1995

The role(s) of arginine-specific mono(ADP-ribosyl)transferase in skeletal muscle cells

Hui-Yu Huang

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Animal Sciences Commons, Cell Biology Commons, Molecular Biology Commons, Physiology Commons, and the Veterinary Physiology Commons

Recommended Citation

Huang, Hui-Yu, "The role(s) of arginine-specific mono(ADP-ribosyl)transferase in skeletal muscle cells " (1995). Retrospective Theses and Dissertations. 10910.

https://lib.dr.iastate.edu/rtd/10910

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
The role(s) of arginine-specific mono(ADP-ribosyl) transferase in skeletal muscle cells

by

Hui- Yu Huang

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
For the Major Department

Signature was redacted for privacy.
For the Graduate College

Iowa State University
Ames, Iowa

1995
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>iv</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>ADP-ribosylation reactions</td>
<td>1</td>
</tr>
<tr>
<td>Prokaryotic mono(ADP-ribosyl)transferases</td>
<td>4</td>
</tr>
<tr>
<td>Eukaryotic mono(ADP-ribosyl)transferases</td>
<td>7</td>
</tr>
<tr>
<td>Inhibitors of ADP-ribosylation reactions</td>
<td>9</td>
</tr>
<tr>
<td>Arginine-specific mono(ADP-ribosyl)transferase</td>
<td>13</td>
</tr>
<tr>
<td>from rabbit skeletal muscle</td>
<td></td>
</tr>
<tr>
<td>Dissertation organization</td>
<td>15</td>
</tr>
<tr>
<td>ADP-RIBOSYLATION OF THE INTERMEDIATE FILAMENT PROTEIN DESMIN AND INHIBITION OF DESMIN ASSEMBLY IN VITRO MUSCLE ADP-RIBOSYLTRANSFERASE</td>
<td>16</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>16</td>
</tr>
<tr>
<td>Summary</td>
<td>16</td>
</tr>
<tr>
<td>Introduction</td>
<td>17</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>19</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>20</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>24</td>
</tr>
<tr>
<td>References</td>
<td>24</td>
</tr>
<tr>
<td>TARGET PROTEINS FOR ARGinine-SPEcIFIC MONO(ADP-RIBOSYL)TRANSFERASE IN CHICK SKELETAL MUSCLE CELLS</td>
<td>39</td>
</tr>
<tr>
<td>Abstract</td>
<td>39</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

ATP    Adenosine triphosphate
DTT    Dithiothreitol
EDTA   [Ethylenedinitrilo]tetraacetic acid
GTP\textsubscript{\textgamma}S Guanosine 5'-O-(3-thiotriphosphate)
HEPES N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
HPLC  High-performance liquid chromatography
MIBG   Meta-iodobenzylguanidine
NAD    Nicotinamide adenine dinucleotide
PAGE  Polyacrylamide gel electrophoresis
PCR    Polymerase chain reaction
SDS    sodium dodecyl sulfate
TCA    Trichloroacetic acid
Tris   Tris(hydroxymethyl)aminomethane
GENERAL INTRODUCTION

ADP-Ribosylation reactions

ADP-ribosylation is a post-translational modification, in which an ADP-ribose moiety is transferred from β-NAD⁺ to specific amino acid residues of proteins by various ADP-ribosyltransferases (1). ADP-ribosylation reactions are classified into two types: poly(ADP-ribosyl)ation and mono(ADP-ribosyl)ation reactions (1). These two types of modification are different with respect to the chain length of ADP-ribose units, the chemical nature of ADP-ribosyl protein linkage, the enzymes involved, and the sites of reaction (1).

(I) Poly(ADP-ribosyl)ation:

Poly-ADPR reaction was first reported by P. Mandel et. al. in 1963. Since then, movement towards better understanding the function of poly-ADPR has been increasingly made. Poly(ADP-ribose)polymerase that catalyses this reaction is primarily associated with the nucleus and is dependent on the presence of DNA nicks or double-strand breaks for its full enzyme activity (2). It is thought to be present in all eukaryotes but not in prokaryotes. The purified poly(ADP-ribose)polymerase consists of three domains: the N-terminal DNA binding, automodification site, and the C-terminal NAD binding domains (3).

The amino acid residues that become poly(ADP-ribosyl)ated are either glutamate or aspartate and their carboxyl group is esterified through the OH-group of C-1 of ADP-ribose (1). The principal endogenous substrate in the cultured cells subjected to DNA damage
appears to be poly(ADP-ribose)polymerase itself (1). Based on the results of inhibitor studies (3-amino-benzamide) (4), and those of cellular mutants with decreased poly(ADP-ribose)polymerase activity (5, 6), and antisense poly(ADP-ribose)polymerase RNA (7), poly(ADP-ribose)polymerase is now believed to play a role in DNA repair and/or cell growth functions. It remains to be established exactly how this enzyme participates in these DNA reactions and what significance the presence of the poly(ADP-ribose) polymer is.

(II) Mono(ADP-ribosyl)ation:

Mono(ADP-ribosyl)ation reactions have been identified in both prokaryotes and eukaryotes. Mono(ADP-ribosyl)ation reactions can be classified into four subtypes according to the amino acid residues that accommodate ADP-ribose,: arginine-, diphthamide-, cysteine-, and asparagine-specific enzymatic ADP-ribosylation (1). Mono(ADP-ribosyl)ation predominates by far (> ten-fold) over poly-ADPR in mammalian cells, particularly in the extranuclear compartments (8), suggesting the importance of this type of reaction.

The linkages between ADP-ribose and the different specific amino acid residue acceptors can be distinguished by their sensitivities to different chemicals (Table I) (9, 10). For instance, the carboxyl-linkage (O-glycoside) is extremely sensitive to acid and hydroxylamine (NH₂OH). The thioglycosidic bond is stable to NH₂OH but is labile to mercuric ions. By contrast, the (ADP-ribosyl)arginine linkage (N-glycoside) is sensitive to NH₂OH with a half-life of 30-60 min. Therefore, the possible structures can be differentiated.
Table I: Stability of protein: ADP-ribose linkages

<table>
<thead>
<tr>
<th>ADP-ribosyl linkage to:</th>
<th>Formic acid (44%)</th>
<th>Hydroxylamine (1M, pH 7.0)</th>
<th>Hg$^{2+}$ (10 mM)</th>
<th>NaOH (1M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylate</td>
<td>Stable</td>
<td>Rapid release</td>
<td>Stable</td>
<td>Released</td>
</tr>
<tr>
<td>Arginine</td>
<td>Stable</td>
<td>Slow release</td>
<td>Stable</td>
<td>Released</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Stable</td>
<td>Stable</td>
<td>Released</td>
<td>Released</td>
</tr>
<tr>
<td>Diphthamide</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>Released</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>Lysine</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
<td>Released</td>
</tr>
</tbody>
</table>
Prokaryotic mono(ADP-ribosyl)transferases

The best studied mono(ADP-ribosyl)transferases are the bacterial toxins, diphtheria toxin, pseudomona exotoxin A, cholera toxin, *E. coli* heat labile enterotoxin, and pertussis toxin. These may be classified as (A-B) subunit ADP-ribosylating toxins (11). In these proteins, the toxic enzymatic activity is generally carried by the A subunit, and the B subunit possesses the cell-membrane receptor binding activity and aids the penetration of the A subunit into host cell cytoplasm (11).

Target proteins for these transferases have been identified and characterized. Diphtheria toxin A subunit ADP-ribosylates a post-translationally modified histidine residue of elongation factor 2 (EF2), called diphthamide. ADP-ribosylation of EF2 inactivates its translocase activity, thereby inhibiting protein synthesis (11). Cholera toxin, produced by *Vibrio cholerae*, ADP-ribosylates an arginine residue of Gsα (12). ADP-ribosylated Gsα can activate adenylate cyclase but is incapable of hydrolyzing GTP (12). Therefore, the adenylate cyclase remains locked in its active state. As a consequence, an increase in the intracellular cAMP concentrations induced by this modification causes the symptoms of cholera. This modification catalyzed by cholera toxin requires certain components of the cytosol that are ADP-ribosylation factors (ARF) acting as GTP-dependent allosteric activators of cholera toxin. Pertussis toxin, produced by *Bordetella pertussis*, has been demonstrated to catalyze the transfer of ADP-ribose to a cysteine residue near the carboxyl terminus of the α-subunit of the inhibitory G-proteins, Goα or Giα (13). In doing so, it prevents Giα from exchanging its
bound GDP for GTP and therefore from inhibiting adenylate cyclase and causes pertussis (whooping cough).

Several Clostridium species produce binary toxins consisting of component II required for cell entry and component I which possesses ADP-ribosyltransferase activity (11). The clostridial ADP-ribosylating toxins can be divided into two groups with respect to their protein substrates. One group of toxins modifies small GTP-binding proteins of the rho family. The other group of transferases belongs to the family of actin-ADP-ribosylating toxins. C3 (14, 15) and C3-like (16-18) exoenzymes ADP-ribosylate an asparagine residue (Asn-41) (19) of rho proteins and cause thereby the disappearance of microfilaments and actinomorphic shape changes (20). Thus, the ADP-ribosylation of a rho protein seems to be responsible for microfilament disassembly.

Monomeric actin is the unique substrate for ADP-ribosylation by the known toxins of type C2 toxin and several clostridia enzymes, including the iota toxins of C. perfringens type E, C. spiroforme toxin and C. difficile, that are distinct from C2 toxin (11). It was shown that the acceptor amino acid for ADPR is arginine-177 (21, 22), and it appears that all these toxins modify actin at the same site. It was shown that actin-ADP-ribosylating toxins disturb the cellular equilibrium between F- and G-actin, thereby resulting in the breakdown of the microfilament network (23, 24).

These bacterial toxins described here turn out to be very useful tools to study the physiological functions of their eukaryotic target proteins, e.g., heterotrimeric G-proteins, actin, and rho proteins etc. One
of the most fascinating questions still unanswered is whether the
targets of toxins are also substrates for ADP-ribosylation by endogenous
ADP-ribosyltransferase.

These bacterial ADP-ribosylating toxins described above are
involved in disrupting eukaryotic cell metabolism or function. Do
bacteria use ADP-ribosylation as a mechanism to control their own
metabolism? Evidence for the ADP-ribosylation of proteins in bacteria
has been reported. The most thoroughly studied example is the
reversible ADP-ribosylation of the dinitrogenase reductase from the
photosynthetic bacterium *Rhodospirillum rubrum* (25, 26). Dinitrogenase
reductase is ADP-ribosylated on arginine-101 by
dinitrogenase reductase ADP-ribosyltransferase (DRAT). ADP-
ribosylation of dinitrogenase reductase occurs in response to fixed
nitrogen sources such as ammonium or glutamine or in response to
darkness. ADP-ribosylation causes a complete inactivation of
dinitrogenase reductase. The enzyme is activated by removal of ADP-
ribose group by dinitrogenase reductase activating glycohydrolase
(DRAG). More recently, the glutamine synthetase III (GSIII) from
*Rhizobium meliloti* was also shown to be a target for ADP-ribosylation
and modification causes the loss of GSIII biosynthetic activity (27).
ADP-ribosylation could therefore play many more important roles in
the endogenous regulation of microbial metabolism.
Eukaryotic mono(ADP-ribosyl)transferases

Mono(ADP-ribosyl)transferase activity has also been detected in a variety of cells and tissues, including turkey and human erythrocytes (28-31), rat and hen livers (32, 33), rabbit and pig skeletal muscle (34, 35), chicken heterophils (36), and a variety of murine cell lines, including lymphocytes (37). Research into endogenous mono(ADP-ribosyl)ation in animal cells has also led to the identification of ADP-ribosyltransferases that are specific not only for arginine, but also for cysteine (38) and diphthamide residues (39).

The physiological role of endogenous mono(ADP-ribosyl)transferases regarding their substrates and cellular functions is still unclear. However, several potential substrates and functions of eukaryotic ADP-ribosyltransferases have been suggested. Soluble turkey erythrocyte arginine-specific mono(ADP-ribosyl)transferase A (40), a 28 kDa single polypeptide activated by salts, has been extensively studied. Ovine brain glutamine synthetase, recombinant Ha-ras-p21, α and β subunits of transducin, and chicken erythrocyte tubulin have been reported to be the substrate proteins for turkey erythrocyte transferase A (41). ADP-ribosylation of those proteins catalyzed by transferase A results in inhibition of activity, GTPase and GTP-binding activities, and microtubule assembly, respectively (41).

