentration response curve for AC7 C_{1a} + AC7 C_2 in the presence or absence of 25µM forskolin. EC50 value was significantly different in the presence of forskolin. EC50 (-) forskolin 188.6 ± 9.8 mM, EC50 (+) forskolin 100.5 ± 6.3 mM, P <0.001.

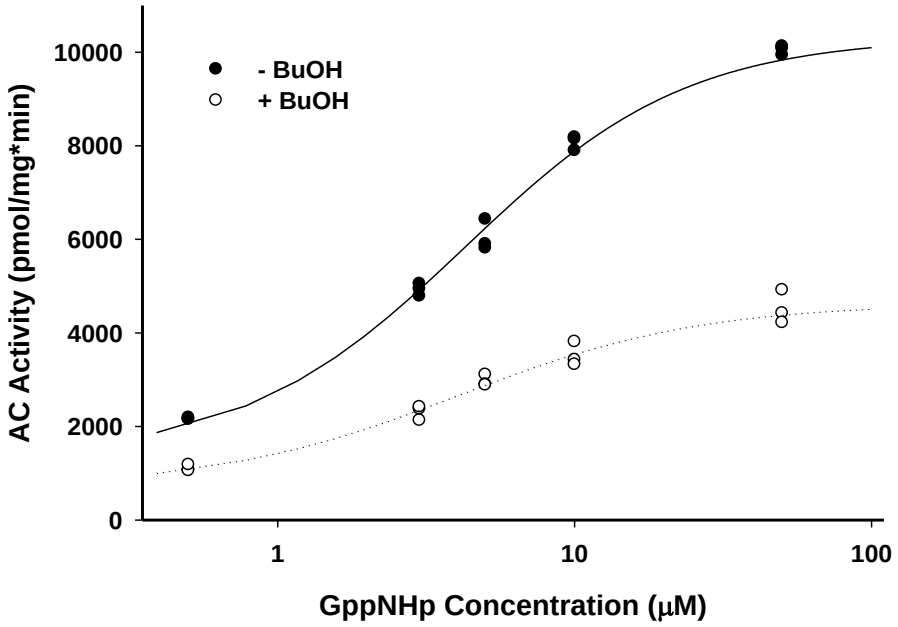

Figure 4.7. GppNHp concentration response curve for AC7 C_{1a} + AC7 C_2 in the presence or absence of 100mM butanol. EC50 value was not significantly different in the presence of butanol. EC50 (-) BuOH 4.3 ± 0.2 (μM), EC50 (+) BuOH 4.1 ± 0.5 (μM), P = 0.667.

To test the effect of Gsα on potency of butanol, we performed a concentration-response experiment using concentrations of butanol ranging from 0-400mM in the presence or absence of Gsα. EC50 for butanol shifted left significantly in the presence of Gsα (Figure 4.8.).

Discussion

It is important to place in context the response of our purified recombinant AC7 to alcohol under some of our experimental conditions. First, let us point out that these experiments are designed as pharmacological tools for understanding the biochemical mechanisms involved in the interaction between AC and alcohol and are not intended to replicate a physiologically relevant system. The use of butanol and other alcohols which are not subject to human use are only meant to provide a tool for accentuating the response of AC to the alcohol. By accentuating