Chicken heterophil arginine-specific ADPRT has a molecular mass of 27.5 kDa and is independent of salts for its activity (36). In permeabilized heterophils, the 33 kDa and to a less extent 43 kDa proteins, corresponding to p33 and cytosolic actin, respectively, were
labeled after incubation with $[^{32}\text{P}]\text{NAD}$, thereby indicating that these two proteins are endogenous targets for chicken transferase (36, 42). It was shown that the partial amino acid sequence of p33, except for one amino acid, matched that of myb-induced myeloid specific protein-1, mim-1. The expression of mim-1 is regulated by the nuclear oncogene product Myb (43), but the function of mim-1 is still not known. ADP-ribosylation of actin inhibited the polymerization of actin monomers, thereby suggesting that ADP-ribosylation of actin catalyzed by heterophil transferase may affect the morphology or movement of the cells by altering the state of actin polymerization.

A link between endogenous mono(ADP-ribosyl)ation and adenylate cyclase activity has been suggested by several findings. Chick spleen cell membrane contains an (ADP-ribosyl)transferase which can catalyze the endogenous GTP-dependent ADP-ribosylation of the arginine residue of $G_{\text{S}\alpha}$, and this modification enhances basal adenylyl cyclase activity in the membrane (44). In addition, measurement of ADP-ribosyltransferase activity in intact human platelets revealed that a protein with a similar molecular mass to $G_{\text{S}\alpha}$ is a substrate for eukaryotic ADP-ribosyltransferase (45). Recently, additional data about canine cardiac sarcolemma has been reported (46), thereby supporting the hypothesis that endogenous transferase modifies the arginine residue of $G_{\text{S}\alpha}$, the acceptor site for cholera toxin-catalyzed ADP-ribosylation, resulting in activation of adenylate cyclase. These findings suggest that endogenous ADP-ribosyltransferases may play an important role in signal transduction involving G-proteins.
Nitric oxide (NO), a novel biological messenger, has been suggested to be a regulator of endogenous intracellular ADP-ribosylation. It was initially found by Brune and Lapetina that NO-generating agents, added to cytosolic extracts from platelets and other mammalian cell types, activated a cytosolic enzyme that mono(ADP-ribosyl)ated a cysteinyl residue of a specific 39-kDa protein (47, 48). The identity of the 39-kDa protein has been revealed as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (49, 50). Recently, however, the conclusion that ADP-ribosylation of GAPDH by an endogenous ADP-ribosyltransferase in the presence of NAD+ and NO was ruled out, because purified GAPDH incubated with NAD+ and NO was modified similarly to that in cell homogenates (49, 50). The hypothesis that GAPDH has an ADP-ribosyltransferase activity was later rejected by finding that the whole NAD+ molecule is bound to GAPDH, not just the ADP-ribose moiety from NAD+ (51).

Inhibitors of ADP-ribosylation reactions

One effective approach to investigate the biological functions of ADP-ribosylation reactions is to modulate the activity of ADP-ribosylating enzymes in vivo with a specific inhibitor and analyze ensuing changes in cellular functions. Many compounds have been shown to inhibit the poly(ADP-ribose)polymerase activity in vitro or in vivo. Among them, the most frequently used are 3-aminobenzamide, nicotinamide, and thymidine. Although their effects are fairly specific on poly(ADP-ribose)polymerase, these inhibitors are accompanied by
various *in vivo* side actions and, therefore, their use has left more or less inconclusiveness to the results. For example, 3-amino-benzamide was reported to inhibit de novo synthesis of DNA (52). Benzamide and its derivatives proved to be potent inhibitors of nicotinamide N-methyltransferase (53), and, in certain cells, to variably inhibit de novo synthesis of DNA (52). Therefore, more specific and potent inhibitors must be found and used to define the physiological roles of ADP-ribosylation reactions in cells.

As listed in Table II (54), many of the inhibitors newly found are highly specific for poly(ADP-ribose)polymerase, whereas some act selectively on mono(ADP-ribosyl)transferase or nonspecifically on both enzymes. The four strongest inhibitors for poly(ADP-ribose)polymerase are 4-amino-1,8-naphthalimide, 6(5H)- and 2-nitro-6(5H)-phenanthridinones, and 1,5-dihydroxyisoquinoline. The 50% inhibitory concentrations, 0.18-0.39 \( \mu \text{M} \), are about 10-20 orders of magnitude lower than that of 3-amino-benzamide (33 \( \mu \text{M} \)). A common structural feature among all potent inhibitors, including 1-hydroxyisoquinoline, chlorthenoxazin, 3-hydroxybenzamide, and 4-hydroxyquinazoline, in addition to the four mentioned above, is the presence of a carbonyl group built in a polyaromatic heterocyclic skeleton or a carbamoyl group attached to an aromatic ring (54).

Four natural compounds have been identified to be very inhibitory to the arginine-specific mono(ADP-ribosyl)transferase from
Table II: Comparison of inhibitory potencies of various compounds on mono(ADP-ribosyl)transferase\textsuperscript{a} & poly(ADP-ribose)polymerase\textsuperscript{b}

<table>
<thead>
<tr>
<th>Compound</th>
<th>mADPRT\textsuperscript{a}</th>
<th>pADPRT\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (\textmu M)</td>
<td>Inhibition</td>
</tr>
<tr>
<td>4-Amino-1,8-naphthalimide</td>
<td>&lt;200</td>
<td>&lt;200</td>
</tr>
<tr>
<td>6(5H)-phenanthridinone</td>
<td>&lt;1000</td>
<td>63</td>
</tr>
<tr>
<td>2Nitro-6(5H)phenanthridinone</td>
<td>83</td>
<td>84</td>
</tr>
<tr>
<td>1,5-Dihydroxyisoquinoline</td>
<td>890</td>
<td>15</td>
</tr>
<tr>
<td>1-Hydroxyisoquinoline</td>
<td>1500</td>
<td>15</td>
</tr>
<tr>
<td>Chlorthenoxazin</td>
<td>#</td>
<td>3</td>
</tr>
<tr>
<td>4-Hydroxyquinazoline</td>
<td>2600</td>
<td>3</td>
</tr>
<tr>
<td>3-Hydroxybenzamide</td>
<td>9000</td>
<td>5</td>
</tr>
<tr>
<td>Benzamide</td>
<td>4500</td>
<td>3</td>
</tr>
<tr>
<td>Oleic acid (C18:1, cis-9)</td>
<td>200</td>
<td>7</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1, cis-9)</td>
<td>200</td>
<td>7</td>
</tr>
<tr>
<td>Arachidonic acid (C20:4)</td>
<td>66</td>
<td>70</td>
</tr>
<tr>
<td>Arachidic acid (C20:0)</td>
<td>4.4</td>
<td>100</td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>6.1</td>
<td>99</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Vitamin K\textsubscript{1}</td>
<td>1.9</td>
<td>&gt;86</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>280</td>
<td>11</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mono(ADP-ribosyl)transferase was abbreviated as mADPRT and was purified from hen heterophils.
\textsuperscript{b} Poly(ADP-ribose)polymerase was abbreviated as pADPRT and was purified from calf thymus.
\# : Not done or not measurable.
\* : Stimulation
hen heterophils (Table II). They are vitamin K₁ and saturated long chain fatty acids (arachidic, steric and palmitic acids). Their IC₅₀ values are between 1.9 and 16 μM. Another natural inhibitor, novobacin, is also very specific for mono(ADP-ribosyl)transferase by competing with NAD⁺. Little has been done with these inhibitors mentioned above to elucidate the biological functions of the N-linked mono(ADP-ribosyl)ation in intact cells.

Recently, an inhibitor of intracellular arginine-specific ADP-ribosyltransferase, meta-iodobenzylguanidine (MIBG) has received more attention. MIBG is a guanidino analogue of the natural neurotransmitter norepinephrine. Radiolabeled [¹³¹I]MIBG is clinically used as a tumor targeting radiopharmaceutical in the diagnosis and treatment of adrenergic tumors (55). MIBG has been demonstrated to be a high-affinity substrate for mono(ADP-ribosyl)transferase of cholera toxin (Kₘ=6.5 μM) and turkey erythrocyte membranes (56) as well as to effectively compete with intracellular acceptors for endogenous enzymes (57). The specificity of MIBG as an inhibitor of mono-, but not poly-, ADP-ribosylation was demonstrated by Smets and coworkers, who showed that MIBG inhibited N-linked mono(ADP-ribosyl)ation with an IC₅₀ of 100 μM in turkey erythrocyte membrane preparations, but did not inhibit poly(ADP-ribosyl)ation in either permeabilized S49 or L1210 leukemia cells (56). Thus, MIBG is both a substrate for mono(ADP-ribosyl)transferases and an inhibitor of protein ADP-ribosylation. Therefore, MIBG has been extensively used as a tool
to study the roles of skeletal muscle ADP-ribosyltransferase in our laboratory.

**Arginine-specific mono(ADP-ribosyl)transferase from rabbit skeletal muscle**

A membrane-associated arginine-specific mono(ADP-ribosyl)transferase, using low molecular weight guanidino compounds (35) or arginine residues in proteins (58-60) as acceptors of ADP-ribose, was detected at relatively high levels in rabbit skeletal muscle and in cultured skeletal muscle cells (61). The transferase can be inactivated by reducing agents (58, 62). The divalent cations Mg$^{2+}$ and Ca$^{2+}$ had no effect on the ADPRT activity (58). High concentrations of Triton X-100 as well as deoxycholate above 0.01% were inhibitory (58). The muscle transferase is highly specific for NAD whereas NADP and NADH are very poor substrates (58). When partially purified transferase from rabbit skeletal muscle was used, Km values for NAD and L-arginine methyl ester (LAME) as substrates were determined to be 0.56 mM and 1.2 mM, respectively (63). By contrast, MIBG was determined to be a much higher affinity substrate for muscle transferase than LAME with Km of 0.2 mM (unpublished data). The kinetic mechanism was defined as random sequential (63).

Arginine-specific ADP-ribosyltransferase from rabbit skeletal muscle has been purified to homogeneity (62, 64, 65). Analysis of the purified transferase by SDS-PAGE revealed the presence of a 38 kDa-protein (62, 64). The enzyme was also cloned from rabbit skeletal
muscle (64). The composite cDNA showed a 981-base-pair open reading frame, encoding a polypeptide of 36,134 Da, which is consistent with the molecular weight determined by SDS-PAGE (64). No extended sequence homology was found between muscle transferase and any of the bacterial toxins, the ADPRT from *Rhodospirillum rubrum*, or poly(ADP-ribose)polymerase (64). It contained two potential sites for N-linked glycosylation (64). The expression of arginine-specific ADPRT appears to be tissue specific, because the mRNA of the enzyme was found primarily in rabbit skeletal and heart muscle but not in smooth muscle, brain, lung, kidney, spleen, or liver (64).

The deduced amino acid sequence of the transferase showed a hydrophilic center region surrounded by a stretch of about 20 very hydrophobic amino acids at both amino and carboxyl termini (64). This suggests that muscle transferase is a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein. GPI-anchored proteins are likely located on the exterior of the plasma membrane or, to a lesser extent, on the luminal face of intracellular vesicles (66, 67). It has been reported that treatment of intact transfected cells with phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* caused almost complete release of the transferase to the medium (68). This finding supports the view that ADPRT is located on the surface of muscle cells. However, several studies also reported that muscle transferase is located in the sarcoplasmic reticulum (59, 65, 69, 70), and the catalytic activity of the transferase is oriented towards
cytoplasm (71). The possibility that muscle ADPRT might exist in isoforms remains to be determined.

Dissertation Organization

This dissertation follows an alternative format in which there are three manuscripts included after the General Introduction. The first paper describes that the intermediate filament protein-desmin as a substrate for muscle ADP-ribosyltransferase and what ADP-ribosylation does to desmin's function in vitro, and has been published in *Biochemical and Biophysical Research Communications*. The second paper concerns the identification of protein substrates for arginine-specific ADP-ribosyltransferase using embryonic chick primary muscle cell cultures as a working model. Results of the second paper will be submitted to *Experimental Cell Research*. The third paper describes the expression and refolding of muscle ADP-ribosyltransferase in *E. coli* and will be submitted to *Protein Engineering*. Following the third paper is a general summary of entire dissertation and an outlook for further work is also discussed. Following the General Summary is the General References in which the references cited in the General Introduction and the General Summary are listed.
ADP-RIBOSYLATION OF THE INTERMEDIATE FILAMENT PROTEIN DESMIN AND INHIBITION OF DESMIN ASSEMBLY IN VITRO BY MUSCLE ADP-RIBOSYLTRANSFERASE


Hui-Yu Huang, Donald J. Graves, Richard M. Robson*, and Ted W. Huiatt*

Department of Biochemistry and Biophysics, *Muscle Biology Group, Molecular Biology Bldg., Iowa State University, Ames, IA 50011

1To whom correspondence should be addressed. Fax: 515-294-0453.

Abbreviations:
ADPRT ADP-ribosyltransferase
ADPR ADP-ribosylation
IF Intermediate filament
PKA cAMP-dependent protein kinase
STI Soybean trypsin inhibitor

Summary
Arginine-specific mono(ADP-ribosyl)transferase purified from rabbit skeletal muscle catalyzes stoichiometric ADP-ribosylation of the intermediate filament protein, desmin. In contrast, cholera toxin catalyzes a much lower level of ADP-ribosylation of desmin.
Modification results in potent inhibition of desmin's ability to assemble into filaments. Phosphorylation of desmin by the catalytic subunit of cAMP dependent protein kinase is also inhibited by ADP-ribosylation. ADP-ribosylation site(s) are located within the N-terminal head domain of desmin.

Introduction

ADP-ribosylation (ADPR) is a covalent post-translational modification of proteins, which involves the transfer of the ADP-ribose moiety from β-NAD+ to specific amino acid side chains of proteins by ADP-ribosyltransferases (ADPRTs) (1, 2). The best understood of the mono-ADPRTs are the bacterial toxins. The bacterial enzymes exert their toxic effects by regulating a variety of cytoplasmic functions in eukaryotic cells, such as protein translation (diphtheria toxin) (3) and adenylcyclase activity at the plasma membrane (cholera and pertussis toxins) (4, 5). Clostridium toxins recently have been shown able to ADP-ribosylate non-muscle actin, skeletal muscle actin and low molecular weight G proteins (6).

Eukaryotic mono-ADPRTs have been identified in many animal tissues (7-13), but their physiological function remains poorly characterized. An arginine-specific mono-ADPRT has been purified from rabbit skeletal muscle in our laboratory (14,15). Recently, we have examined the effect of a novel, specific inhibitor of cellular arginine-specific mono-ADPRTs, meta-iodobenzylguanidine (MIBG) (16,17), on differentiation of cultured embryonic chick skeletal
myoblasts (18). We found that MIBG reversibly inhibited both proliferation and differentiation of cultured myoblasts. In addition, the total arginine-specific mono-ADPRT activity increased with differentiation in the myogenic cell cultures, and this increase was also blocked by MIBG treatment. Therefore, these findings suggested a possible role for this type of enzymatic reaction in myogenesis.

Intermediate filaments (IFs), which are part of the cytoskeleton and the nuclear lamina of most eukaryotic cells (19,20), average 10 nm in diameter. The overall structure of IF proteins consists of an α-helical central rod domain surrounded by non-helical N-terminal head and C-terminal tail regions. Recently it has been shown that IFs are much more dynamic cellular structures than originally believed, and the IF proteins are subject to covalent modification by phosphorylation that alters their assembly-disassembly properties (19). The head domain, characterized by a large number of arginine residues, is highly basic. The functional importance of arginine residues has been suggested by the inhibition of filament formation following removal of the head domain by proteolysis (21) and by modification of arginine residues in the head domain of the protein (22, 23). Purified IF proteins undergo assembly to form filaments when the ionic strength is increased or pH is decreased to 7 (24). The muscle-specific type III IF protein, desmin, with a molecular mass of 53 kDa, has been shown to be expressed early in myogenesis, and may help align and tie together adjacent myofibrils during myofibrillogenesis (25), but the details of desmin's role in this process remain to be shown.
Because ADPR may play a role in myogenesis and because desmin is one of the earliest muscle specific gene products expressed, we determined if desmin is a substrate for purified skeletal muscle ADPRT. We show that desmin is an excellent substrate for the muscle ADPRT and that the resulting covalent modification markedly alters desmin's ability to assemble into IFs.

Materials and Methods

Purification of proteins - Arginine-specific mono-ADPRT obtained from the Con A elution step was purified from rabbit skeletal muscle (15). Purified desmin was prepared from turkey gizzard (26). Skeletal muscle G-actin was purified as described (27). The catalytic subunit of PKA was prepared as described (28).

ADP-ribosylation - Before desmin was used for experiments, it was dialyzed overnight against two changes of 10 mM Tris-HCl, pH 8.5, at 4 °C and then clarified by centrifugation at 100,000 x g for 30 min. Clarified desmin (0.25 mg/ml) was routinely ADP-ribosylated by incubation with 20 μg/ml muscle ADPRT, 1 mM [32P]NAD, 10 mM Tris-HCl, pH 8.5, at 30 °C, except as specified in Fig. 1. Soybean trypsin inhibitor (STI) (Sigma) and skeletal muscle G-actin at concentrations of 0.25 mg/ml were used in the indicated experiments. ADPR by cholera toxin A subunit was carried out in a reaction mixture containing 200 mM potassium phosphate, pH 7.0, 20 mM DTT, and 10 μg/ml activated cholera toxin A subunit at 30 °C. ADPR reaction was stopped by removing unreacted [32P]NAD by spin dialysis with Amicon centicon
30. The incorporation of ADP-ribose in proteins was measured by the filter paper assay (29).

**Phosphorylation** - Control and ADP-ribosylated desmin at concentrations of 0.25 mg/ml were phosphorylated by incubation with 30 μg/ml catalytic subunit of PKA in 10 mM Hepes buffer, pH 7.5, 0.3 mM MgCl₂, 0.5 mM DTT, and 1 mM [γ-³²P]ATP at 30 °C.

**Electron microscopy** - Electron microscope observations were made on negatively stained samples as described (26) and examined in a JEOL 100 CX-II electron microscope operated at 80 kV.

**IF Assembly** - Control and ADP-ribosylated desmin were dialyzed against assembly buffer containing 10 mM Tris-HCl, pH 7.5, 170 mM NaCl, 5 mM DTT, and 0.2 mM MgCl₂ at 4 °C for 20 hr to promote filament formation (24, 26).

**Protein concentration** - Protein concentration was determined by using Pierce BCA protein assay reagent.

**Domain assignment of ADP-ribosylated desmin** - ADP-ribosylated desmin was digested with lysyl endopeptidase according to the conditions described by Kusubata et al. (30). Ten μg of the digest and of ADP-ribosylated desmin were subjected to 15 % SDS-PAGE followed by Coomassie blue staining and autoradiography.

**Results and Discussion**

Muscle specific desmin was treated with arginine-specific mono-ADPRT under conditions selected to inhibit or promote IF assembly. As shown in Fig. 1, we found that muscle ADPRT catalyzed the ADPR of
about 2-2.5 mol of arginine residues per mol of 53 kDa-subunit of soluble desmin in 10 mM Tris-HCl, pH 8.5, after 4-h incubation. Less incorporation of ADP-ribose was obtained when desmin was incubated with ADPRT under conditions selected to partly promote assembly of desmin into 10-nm filaments (low ionic strength at pH 7.5), and much less under conditions that favor additional assembly (pH 8.5 with 150 mM NaCl).

We also tested whether desmin can serve as good a substrate for a different transferase, namely cholera toxin. Unlike the significant level of ADP-ribose incorporation catalyzed by muscle ADPRT, cholera toxin catalyzed a very low stoichiometry of less than 0.06 mol ADP-ribose per mol desmin as shown in the Table I. To further address whether the difference in ADP-ribosylation of desmin catalyzed by these two enzymes is due to their different substrate specificity, STI which contains the potential arginine residues targeted for deimination (31), was tested. As shown in Table I, STI was a very good substrate for cholera toxin, but it was a poor substrate for muscle ADPRT. ADPR of skeletal muscle G-actin by clostridium perfringens iota toxin has been reported, and the modification caused inhibition of actin polymerization (32, 33). We examined the possibility of skeletal muscle G-actin being a target substrate for muscle ADPRT. A very low extent of ADPR (i.e., 0.06 mol ADP-ribose/mol G-actin) was observed (Table I). That muscle ADPRT and bacterial toxins showed different substrate specificity is not unexpected because no sequence homology has been found between rabbit skeletal muscle ADPRT and bacterial toxins (34).
To examine what ADPR does to a physiological-like function of desmin, the effect of ADPR on the assembly of desmin was investigated by using both a sedimentation assay and electron microscopy. Fig. 2 shows that the amount of soluble desmin remaining in the supernatant increased as the extent of ADPR increased. Electron microscopy (Fig. 3) demonstrated that ADPR inhibited assembly of desmin into filaments. Control desmin, with no ADP-ribose incorporation (Fig. 3A), assembled into 10-nm diameter filaments after dialysis against assembly buffer. After ADPR, desmin did not form typical 10 nm filaments (Fig. 3B, 3C), and these samples consisted primarily of assembly intermediates, along with a few short filaments and some non-filamentous aggregates. The number of short filaments and aggregates decreased further with increasing ADP-ribose incorporation. These results indicated that ADPR of desmin by muscle ADPRT caused a marked inhibition of desmin's ability to self assemble.

It has been shown that ADPR of arginyl residues near phosphorylation sites inhibited phosphorylation by PKA (35-37). Because ADPR is a reversible reaction, it has been suggested that ADPR could regulate specific phosphorylation reactions (36,38). Phosphorylation of desmin by PKA and protein kinase C occurs in the N-terminal head domain and inhibits filament assembly (39, 40). The phosphorylation competence of ADP-ribosylated and control desmin was analyzed. As shown in Fig. 4, phosphorylation of soluble desmin by the catalytic subunit of PKA was significantly inhibited by previous ADPR. When desmin with 1.3 mol ADP-ribose per mol 53-kDa protein
was used for phosphorylation, about 75% inhibition was observed. This result also suggested that the head domain is the target for both ADPRT and PKA. This result lends support to the view that ADPR may be a mechanism modulating phosphorylation. To locate the ADPR site(s), [\(^{32}\)P]NAD labeled desmin with 1.2 mol of ADP-ribose per mol of 53-kDa desmin was cleaved with a lysine-specific protease and analyzed by SDS-PAGE followed by autoradiography. The head domain of desmin contains no lysine residues; thus, digestion with lysyl endopeptidase produces a small fragment of about 12-14 kDa, which includes and consists mainly of the intact head domain (30). As shown in Fig. 5, virtually all of the radioactivity associated with the ADP-ribosylated desmin was found in the small fragment migrating to a position corresponding to about 12-14 kDa. This result confirmed that the ADPR site(s) were located in the head domain. Work is in progress in our laboratory to define the sequence of the modified site(s).

In summary, we have shown that the muscle-specific IF protein, desmin, is covalently modified by muscle arginine-specific mono-ADPRT in vitro. ADPR takes place within the head domain of desmin and results in inhibition of desmin self-assembly. ADPR of desmin by muscle ADPRT causes subsequent inhibition of phosphorylation by the catalytic subunit of PKA. These findings, coupled with the observations that (a) ADP-ribosylarginine hydrolase that catalyzes the reverse reaction of ADPR has been identified in many animal tissues, including skeletal muscle (41), (b) skeletal muscle ADPRT is a glycosyl-phosphatidylinositol-linked membrane protein (15, 34), and (c) desmin
interacts with, or is anchored, via its N-terminal head domain to ankyrin at the plasma membrane (42), suggest that ADPR might play an important role in regulating desmin filaments and functions.

Acknowledgments

This research was supported in part by grants from the National Institutes of Health (GM-34262), Muscular Dystrophy Association, American Heart Association, Iowa Affiliate, and USDA Competitive Grants Program Award 92-37206-8051. This is Journal Paper J-15581 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Projects 2677, 2921, 3169, and 2127.