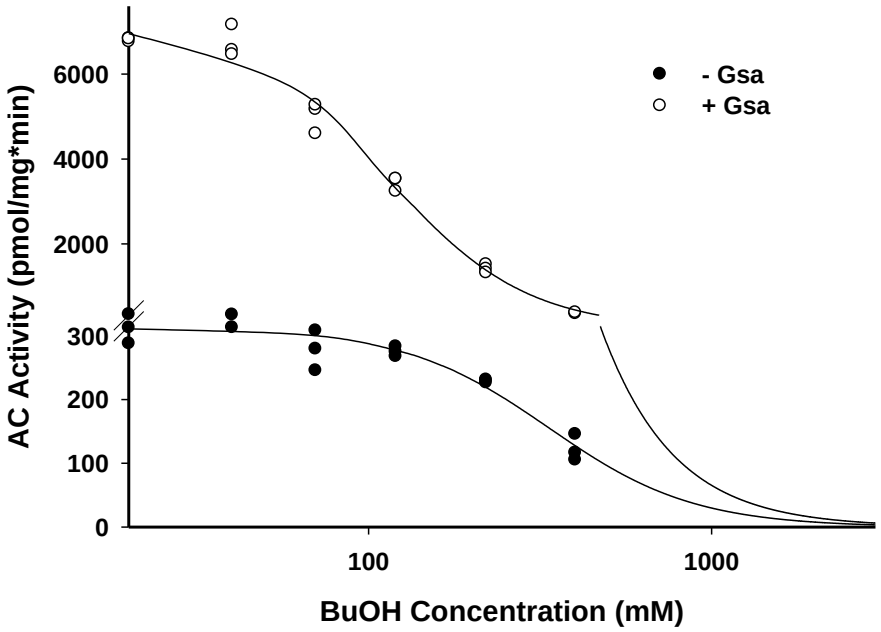

Figure 4.8. Butanol concentration response curve for AC7 C_{1a} + AC7 C_2 in the presence or absence of 4μg Gsα. EC50 value was significantly different in the presence of Gsα. EC50 (-) Gsα 334.7 ± 22.8 mM, EC50 (+) Gsα 113.4 ± 4.9 mM, P <0.001.

the AC response to alcohol we are provided with greater flexibility in manipulating the AC response with other AC modulators such as $MnCl_2$, forskolin, and Gsα.

Under conditions with multiple AC activators, the effect of alcohol on AC7 appears to reverse from enhancement of AC activity to inhibition of AC activity. There are several potential explanations for this inhibition. As discussed, the use of multiple AC activators, especially drugs that are not endogenous activators of AC, may result in a conformational organization that does not normally occur *in vivo*. This may be the explanation for the high AC activity observed when $MnCl_2$, forskolin, and Gsα are used together to activate AC. Furthermore, while our chimeric studies determined that the membrane-spanning domains were not required for the alcohol effect on AC, they may provide an anchor to ensure that the AC structure maintains the appropriate conformation. Outside of the constraints of the membrane or

covalent linkage indicated for AC7sol, our purified recombinant AC may exist in a slightly altered conformation or alcohol may cause conformational changes different from that in native AC7 or AC7sol. In fact, AC7sol also exhibited inhibition by alcohol under activation conditions containing Gsα or Gsα + forskolin.

To ensure that this novel observation of inhibition of AC activity by alcohol did not negate our previous findings, we designed an alcohol cutoff experiment to test if the inhibition still followed the same alcohol cutoff as in previous studies where alcohol enhanced AC activity. Under the condition of AC activation by $MnCl_2$, forskolin, and Gsα, in which the activity of AC and effect of alcohol were both highest, we examined the effect of various concentrations of *n*-alkanols: ethanol, butanol, pentanol, and hexanol. This experiment found that pentanol was the alcohol cutoff point for AC7 $C_{1a} + C_2$ under these conditions; the inhibition of AC activity grew more potent with increasing *n*-alkanols up to pentanol, beyond pentanol there was no further increase in inhibition. These results suggest that the fundamental interaction of AC and alcohol remains the same regardless if the effect of alcohol is inhibition or enhancement of AC activity. Thus, we determined that the use of these AC activation conditions by $MnCl_2$, forskolin, and Gsα would be a valid means for performing a concentration response study in an effort to uncover a potential interaction mechanism for alcohol and AC.

The result showing that the alcohol effect is dependent on the combination of AC activators suggests that one of these activators and alcohol may be acting allosterically in the regulation of AC activity. Initially, we suspected that alcohol might be competing with either forskolin or Gsα for an allosteric site; essentially interfering with the ability of forskolin or Gsα to enhance AC activity. However, our data did not support that hypothesis. Instead, the data

suggests that butanol slightly decreased the potency of forskolin while having no effect on the potency of Gsα.

The regulation of AC by forskolin and alcohol was measured in two ways. Concentration-response curves were generated for forskolin (analogue NKH477) in the presence or absence of 100mM butanol and butanol response curves in the presence or absence of 25μM forskolin. The forskolin concentration-response curve suggests that addition of butanol shifts the curve to the right, thus decreases the potency of NKH477. The butanol concentration-response curve indicated that addition of forskolin did increase the potency of butanol on AC activity.

The regulation of AC by Gsα and alcohol was measured in two ways. Rather than shifting the concentration of Gsα in a concentration-response curve, we shifted the concentration of the G protein activator, GppNHp. The concentration-response experiment with GppNHp essentially controls the concentration of active Gsα in the reaction. There was no significant change in the EC50 value for GppNHp suggesting that alcohol does not affect the potency of Gsα. In the second experiment, a concentration-response curve was generated for butanol in the presence or absence of Gsα activated by 20μM GppNHp. This experiment suggests that Gsα does enhance the effect of butanol on AC activity. Furthermore, comparing to forskolin the effect of Gsα on the butanol effect is much greater (>3fold change in EC50) under the conditions used.