References


Table I
ADP-ribosylation by muscle transferase and *cholera* toxin A subunit

<table>
<thead>
<tr>
<th>Protein</th>
<th>Muscle transferase</th>
<th>Cholera toxin A subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmin</td>
<td>2-2.5</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>STI</td>
<td>0.23</td>
<td>1-2</td>
</tr>
<tr>
<td>G-actin</td>
<td>0.06</td>
<td>ND</td>
</tr>
</tbody>
</table>

*1Data are expressed as mole ADP-ribose per mol protein
ND: Not determined*
Figure 1. Time course of ADPR of desmin by muscle ADPRT. Purified desmin (0.25 mg/ml was ADP-ribosylated by incubation in 10 mM Tris-HCl, pH 8.5 (●), or 10 mM Tris-HCl, pH 7.5 (●), or 10 mM Tris-HCl, pH 8.5, 150 mM NaCl (▲), with [³²P]NAD and muscle ADPRT at 30 °C.
Figure 2. The percentage of desmin remaining unassembled after ADPR. Control (reaction mixture stopped at time 0 min with no incorporation) and ADP-ribosylated desmin with different levels of incorporation (0.82, 1.2, and 1.6 mol ADP-ribose/mol desmin) were induced to form filaments under assembly conditions. The amounts of desmin recovered in the supernatant were measured after sedimentation at 100,000 x g for 30 min.
Figure 3. Electron microscopy of the negatively stained control (A) and ADP-ribosylated (B, C) desmin after incubation under filament-forming conditions (samples selected from Fig. 2 before sedimentation). The samples were taken at (A) 0 min (no incorporation), (B) 90 min (0.82 mol/mol), and (C) 240 min (1.6 mol/mol) after ADPR. Bar=0.25 μm.
Figure 4. Time course of phosphorylation by the catalytic subunit of PKA of control (■) and ADP-ribosylated desmin (1.3 mol ADP-ribose/mol desmin) (●).
Extent of phosphorylation
(mol Pi/mol desmin)
Figure 5. Identification of ADP-ribosylated desmin domain. Labeled proteins before and after lysyl endopeptidase treatment, were analyzed by SDS-PAGE. (A) the gel patterns and (B) the corresponding autoradiogram. Lane 1, ADP-ribosylated desmin; lane 2, ADP-ribosylated desmin after cleavage with lysyl endopeptidase. Lane 3, molecular mass markers of 97.4, 66.2, 42.7, 31, 21.5, and 14.4 kDa.
TARGET PROTEINS FOR ARGinine-SPECIFIC MONO(ADP-RIBOSyl)TRANSFERASE IN CHICK SKELEtal MUSCLE CELLS

A paper to be submitted to the Experimental Cell Research

Hui-Yu Huang, Ted W. Huiatt, Hao Zhou, and Donald J. Graves*

Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011

Running Title: Target Proteins for Muscle ADP-ribosyltransferase

*To whom correspondence should be addressed:
Dr. Donald J. Graves
Department of Biochemistry and Biophysics
Iowa State University, Ames, IA 50011
Phone: 515-294-8961 Fax: 515-294-0453

Abstract

In previous studies, we have shown that a specific inhibitor of cellular arginine-specific mono(ADP-ribosyl)transferase, meta-iodobenzylguanidine (MIBG), reversibly inhibited both proliferation and differentiation of cultured embryonic chick primary muscle myoblasts. In addition, we observed that arginine-specific ADP-ribosyltransferase activity increased with muscle cell differentiation in cultures. This
increase in transferase activity suggests an important role for the enzyme in the myotubes. Therefore, muscle cell cultures, especially the 96-h myotube cultures which contain the highest levels of ADP-ribosyltransferase, were used as a working system to determine the cellular protein substrates for arginine-specific ADP-ribosyltransferase.

When membrane fractions extracted from 96-h chick myotubes were incubated with $[^{32}\text{P}]{\text{NAD}}$ at 30 °C for 30 min, only a few proteins were labeled under conditions favorable for mono(ADP-ribosyl)ation to occur. The labeling of two proteins of 36 k and 56 kDa was blocked in the presence of arginine-specific mono(ADP-ribosyl)transferase inhibitors, MIBG and novobiocin. To prove that these proteins are indeed the targets for arginine-specific mono(ADP-ribosyl)ation, active recombinant muscle ADP-ribosyltransferase was incubated with membrane proteins under the same conditions. ADP-ribosylation of these two membrane proteins, as seen in the endogenous reactions, was also catalyzed by the added muscle transferase. ADP-ribosylation of these two proteins was greatly enhanced in the presence of exogenous muscle transferase as compared to endogenous ADP-ribosylation, and also inhibited by inhibitors. Effects of MIBG and novobiocin on protein labeling suggested that 56 k and 36 kDa proteins are the endogenous target substrates for muscle transferase. Immunoblot analysis demonstrated that the 56 kDa protein reacted with antibody specific for desmin, indicating that desmin might be an in vivo target for muscle transferase.
Introduction

ADP-ribosylation is a covalent post-translational modification of proteins, which involves the transfer of the ADP-ribose moiety from β-NAD$^+$ to proteins by specific ADP-ribosyltransferases (1). In eukaryotic cells, two classes of ADP-ribosylation reaction can be distinguished, namely, mono and poly(ADP-ribosyl)ation (1). These two types of modification differ not only in the length of ADP-ribose chain, but also with respect to the chemical nature of ADP-ribosyl protein bond, the enzymes involved, and the site of reaction. Poly(ADP-ribose)polymerase is a nuclear protein and is suggested to be involved in DNA repair. On the other hand, mono(ADP-ribosyl)transferases are found mainly in the cytoplasm and cell membranes.

Endogenous mono(ADP-ribosyl)transferases specific for ADP-ribosylation of arginine, cysteine, and diphthamide residues have been identified in many tissues of different animal species (2-11). Among them, arginine-specific ADP-ribosyltransferases are the best characterized transferases. This type of transferase includes four distinct transferases purified from cytosol, membrane fraction, and nucleus of turkey erythrocytes (12, 4, 5), and a transferase from skeletal muscle (9, 13). An arginine-specific mono-ADPRT transferase was purified to ≥ 95% homogeneity from rabbit skeletal muscle microsomes by a combination of DE-52 cellulose, concanavalin A agarose, 3-aminobenzamide-agarose, and size-exclusion HPLC in our laboratory (14). Two different molecular masses of the enzyme, 39,000 ± 500 (α form) and 38,500 ± 500 (β form), were isolated indicating that
isoforms of the muscle transferase might be present in different compartments (14). In contrast to the cholera toxin A subunit, which requires reduction for its activity, the muscle transferase was found to be inactivated by reducing agents (14), thereby suggesting intramolecular disulfide(s) are required for its full activity. Alternative methods of purification of the rabbit skeletal muscle ADP-ribosyltransferase have also been reported (15, 16). This enzyme was also recently cloned and shown to be a glycosylphosphatidylinositol (GPI)-anchored membrane protein in rabbit skeletal muscle cells (15).

It has been suggested that ADP-ribosyltransferase plays a role in signal transduction involving G-proteins. ADP-ribosylations of $G_{S\alpha}$ catalyzed by a transferase from chicken spleen membrane (17), human platelet (18), and canine cardiac sarcolemma (19), resulting in inactivation of adenylate cyclase activity, have been reported. Also, Ca$^{2+}$-ATPase in the sarcoplasmic reticulum (SR) was shown to be ADP-ribosylated (20). Modification of Ca$^{2+}$-ATPase inhibited the ATP hydrolyzing activity implicating a role in the regulation of Ca$^{2+}$ transport in the SR (21). Recently, skeletal muscle integrin $\alpha 7$ has been shown to be ADP-ribosylated when intact mouse muscle C2C12 cells were incubated with extracellular [32P]NAD (22).

Recent data collected from our laboratory demonstrated that meta-iodobenzylguanidine (MIBG), both a substrate for arginine-specific mono(ADP-ribosyl)transferase and a competitive inhibitor of cellular protein ADP-ribosylation, reversibly inhibited both proliferation and differentiation of cultured embryonic chick skeletal myoblasts (23).
The conclusions were based on the effects of MIBG on cell fusion, creatine kinase activity, and protein and DNA contents. Arginine-specific ADP-ribosyltransferase was found to be predominately associated with the cell membrane, and its activity increased with differentiation in the primary myogenic cell cultures. A similar finding was also reported for mouse skeletal muscle C2C12 cells (22). The increase in transferase activity suggests to us an important role for the enzyme in the myotubes. But, it is not certain what substrate proteins can be modified in the muscle cell by ADP-ribosylation. Therefore, embryonic chick primary muscle cell cultures, especially the 96-h myotube cultures which contain the highest levels of ADP-ribosyltransferase, were used as a working system to determine the cellular protein substrates for arginine-specific ADP-ribosyltransferase.

Materials and Methods

Cell cultures - Myogenic cells were isolated from leg and thigh muscle of 12-day chick embryos according to the procedure of Ridpath et al. (24). Cultures were plated on collagen-coated Falcon tissue culture dishes at a density of about 2 X 10^6 cells per ml and a volume of 3 ml on 60-mm or 15 ml on 150-mm plates. Cells were maintained by daily feeding with medium consisting of 86.5% (v/v) Dulbecco's MEM supplemented with nonessential amino acids, 10% horse serum, 2.5% chick embryo extract, 50 IU penicillin/100 ml, and 1% Fungizone and incubated at 37 °C in an atmosphere of 5% CO₂ and 95% air.
Proteins - Active muscle arginine-specific mono(ADP-ribosyl)transferase was expressed and renatured according to the procedure described in Section III. ADP-ribosylarginine hydrolase was partially purified from rat brains (25). The protein concentrations were determined by the Bradford assay (26) using commercially prepared reagent from Bio-Rad.

Subcellular fractionation - 96-h culture dishes were rinsed three times with PBS and cells were scraped off with a rubber policeman with PBS. Cells were collected by low-speed centrifugation (800 g, 5 min) and resuspended in hypotonic buffer (10 mM Tris, 0.2 mM MgCl₂, pH 7.4) (27). After a 10-15 min incubation on ice, cells were disrupted by 25-30 strokes in a Dounce homogenizer. The homogenates were adjusted to a final concentration of 0.25 M sucrose and 1 mM EDTA and centrifuged at 1,000 g for 10 min at 4 °C. The supernatant was removed immediately and saved. The pellet was resuspended in 0.25 M sucrose, 1 mM EDTA, 10 mM tris, pH 7.4, 0.2 mM MgCl₂ by five strokes in a Dounce homogenizer. This suspension was spun at 1,000 g for 10 min, after which the supernatant was removed and combined with the supernatant from the first 1,000 g spin. The pooled 1,000-g supernatants were centrifuged at 100,000 g for 1.5 h at 4 °C in a Beckman Ti 70.1 rotor. The resulting pellet (membrane fractions or P100) was resuspended in buffer L (50 mM HEPES, pH 7.5, 0.5 mM MgCl₂, 137 mM NaCl, 1 mM PMSF, 2μg/ml each of aprotinin, leupeptin, pepstatin, and E-64), containing 0.25% Nonidet P-40 and assayed immediately.
ADP-ribosylation of intact embryonic chick muscle cells - 96-h myotube cells attached to 150-mm plates were rinsed once with PBS and twice with buffer L. Ten ml of buffer L containing 20 mM isoniazid, 10 mM thymidine, and 5 μM [32p]NAD was added to culture dishes. The reaction was carried out at 37 °C for 2 h and stopped by washing the culture dishes three times with PBS. Various cellular fractions were then prepared as described previously. Proteins were separated on a 10% SDS-PAGE under reducing conditions, followed by autoradiography.

ADP-ribosylation of embryonic chick muscle cell membrane proteins - Membrane fractions extracted as described above at concentrations of 1 mg/ml were preincubated with buffer L containing 20 mM isoniazid, and 10 mM thymidine at 30 °C for 5 min. Different ligands, including inhibitors and active recombinant muscle ADP-ribosyltransferase used as indicated, were also preincubated in the reaction mixture as described above. ADP-ribosylation was initiated by the addition of [32P]NAD (final concentration of 5 μM with specific activity of 25 μCi) and was allowed to proceed at 37 °C for 30 min. After incubation, the reaction was terminated by adding equal volume of 10% TCA. The TCA precipitates were washed once with 10% TCA and twice with water saturated ether and resuspended in SDS loading buffer. Proteins were analyzed by electrophoresis on 8% polyacrylamide SDS gels under either non-reducing or reducing conditions and autoradiography.

Identification of the ADP-ribosylarginine linkage - Hydrolysis of the ADP-ribosylarginine linkage was analyzed by incubation of 100 μg
of prelabeled membrane proteins with partially purified ADP-ribosylarginine hydrolase from rat brain in a total volume of 200 μl containing 20 mM potassium phosphate, pH 7.5, 10 mM DTT, and 15 mM MgCl₂ at 30 °C. The reaction was terminated by adding equal volume of 10 % TCA. TCA precipitated protein pellets were then washed and analyzed as described previously.