These results suggest that there are some interactions between alcohol and forskolin or Gsα, however, in comparison to the magnitude of the alcohol effect they are minor. The observed inhibitory effect of alcohol when AC is activated by $MnC1_2$ + forskolin + Gsα is not due to the change in binding affinity of forskolin or Gsα. It appears that the alcohol effect is directly on AC molecules causing conformational changes resulting in inhibition and mainly

independent of the activator (forskolin or Gsα) effect. Future kinetic studies will determine the effect of alcohol on K_m and V_{max} as determined for AC7sol.

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CHAPTER 5.
CONCLUDING REMARKS

Overall Summary of Findings

Ample research has established an effect of alcohol on the cAMP cell signaling system. Changes to cAMP signaling following alcohol exposure have been observed in clinical alcoholic patients as well as experimental animal models of alcohol use (Diamond, Wrubel, Estrin, & Gordon, 1987; Nagy, Diamond, & Gordon, 1988; Kirstein & Tabakoff, 2001; Donohue, Hoffman, & Tabakoff, 2005) Furthermore, a specific component of cAMP signaling, adenylyl cyclase has been proposed as the direct target of the alcohol effect on cAMP signaling (Menninger, Baron, & Tabakoff, 1998; Menninger et al., 2000; Maas, Jr. et al., 2005; Kim, Kim, Baek, Lee, & Han, 2011; Morales-Mulia et al., 2012). The primary objective in our laboratory has been to characterize the effect of alcohol on adenylyl cyclase at the molecular level. Thus far, our lab has established that the alcohol effect is isoform specific with AC7 most enhanced by ethanol and ethanol responsive domains are located in the cytoplasmic regions of the AC protein (Yoshimura & Tabakoff, 1995; Yoshimura & Tabakoff, 1999; Yoshimura, Pearson, Kadota, & Gonzalez, 2006; Kou & Yoshimura, 2007; Hasanuzzaman & Yoshimura, 2010; Gupta, Qualls-Creekmore, & Yoshimura, 2013). We have also generated a recombinant AC protein, AC7sol, containing only the cytoplasmic regions of AC7, and characterized the alcohol effect on this protein in a bacterial lysate preparation (Dokphrom, Qualls-Creekmore, & Yoshimura, 2011). The current study expanded the design of recombinant AC to include isoforms AC2 and AC9, and optimized conditions for the expression and purification of these proteins. The purification of AC constructs allowed us the ability to study the biochemical mechanism of the alcohol effect

on AC in a manner not previously possible in our other model systems, such as live cells transfected with specific components of cAMP signaling system.

In chapter 2, we designed recombinant proteins derived from mammalian AC including AC2sol, AC7sol, AC7 C_{1a}, AC7 C_2, AC2 C_{1a}, AC2 C_2, AC9 C_{1a}, and AC9 C_2. We optimized the expression of these recombinant proteins through manipulations in expression conditions including choice of plasmid vector, *E. coli* cell line for expression, culture growth medium, growing conditions for temperature, time, and agitation speed, cell growth density (A600) at time of protein expression induction by IPTG, and concentration of IPTG used for induction. We optimized the purification of our recombinant AC proteins by modifying our choice of affinity chromatography column and elution conditions. We determined that the optimal condition were the use of pQE82L plasmid vector, transformation in BL21 (DE3) *E. coli* cell line, culture growth in T7 medium with induction at A600 = 0.6 with 100μM IPTG and continued culture growth at 25ºC, 100rpm, for 19hr. The optimal protein purification protocol included the use of a cobalt metal affinity column, HisPur (by Pierce), with the addition of selected protease inhibitors to all working buffers and glycerol in the elution buffer, followed by ion exchange chromatography. The expression and purity of each recombinant protein was visualized by SDS-PAGE analysis. Ultimately, AC2sol and AC7sol did not achieve sufficient expression to warrant further enzymatic analysis. All other recombinant AC proteins, with the exception of AC2 C_{1a}, were expressed and purified to a quality acceptable for further analysis.

In chapter 3, we examined the AC activity of our various AC recombinant proteins. Through analysis of our primary isoform of interest, AC7, we found that the separate expression and purification of the cytoplasmic regions of AC can be combined to reconstitute catalytic activity *in vitro*. Our use of purified protein confirms that the alcohol effect is not reliant upon

any other proteins. We provided support for our previous work indicating that the alcohol response of AC is isoform specific. Through chimeric combinations of AC C_{1a} and C_2 regions, we were able to determine that the AC7 C_{1a} region contributes more strongly to the AC alcohol effect than does AC7 C_2.

In chapter 4, we measured the response of AC7 C_{1a} + AC7 C_2 to alcohol under various conditions of AC activation. Concentration-response curves were generated for butanol in the presence or absence of forskolin and Gsα. Dose-response curves were also generated for forskolin and Gsα in the presence or absence of butanol. Our initial expectation was that alcohol was competing with Gsα or forskolin, leading to the inhibition observed when AC is activated by $MnCl_2$ + forskolin + Gsα in the presence of alcohol. However, the dose-response experiments suggest that there is no competition for binding sites between alcohol and either forskolin or Gsα. While there are some interactions between alcohol and forskolin/Gsα, these interactions are minor and not likely the cause of the AC inhibition observed in the presence of alcohol. It seems that alcohol interacts with AC protein directly and causes conformational changes resulting in either enhancement or inhibition depending on the stimulators present.

Significance of Research

Adenylyl cyclase is a key component of the cAMP signaling system. cAMP signaling initiates many cellular events including gene expression, cell growth, myocardial contraction, cytokine production, glycolysis, fatty acid metabolism, and is also implicated in learning and memory. It is possible that the effect of alcohol on AC may have downstream effects on any of these cAMP-linked functions. Therefore, understanding how alcohol affects AC may also help to explain many of the physiological consequences of alcohol use in both acute and chronic exposure.

While research on the clinical and experimental effects of alcohol use continues to expand, there remains a great deal of unknown when it comes to the specific biochemical effect of alcohol. Much of the research remains at the observational/descriptive level. A few research groups have investigated the specific mechanism of action for alcohol within the cell. Crystal structures have been resolved for LUSH, an odorant binding protein in Drosophila responsible for detecting alcohol, in complex with ethanol, propanol, and butanol (Kruse, Zhao, Smith, & Jones, 2003). Other structural studies have suggested alcohol binding sites on ion channels (Howard et al., 2011), but these studies have used much larger, synthetic types of alcohol. While many descriptive studies have indicated a role for AC in the alcohol response, ours is the first to attempt to understand the direct interaction between AC and alcohol. We are the first to ever investigate the effects of alcohol on purified AC. Identifying the AC regions required for the alcohol effect can help reveal a mechanism for how alcohol affects protein function. While our current studies do not provide the definitive answers provided by structural analysis, we have been able to narrow down the potential sites for alcohol action on AC. There are not many direct alcohol binding mechanisms known, and the interaction between alcohol and AC could represent a new alcohol binding motif. Ultimately, elucidating the mechanism through which alcohol interacts with AC will have great implications for the field of alcohol research and may lead to the development of clinical tools for the diagnosis and treatment of alcohol-related disorders.

Future Directions

The results presented here provide clear evidence for the direct effect of alcohol on AC within the constraints of the cytoplasmic domains, C_{1a} and C_2. However, the exact location of alcohol interaction with AC cannot be determined from these data. Additional kinetic studies

will be performed to determine the effect of alcohol on K_m and V_{max}. The kinetic values will determine any effect of alcohol on the binding of AC substrate ATP.

The next milestone in this research will be the attainment of structural data for the interaction of AC with alcohol. The primary limiting factor for the accomplishment of this step is the expression and purification of the target protein. Structural analysis requires not only pure protein, but also a high concentration of protein. To date in our lab, we have attempted structural analysis of an AC/alcohol complex through nuclear magnetic resonance (NMR) spectroscopy and circular dichroism (CD). NMR uses the magnetic properties of atoms to make inferences about the structure of the protein. CD measures the absorption of circularly polarized light to make inferences about the secondary structure of the protein and can measure changes in that structure under different conditions. We have experimented with both NMR and CD in our lab. Preliminary data has been obtained by NMR which was capable of resolving the structure of AC7 C_2, observing a folded and monomeric protein. However, successive attempts to reproduce this data in the presence of alcohol have not been productive. CD analysis was carried out on AC7 C_{1a} + AC7 C_2 alone in a buffer solution and under AC activation conditions of $MnCl_2$ and $MnCl_2$ + ethanol. We were able to resolve a CD profile with relatively low background noise. Differential CD profiles were observed between the protein complex alone and with $MnCl_2$ added, however, no clear difference was detected with the addition of ethanol. Successful analysis at the structural level will require a more highly expressed protein and increased homogeneity of the sample, especially in the case of AC7 C_{1a}, which remains a challenge. Additional measures of purification are planned, including size-exclusion chromatography and a more sophisticated ion-exchange chromatography. However, it is also important to minimize the

exposure of the protein during purification in order to maintain the enzymatic integrity of the protein.

In conclusion, these studies have provided the means for examining the effect of alcohol on purified adenylyl cyclase proteins. We have begun to narrow down the potential mechanisms for alcohol-AC interaction and have paved the way for future structural studies that may have the capability to resolve the exact binding motif for alcohol on adenylyl cyclase.

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THE VITA

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