**Immunoblotting** - Prelabeled membrane proteins were separated on an 8% SDS-polyacrylamide minigel. The gel was equilibrated in 25 mM Tris, 200 mM glycine, pH 8.8, for 15 min, and the proteins were transferred to Zeta-probe nylon membrane (Bio-Rad) at 60V on ice for 2h. The membrane was blocked with 1% blotto in TBS (100 mM Tris-HCl, pH 8.0, 150 mM NaCl), reacted with rabbit polyclonal antibody specific for desmin or muscle ADP-ribosyltransferase, followed by alkaline phosphatase conjugated goat anti-rabbit immunoglobulin (Sigma). Immunoreactive proteins were visualized with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂.

**Results and Discussion**

Initially, we labeled the membrane proteins extracted from chick myotube cultures or intact cells with 1 mM [³²P]NAD in a neutral buffer at 30 °C for two hours, and we found that many proteins ranging from 20 k to 230 k were labeled. However, in doing so, the labeling results were not very consistent from time to time. To eliminate the possibility of non-specific reactions and NAD⁺ degradation, a modified method in
which thymidine, isoniazid were included in the reaction mixture was used (28). Thymidine and isoniazid are the inhibitors of poly(ADP-ribose)polymerase and NAD glycohydrolase, respectively. We found that when membrane fractions extracted from 96-h myotubes were incubated with 5 μM [32P]NAD under conditions described in "Methods", only a few proteins were labeled (Fig. 1A). An inhibitor of arginine-specific mono(ADP-ribosyl)ation, novobiocin, was used to identify the substrate proteins that were endogenously ADP-ribosylated. As shown in Fig. 1A, labeling of proteins with molecular masses of 56 kDa and 36 kDa, but not 127 kDa, were inhibited in the presence of 1 mM novobiocin. In a parallel experiment, ADP-ribosylation of membrane proteins by the active recombinant muscle ADP-ribosyltransferase was done under the same conditions. We found that the same two proteins, 56 k and 36 kDa, as seen in endogenous ADP-ribosylation, were the only targets to be labeled by active recombinant muscle transferase (Fig. 1B). The radioactivity associated with these two proteins were greatly enhanced (24h exposure in Fig. 1A vs. 30 min exposure in 1B). Again, novobiocin blocked the labeling of these two proteins. These results from the inhibitor study on both endogenous and exogenous ADP-ribosylation of cell membrane proteins provided evidence that the modification was arginine-specific mono(ADP-ribosyl)ation.

Another class of arginine-specific mono(ADP-ribosyl)transferase inhibitor was also used as a tool to identify protein substrates. As expected (Fig 2, lane 1), MIBG blocked the labelings of 56 k and 36 kDa proteins. The results from the inhibitor study (lane 1 and 3) indicated
that novobiocin was a more potent inhibitor than MIBG was. Since endogenous ADP-ribosylation of $G_{\text{S} \alpha}$ in the presence of GTP$_{\gamma}$S or AlF$_4^-$ has been shown to occur in highly purified canine cardiac sarcolemma (28), we tested whether one of the proteins identified in our system could be $G_{\text{S} \alpha}$. Unexpectedly, we found that 100 $\mu$M GTP$_{\gamma}$S inhibited the labelings of 56 kDa protein with the inhibition percentage of 61%, and to a lesser extent 36 kDa protein (33%), (Fig. 2, lane 4). However, AlF$_4^-$ had no effect on labeling of either protein (data not shown).

We also examined the effect of ATP on $[^{32}\text{P}]$ADP-ribosylation of membrane proteins. Interestingly, unlike GTP$_{\gamma}$S, ATP exerted a dramatic inhibitory effect on ADP-ribosylation of only the 56 kDa protein (about 91% inhibition), but did not inhibit labeling of the 36 kDa protein (Fig. 2, lane 5). This different effect of GTP$_{\gamma}$S and ATP on the endogenous $[^{32}\text{P}]$ADP-ribosylation of these two proteins suggested that 56 k and 36 kDa proteins are probably two distinct polypeptides and not the reduced fragments of one single polypeptide. The latter view was proven to be true because no changes on the mobility of the labeled proteins were observed when electrophoresis was performed under non-reducing condition (Fig. 3). The inhibition of $[^{32}\text{P}]$labeling of the 56 kDa protein by ATP also implied that endogenous phosphorylation of this particular protein might have occurred, thereby inhibiting subsequent ADP-ribosylation.

Muscle ADP-ribosyltransferase can be inactivated by reducing agents (14). Therefore, the effect of DTT on the labeling of muscle membrane proteins was investigated. We found that the radioactivities
associated with 56 k and 36 kDa proteins, but not 127 kDa protein, were reduced in the presence of 10 mM DTT (Fig. 2, lane 6). This result further demonstrated that the modification of these two proteins was catalyzed by arginine-specific mono(ADP-ribosyl)transferase in myogenic cell cultures.

To conclusively demonstrate that the modification of the 56 k and 36 kDa proteins is really arginine-specific mono(ADP-ribosyl)ation reaction, chemical stability or hydrolysis by ADP-ribosylarginine hydrolase of the ADP-ribosyl protein linkage was investigated. As shown in Fig. 4, when prelabeled membrane proteins catalyzed by active recombinant muscle transferase were incubated with partially purified ADP-ribosylarginine hydrolase from rat brain, the radioactivity associated with 56 and 36 kDa proteins declined as incubation time increased.

Our previous studies showed that the muscle specific intermediate filament protein, desmin (53 kDa/subunit), was an excellent in vitro substrate for purified muscle arginine-specific mono(ADP-ribosyl)transferase and that ADP-ribosylation of desmin caused a marked inhibition of desmin self-assembly (29). Additionally, ADP-ribosylation inhibited phosphorylation of desmin by cAMP-dependent protein kinase (29). It is known that phosphorylation of desmin also alters its assembly-disassembly properties (30). Since the 56 kDa protein identified here could be endogenously phosphorylated and has a molecular mass similar to that of desmin, immunoblot analysis was conducted to test if 56 kDa protein is desmin. As shown in Fig 5., we
found that 56 kDa protein that was both endogenously (lane 1 and 2) and exogenously (lane 3) ADP-ribosylated was recognized by rabbit antiserum specific for desmin. This result strongly suggests that desmin might be an in vivo target for muscle ADP-ribosyltransferase.

The 36 kDa protein might be ADP-ribosyltransferase. Because of the similarity of molecular mass between 36 kDa protein and muscle transferase (38 kDa), and the fact that ADP-ribosyltransferase can auto-ADP-ribosylate itself (14), this possibility is suggested. Work is in progress to show whether the 36 kDa band can act to ADP-ribosylate another substrate, e.g. MIBG. A gel assay developed by Peterson et. al. was used earlier to show that the 38 kDa protein prepared from rabbit skeletal muscle was arginine-specific mono(ADP-ribosyl)transferase (14).

Recently, skeletal muscle integrin α7 has been identified as an endogenous substrate for muscle ADP-ribosyltransferase when intact mouse C2C12 muscle cells were incubated with extracellular [³²P]NAD at 37 °C for 2h (22). The difference between our system and theirs was that we utilized the extracted membrane fractions from cultured embryonic chick primary muscle cells instead of intact mouse C2C12 muscle cells. Skeletal muscle integrin α7 is a membrane protein and consists of N-terminal large extracellular, transmembrane, and C-terminal cytoplasmic domains. It has been shown that a monoclonal antibody 05, specific for the 35 kDa proteolytic fragment, recognizes bands at 120 k and 70 kDa in the non-reducing L8E63 myotube lysate and at 35 k and 100 kDa upon reduction (31). With our working
system, we did not find that a protein with the molecular mass similar to integrin α7 was predominately labeled. Also, no changes on gel mobility of the labeled proteins were observed under either non-reducing or reducing conditions (Fig. 3) ruling out the possibility that these two proteins identified here were fragments of integrin α7.

Labeling of intact chick primary myotube cultures with extracellular [³²P]NAD under the same conditions used in this study showed that proteins derived from nuclear pellet, cytosolic, and membrane fractions ranging from 14 k to 200 kDa were labeled (Fig. 6, lane 1-2: nuclear pellet, lane 3-4: cytosolic fractions, and lane 5-6: membrane fractions). The labeling of these proteins was almost completely inhibited in the presence of 2 mM novobiocin (lane 2, 4, and 6). β-NAD⁺ is supposedly not able to cross the cell membrane due to its highly hydrophilic property and no known receptors on the cell surface have been reported. Surprisingly, we observed that proteins derived from nuclear pellet and cytosol (lane 1, and 3) were labeled, indicating that [³²P]NAD might have gotten into cells by an unknown mechanism. As found earlier, novobiocin was a potent inhibitor of the labeling with the exception of the labeling of one band at approximately 36 kDa. The significance of this result is not known.

The discrepancies between our findings and others can be explained in part by the possibility that muscle arginine-specific mon(ADP-ribosyl)transferase present in cultured chick myogenic cells is a mixed-type membrane protein with different isoforms; some are GPI-anchored and some are transmembrane proteins. The membrane
fraction that we extracted from myotubes is composed of several cellular membranes. Therefore the enzyme and the protein substrates are not necessarily restricted to the cell surface. Another possibility is that arginine-specific ADP-ribosyltransferase present in chicken skeletal muscle is simply different in the properties of structure and substrate specificity from the enzyme found in mouse skeletal muscle.

In summary, we identified two potential protein substrates, of 56 k and 36 kDa, for muscle arginine-specific mono(ADP-ribosyl)transferase in cultured myogenic cells. This finding was based on (1) the studies of two inhibitors, novobiocin and MIBG, on both endogenous and exogenous ADP-ribosylation of membrane proteins; (2) the inhibitory effect of DTT on the protein labelings. We also found that GTP\textsubscript{S} and ATP exerted different inhibitory effects on the labeling of these two proteins. Immunoblot analysis showed that antibody specific for desmin identified the 56 kDa band. This finding strongly suggests that desmin might be an \textit{in vivo} substrate for muscle transferase.

Acknowledgments

We thank Dr. Richard Robson for providing the rabbit antibody specific for desmin and for a critical reading of this manuscript. We are grateful to Jo Philips for preparing the cell cultures. This work was supported by Research Grant from the Muscular Dystrophy Association. This a Journal Paper No. J- of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project 2677.
References


Figure 1. Endogenous (A) and exogenous (B) ADP-ribosylation of muscle cell membrane proteins. Membrane proteins at concentrations of 1 mg/ml were preincubated with buffer containing 20 mM isoniazid, 10 mM thymidine and protease inhibitors (A) or in the presence of 20 μg/ml of active recombinant muscle transferase (B) at 30 °C for 5 min. ADP-ribosylation was initiated by the addition of 5 μM [32P]NAD and continued for 30 min at 30 °C. Reaction was terminated by TCA precipitation. Proteins were separated on 8% SDS-PAGE under reducing conditions followed by autoradiography. (A) was exposed for 24 h and (B) was exposed for 30 min. Lane C: in the absence of 1 mM novobiocin; lane N: in the presence of 1 mM novobiocin.
Figure 2. Effect of different ligands on the endogenous ADP-ribosylation of muscle cell membrane proteins. Different ligands added were also preincubated with the reaction mixture at 30 °C for 5 min and ADP-ribosylation was carried out under conditions as described in "Methods". Lane 1: 1 mM MIBG; lane 2: control without ligand addition; lane 3: 2 mM novobiocin; lane 4: 100 μM GTPγS; lane 5: 100 μM ATP; lane 6: 10 mM DTT.
Figure 3. Comparison of gel mobility of the labeled membrane proteins under non-reducing (lane 1) or reducing conditions (lanes 2 and 3). Labeled membrane proteins were separated on 8% SDS-PAGE followed by autoradiography. Novobiocin at a concentration of 2 mM (lane 2) was utilized as a tool to identify ADP-ribosylated proteins. Lane C: control, in the absence of novobiocin; lane N: in the presence of novobiocin.
Figure 4. Identification of ADP-ribosyl protein linkage. [32P]NAD labeled membrane proteins catalyzed by active recombinant muscle transferase were incubated with 20 µg/ml partially purified ADP-ribosylarginine hydrolase at 30 °C. Lane 1: 0 min; lane 2: 30 min; lane 3: 120 min; lane 4: 240 min; lane 5: 240 min control without hydrolase.
Figure 5. Immunoblot analysis of [32P]NAD labeled membrane proteins. The labeled membrane proteins were resolved on 8% SDS-PAGE under reducing conditions and were transferred to Zeta-probe nylon membrane and immunoblotted with rabbit antibody specific for desmin (A) followed by autoradiography (B). Lane 1: endogenous ADP-ribosylation in the presence of 2 mM novobiocin; lane 2: endogenous ADP-ribosylation; lane 3: ADP-ribosylation in the presence of 20 μg/ml of active recombinant muscle transferase. ADP-ribosylated desmin was run as a control (lane 4).
Figure 6. [32P]NAD labeling of the intact embryonic chick primary myogenic cells in cultures. 96-h myotube cells attached to 150-mm plates were labeled with extracellular [32P]NAD (5 μM) in the absence (lane 1, 3, and 5) or in the presence of 2 mM novobiocin (lane 2, 4, and 6) at 37 °C for 2 h. Various cellular fractions (lanes 1-2: nuclear pellets; lanes 3-4: cytosolic fractions; lanes 5-6: membrane fractions) were extracted as described in "Methods". Proteins were run on a 10% SDS-PAGE under reducing conditions followed by autoradiography. Labeled membrane proteins as shown previously without (lane 7) or with (lane 8) novobiocin were used as comparison.
EXPRESSION AND REFOLDING OF ARGinine-SPECIFIC MONO(ADP-RIBOSYL)TRANSFERASE IN E. COLI

A paper to be submitted to Protein Engineering

Hui-Yu Huang, and Donald J. Graves*

Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011, USA

*To whom correspondence should be addressed:
Dr. Donald J. Graves
Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011
Phone: 515-294-8961 Fax: 515-294-0453

Running Title: Expression and Refolding of ADP-ribosyltransferase.

Key words: arginine-specific mono(ADP-ribosyl)transferase/bacterial expression/glutathione/inclusion body/refolding

Introduction

The presence of arginine-specific mono(ADP-ribosyl)transferase in vertebrates was first demonstrated by Moss and Vaughan in 1978 (1). The enzyme, found in turkey erythrocytes, utilized β-NAD+ as an ADP-
ribose donor, and modified arginine and its derivatives. Since then, similar ADP-ribosyltransferase activity was found in many different animal tissues, including rat liver (2), chicken liver nuclei (3) and rabbit skeletal muscle (4). Endogenous mono(ADP-ribosyl)ation reactions specific for cysteine (5) and diphthamide (6) residues have been identified. However, arginine-specific ADP-ribosyltransferases are the more extensively studied enzymes.

Our laboratory has previously identified a membrane-associated arginine-specific mono(ADP-ribosyl)transferase from rabbit skeletal muscle (4). This muscle transferase was extracted from rabbit microsomes with deoxycholate and purified to homogeneity by successive DE-52, concanavalin A agarose, 3-aminobenzamide agarose, and size-exclusion HPLC chromatographies (7). Study of this transferase revealed that it consists of a single subunit with a molecular mass of about 38 kDa. The binding of this transferase to concanavalin A agarose suggests that muscle ADP-ribosyltransferase is a glycoprotein. Additionally, the transferase activity can be inhibited by reducing agents such as DTT, suggesting that disulfide bond(s) might be required for the biological activity of muscle transferase. Recently, other methods for the purification of muscle transferase have also been reported, resulting in a similar transferase activity (8, 9).

The cDNA for rabbit skeletal muscle arginine-specific ADP-ribosyltransferase was cloned (8). Expression of the transferase was shown to be tissue-specific, because its mRNA was detected primarily in skeletal and heart muscle (8). Based on the deduced amino acid
sequence, ADP-ribosyltransferase was postulated to be a glycosylphosphatidylinositol-anchored protein (8). This hypothesis was supported by the finding that treatment of intact cells or cell suspensions with phosphatidylinositol specific phospholypase C resulted in the release of transferase from the cell membrane (8, 10). No extensive sequence homology can be found between muscle ADP-ribosyltransferase and any of the bacterial toxins, the ADPRT from Rhodospirillum rubrum, or poly(ADP-ribose)polymerase. This finding implied unique structural properties, and possibly different substrate specificity, for the muscle transferase.

To better understand muscle arginine-specific ADP-ribosyltransferase, its structure, catalytic properties, and substrate specificity, it is necessary to develop a practical method to provide adequate quantities of enzyme needed for the biochemical characterizations. Here we describe the expression of muscle transferase in E. coli, and the approach we used for the refolding of active recombinant muscle ADP-ribosyltransferase.

Materials and Methods

DNA manipulation

Rabbit skeletal muscle ADP-ribosyltransferase, subcloned into pBluescript plasmid and containing the full coding region, was obtained from Dr Moss. The transferase cDNA was amplified by PCR using 5' NdeI and 3' BamHI primers that were synthesized in the Nucleic Acid Facility at Iowa State University. Amplification was performed at a
melting temperature 94 °C for 7 min and 30 cycles of 94 °C for 1 min/55 °C for 2 min/72 °C for 3 min, followed by extension at 72 °C for 10 min in a volume of 100 μl. The PCR product was gel-purified using the glass milk method (11), and digested with NdeI and BamHI (New England Biolabs). The resulting fragment contained about 843 bp that corresponded to a truncated form of transferase (amino acids 24-304). The fragment was then ligated to NdeI- and BamHI-digested pET15b expression vector (Novagen) with T4 DNA ligase. The nucleotide sequence of the coding region was confirmed by sequencing and showed no alteration. The expression plasmid was transformed into E. coli strain BL21(DE3) (Novagen).

For use in the ThioFusion expression system (a complete expression kit from Invitrogen), full-length or truncated forms of transferase cDNA with 5' kpnl and 3' BamHI restriction sites were also amplified by PCR. pTrxFus expression vector was used. Digestion and purification of DNAs and transformation were performed according to the standard procedures described previously. Two E. coli strains, GI724 for expression at 30-37 °C, and GI698 for expression at less than 30 °C were used.

Expression of ADP-ribosyltransferase

For the pET His-Tag expression system, E. coli BL21(DE3) cells containing the expression plasmid were first cultured in 5 ml of LB containing 100 μg/ml ampicillin (Amp) at 37 °C with shaking overnight and then diluted 1:100 in 500 ml of LB/Amp, grown at 37 °C with constant shaking at 200 rpm. When an O.D.600 of cell cultures was
reached, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cell culture to a final concentration of 1 mM. The induction of transferase was allowed to proceed at 37 °C for 1h or 3h. The cells were harvested by centrifugation at 5,000 g for 5 min and the cell pellets were stored at -20 °C until use.

For use in the ThioFusion expression system, GI724 or GI698 E. coli cells with expression plasmids were grown in media containing casamino acid at the temperature indicated. Expression of the transferase fusion protein was induced by adding tryptophan. Lysis of cells and purification of the fusion protein by osmotic shock of whole cells were done according the manufacturer's instruction (Invitrogen).

*Purification of the inclusion bodies*

The cell pellets were suspended in cold deionized water and lysed by passage through a French pressure cell two to three times at 5000 lb/in. pressure. The cell lysate was centrifuged at 12,000 g for 15 min at 4 °C. Purification of the collected pellet was carried out by sequential extraction steps using lysozyme, deoxycholate, DNase, RNase, and STET buffer (8% sucrose/50 mM Tris-HCl, pH 8.0/50 mM EDTA/5% Triton X-100) as described (12). The final washed pellet was suspended in 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA for storage at 4 °C.

*Purification of the His-tagged ADP-ribosyltransferase*

Cell lysate derived from BL21(DE3) was prepared as described previously and was applied onto Ni²⁺-agarose. The protein was washed and eluted with imidazole according to manufacturer's manual.

*ADP-ribosyltransferase activity assay*
Cell lysates purified from Ni\textsuperscript{2+} ion affinity column or renatured proteins were assayed for ADP-ribosyltransferase activity. The reaction mixture contained Tris-HCl, pH 9.0, 1 mM MIBG, 1 mM NAD\textsuperscript{+}, and an aliquot of refolded protein in a total volume of 300 µl. After incubation at 30 °C for 30 min, the reaction was stopped by adding an equal volume of 10% TCA and subjected to HPLC analysis as described (13).

**Protein concentration**

Protein concentrations were determined by the Bradford assay (14) using commercially prepared reagent from Bio-Rad.

**Results and Discussion**

We have used two different *E. coli* expression systems, pET-15b His-Tag and pTrxFus ThioFusion systems, in our attempt to express active muscle arginine-specific ADP-ribosyltransferase. In the pET His-Tag expression system, a stretch of 6 consecutive histidine residues is placed at the N-terminus of the truncated transferase and can be separated from the protein, if necessary, by thrombin cleavage (Fig. 1A). This His-Tag sequence provides a one-step rapid purification by binding to divalent cations (e.g. Ni\textsuperscript{2+}) immobilized on the metal chelation resin. The expression of His-tagged muscle transferase is under the control of T7lac promoter and can be induced by 1 mM IPTG according to the manufacturer’s recommendation. In the ThioFusion expression system, both full-length and truncated forms of muscle ADP-ribosyltransferase are expressed as thioredoxin fusions. Due to several characteristics of thioredoxin, including its solubility, thermostability
and accumulation at adhesion zones in E. coli, fusion protein expressed in this system is likely to be soluble and easily purified by osmotic shock and heat treatment. The expression of transferase as a fusion to thioredoxin is driven by the bacteriophage P1 promoter and induced by the addition of tryptophan to growth medium (Fig. 1B).

The high-level expression of eukaryotic proteins in E. coli sometimes results in the formation of insoluble inclusion bodies as non-native protein aggregates. Unfortunately, the vast majority of the muscle transferase expressed in either system at 37 °C was present in inculsion bodies based on the results from transferase activity assay and polyacrylamide gel analysis (Fig. 2). Various growth parameters, e.g. the temperature for induction in either system, were tested to determine if active soluble muscle transferase could be obtained as summarized in Table 1.

In the expression system with pTrxFus vector, the attempts to obtain active transferase with all conditions tried met failure (Table 1). No ADP-ribosyltransferase activity could be detected from either cell lysate or the osmotic shock fluid (data not shown). To test if thioredoxin interfered with ADP-ribosyltransferase activity, inclusion bodies of both full-length and truncated forms of transferase fusion proteins (Fig. 2C) were renatured as described in the section on refolding. We found that the truncated form of transferase fusion regained its activity, indicating that this small thioredoxin protein (12 kDa) did not affect transferase activity assay or its refolding. However, the full-length form of transferase fusion protein still remained inactive.
after its renaturation. In the pET His-Tag expression system, a very low level of ADP-ribosyltransferase activity was detected from cell lysates purified by Ni\textsuperscript{2+} ion affinity chromatography when cells were cultured at 22 °C with M9ZB media. This low expression of soluble transferase (<1% of bacterial proteins) made the purification of muscle transferase difficult. It is very tempting to use inclusion bodies as the source of muscle transferase, because of the high yield and easy purification of inclusion bodies. Therefore, a truncated form of muscle ADP-ribosyltransferase (amino acids 24-304) expressed with pET His-Tag vector at 37 °C (Fig. 2D) as inclusion bodies was utilized in this study.

Because no ADP-ribosyltransferase activity was found in the inclusion bodies, a procedure for protein refolding was essential. Previously, our laboratory observed that purified muscle ADP-ribosyltransferase was inactivated upon DTT treatment (7). This phenomenon suggested the likely presence of intramolecular disulfide(s) are required for full activity. The expressed truncated form of transferase contains of 6 cysteinyl residues. Thus, the redox system for disulfide formation was taken into account when a protein refolding strategy was designed. In addition, several factors e.g. denaturant, temperature, protein concentration, and refolding assistants, that could increase protein solubility were tested.

Our initial study on the refolding of muscle ADP-ribosyltransferase used a procedure described by Huang et. al. (15) in which a fully refolded truncated form of phosphorylase kinase γ subunit was successfully obtained. Inclusion bodies of muscle ADP-
ribosyltransferase were dissolved in 5 M guanidine-HCl/50 mM Tris-HCl, pH 8.0/5 mM EDTA/2 mM DTT. Undissolved particulate was removed by centrifugation. The denatured and reduced transferase was diluted to 0.3 mg/ml with the same guanidine-HCl buffer. The renaturation of the recombinant muscle transferase was initiated by diluting the solution 10-fold with RE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM DTT, 1% CHAPS and protease inhibitors) and was allowed to proceed at 0 °C for overnight (about 12 hours). The precipitates were removed from the solution by centrifugation. Unfortunately, no detectable ADP-ribosyltransferase activity was found in the supernatant and the vast majority of transferase precipitated out during this process (Fig 3).

A two-step renaturation procedure described by Hirose et. al. (16) has been successfully used in the folding of ovotransferrin that is a 78-kDa single polypeptide containing 15 intramolecular disulfide bonds(16). In their first step, the reduced and denatured form of the protein was incubated at 0 °C in a nondenaturing buffer containing reduced glutathione (1 mM); in the second step, the reduced form was reoxidized at 22 °C in the presence of both reduced and oxidized glutathione (1 mM /0.5 mM ). The rationale for two separate steps in the folding process is to avoid protein aggregation by decreasing the loss of conformational entropy upon folding of a reduced form at a lower temperature and by allowing the covalent process of disulfide formation to proceed rapidly at a higher temperature. By following the procedure, we were able to obtain active transferase as shown in Table
2. Also, we observed that when urea was used as a denaturant, the recovery of ADP-ribosyltransferase activity was about 10 fold higher than that when guanidine-HCl was used (Table 2), but the specific activity of the enzyme was lower. Although 10% glycerol used as a refolding assistant slightly improved both protein and activity recovery (Table 3), the precipitation of the proteins could not be eliminated under those conditions, resulting in very poor recovery of refolded transferase.

To avoid protein aggregation and to increase solubility during the folding process, we then adapted a method described by Albert Light (17) by introducing disulfide bridges between glutathione and the fully reduced, denatured muscle transferase as shown in Fig. 4. As the mixed disulfide, the thiol groups of the transferase were protected in a disulfide bridge and were stable and unreactive until refolding and disulfide interchange were both initiated. Inclusion bodies of muscle ADP-ribosyltransferase were solubilized in DE buffer (100 mM Tris, pH 8.5, 1 mM EDTA, 8 M urea) containing 5 mM DTT and held at room temperature for 1-2 h. The concentration of the denatured, reduced mixture was then diluted to 0.2 mg/ml with DE buffer containing 25 mM oxidized glutathione (GSSG) and the solution was allowed to stand at room temperature for overnight (about 12 h). Renaturation of muscle transferase was initiated by diluting the protein solution 10-50 fold, as indicated, with RE buffer (100 mM Tris, pH 8.5, 1 mM EDTA, 200 mM NaCl, and either glucose or sucrose) containing 4 mM reduced glutathione (GSH), at 4 °C. All the solutions were degassed under a
nitrogen atmosphere before use. The renaturing mixture was concentrated by ultrafiltration with Amicon PM30 membrane and dialyzed against RE buffer for overnight.

We successfully refolded muscle ADP-ribosyltransferase with this approach and we thereafter designated this approach as "protocol-AL". The recovery of ADP-ribosyltransferase activity increased rapidly within 30 min and reached maximum 4 hours after renaturation was initiated at 4 °C (Fig. 5). However, if incubation was continued, the recovery of activity decreased. Similar recovery of transferase activity was obtained with protein concentrations in the folding process up to 20 μg/ml (Table 4). The average of yield of protein after renaturation was about 6% in the presence of either glucose (10-20%) or sucrose (10%) (Table 4). On the other hand, glycerol (10%), as compared to glucose and sucrose, was less effective in improving the yield of refolded transferase (Table 4). The gel profile of refolded muscle transferase was shown in Fig. 6.

The specific activity of ADP-ribosyltransferase activity of the refolded protein obtained with the protocol-AL is the best thus far and is about 4-5 fold higher than the specific activity of enzyme prepared from rabbit skeletal muscle through the Con A affinity chromatography step. One problem not completely resolved is how to eliminate protein precipitation during protein folding. To this regard, a new approach developed by Rozema & Gellman (18) using a pair of low molecular weight folding assistants, a nonionic detergent POE(10)L and a cyclodextrin (β-CD) was applied in conjunction with protocol-AL
described previously to obtain active muscle ADP-ribosyltransferase (designated as "protocol-RG). The action of the detergent and cyclodextran mimics the mechanism of GroEL/S chaperone system on protein refolding. First, the detergent forms a complex with the denatured protein, thereby preventing protein self-association upon dilution of denaturant. Secondly, the cyclodextrin strips the amphiphile away from the protein, thus initiating protein folding.

We used the specific activity of refolded recombinant muscle transferase obtained with the protocol-AL as 100% for comparison with other methods. Dilution of the mixed-disulfide transferase in the presence of POE(10)L followed by further dilution with β-CD and reduced glutathione (protocol-RG) led to the same recovery of ADP-ribosyltransferase activity (Fig. 7, 2nd and 3rd column for 4h and 2h incubation, respectively) as compared to the specific activity of transferase obtained with protocol-AL (1st column), and to about 10% protein recovery. Only 26% activity was obtained (4th column) but with 70% protein recovery if β-CD was omitted. This result indicated that transferase was not able to refold from the detergent-complexed state unless the detergent was removed away from the protein by β-CD. Additionally, it supports the view that POE(10)L, an amphiphile, and β-CD, a stripping agent, mimic the action of the GroEL/S chaperone system. On the other hand, if the redox system (GSSG/GSH) was eliminated, even still in the presence of POE and β-CD, much less recovery of transferase activity (14.5%) was obtained (last column).
The arginine-specific mono(ADP-ribosyl)transferase purified from rabbit skeletal muscle was shown to be a glycosyl phosphatidylinositol-anchored membrane protein (8). However, the expressed muscle transferase as inclusion bodies from *E. coli* was no longer glycosylated. High ADP-ribosyltransferase activity can be recovered from its non-glycosylated form, indicating that glycosylation of transferase is not essential for its full activity. Also, the hydrophobic N- and C-termini that were postulated to be a signal peptide and recognition site for glycosylation, respectively, were not involved in catalytic activity, because the expressed muscle transferase was a truncated form (amino acid 24-304). Based on the effect of DTT on the transferase activity and the redox system required on the transferase refolding, it strongly suggests that disulfide(s) are essential for its full function. Therefore, it is important to determine how many and which cysteinyl residues in transferase are involved in forming disulfide bonds.

In conclusion, we have expressed muscle ADP-ribosyltransferase in *E. coli* in the form of inclusion bodies. An experimental approach for transferase refolding has been developed. It consists of several key steps: (1) solubilization of inclusion bodies by 8 M urea; (2) full reduction of transferase by DTT; (3) introduction of the mixed disulfide to transferase by oxidized glutathione; (4) first dilution of the mixed disulfide in the presence of detergent, POE(10)L; (5) further dilution with reduced glutathione and β-CD. The highly active recombinant muscle ADP-ribosyltransferase obtained with the protocol described here has been used in some studies like cell labeling and
characterization of ADP-ribosylated desmin in our laboratory. We believe this method should provide a precedent for further study of the skeletal muscle ADP-ribosyltransferase.

Acknowledgments

This work was supported by Research Grant from the Muscular Dystrophy Association. This a Journal Paper No. J- of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project 2677.

References


Figure 1. Diagrams illustrating the main features of (A) the His-Tagged muscle
ADP-ribosyltransferase expression vector, pET-15, and (B) the
thioredoxin gene fusion vector, pTrxFus, as well as the expression
system. (A) shows the DNA sequence for the 3'-end of the lacI gene,
T7lac promoter, His-Tag leader, protease cleavage site, cloning sites,
and T7 terminator. The truncated form of transferase (amino acid 24-
304) was cloned into the vector with 5'NdeI and 3'BamHI restriction
sites. (B) shows that the entire thioredoxin gene is placed behind the
bacterial phage P_l promoter in the pTrxFus followed by an
enterokinase cleavage site. A full-length or truncated form of
transferase was inserted in a polylinker region with 5'KpnI and
3'BamHI sites. The regulation of the expression of transferase fusion
is shown in this flow chart.
A

T7 promoter primer #69346-1

BglII T7 promoter lac operator XbaI rbs

AGATCTCGATCCGGAAATTAATACGACTCACTATAGGGGACACCGAGCTCGAGGATCCGGCTAACAAAGCCCQA

NcoI HisTag

TATACCAAGGTCAGACCATCATCAATCGACGCGCCTCCATGGCGCGCGCAGCC.M

MetGlySerHisHisHisHisHisSerGlyLeuGluProArgGlySerHisMetLeuGluAspProAlaAlaAsnLysAlaArg

NcoI

T7 terminator

AAGGAAGCTGAGGTnGClGCTRCCACCGCTnARCAATAACTAr.CAIAACCCCTLGGGrXCTCIAAACaRGICnCARGGnrilTTlG

LysGluAlaGluLeuAlaAlaThrAlaGluGluInEnd

BglII

thrombin

BglII

T7 terminator primer #69337-1

pET-15b
B

1. Clone gene of interest into pTrxFus in frame with the thioredoxin start codon.

2. Transform GI724 or GI699 E. coli cells. Isolate positive and grow cells.

3. Induce expression of the fusion protein by adding tryptophan.

4. Fusion protein expressed in E. coli cell. Some fusions may localize at adhesion zones.

5. Purify fusion protein by osmotic shock of whole cells or heat treatment of cell lysate.

6. Optional. Cleave the fusion protein with enterokinase to release the native protein.

7. Transcription is stopped by repression.
Figure 2. Inducible expression of rabbit skeletal muscle ADP-ribosyltransferase at 37 °C in bacteria. 10 μl of either soluble (S) or insoluble (inclusion bodies) (P) fractions derived from E. coli cells expressing thioredoxin-muscle ADP-ribosyltransferase for 0, 1, 2, 3, and 4 h was loaded onto a 10% SDS-PAGE gel. The full-length transferase fusion protein is shown in (A) and the truncated form is shown in (B). (C) shows the inclusion bodies of full-length (lane 1) and truncated forms (lane 2) of transferase fusions derived from 4h-expression cells. Purified inclusion body of His-tagged truncated muscle transferase as described in "Methods" is shown in (D). Lane 1: 1.5 h and lane 2: 3.0 h expression. M: molecular markers.
Table 1. Summary of Muscle ADP-ribosyltransferase Bacterial Expression

<table>
<thead>
<tr>
<th>Vector</th>
<th>pTrxFus</th>
<th></th>
<th>pET-15b His-Tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp'ed ADPRT</td>
<td>Full-length &amp; Truncated</td>
<td></td>
<td>Truncated form</td>
</tr>
<tr>
<td>E. coli strains</td>
<td>GI724</td>
<td>GI724</td>
<td>GI698</td>
</tr>
<tr>
<td>Temperature</td>
<td>37 °C</td>
<td>30 °C</td>
<td>22 °C</td>
</tr>
<tr>
<td>IPTG (1 mM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tryptophan (100 µg/ml)</td>
<td>I.B.a</td>
<td>I.B.</td>
<td>I.B.</td>
</tr>
</tbody>
</table>

a: Expressed His-tagged muscle transferase or thioredoxin fusion of transferase was synthesized as a non-native aggregates (inclusion bodies (I.B.)) in both expression systems under conditions described after appropriate time periods of induction.
Figure 3. Recombinant muscle ADP-ribosyltransferase derived from the supernatant (S) and precipitation (P) fractions after renaturation. Inclusion bodies of His-tagged truncated ADP-ribosyltransferase were dissolved in 5 M guanidine-HCl, 2 mM DTT buffer and renatured under conditions described in the text. After an overnight incubation at 0 °C, the solution was concentrated. The supernatant was separated from the precipitate by centrifugation. Shown is a 10% SDS-PAGE gel, stained with Coomassie blue. I: inclusion bodies; M: molecular markers.
Table 2. Refolding of Muscle ADP-ribosyltransferase Under 2-step Conditions Using Different Denaturants

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>Urea</th>
<th>Guanidine-HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>ADPRT(SH)₆</td>
<td>ADPRT(SH)₆</td>
</tr>
<tr>
<td>Conc. (µg/ml)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mercaptan</td>
<td>GSH/GSSG</td>
<td>GSH/GSSG</td>
</tr>
<tr>
<td>Protein Yield (%)</td>
<td>0.55</td>
<td>2.8</td>
</tr>
<tr>
<td>Activityᵃ</td>
<td>13.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Inclusion bodies were dissolved in either 8 M urea or 5 M guanidine-HCl in the presence of 5 mM DTT. The fully reduced, denatured proteins were diluted 100 fold at 0°C with RE buffer (0.1 M Tris-HCl, pH 8.2, 1 mM EDTA, and 0.15 M NaCl) containing 1 mM GSH. After a 5-min incubation, the reduced proteins were incubated at 22°C in the presence of GSH and GSSG (final concentrations of 1 mM and 0.5 mM, respectively) overnight. Proteins were concentrated and dialyzed against RE buffer and then subjected to protein determination and activity assay.

ᵃ: Activity was expressed as nmol (ADP-ribose)MIBG/min/mg.
Table 3. Refolding of Muscle ADP-ribosyltransferase under 2-step conditions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc. (mg/ml)</th>
<th>Time (h)</th>
<th>Activity $^a$</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADPRT(SH)$_6$</td>
<td>20</td>
<td>4</td>
<td>16.4</td>
<td>2.3</td>
</tr>
<tr>
<td>ADPRT(SH)$_6$</td>
<td>20</td>
<td>12</td>
<td>24.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Denaturation and 2-step renaturation of transferase were performed according to the conditions described in Table 2 with some modifications. Urea was used as a denaturant. 10% glycerol was included in the RE buffer.

$a$: Activity was expressed as nmol (ADP-ribose)MIBG/min/mg.
Figure 4. The principle of muscle ADP-ribosyltransferase refolding. Denaturation and reduction of transferase were achieved by 8 M urea and 5 mM DTT. The mixed disulfide was introduced to muscle transferase by adding excess GSSG. After an overnight incubation at room temperature, protein folding was initiated by dilution with RE buffer in the presence of 4 mM GSH and was performed at 4 °C.
Formation 1
\[ \text{Reduced & denatured} \quad \rightarrow \quad \text{Mixed-disulfide} \]
\[ + \text{GSSG} \]

Rearrangement 3
\[ \text{Folded protein} \quad \rightarrow \quad \text{Mixed-disulfide} \]
\[ + \text{GSH} \]

2
\[ \text{Breakage} \]

GSH
Figure 5. Time course of muscle ADP-ribosyltransferase refolding. The denatured and reduced transferase was incubated with 25 mM GSSG in DE buffer at room temperature overnight. The solution was diluted with RE buffer containing 4 mM GSH. After incubation at 4 °C, protein was concentrated and dialyzed and ADP-ribosyltransferase activity was assayed.
Table 4. Comparison of Refolding Parameters for the Renaturation of Muscle ADP-ribosyltransferase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc. (µg/ml)</th>
<th>Assistant</th>
<th>Time (h)</th>
<th>Activity$^a$</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ADPRTssG</td>
<td>4</td>
<td>20% glucose</td>
<td>4</td>
<td>1020.6</td>
<td>8.6</td>
</tr>
<tr>
<td>2. ADPRTssG</td>
<td>18</td>
<td>20% glucose</td>
<td>4.5</td>
<td>825.3</td>
<td>4.6</td>
</tr>
<tr>
<td>3. ADPRTssG</td>
<td>18</td>
<td>10% glucose</td>
<td>4</td>
<td>917.2</td>
<td>5.3</td>
</tr>
<tr>
<td>4. ADPRTssG</td>
<td>5</td>
<td>10% glucose</td>
<td>4</td>
<td>1145.8</td>
<td>5.9</td>
</tr>
<tr>
<td>5. ADPRTssG</td>
<td>5</td>
<td>10% glucose</td>
<td>8</td>
<td>663.3</td>
<td>5.2</td>
</tr>
<tr>
<td>6. ADPRTssG</td>
<td>2.75</td>
<td>10% sucrose</td>
<td>4</td>
<td>977</td>
<td>6.3</td>
</tr>
<tr>
<td>7. ADPRTssG</td>
<td>4.7</td>
<td>10% sucrose</td>
<td>8</td>
<td>733</td>
<td>2.3</td>
</tr>
<tr>
<td>8. ADPRTssG</td>
<td>2.6</td>
<td>30% sucrose</td>
<td>12</td>
<td>545</td>
<td>13.7</td>
</tr>
<tr>
<td>9. ADPRTssG</td>
<td>4.7</td>
<td>10% glycerol</td>
<td>8</td>
<td>290</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The urea-denatured and DTT-reduced transferase was diluted with denaturing buffer containing 25 mM GSSG to 0.2 mg/ml. After incubation at room temperature overnight, the protein solution was further diluted with RE buffer (0.1 M Tris-HCl, pH 8.5, 1 mM EDTA, 0.2 M NaCl, and assistant as indicated) for time indicated at 4 °C.

$a$: Activity was expressed as nmol(ADP-ribose)MIBG/min/mg
Figure 6. The active recombinant muscle ADP-ribosyltransferase remaining in the solution after refolding process. 10 μg of refolded muscle transferase was taken from condition 3 (lane 1) or condition 4 (lane 2) in Table 4 and analyzed by a 10 % SDS-PAGE gel. Proteins were visualized with Coomassie blue. M: molecular markers.
Figure 7. Comparison of the effects of different refolding assistants on the renaturation of muscle ADP-ribosyltransferase. Dilution of the mixed disulfide or the fully reduced transferase with RE buffer in the presence or absence of a detergent, POE(10)L. After an one-hour incubation at 4 °C, protein refolding was allowed to proceed by further dilution with or without β-CD and GSH to give a final dilution of 10-fold at 4 °C for 2h or 4h as indicated. RE A: 100 mM Tris-HCl, pH 8.5/1 mM EDTA/200 mM NaCl/10% glucose. RE B: 167 mM Tris-HCl, pH 8.5/1.7 mM EDTA/334 mM NaCl.
P-S-S-G  +  +  +  +  -
P-SH    -  -  -  -  +
RE A    -  +  +  +  +
RE B    +  -  -  -  -
POE     -  +  +  +  +
β-CD    -  +  +  -  +
GSH     +  +  +  +  -
4h      +  +  -  +  +
2h      -  -  +  -  -
ADP-ribosylation is one of the reversible post-translational modifications of cellular proteins. In this modification, the ADP-ribose moiety of β-NAD is transferred to various acceptor proteins with a concomitant release of nicotinamide. Poly(ADP-ribosyl)ation reactions are associated with eukaryotic nuclei and dependent on the presence of DNA breaks. The biological roles suggested for this reaction include DNA repair, cell differentiation, cell growth, and alteration of chromatin architecture. Most of these findings were obtained from experiments employing inhibitors of the poly(ADP-ribose)polymerase such as 3-amino-benzamide (3-ABA). Mono(ADP-ribosyl)ation reactions are found in both prokaryotes and eukaryotes. Four types of ADP-ribosylating toxins produced by bacteria, arginine-, cysteine-, diphthamide-, and asparagine-specific, are well characterized and their functions are well established. Evidence from the studies on prokaryotes such as *R. rubrum* and *Rhizobium meliloti* support the conclusion that ADP-ribosylation plays a role in the endogenous regulation of microbial metabolism. Several eukaryotic ADP-ribosylating enzymes similar to the bacterial toxins have been identified and characterized. Among these, arginine-specific ADP-ribosyltransferases are the most extensively studied. The physiological functions of these eukaryotic transferases are not yet well defined.

This dissertation describes a study of what role(s) arginine-specific mono(ADP-ribosyl)transferase may have in skeletal muscle cells
by finding its protein substrates and by studying what ADP-ribosylation does to a protein's function. It also describes the study of expression and the refolding of muscle arginine-specific ADP-ribosyltransferase.

Previously, we showed that an inhibitor of arginine-specific mono(ADP-ribosyl)transferase, MIBG, reversibly inhibits both differentiation and proliferation of cultured embryonic chick myoblasts. Additionally, we observed that arginine-specific mono(ADP-ribosyl)transferase activity predominately is associated with the cell membrane and increases with cell differentiation. These findings suggested to us that this enzyme might play an important role in myogenesis and led us to screen the in vitro substrate proteins for the enzyme that may be important in the development of skeletal muscle.

The muscle specific type III intermediate filament protein, desmin, was chosen for the in vitro substrate study because it has been shown to be expressed early in myogenesis. Monomeric actin appears to be a target for either clostridial toxins or an endogenous ADP-ribosyltransferase found in chicken heterophils. These findings also led us to investigate if G-actin can be modified by skeletal muscle ADP-ribosyltransferase. We found that muscle transferase selectively ADP-ribosylated desmin but not G-actin (2 mol/mol vs 0.06 mol/mol). However, desmin was a very poor substrate for cholera toxin. Although cholera toxin and muscle ADP-ribosyltransferase exert similar enzymatic activity, they appear to have different protein substrate specificity. ADP-ribosylation of desmin catalyzed by muscle transferase resulted in the inhibition of desmin's ability to assemble. Desmin is also
subject to modification by phosphorylation. We demonstrated that phosphorylation of desmin by the catalytic subunit of PKA was inhibited by previous ADP-ribosylation, therefore suggesting the arginyl residue(s) targeted for muscle transferase are N-terminal to, and close to, the phosphorylation sites and are located in the head domain. More recently, the tryptic fragments derived from the modified head domain of desmin have been purified and are being sequenced. The sequence information on the ADP-ribosylation site should help us understand what features in the protein are important for modification by muscle arginine-specific ADP-ribosyltransferase.

To further demonstrate that this regulation by ADP-ribosylation of desmin is possible in vivo, a $[^{32}P]NAD$ labeling method was used with cultured myoblasts as a working system because both ADP-ribosyltransferase and ADP-ribosylhydrolase have been detected. Inhibitors specific for arginine-type mono(ADP-ribosyl)transferase, novobiocin and MIBG, were utilized as a tool to identify protein substrate(s) for muscle transferase. Two potential proteins with a molecular mass of 56 k and 36 kDa were identified in the membrane fractions of 96h myotube cultures. Importantly, we found that ATP almost completely inhibited the labeling of the 56 kDa protein, thereby suggesting that endogenous phosphorylation of 56 kDa protein might have occurred and this modification of desmin suppressed subsequent ADP-ribosylation. This observation, along with the results from immunoblot assays where an antibody specific for desmin reacted with the 56 kDa polypeptide, strongly suggest the possibility that the 56 kDa
protein is desmin and that it is an endogenous target for muscle ADP-ribosyltransferase.

Although a purification scheme for muscle transferase has been reported by three laboratories including ours, the time consuming nature of the purification process and its low yield of pure protein makes the biochemical study of the enzyme very difficult. Therefore, an expression system to generate adequate active recombinant muscle ADP-ribosyltransferase is needed. Both full-length and truncated forms of muscle transferase were expressed as thioredoxin fusion proteins, and another truncated form was expressed as a His-tagged protein in which a stretch of 6 histidine residues was placed N-terminal to transferase (amino acids 24-304). In either system, muscle ADP-ribosyltransferase was expressed as inclusion bodies in which the enzyme was inactive.

The advantages of working with inclusion bodies are that the protein is expressed in a very high yield and that there are less contaminants in the inclusion body. We therefore chose the inclusion body of truncated His-tagged muscle transferase as our protein source for further study. A protocol with a combination of the GSSG/GSH redox system and a pair of refolding assistants, POE(10)L and β-cyclodextrin, was successfully established for the renaturation of transferase. Based on the effect of refolding parameters e.g. GSSG/GSH, and DTT on the recovery of ADP-ribosyltransferase activity, it strongly indicates that disulfide bonding in the muscle transferase is a critical determinant for enzyme activity. However, we at this point are not certain how many
pairs of disulfide are involved in maintaining the correct conformation of muscle transferase and which cysteiny1 residues are involved in forming correct disulfide bridge(s). The answers to these questions would be very helpful for us to understand the structural or catalytic properties of muscle arginine-specific mono(ADP-ribo3yl)transferase. Mutagenesis of the six cysteine residues in muscle transferase would be the best choice in the future.
GENERAL REFERENCES


ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my major professor, Dr. Donald J. Graves, for his guidance, support, and encouragement during the period of my graduate studies. His enthusiasm and his many "crazy ideas" made the research in the lab very exciting. I am very thankful for being in his laboratory.

I greatly thank Dr. Richard Robson and Dr. Ted Huiatt, with whom we collaborated on desmin and cell culture projects, for their helpful assistance and suggestions through the years. I would like to thank Jo Philips for growing the muscle cells for me.

Special thanks go to Dr. Bruce Martin for his encouragement and beneficial advice in my research and in my graduate student life. Thanks are also extended to my labmates at Iowa State University for their friendship and assistance.

Finally, I give my truly gratitude to my dearest parents for everything they have done to me, and to my husband, JinJun, for his love, support and sacrifice.